Carbonic anhydrase subunits form a matrix-exposed domain attached to the membrane arm of mitochondrial complex I in plants

Stephanie Sunderhaus\textsuperscript{1}, Natalia Dudkina\textsuperscript{2}, Lothar Jänsch\textsuperscript{3}, Jennifer Klodmann\textsuperscript{1}, Jesco Heinemeyer\textsuperscript{1}, Mariano Perales\textsuperscript{4}, Eduardo Zabaleta\textsuperscript{4}, Egbert Boekema\textsuperscript{2} and Hans-Peter Braun\textsuperscript{1,5}

Running title: Carbonic anhydride subunits of complex I

\textsuperscript{1} Institut für Angewandte Genetik, Universität Hannover, Herrenhäuser Str. 2, D-30419 Hannover, Germany, \textsuperscript{2} Department of Biophysical Chemistry, GBB, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands, \textsuperscript{3} Gesellschaft für Biotechnologische Forschung, Mascheroder Weg 1, D-38124 Braunschweig, Germany, \textsuperscript{4} Instituto de Investigaciones Biológicas, FCEyN, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina, \textsuperscript{5} corresponding author: Hans-Peter Braun, e-mail: braun@genetik.uni-hannover.de

Complex I of Arabidopsis includes five structurally related subunits representing γ-type carbonic anhydrases termed CA1, CA2, CA3, CAL1 and CAL2. The position of these subunits within complex I was investigated. Direct analysis of isolated subcomplexes of complex I by liquid chromatography linked to tandem mass spectrometry allowed to assign the CA subunits to the membrane arm of complex I. Carbonate extraction experiments revealed that CA2 is an integral membrane protein which is protected upon protease treatment of isolated mitoplasts, indicating a location on the matrix-exposed side of the complex. A structural characterization by single particle electron microscopy of complex I from the green alga Polytomella and a previous analysis from Arabidopsis indicate a plant-specific spherical extra-domain of about 60 Å in diameter, which is attached to the central part of the membrane arm of complex I on its matrix face. This spherical domain is proposed to contain a heterotrimer of three CA subunits, which are anchored with their C-termini to the hydrophobic arm of complex I. Functional implications of the complex I-integrated CA subunits are discussed.

Carbonic anhydrases are zinc-containing enzymes that catalyse the reversible interconversion of \( \text{CO}_2 \) and \( \text{HCO}_3^- \). In higher plants, carbonic anhydrases have been localized to the chloroplast stroma and to the cytoplasm, in algae additionally to the mitochondrion (reviewed in Moroney et al. 2001 (1)). Chloroplast carbonic anhydrases are important for efficient delivery of \( \text{CO}_2 \) to Rubulose-bisphosphate Carboxylase/Oxygenase (RubisCO). In contrast, the physiological role of carbonic anhydrases of other subcellular compartments is still a matter of debate. Structurally, carbonic anhydrases can be divided into three families termed \( \alpha \), \( \beta \) and \( \gamma \), which evolved independently (2). The chloroplast carbonic anhydrases belong to the \( \beta \)-family. Homologs of carbonic anhydrases from all three families were identified in the course of the Arabidopsis genome sequencing project (1, 3) but so far have not been physiologically characterized.

Recently, γ-type carbonic anhydrases were found to be localized within plant mitochondria attached to complex I of the respiratory chain. This complex was first purified about 10 years ago from plants (4-8, reviewed in Rasmusson et al. 1998 (9)). Many of the characterized subunits were found to be homologous to subunits of complex I from fungi and animals, but some did not exhibit any significant sequence identity, e.g. an “unknown 29 kDa protein” of potato complex I (5). Systematic identifications of complex I proteins in Arabidopsis, rice and Chlamydomonas led to the discovery of up to 5 subunits related to the “unknown 29 kDa protein” within this respiratory complex of these organisms (10, 11, H.P.Braun, unpublished results). Based on sequence comparisons, these subunits were initially proposed to be called “ferrirypochelin-binding proteins”. However, later it became clear that these identifications were based on a falsely annotated database entry (12). Instead, significant sequence identity was discovered to the prototype γ-carbonic anhydrase of the
archaeobacterium Methanosarcina thermophila (12). Computer modelling using the crystal structure of the archaeobacterial γ-carbonic anhydrase revealed that at least three of the five plant-specific complex I subunits of Arabidopsis have a conserved active site (12). They were suggested to be called carbonic anhydrase 1 (CA1) (At1g19580), CA2 (At1g47260) and CA3 (At5g66510) in Arabidopsis. Two further related complex I subunits of Arabidopsis have a less conserved primary structure and are termed carbonic anhydrase-like protein 1 (CAL1) (At5g63510) and CAL2 (At3g48680) (13).

