The carbonic anhydrase domain of plant mitochondrial complex I

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The mitochondrial NADH dehydrogenase complex (complex I) consists of several functional domains which independently arose during evolution. In higher plants, it contains an additional domain which includes proteins resembling gamma-type carbonic anhydrases. The Arabidopsis genome codes for five complex I-integrated gamma-type carbonic anhydrases (γCA1, γCA2, γCA3, γCAL1, γCAL2), but only three copies of this group of proteins form an individual extra domain. Biochemical analyses revealed that the domain is composed of one copy of either γCAL1 or γCAL2 plus two copies of the γCA1/γCA2 proteins. Thus, the carbonic anhydrase domain can have six distinct subunit configurations. Single and double mutants with respect to the γCA/γCAL proteins were employed to genetically dissect the function of the domain. New insights into complex I biology in plants will be reviewed and discussed.

Abbreviations – γCA, gamma-type carbonic anhydrase; γCAL, gamma-type carbonic anhydrase like; CCM, CO_2 concentrating mechanism; EM, electron microscopy; complex I, NADH dehydrogenase complex; NADH, Nicotinamide adenine dinucleotide; OXPHOS, oxidative phosphorylation.

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Introduction

The NADH dehydrogenase complex is the first protein complex of the mitochondrial Oxidative Phosphorylation (OXPHOS) system and the main site of electron insertion into the respiratory chain (Hirst 2013). It is composed of two large and longish domains called "arms": the membrane arm, which mainly is inserted into the inner mitochondrial membrane, and the peripheral arm, which protrudes into the mitochondrial matrix. The domains are put together end-by-end, forming an L-like particle. The structure was first described by electron microscopy (Hofhaus et al. 1991, Weiss et al. 1991) and later by x-ray crystallography (Hunte et al. 2010, Zickermann et al. 2015). The most detailed structure has been resolved for complex I of the bacterium *Thermus thermophilus*, which is only composed of 16 subunits (Baradaran et al. 2013). In contrast, mitochondrial complex I is larger and includes more than 40 subunits (Balsa et al. 2012, Carroll et al. 2006). The structure of *Bos taurus* complex I has been recently resolved by cryo electron microscopy (Vinothkumar et al. 2014).

Ten years ago the first EM images of complex I from plants have been published (Dudkina et al. 2005). It also has an L-like shape but surprisingly turned out to have a second matrix-exposed domain which is attached to the membrane arm at a central position (Fig. 1). Besides complex I from the model plant *Arabidopsis thaliana*, this extra domain meanwhile also has been described for potato, maize and the alga *Polytomella* (Bultema et al. 2009, Peters et al. 2008, Sunderhaus et al. 2006) and is considered to represent a general feature of plant complex I (Braun et al. 2014). Since its discovery it was speculated that this extra domain is composed of additional subunits which do not form part of complex I in animals and fungi. Indeed, proteomic analyses of complex I subunits of plants have revealed a number of plant-specific subunits (Cardol et al. 2004, Heazlewood et al. 2003, Sunderhaus et al. 2006). Forty-nine different types of subunits have meanwhile been described for *Arabidopsis thaliana*, some of which occur in different isoforms (Braun et al. 2014, Peters et al. 2013). Of the extra-subunits in plants, most remarkable is a group of five structurally related proteins which resemble γ-type carbonic anhydrases (Parisi et al. 2004, Perales et al. 2004).

The carbonic anhydrase subunits of complex I in plants

Three of the five gamma-type carbonic anhydrase subunits of *Arabidopsis thaliana* have a largely conserved active site with respect to the archaebacterial gamma-type carbonic anhydrases of *Methanosarcina thermophila* (Ferry 2010, Parisi et al. 2004) and are named γCA1 (AGI code: At1g19580), γCA2 (At1g47260) and γCA3 (At5g66510). The two remaining proteins lack two of the histidines essential for zinc binding and therefore are called γ-carbonic anhydrase like proteins, γCAL1 (At5g63510), and γCAL2 (At3g48680). Amino acid sequence conservation is in the range of 70–75% for the γCAs and even 91% for the two γCALs (Wang et al. 2012). The latter proteins therefore most likely represent isoforms. All five proteins are nuclear encoded and post-translationally transported into mitochondria. The γCA1/γCA2/γCA3 proteins lack cleavable mitochondrial presequences, while the γCAL1/γCAL2 proteins have short presequences of 12 (γCAL1) and 15

(γCAL2) amino acids (Braun et al. 2014). The mature γCA1 and γCA2 proteins have masses of 30 kDa, γCA3 has a mass of 28 kDa and the two γCAL proteins of 25 kDa, respectively. Despite extensive attempts, carbonic anhydrase activity to this day has not been proven for any of the γCA subunits of plant complex I. It has been speculated that carbonic anhydrase activity of the γCA proteins might depend on attachment to native complex I (Braun and Zabaleta 2007, Zabaleta et al. 2012). However, γCA2 overexpressed in *E. coli* has been proven to bind CO_2/bicarbonate (Martin et al. 2009).

