Special Features of Cellular Respiration in *Viscum album*

Besonderheiten der Zellatmung von *Viscum album*

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Summary

Background: Cellular respiration depends on the enzymes of the mitochondrial respiratory chain, particularly on the so-called complexes I–IV. Together, these protein complexes catalyze the transfer of electrons from reduced organic compounds onto molecular oxygen (which is reduced to water). The NADH dehydrogenase complex (complex I) is of special importance because it is the main site for electron insertion into the respiratory chain. Complex I deficiencies cause drastic complications in humans, animals, fungi and plants. Recent investigations in European mistletoe (*Viscum album*) surprisingly revealed that this species lacks mitochondrial complex I. This is the very first example of a multicellular organism that naturally can exist despite a major truncation of the respiratory chain. How is this compatible with cellular life? Indeed, intactness of the mitochondrial respiratory chain is of prime importance for the efficient generation of adenosine triphosphate (ATP) which drives many cellular processes. How is energy metabolism maintained in *V. album*?

Methods: A procedure was developed for purifying mitochondria from *V. album* leaves, which is based on differential centrifugation and density gradient centrifugation. Membrane bound proteins and protein complexes are solubilized by a mild non-ionic detergent. Mitochondrial proteins and protein complexes are finally separated by native gel electrophoresis and identified by mass spectrometry.

Results and conclusions: The respiratory chain of *V. album* is rearranged in a very sophisticated way. The complexes III and IV form a stable respiratory supercomplex and numerous so-called alternative oxidoreductases occur. As a consequence, the respiratory chain maintains a basic but reduced functionality. Furthermore, other subcellular compartments seem to compensate for

reduced ATP formation by the mitochondria. In conclusion, energy metabolism in *V. album* follows unique routes, which may contribute to the extraordinary biochemical properties of this species.
Keywords: *Viscum album*, cell respiration, mitochondria, respiratory chain, ATP formation

Zusammenfassung
Hintergrund: Die mitochondriale Atmungskette, insbesondere die an ihr beteiligten Enzymkomplexe I–IV, werden als unbedingte Voraussetzung für die Zellatmung angesehen. In ihrer Gesamtheit katalysieren diese Proteinkomplexe den Elektronentransport von organischen Verbindungen auf molekularen Sauerstoff (der dadurch zu Wasser reduziert wird). Der NADH Dehydrogenase Komplex (Komplex I) ist dabei als besonders wichtig anzusehen, da er als Haupteintrittsstelle für Elektronen in die Atmungskette fungiert. Defekte innerhalb dieses Proteinkomplexes haben bekanntermaßen gravierende Auswirkungen in Menschen, Tieren, Pilzen und Pflanzen. Überraschenderweise haben jüngste Untersuchungen an der Weißbeerigen Mistel (*Viscum album*) ergeben, dass der mitochondriale Komplex I in dieser Pflanze fehlt. Dabei handelt es sich um das erste berichtete Beispiel überhaupt, dass ein mehrzelliger Organismus natürlicherweise ohne Komplex I auskommen kann. Bisher ist unklar, wie die Zellen der Weißbeerigen Mistel dennoch lebensfähig sind, da die mitochondriale Atmungskette eine Voraussetzung für die Bildung des Adenosintriphosphats (ATP) in den Mitochondrien ist. Fast alle Lebensprozesse werden direkt oder indirekt durch ATP angetrieben. Wie kann der Energiestoffwechsel in *Viscum album* dennoch funktionieren?
Methoden: Mitochondrien aus Blättern der Weißbeerigen Mistel wurden mithilfe einer differentiellen Zentrifugation und einer Dichtegradientenzentrifugation aufgereinigt. Die Proteinkomplexe der Mitochondrien wurden nachfolgend durch Behandlung mit einem milden Detergens aus den mitochondrialen Membranen herausgelöst. Schließlich wurden die mitochondrialen Proteine und Proteinkomplexe mittels einer Blau-nativen Gelelektrophorese aufgetrennt und massenspektrometrisch analysiert.
Ergebnisse und Schlussfolgerungen: Die Atmungskette der Weißbeerigen Mistel ist in ungewöhnlicher Weise umgestaltet. Die Komplexe III und IV bilden einen stabilen Superkomplex aus. Ferner kommen zahlreiche sogenannte alternative Oxidoreduktasen vor. Auf diese Weise wird eine zwar verminderte, aber insgesamt ausreichende Funktionalität der Atmungskette gewährleistet.

Darüber hinaus sind möglicherweise andere subzelluläre Kompartimente daran beteiligt, die verringerte ATP-Bildung der Mitochondrien zu kompensieren. Die Atmungskette der Weißbeerigen Mistel weist somit außergewöhnliche Merkmale auf. Diese Einblicke erweitern unser Wissen um die biochemischen Besonderheiten der Weißbeerigen Mistel um ein neues Kapitel.
Schlüsselwörter: *Viscum album*, Zellatmung, Mitochondrien, Atmungskette, ATP Biosynthese

Introduction

Plants are photoautotrophic organisms. Photosynthesis, the formation of energy-rich organic compounds from simple inorganic compounds driven by light energy, is in the very center of the plant energy metabolism. In its quantitative most relevant mode, carbon dioxide and water are converted into carbohydrates in a process that is linked to the liberation of oxygen. However, besides photosynthesis, plant cells also carry out cellular respiration, the oxidation of organic compounds, which is coupled to the formation of adenosine triphosphate (ATP). Main products of cellular respiration are carbon dioxide and water. On a global scale, about 50 % of the atmospheric carbon fixed by photosynthesis is directly re-liberated into the atmosphere by cellular respiration. Photosynthesis takes place in the chloroplasts and cellular respiration in the mitochondria. Mitochondria and chloroplasts tightly interact. Indeed, the processes of photosynthesis and cellular respiration are metabolically linked in plant cells on several levels (Braun 2020).