The physiological role of CA2 and CA3 in plant mitochondria was addressed by the use of Arabidopsis knock-out lines (14). Surprisingly, the phenotype of the mutants was not distinguishable from a wild-type line under all conditions tested, which might be a consequence of redundant activities of the five related complex I subunits. However, suspension cell cultures of the knock-out lines had a reduced growth rate. Furthermore, complex I levels were clearly reduced in mutant lines, indicating that the CA subunits are important for complex I assembly. The genes encoding CA1 and CA2 were found to be down-regulated if Arabidopsis plants are grown under elevated CO\(_2\) concentrations, supporting a role of these proteins in mitochondrial one-carbon metabolism. Possibly the CA subunits play important roles in the context of photorespiration, which leads to the liberation of large amounts of CO\(_2\) in plant mitochondria, especially under high light conditions (14).

The location of the carbonic anhydrase subunits within mitochondrial complex I so far is unknown. The CAL1 and CAL2 subunits of Arabidopsis were shown to interact with CA2 using the yeast-two-hybrid system (13). Very recently, the projection structure of Arabidopsis complex I was resolved by electron microscopy and single particle analysis (15). Interestingly, it shows an extra matrix-exposed domain, which is attached to the membrane arm of this complex. Here we present evidence that CA2 forms part of this extra-domain: CA2 can not be detached from mitochondrial membranes by carbonate extraction, was identified by mass spectrometry to form part of the membrane arm of complex I and is shown to be localized on the matrix-exposed side of this arm by protease protection experiments with isolated mitoplasts. Location of the carbonic anhydrase domain of complex I was confirmed by a structural analysis of this complex from Polytomella by electron microscopy and single particle analysis.

**Materials and Methods**

**Cultivation of Arabidopsis and Polytomella**

Arabidopsis cell suspension cultures were established as described by May and Leaver 1993 (16). Cells were cultivated in 500 ml flasks containing 100 ml medium [0.316 % (w/v) B5 medium, 3% (w/v) sucrose, 0.0001% (w/v) 2,4 dichlorophenoxyacetic acid, 0.00001 % (w/v) kinetin, pH 5.75] at 25°C, and 90 rpm in the dark. Cells were transferred into fresh medium all 7 days. Starting material for mitochondrial isolations were approximately 100 g cells.

Polytomella spp. (198.80, E.G. Pringsheim) was obtained from the “Sammlung von Algenkulturen der Universität Göttingen” (SAG) ([http://epsg.uni-goettingen.de/html/sag.html](http://epsg.uni-goettingen.de/html/sag.html)). Cells were cultivated in 2.5 liter culture flasks containing 1200 ml medium [0.2 % (w/v) sodium acetate, 0.1 % (w/v) yeast extract, 0.1 % (w/v) tryptone] for 4-5 days at 25°C in the dark without shaking. Starting material for mitochondrial isolations were approximately 100 g cells.

**Isolation of mitochondria from Polytomella**

Mitochondria from Polytomella were purified as outlined in Dudkina et al. 2005b (17). Organelles were resuspended in Resuspension Buffer (0.4 M Mannitol, 1 mM EGTA, 10 mM Tricine, 0.1 mM PMSF, pH 7.2) at a protein concentration of 10 mg /ml, divided into aliquots à 100µl and stored at –80°C.

**Isolation and subfractionation of mitochondria from Arabidopsis**

Mitochondria from Arabidopsis were purified as outlined in Werhahn et al. 2001 (18). Organelles were resuspended in Resuspension Buffer (0.4 M Mannitol, 1 mM EGTA, 10 mM Tricine, 0.2 mM PMSF, pH 7.2) and either stored at –80°C or directly used for the
generation of mitochondrial subfractions. In the latter case, mitochondria were sedimented by centrifugation for 10 minutes at 12,000 xg and resuspended in Resuspension Buffer without Mannitol at a protein concentration of 10 mg / ml. Mitochondria were broken by sonication with a Ultrasonic cell disruptor (Misonix Inc, NY, USA) by four intervals of 10 seconds. Unbroken mitochondria were sedimented by centrifugation for 7 minutes at 5000 xg and discarded. The supernatant was centrifuged at 150,000 xg for 90 minutes. The resulting pellet contains the mitochondrial membranes while the supernatant contains the soluble proteins of the mitochondrial matrix and the intermembrane space. The pellet was resuspended with Resuspension Buffer at a protein concentration of 10 mg / ml. Membrane and soluble fractions were divided into aliquots and stored at –80°C. The purity of subfractions was analysed by 2D Blue-native PAGE (see below) or by immune-blotting experiments using antibodies directed against marker proteins of the mitochondrial membrane and soluble fractions (see results section).