All genomes of higher plants code for complex I-integrated carbonic anhydrases. However, genomes of several plants like rice, maize and poplar only comprise one gene encoding γCAL (Meyer 2012), further supporting that the Arabidopsis γCAL1 and γCAL2 proteins represent isoforms. Moreover, some plant genomes only code for two γCA subunits (maize, sorghum), while other code for three (Arabidopsis, rice, poplar) (Meyer 2012). As a consequence, a minimal set of complex I integrated γ-carbonic anhydrase subunits might consist of one γCAL and two γCA proteins.

The carbonic anhydrase domain of plant complex I

Extensive evidence has been presented that the extra matrix-exposed domain of plant complex I indeed is composed of the γCA/γCAL proteins. Looking at EM images of plant complex I, the extra domain has a spherical shape and a diameter of about 60 Å (Dudkina et al. 2005, Peters et al. 2008). This allows estimating a molecular mass of 75 kDa, plus some mass for anchoring the domain within the membrane arm of complex I. The prototype γ-carbonic anhydrase of *Methanosarcina thermophila*, which has been characterized by x-ray crystallography (Iverson et al. 2000), is a spherical homotrimer of very similar dimensions and shape (Peters et al. 2008). Localization of the γCA/γCAL subunits at the matrix-exposed side of the membrane arm was furthermore demonstrated by protease protection experiments (Sunderhaus et al. 2006). Finally, systematic dissection of the membrane arm of isolated complex I from Arabidopsis by treatment with low concentrations of SDS allowed separating an 85 kDa subcomplex from the membrane arm (Klodmann et al. 2010). It has been demonstrated by mass spectrometry that this domain includes the γCA1, γCA2, γCAL1 and γCAL2 proteins (γCA3 was not detected). A smaller version of the membrane arm is detectable upon detachment of the 85 kDa subcomplex which includes all known subunits of the membrane arm except for the γCA/γCAL proteins. It was concluded that detachment of the γCA/γCALs does not destabilize the membrane arm (Klodmann et al. 2010). In summary, experimental data indicate that the extra domain of plant complex I consists of three γCA/γCAL proteins and has a molecular mass of approximately 85 kDa.

Subunit composition of the carbonic anhydrase domain

The genome of Arabidopsis codes for five complex I integrated γCA/γCAL proteins but the carbonic anhydrase domain of complex I only includes three of these proteins. How can this be explained? Careful inspection of 2D Blue native / SDS gels separating complex I dissection products revealed that

the 85 kDa complex includes the γCA1, γCA2, γCAL1 and γCAL2 subunits, but that the latter two proteins are not exactly aligned on a vertical line as required for subunits forming part of the same protein complex (Fig. 2). It therefore was concluded that two ~ 85 kDa complexes closely co-migrate on the 2D gels, both including two copies of the γCA1/γCA2 proteins and additionally *either* γCAL1 *or* γCAL2. These findings are nicely supported by yeast-two hybrid data: It has been found that γCA2 interacts with γCA2, γCAL1 and γCAL2 (Braun et al. 2011, Perales et al. 2004). Interaction of γCAL1 and γCAL2 has not been found by yeast-two-hybrid analyses. Again, no interaction data could be obtained for γCA3.