Carbonate extraction

For carbonate extraction, mitochondrial membranes were resuspended in Carbonate Buffer (0.1 M Na₂CO₃, 0.1 mM PMSF, pH 11.5) at a protein concentration of 10 mg / ml and incubated for 5 minutes at 4°C. Afterwards the membranes were again sedimented by ultracentrifugation for 90 minutes at 150,000 xg. The pellet was finally resuspended in Resuspension Buffer at a protein concentration of 10 mg / ml and divided into aliquots (= membranes with integral membrane proteins); the supernatant was directly divided into aliquots (= peripheral membrane proteins). All aliquots are stored at –80°C.

Protease protection experiments

Mitoplasts (mitochondria lacking the outer membrane) were generated from isolated mitochondria to obtain topological information on inner membrane proteins: first mitochondria were sedimented by centrifugation and resuspended in Swelling Buffer (5 mM potassium phosphate, pH 7.2) at a protein concentration of 5 mg / ml. Subsequently the outer mitochondrial membrane was selectively ruptured by treatment with a Teflon homogenizer (20 strokes). Resulting mitoplasts and outer membrane fragments were next separated by sucrose gradient ultracentrifugation (step gradient of 60%, 32% and 15% sucrose in 1 mM EDTA, 10 mM MOPS, pH 7.2) for 1 h at 92,000 xg. Mitoplasts are enriched at the 32% / 60 % interphase of the gradients. They were purified from sucrose by dilution with Resuspension Buffer, sedimentation by centrifugation for 10 minutes at 12,000 xg and again resolution in Resuspension Buffer at a protein concentration of 10 mg / ml. For protease protection experiments, 250 µl mitoplasts are combined with 10 µl Protease Solution (0.1 % Proteinase K in 10 mM Tris-HCl, pH 7.2) and incubated for 30 minutes on ice. The reaction is stopped by addition of 2.5 µl PMSF Solution (200 mM PMSF). Fractions were either directly analysed by 1D SDS-PAGE and immune-blotting (see below) or stored at –80°C.

Gel electrophoresis procedures and immunoblotting

1D SDS PAGE was carried out according to Schägger et al. 1987 (19), 1D Blue-native PAGE and 2D Blue-native / SDS PAGE according to Heinemeyer et al. 2005 (20) and 2D Blue native / Blue native PAGE as outlined by Sunderhaus et al. 2005 (21). Proteins were either visualized by Coomassie colloidal staining (22, 23), in-gel NADH dehydrogenase activity-staining (24) or blotted onto nitrocellulose filters. Blots were incubated over night with different antibodies directed against the mitochondrial adenine nucleotide translocator (ANT), the mitochondrial superoxide dismutase (SOD) and the C-terminal half of the mitochondrial carbonic anhydrase (CA; At1g47260) from Arabidopsis (14). Visualization of immune-positive bands was performed using biotinylated secondary antibodies, avidin and horseradish peroxidase (Vectastain ABC kit, Vector laboratories, CA, USA). Selected protein complexes were cut out from Coomassie-stained 2D Blue-native / Blue native gels and subunits were identified by mass spectrometry (25).

Purification of complex I from Polytomella

Complex I from Polytomella was purified as outlined in Dudkina et al. 2005a (15) for the corresponding protein complex from Arabidopsis: About 1 mg Polytomella
mitochondria (100 µg mitochondrial protein) was resolved in Digitonin Solubilization Buffer (5.0 % digitonin, 30 mM HEPES, 150 mM potassium acetate, pH 7.4). Solubilized protein complexes were subsequently separated by sucrose gradient ultracentrifugation (gradients of 0.3 – 1.5 M sucrose / 15 mM Tris base, pH 7.0, 20 mM KCl / 0.2 % digitonin) for 20 hours at 150 000 xg. Fractions were removed from the gradient from bottom to top. The protein complex content of the fractions was analysed by 1D Blue-native PAGE and 2D Blue-native / SDS PAGE (see above). Complex I-containing fractions were used for EM analyses.

Electron microscopy and image analysis

Negatively stained specimens were prepared with 2% uranyl acetate on glow-discharged carbon-coated copper grids. Images were recorded with a Gatan 4K slow-scan CCD camera on a Philips CM12 electron microscope. 2000 x 2000 pixel images were recorded at 77,400 x magnification with a pixel size of 15 µm and a binning factor of 2, corresponding to a size of 3.85 Å at the specimen level. Single particle projections were extracted from images and analysed with Groningen image processing (“Grip”) software on a PC cluster. Images were subsequently subjected to multireference alignment, multivariate statistical analysis and hierarchical classification as described before (15). Resolution of averaged single particle 2D projections was measured according to van Heel (26).

Results

The carbonic anhydrase CA2 of Arabidopsis is an integral membrane protein.

Complex I of plants purified by chromatographic or electrophoretic procedures was shown to include carbonic anhydrase subunits. Deletion of the gene encoding CA2 drastically reduces complex I levels in Arabidopsis, indicating an integral position of this protein within this respiratory complex. However, a more loosely binding of the carbonic anhydrase subunits to complex I so far can not be excluded. Therefore, localization of the carbonic anhydrases was immunologically tested using submitochondrial fractions. The following results are based on an antibody directed against the C-terminal half of CA2 (At1g47260), which previously was shown to be mono-specific for the CA2 protein (14).

Mitochondria from Arabidopsis were subfractionated into a membrane (M) and a soluble (S) fraction as described in the Material and Methods section. The purity of the generated fractions was tested by 2D Blue-native PAGE (Figure 1, part A). The respiratory protein complexes, which were identified on the basis of their subunit compositions (27), are exclusively present in the membrane fraction. In contrast the formate dehydrogenase complex, the HSP60 complex and the superoxide dismutase (SOD) of the mitochondrial matrix are absent in this fraction but present in the soluble fraction. The purity of the mitochondrial subfractions was confirmed by 1D SDS PAGE and immune-blotting using antibodies directed against the adenine nucleotide translocator (ANT, marker for the mitochondrial membrane fraction) and the superoxide dismutase (SOD, marker for the soluble mitochondrial fraction) (Figure 1, part B). ANT exclusively was recognized in the membrane fraction, SOD in the soluble fraction. In conclusion, the generated subfractions can be considered to be very pure.

In a parallel immune-blotting experiment, CA2 was only detectable in the membrane fraction. Probing the 12 µg sample of the membrane fraction gave a clear signal on the immune-blot, whereas no signal was observed in the corresponding 100 µg sample of the soluble fraction (Figure 1, part B, right immune blot). This suggests that CA2 is a membrane protein.

In a next step carbonate extraction was employed to test whether CA2 is an integral membrane protein or associated to the surface of the membrane. Mitochondria were isolated from Arabidopsis and subfractionated into a soluble fraction (SP), a fraction containing peripheral membrane proteins (PMP) and a fraction containing integral membrane proteins (IMP) as described in the Materials and Methods section. All three subfractions have a very distinct composition of proteins as monitored by 1D SDS PAGE in combination with Coomassie-staining (Figure 2). Parallel immune-blotting experiments were carried out to monitor the purity of the generated
subfractions. As expected, SOD only is present in the soluble fraction and ANT in the fraction of the integral membrane proteins. Similarly, CA2 was nearly exclusively present in the fraction of the integral membrane proteins. We conclude that CA2 is an integral membrane protein.

The carbonic anhydrase CA2 forms part of the membrane arm of complex I

Complex I has an L-like shape. The “matrix arm”, which is responsible for NADH oxidation, protrudes into the matrix. It is attached to a “membrane arm” responsible for the proton translocation activity of complex I. The location of CA2 with respect to these two arms was investigated by Blue-native / Blue-native PAGE in combination with in-gel ADH-oxidation activity-staining and mass spectrometry. Proteins normally are localized on a diagonal line on gel systems if the same buffer- and detergent conditions are used for both dimensions. However, in an alternative approach of Blue-native / Blue-native PAGE, the first gel dimension is carried out in the presence of digitonin, which is most mild for protein solubilization, and the second gel dimension in the presence of dodecylmaltoside, which is slightly less mild (28). Protein complexes specifically destabilized in the presence of the second detergent are dissected on this 2D gel system into subcomplexes of enhanced electrophoretic mobility, which are visible beneath the diagonal line. Using this gel system, complex I of Arabidopsis (1000 kDa) partially becomes dissected into a 600 and a 400 kDa subcomplex (Figure 3). For unknown reasons, the 400 kDa complex partially gets further dissected into a 380 kDa complex. The 400/380 kDa complexes represent the matrix arm of complex I as shown by in-gel NADH-oxidation activity-staining (Figure 3). We conclude that the 600 kDa complex must represent the membrane arm.

To localize CA2 within complex I, the two 600 and 400 kDa subcomplexes were directly cut out from the 2D Blue-native / Blue-native gel, trypsinated and analysed by liquid-chromatography in combination with tandem mass spectrometry (Table 1). 19 different proteins could be identified forming part of the 600 kDa complex, 16 of which were previously known ((10) and references within) and 3 of which were described for the first time (At1g14450, At4g00585, At1g67350). As expected, several of the subunits forming part of the 600 kDa subcomplex of complex I from Arabidopsis are homologous to complex I subunits from beef and Neurospora known to be present in the membrane arm. Furthermore, 4 of the 5 CA subunits (γCA1, γCA2, γCA3, γCAL2) were identified forming part of the 600 kDa complex. In contrast, no CA subunits were identified within the 400 kDa subcomplex, which includes 4 known proteins of the matrix arm (Table 1). We conclude that the CA subunits of complex I from Arabidopsis are integral membrane proteins, which form part of the membrane arm of this complex. This result was confirmed by immune-blotting experiments using 2D Blue-native / Blue native gels (data not shown).