In summary, location of γCA3 within the γCA domain of complex I remains elusive. Several lines of evidence indicate that γCA3, although detectable by MS in total complex I fractions, is of very low abundance and unclear localization: (i) few γCA3-specific peptides were detected in the course of proteomic analyses of intact complex I (Peters et al. 2013), (ii) γCA3 was not detectable by MS in the 85 kDa domain (Klodmann et al. 2010), and (iii) interaction of γCA3 with any of the other γCA/γCAL proteins was not detected by yeast-two-hybrid screens. We conclude that γCA3 might not be included in the γCA domain (Fig. 3). Furthermore, γCAL1 and γCAL2 do not simultaneously form part of individual complex I particles, but alternatively occur within two distinct 85 kDa subcomplexes. Finally, two copies of the γCA proteins form part of the 85 kDa subcomplexes. Since yeast-two-hybrid data indicate that γCA2 also can interact with γCA2, we speculate that the γCA pair can be either homo- or heteromeric (γCA1+γCA2, or γCA1+γCA1, or γCA2+γCA2). However, further experiments have to be carried out to prove that all three possible CA1/CA2 pairs indeed occur. Taken together, available data are compatible with occurrence of up to six subunit configurations for the gamma carbonic anhydrase domain as illustrated in Fig. 4.

Function of the carbonic anhydrase domain

Single and double knock-out mutants with respect to the γCA/γCAL proteins were employed to genetically dissect the function of the γCA domain in *Arabidopsis thaliana*. Plants deficient in γ*CA*2 had no visible phenotype at the conditions tested (Perales et al. 2005). However, a suspension cell culture of γ*ca*2-deficient plants had reduced growth and oxygen-uptake rates. Analyses of the respiratory chain by 2D Blue native / SDS PAGE revealed drastic reduction in complex I amount and activity (Perales et al. 2005). However, single particle electron microscopy showed that complex I particles from mutant cells had a normal shape and included the characteristic γCA domain (Sunderhaus et al. 2006). It was concluded that γCA2 could be replaced by other γCA/γCAL proteins, most likely γCA1. In contrast to plants deficient in γ*CA*2, deletion of γ*CA*3 had less influence on complex I amount (Perales et al. 2005) further supporting a more cryptic role of this member of the γCA/γCAL protein family. Meanwhile, knock out mutants for all five γ*CA*/γ*CAL* genes have been analyzed in Arabidopsis. They all lack visible phenotypes (Wang et al. 2012). It was concluded that reciprocal substitutions can compensate depletion of single γCA/γCAL proteins. However, complex I

amount and activity was reduced in the mutants indicating that the γCA/γCAL subunits are required for complex I assembly or stability. Nevertheless, they should not be considered representing assembly factors since they clearly form part of mature complex I, constituting the very significant extra domain. A role of the γCA/γCAL subunits in early stages of complex I assembly has been demonstrated by analysis of complex I subcomplexes in Arabidopsis mutant lines (Meyer et al. 2011) and by ^{15}N-labeling experiments (Li et al. 2013). Interestingly, genomes of some protists also code for the complex I-integrated γCA/γCAL proteins (Gawryluk and Gray 2010), possibly indicating their involvement in ancient complex I assembly processes which became lost during evolution in animal and fungal lineages.

Beside their importance for complex I assembly, the structural significance of the γCA domain clearly points to another specific role of the γCA/γCAL protein family. Even though carbonic anhydrase activity could not be demonstrated up to date, active site conservation with respect to the prototype γCA from *Methanosarcina thermophila* much supports a role of these proteins in mitochondrial CO_2–bicarbonate conversion. Alternatively, since γCA proteins form part of a larger protein superfamily which also includes acetyltransferases (Parisi et al. 2004), a role in mitochondrial protein acetylation has been proposed but also could not be experimentally demonstrated (Wang et al. 2012). Finally, sequence similarity of the γCA/γCAL proteins to the M-subunit of the cyanobacterial "carbon concentration mechanism" (Ccm) machinery has been recognized (Parisi et al. 2004). Some of the CcmM proteins exhibit carbonic anhydrase activity, but others do not (Peña et al. 2010). The CcmM protein is important for efficient carbon fixation in the carboxysomes of cyanobacteria (Rae et al. 2013).