The topological localization of CA2 within the membrane arm of complex I was addressed by protease protection experiments using isolated mitoplasts (mitochondria lacking the outer mitochondrial membrane). Mitoplasts of Arabidopsis were incubated with Proteinase K as outlined in the Material and Methods section. Subsequently, proteins of protease-treated and untreated mitoplasts were analysed by SDS-PAGE and immune-blotting (Figure 4). As expected, SOD was protease-protected, because it is localized within the mitochondrial matrix. ANT was accessible to protease digestion. As known from the crystal structure of this protein from beef, ANT is composed of six membrane-spanning helices (29). The N- and the C-terminus as well as two internal loops are exposed to the intermembrane space. Therefore, degradation of ANT upon protease-treatment of mitoplasts into several distinct fragments is expected. In contrast, the 30 kDa CA2 proved to be protease-protected. Only one very faint degradation product is visible on the corresponding immune-blot at 28 kDa (Figure 4). We conclude that CA2 is an integral membrane protein of the membrane arm of complex I, which is localized on the matrix side of this arm. Possibly a small part (2 kDa) of the protein protrudes into the intermembrane space.
Polytomella also has the matrix-exposed extra-domain of complex I

Single particle electron microscopy is a suitable technique for a direct structural characterization of CA subunits within complex I. Previously, EM data from Arabidopsis revealed a spherical extra matrix-exposed domain, which is attached to the central part of the membrane arm (15). Most likely the extra domain represents the carbonic anhydrase subunits of complex I. This hypothesis was tested with an alga species. The green algae Chlamydomonas was recently reported to also include homologous CA subunits within complex I (11). It therefore should also have an extra CA domain. This was investigated using purified mitochondria from *Polytomella* spp., a non-green alga of the Chlamydomonaceae closely related to *Chlamydomonas reinhardtii*. Analysis of the mitochondria by 2D Blue-native / SDS PAGE allowed to identify the known respiratory protein complexes of Polytomella (30-32): dimeric complexes III and V, two forms of complex IV and complex I. Complex I is partially dissected into a smaller form which lacks the large subunits of the NADH oxidation domain (complex I* in Figure 5).

For EM analysis of complex I, mitochondrial protein complexes were solubilized using digitonin and separated by sucrose density ultracentrifugation. Complex I containing fractions of the gradient were directly analysed by EM in combination with single particle analysis (Figure 6). A subset of 6,500 side-view projections was analyzed. Four different projection views could be distinguished, which represent the larger and the smaller form of complex I from both sides, respectively (Fig. 6a, b, d, e). In these views the stronger stain-excluding membrane-bound arm with a length of 210 Å runs horizontal and the hydrophilic arm almost vertical. The smaller particle which lacks part of the matrix arm (Fig. 6a,b) is more abundant than the intact particle (Fig. 6d,e). A fifth projection class represents a top view of Polytomella complex I (Fig. 6c). From this perspective, the membrane arm is slightly bent, which previously was reported for the Arabidopsis supercomplex consisting of complex I and dimeric complex III (Fig. 6f; (15)). The best projection maps of Figs. 6b,c have a resolution of about 17 Å.

All the side view projections of Polytomella complex I show the matrix-exposed extra-domain at the centre of the membrane arm of the complex (Fig. 6a-f, white arrows). The side views indicate that this spherical domain has a diameter of about 60 Å in both Polytomella (Fig. 6a,e) and Arabidopsis (Fig. 6g,h). In our hypothesis it contains a heterotrimer of different carbonic anhydrase subunits, as will be discussed below. In order to verify this hypothesis we did an additional single particle electron microscopy study on purified complex I from an Arabidopsis knockout mutant of gene At1g47260, encoding the CA2 subunit. The total sum of 318 projections represents a complex I particle which is very similar or identical to the wild type (Fig. 1, i). Most likely, other structurally related CA subunits of complex I are compensating the absence of CA2.

**Discussion**

The subunit composition of complex I is well known for several organisms (33-35). In eukaryotes, 40 – 45 subunits form part of this complex, many of which have a conserved primary structure in fungi and animals. In contrast, complex I from plants includes quite a number of plant-specific subunits, most strikingly a group of 3-5 structurally related γ-carbonic anhydrases (10, 11). Using a mono-specific antibody directed against CA2, this protein was shown to be an integral membrane protein, which is attached to the membrane arm of complex I on its matrix-side. These results nicely fit to a special structural feature of plant complex I. In contrast to bacteria, fungi and animals, Arabidopsis complex I has an extra spherical matrix-exposed domain attached to the central part of its membrane arm (15). This extra-domain now was confirmed for green algae, which also are known to include the plant-specific carbonic anhydrase subunits (11).

**Topology of carbonic anhydrase subunits of Arabidopsis complex I**

Based on carbonate extraction, CA2 is anchored to the inner mitochondrial membrane by hydrophobic interaction. Protease protection experiments reveal anchoring of the protein by a region close to
one of its termini. Since the N-terminal two-thirds of the protein have clear homology to the hydrophilic carbonic anhydrase domain of the prototype gamma carbonic anhydrase from *Methanosarcina thermophila*, anchoring of CA2 to the membrane most likely is mediated by its C-terminus. Strikingly, compared to the *Methanosarcina thermophila* enzyme, CA1, CA2 and CA3 have a C-terminal extension of 27 to 47 amino acids (Supplementary Figure 1). The 2 kDa segment of CA2 accessible by proteolysis during protease protection experiments probably is represented by this C-terminal extension, which is exposed to the mitochondrial intermembrane space. In contrast, the main part of the 30 kDa CA2 protein is not degraded during protease protection experiments and seems to form a carbonic anhydrase domain of about 25 kDa on the matrix side of the inner membrane.

We speculate that the spherical extra domain of Arabidopsis complex I represents the plant specific carbonic anhydrase subunits of this complex. The extra domain has a diameter of about 6 nm, corresponding to a molecular mass of approximately 75 kDa, which could include three carbonic anhydrase domains. X-ray crystallography of the γ-carbonic anhydrase of the archaon *Methanosarcina thermophila* has revealed a homo-trimeric structure of this enzyme with dimensions of 65 x 65 x 70 Å (36), compatible to the diameter of the extra domain found by EM. The γ-carbonic anhydrase subunits of Arabidopsis have a rather high homology to the γ-carbonic anhydrase of *M. thermophila* and even show immune cross-reactivity to antiserum raised against this γ-CA (12). It therefore is very likely that the complex I-integrated γ-carbonic anhydrase domain of Arabidopsis also forms a trimeric structure. However, information on the precise anchoring of the CA subunits to complex I in plants has to await further structural investigation.

Upon EM analysis, the spherical extra domain is the only visible difference between complex I from plants and other eukaryotes on the matrix-side of the membrane arm of complex I. It therefore is unlikely that the CA subunits are represented by a different region of the membrane arm on its matrix surface. However, EM analysis of complex I of an Arabidopsis knockout mutant of the gene encoding CA2 yielded a complex I particle which is (almost) identical to the wild type (Fig. 6, i). We interpret that absence of one of the five complex I included CA or CAL subunits can be compensated by the remaining proteins. Indeed, cells of the Arabidopsis knockout mutant clearly have reduced levels of complex I, which nevertheless has a normal subunit composition (14). We speculate that the CA subunits form a hetero-trimeric domain and that different CA or CAL subunits can replace each other. The biological reason for the occurrence of five structurally related CA or CAL subunits within Arabidopsis complex I remains to be established.

In summary we conclude that Arabidopsis complex I is composed of three different subcomplexes, the main matrix-exposed domain constituting the NADH oxidation activity, the membrane arm involved in proton translocation across the inner mitochondrial membrane and the spherical extra matrix-domain most likely constituting a γ-type carbonic anhydrase domain (Figure 7).

**Physiological role of carbonic anhydrases in plant mitochondria**

Although the spherical domain is attached to complex I by a very thin stalk, this extra domain is very tightly attached to the respiratory complex and not ripped off by biochemical extractions like carbonate treatment. Interestingly, complex I from Arabidopsis has a cavity directly in opposite of the point of attachment of the spherical domain. Possibly the γ-carbonic anhydrase domain of complex I is physiologically linked to a pore-like structure within complex I involved in proton translocation. In cyanobacteria, CO₂ hydration also takes place at the cytoplasmic face of a homologous NADH dehydrogenase complex and is thought to be linked to proton translocation activity of this complex (reviewed in Badger and Price 2003 (37)). This NADH linked carbonic anhydrase constitutes an important part of a CO₂ concentrating mechanism in cyanobacteria, which is very important for...
photosynthesis. In contrast, plant mitochondria rather have to handle excess of CO₂, which is liberated during the citric acid cycle and other catabolic reactions within these organelles and additionally during photorespiration. Indirect involvement of mitochondrial CAs in photorespiration is supported by the down-regulation of CA genes if plants are cultivated in the presence of elevated CO₂ concentration (14). It will be interesting to further explore the physiological role of the complex I-integrated mitochondrial γ-carbonic anhydrases in higher plants.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (grant Br1829-7/1).
Literature cited