In analogy to the cyanobacterial carbon concentration mechanism it has been proposed that complex I integrated γCAs in plants participate in an inner-cellular carbon transfer mechanism that allows recycling of mitochondrial CO_2 released by matrix-localized catabolic processes (including glycine–serine conversion during photorespiration) for carbon fixation in chloroplasts (Braun and Zabaleta 2007, Zabaleta et al. 2012). This hypothesis is supported by some experimental evidence (summarized in Fromm et al. 2016): (i) When photosynthetic rates were compared between protoplasts and isolated chloroplasts, the protoplasts performed better at low CO_2 suggesting that an internal CO_2 source was available in protoplasts, but not in isolated chloroplasts (Riazunnisa et al. 2006). Higher photosynthetic rates in protoplasts were not detectable in the presence of inhibitors of carbonic anhydrases. (ii) Transcription of genes encoding the γCA/γCAL proteins is reduced if plants are cultivated at high CO_2 (Perales et al. 2005). (iii) The tobacco "CMSII" mutant, which has drastically reduced amounts of complex I, exhibits diminished steady-state photosynthesis. Inhibition of photosynthesis was reduced if plants were cultivated at conditions diminishing photorespiration (Dutilleul et al. 2003). However, the above listed experimental results also could be interpreted representing general effects of complex I dysfunction on photorespiration. Altered mitochondrial NAD^+/NADH rations caused by complex I depletion might directly affect GDC function (Bykova et

al. 2014).

Characterization of double mutants for investigating γCA/γCAL function

Until now, conclusive evidence for involvement of the γCA/γCAL proteins in recycling mitochondrial CO_2 for carbon fixation by photosynthesis so far has not been presented. Very recently, double mutants with respect to the γCA/γCAL proteins have been analyzed for investigating the function of the γCA domain in Arabidopsis (Fromm et al. 2016, Soto et al. 2015, Wang et al. 2012). Overall, four different double mutants have been characterized so far:

(i) Δγ*ca*1/Δγ*ca*3 (Wang et al. 2012). Genes encoding γCA1 and γCA3 are deleted in this Arabidopsis mutant line. Plants do not show a visible phenotype under the conditions tested and are not distinguishable form a γ*ca*1 single mutant. The biological role of γCA3 therefore has to be further studied.

(ii) Δγ*cal*1/γ*cal*2i (Wang et al. 2012). Since deletion of either γ*CAL*1 or γ*CAL*2 did not reveal any phenotypic alterations, mutant lines were crossed to generate γ*cal* double mutants. However, resulting seeds turned out to arrest in development. To obtain viable plants, the γ*CAL*2 gene was down-regulated by RNAi in the background of a homozygous γ*CAL*1 knockout (Fig. 5). Δγ*cal*1/γ*cal*2i plants showed delayed germination and significantly postponed development. In the light, Δγ*cal*1/γ*cal*2i plants developed a short hypocotyl phenotype. The gene encoding chalcone synthase, a key enzyme of anthocyanin synthesis, was induced in the mutant lines. It was concluded that γCAL1 and γCAL2 play important roles in light-dependent growth and development in Arabidopsis (Wang et al. 2012). Meanwhile, Δγ*cal*1/γ*cal*2i plants have been characterized with respect to the mitochondrial compartment (Fromm et al. 2016). Complex I amount was reduced by 90–95% and oxygen consumption of isolated mitochondria is much diminished. Comparative proteome analyses revealed several changes in the mutant which not only refer to complex I subunits but also point to specific alterations of central mitochondrial metabolism, e.g. pyruvate, glutamate and stress metabolism. However, it still is difficult to dissect molecular consequences caused by complex I depletion on the one side and diminishment of γCAL proteins on the other.

(iii) Δγ*ca*2/Δγ*cal*1 and Δγ*ca*2/Δγ*cal*2 (Soto et al. 2015). These double mutants lack the γ*CA*2 gene and additionally either γ*CAL*1 or γ*CAL*2. This only allows for assembly of one out of six possible subunit configurations of the γCA domain (Fig. 5). Both mutant lines are clearly delayed in growth and development. Interestingly, this effect is reverted if plants are cultivated in the presence of high CO_2, indicating that γCA function might be especially relevant in the presence of photorespiration. Reduction of complex I (about 80%) is similar to the degree of complex I reduction in γ*ca*2 single mutants. However, the γ*ca*2 mutant did not exhibit a growth phenotype. The double mutants also exhibit altered glycine metabolism. It is concluded that γCA/γCAL function is important in the context of photorespiration, but the precise role of the γCA/γCAL proteins still remains elusive.

Outlook

For future research, precise differentiation between general complex I function and specific function of the γCA/γCAL subunits will be of great importance. Very recently, a Δγ*ca*1/Δγ*ca*2 mutant has been generated (Fromm, Braun and Peterhänsel, unpublished results and Córdoba, Soto and Zabaleta, unpublished data). This mutant, which lacks both main γCA proteins of the γCA domain (presence of γCA3 within this domain is questionable) is severely impaired in development. Plants can be rescued but development is extremely delayed. As predicted (Fig. 5), complex I is completely absent. This double mutant was genetically transformed with γ*CA* genes encoding modified γCA proteins which allow reconstitution of complex I assembly, but lack their specific enzymatic function. Analysis of resulting plant lines might help to finally uncover the significance of the γCA domain of plant complex I.