Figure legends:

Figure 1:
Submitochondrial localization of the carbonic anhydrase CA2. Mitochondria were isolated and subfractionated into a membrane and a soluble fraction as described in the Material and Methods section. A: Documentation of the purity of the subfractions by 2D Blue-native / SDS PAGE. T: total mitochondrial protein; M: protein of the membrane fraction; S: protein of the soluble fraction. Identities of membrane-bound protein complexes are given above the gels (I: complex I; II: complex II, III2: dimeric complex III, IV: complex IV; V: ATP synthase complex; I+III2 supercomplex formed by complex I and dimeric complex III) and identities of soluble protein complexes directly on the 2D gels (HSP60: heat stress protein 60, FDH: formate dehydrogenase; SOD: superoxide dismutase). The molecular masses of standard proteins are given to the right (in kDa). B: Immunological localization of carbonic anhydrase. Varying amounts of protein (indicated above the gels in µg) of the soluble (S) and the membrane (M) fraction were resolved by 1D SDS PAGE and Coomassie-stained (Coo) or blotted onto Nitrocellulose. Proteins of interest were detected using antibodies directed against the adenine nucleotide translocase (ANT; membrane marker), the superoxide dismutase (SOD; matrix marker) and carbonic anhydrase (CA). The molecular masses of standard proteins are given to the right of the gels (in kDa).

Figure 2:
Analysis of the anchoring of carbonic anhydrase CA2 within the inner mitochondrial membrane by carbonate extraction. Peripheral and integral membrane proteins were separated by carbonate treatment and subsequent centrifugation as described in the Experimental Procedures section. Two different protein amounts (indicated above the gels in µg) of fractions containing soluble mitochondrial proteins (SP), integral membrane proteins (IMP) and peripheral membrane proteins (PMP) were separated by 1D SDS PAGE and Coomassie-stained (Coo) or blotted onto Nitrocellulose. Proteins of interest were immune-detected using antibodies directed against SOD (superoxide dismutase - matrix marker), ANT (adenine nucleotide translocase - membrane marker) and CA (carbonic anhydrase). The molecular masses of standard proteins are given to the left of the Coomassie-stained gel.

Figure 3:
Partial dissection of complex I from Arabidopsis into two subcomplexes. Total mitochondrial membrane protein of Arabidopsis was resolved by 2D Blue-native / Blue-native PAGE. First dimension Blue-native PAGE was carried out in the presence of digitonin, second dimension Blue-native PAGE in the presence of dodecylmaltoside. A: Coomassie-stained 2D gel, B: complex I activity-stained gel (NADH oxidation). Identities of protein complexes are given on top of the gels and to the left of the Coomassie-stained gel, molecular masses of standard protein complexes are given to the right of the activity-stained gel. Complex I and its subcomplexes of 600 and 400 kDa (arrows) are marked by a box.

Figure 4:
Topographical analysis of carbonic anhydrase CA2 by protease protection experiments. Mitoplasts (mitochondria lacking the outer membrane) were incubated with Proteinase K as described in the Materials and Methods section. Total mitochondrial protein of untreated (-) and treated (+) fractions was resolved by SDS-PAGE and blotted onto Nitrocellulose. Proteins of interest were identified by immune-detection using antibodies directed against ANT (adenine nucleotide translocase – marker for an integral inner membrane protein accessible for protease digestion), SOD (superoxide dismutase – marker for a matrix protein inaccessible for protease digestion) and CA (carbonic anhydrase).

Figure 5:
Two-dimensional resolution of mitochondrial protein complexes from Polytomella. Isolated mitochondria from Polytomella were solubilized with 5% digitonin as described in the Material and Methods section. Proteins were separated by 1D Blue-native PAGE (gel stripe on top) and subunits of the protein complexes by a second gel dimension in the presence of SDS. Molecular masses of standard proteins are given to the right of the gel and identities of protein complexes on top: V$_2$: dimeric ATP synthase; I: complex I; I*: subcomplex of complex I lacking subunits of the NADH oxidizing domain; III$_2$: dimeric complex III; IV: complex IV; IV*: subcomplex of complex IV.