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Figure legends

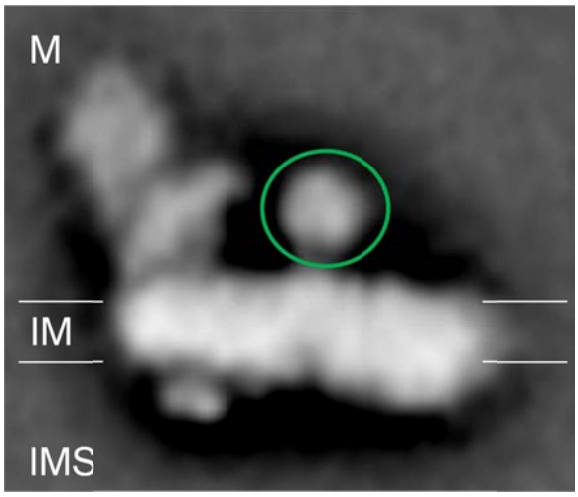

Fig. 1. Structure of mitochondrial complex I from *Arabidopsis thaliana* as revealed by single particle electron microscopy. The extra domain, which is absent in complex I from animals and fungi, is indicated by a green circle. From: Sunderhaus et al. 2006, modified.

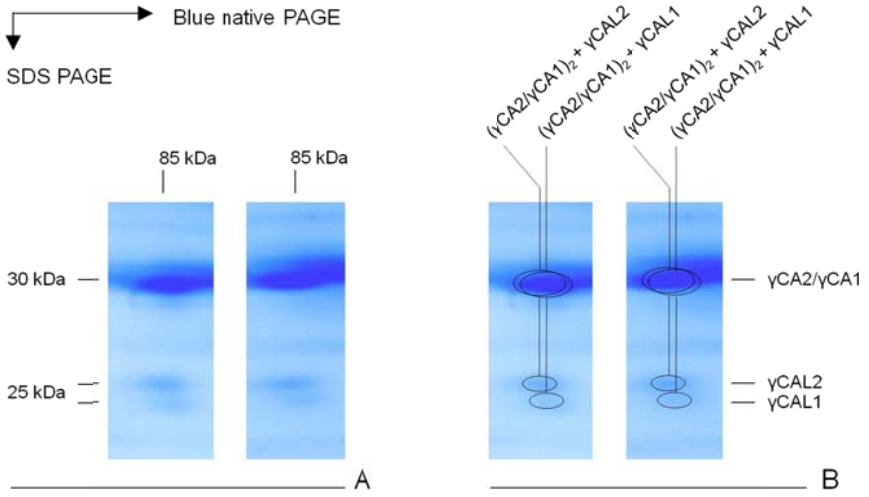

Fig. 2. Subunit composition of the carbonic anhydrase domain. Dissection products of complex I were analyzed by 2D Blue native / SDS PAGE (Klodmann et al. 2010; dissection by pre-treatment of isolated complex I with low concentrations of SDS). Only small areas of the 2D gels are shown (native [horizontal] gel dimension: ~ 75–100 kDa, SDS [vertical] gel dimension: ~ 20–40 kDa). A: results of two different experiments (Coomassie-stained 2D gels). B: same gels including identified proteins (Klodmann et al. 2010; protein identifications by mass spectrometry). An interpretation with respect to the subunit composition of the carbonic anhydrase domain is given above the gels in B.

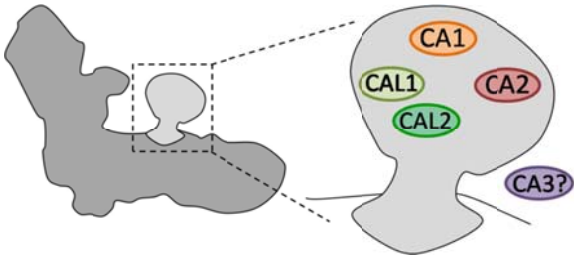

Fig. 3. Scheme of the subunit composition of the carbonic anhydrase domain of complex I in *Arabidopsis thaliana.* CAL subunits are given in light or dark green, CA subunits in orange red and purple. The CA3 protein was not found to be part of the carbonic anhydrase domain.