**Figure 6:**
Structure of complex I from Polytomella spp. A data set of 11 000 single particle projections was analysed by multi-reference alignment and multivariate statistical analysis. A total of 6581 projections could be assigned to side views of the larger and smaller form of complex I in two different positions, respectively (a: average of 1449 projections, b: average of 4608 projections, d: average of 512 projections, e: average of 16 projections). 1366 projections represent a top view (c). f – averaged projection map of top-view projections of I-III$_2$ supercomplex particles from *Arabidopsis*; g, h – average of side-view projections of a complete complex I and complex I lacking a part of the NADH-oxidizing domain from *Arabidopsis* (taken from Dudkina et al. 2005 (15), Fig. 2, panels d and e); i – total sum of 318 projections of complex I in a side-view position from an *Arabidopsis* mutant lacking the At1g47260 gene encoding CA2. The plant-specific carbonic anhydrase domain is indicated by an arrow. The bar equals 10 nm.

**Figure 7:**
Domain structure of plant complex I.
Table 1: Identified subunits of the 600 and 400 kDa subcomplexes of complex I

<table>
<thead>
<tr>
<th>Protein complex</th>
<th>Accessions</th>
<th>Proteins</th>
<th>calc MW</th>
<th>MOWSE score</th>
<th>Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>600 kDa</td>
<td>At1g47260</td>
<td>NADH-DH carbonic anhydrase subunit</td>
<td>30.0</td>
<td>391</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>At3g48680</td>
<td>NADH-DH carbonic anhydrase subunit</td>
<td>28.0</td>
<td>153</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>At2g33220</td>
<td>NADH-DH B16.6 subunit (N. crassa)</td>
<td>16.1</td>
<td>124</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>At1g04630</td>
<td>NADH-DH B16.6 subunit (N. crassa)</td>
<td>16.1</td>
<td>16.1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>At2g27730</td>
<td>NADH-DH 16 kDa (S. tuberosum)</td>
<td>11.9</td>
<td>123</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>At5g66510</td>
<td>NADH-DH carbonic anhydrase subunit</td>
<td>27.8</td>
<td>105</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>At2g02050</td>
<td>NADH-DH CI-B18 (beef)</td>
<td>11.7</td>
<td>88</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>At1g14450</td>
<td>unknown protein</td>
<td>8.2</td>
<td>85</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>At1g19580</td>
<td>NADH-DH carbonic anhydrase subunit</td>
<td>30.0</td>
<td>84</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>At4g34700</td>
<td>NADH-DH chain CI-B22 (N. crassa)</td>
<td>13.6</td>
<td>73</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>At4g16450</td>
<td>NADH-DH 20.9K chain (N. crassa)</td>
<td>11.4</td>
<td>73</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>At1g67350</td>
<td>unknown protein</td>
<td>15.2</td>
<td>59</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>At2g31490</td>
<td>NADH-DH subunit (A. thaliana)</td>
<td>8.2</td>
<td>57</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>At3g18410</td>
<td>NADH-DH 14 kD subunit (S. tuberosum),</td>
<td>12.4</td>
<td>56</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>At1g49140</td>
<td>NADH-DH PDSW subunit (beef)</td>
<td>12.5</td>
<td>56</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>At2g47690</td>
<td>NADH-DH 15 KD subunit (beef)</td>
<td>14.0</td>
<td>55</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>AtMg01120</td>
<td>NADH-DH, subunit 1</td>
<td>36.0</td>
<td>49</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>At4g00585</td>
<td>unknown protein</td>
<td>9.8</td>
<td>49</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>At2g42310</td>
<td>NADH-DH subunit (A. thaliana)</td>
<td>12.6</td>
<td>41</td>
<td>2</td>
</tr>
<tr>
<td>400 kDa</td>
<td>At5g37510</td>
<td>NADH-DH, 76K chain precursor protein</td>
<td>81.5</td>
<td>187</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>At2g20360</td>
<td>NADH-DH, 40K chain (N. crassa) = 39 K prot</td>
<td>43.9</td>
<td>104</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>nad7</td>
<td>NADH-DH, subunit 7 = nuoD</td>
<td>44.6</td>
<td>72</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>At5g52840</td>
<td>NADH-DH 22.5 KD subunit (S. tuberosum)</td>
<td>19.2</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13 KD-B subunit (B13) (beef)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Protein complexes were directly excised from the Coomassie-stained BN / BN gel shown in Figure 3
2 Accession numbers corresponding to TAIR (http://www.arabidopsis.org/)
3 Protein identities based on sequence identity to complex I subunits from other organisms as revealed by BLAST searches
4 Calculated molecular mass of the identified precursor proteins (in kDa)
5 MOWSE score from the MASCOT software package (http://www.matrixscience.com/)
6 Number of matching peptides
Fig. 4

SOD_+ CA_+ 46 30 21

ANT SOD CA

- + - + - +

46 30 21
Fig. 5