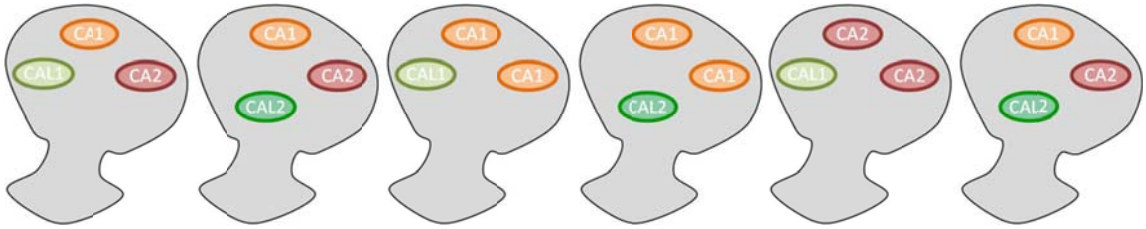

Fig. 4. The six subunit arrangements of the carbonic anhydrase domain.

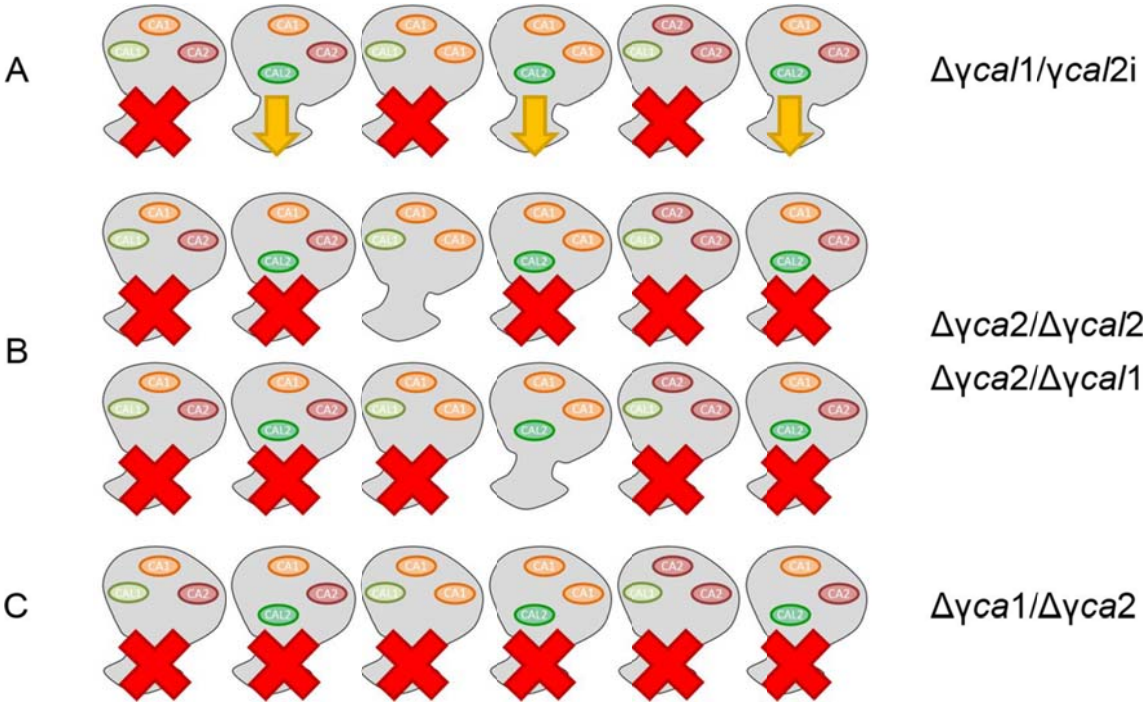

Fig. 5. Consequences of mutations with respect to the γCA/γCAL proteins on the six possible subunit arrangements of the carbonic anhydrase domain (Fig. 4). The following mutants were generated: A) RNAi-mediated depletion of γ*CAL*2 in the background of a γ*cal*1 knock-out (Δγ*cal*1/γ*cal*2i), B)

γ*ca*2/γ*cal*1 and γ*ca*2/γ*cal*2 knock outs (Soto et al. 2015). C) a γ*ca*1/γ*ca*2 double knock out (Fromm, Braun and Peterhänsel, unpublished results). Red cross: complex I assembly not possible anymore; yellow arrow: complex I assembly depleted.