Cellular respiration is a central process in nearly all eukaryotic cells. On a molecular scale, it is based on three steps: (i) import of organic compounds into the mitochondria and their oxidation, e.g. by the enzymes of the citric acid cycle. Many of the occurring reactions are coupled to the formation of "reducing equivalents", e.g. nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). (ii) Re-oxidation of the reducing equivalents by the enzymes of the respiratory electron transfer chain (ETC). These enzymes are located in the inner mito-

chondrial membrane, which, in contrast to the outer mitochondrial membrane, forms invaginations called "cristae"; electrons are finally transported by the ETC onto molecular oxygen (O_2), which is converted into water. Respiratory electron transport, which is an exergonic process, is linked to formation of a proton gradient across the inner mitochondrial membrane. (iii) In the last step, this proton gradient drives the formation of ATP from adenosine diphosphate (ADP) and phosphate. This reaction is catalyzed by the ATP synthase complex, which also is located in the inner mitochondrial membrane. The whole process is called "Oxidative Phosphorylation" (OXPHOS), because formation of ATP by phosphorylation of ADP is coupled to the consumption of oxygen. The enzymes of the ETC and the ATP synthase complex altogether are called the OXPHOS system.

The OXPHOS system is likewise present in the mitochondria of nearly all eukaryotes. It consists of the four enzyme complexes of the ETC (the complexes I to IV) and the ATP synthase complex (complex V). Complex I is a NADH-ubiquinone oxidoreductase. It is the main site of electron insertion into the ETC and much contributes to the formation of the proton gradient across the inner mitochondrial membrane. Complex II is a $FADH_2$-ubiquinone oxidoreductase, which does not contribute to the proton gradient. Complex III, the cytochrome c reductase, transfers electrons from ubiquinol (the reduced form of ubiquinone) onto a small protein called cytochrome c. Finally, complex IV, the cytochrome c oxidase, transfers electrons from cytochrome c onto molecular oxygen. Electron transport by the latter two protein complexes also contributes to the proton gradient across the inner mitochondrial membrane.

The OXPHOS system is highly conserved in animals, fungi and plants, which reflects its outstanding importance for cellular energy metabolism. Indeed, no multicellular species has ever been reported to lack any of the five protein complexes of the mitochondrial OXPHOS system. However, recent genetic findings indicated that *Viscum album* might be an exception (Petersen et al. 2015, Skippington et al. 2015, Skippington et al. 2017). *V. album* has a very remarkable life cycle. It is an obligatory hemiparasitic flowering plant that grows on branches of various trees. It is supplied with water, minerals and organic compounds by its host but at

the same time can synthesize energy-rich compounds by its own photosynthesis. However, the energy metabolism of *V. album* is largely unknown so far.

What is the nature of the genetic findings pointing to an unusual cellular respiration of *V. album*? According to the endosymbiont theory on mitochondrial origin, mitochondria descend from free-living bacteria. One key proof for this theory is the presence of a genome in the mitochondria of present-day cells. Mitochondrial genomes have features resembling bacterial genomes. However, during evolution, the mitochondrial genomes became very much reduced. Today, only a few mitochondrial proteins are encoded by the mitochondrial genome, whereas most proteins are encoded by the genome of the cell nucleus, synthesized on cytoplasmic ribosomes and afterwards transported into the mitochondria. Several of the genes still present on the mitochondrial genome encode protein subunits of the complexes I to V of the OXPHOS system.

Surprisingly, it has been discovered that some genes encoding OXPHOS subunits are lacking on the mitochondrial genome of *Viscum* species (Petersen et al. 2015, Skippington et al. 2015, Skippington et al. 2017). Particularly, genes encoding subunits of mitochondrial complex I are absent. It never has been reported before that a multicellular species lacks the genes encoding complex I-subunits in the mitochondrial genome. How can this finding be interpreted? Three hypotheses have been discussed: (i) the complex I genes might have been overlooked in the mitochondrial genome due to sequence divergence. Indeed, it has been found that the mutation rate of the mitochondrial genome is exceptionally high in *Viscum* (Skippington et al. 2015). (ii) The genes encoding complex I subunits have been transferred to the nuclear genome. This has occurred for numerous other mitochondrial genes during evolution. However, it has not occurred for a set of complex I genes, which encode especially hydrophobic subunits, in any multicellular species investigated so far. (iii) *V. album* has no complex I. This hypothesis seemed to be rather unlikely, because, as mentioned above, complex I is the main site for electron insertion into the respiratory chain.

We here investigated cellular respiration in *V. album*.

Materials and Methods

Isolation of mitochondria from *V. album* turned out to be challenging; it did not work using standard protocols for purifying mitochondria from plants. Particularly, various viscous compounds, which not only occur in the berries, but also in stems and leaves, formed aggregates, which co-sedimented with organelles upon centrifugation. These had to be removed between the different centrifugation steps. A procedure for purifying mitochondria from *V. album* leaves is given in Senkler et al. 2018 (detailed information on all methods is provided at https://www.cell.com/current-biology/fulltext/S0960-9822(18)30379-8).

Results

Transmission electron microscopy of ultra-thin leaf sections was employed to obtain insights into shape and ultrastructure of *V. album* mitochondria. The leaf cells include numerous mitochondria, which have a rounded shape (Fig. 1). Compared to other plants, invaginations of the inner mitochondrial membrane are less pronounced. *V. album* mitochondria include ribosomes, indicating that protein biosynthesis can take place inside these organelles.

Next, the composition of the OXPHOS system was analyzed (Senkler et al. 2018). Mitochondrial membranes from *V. album* leaves were carefully solubilized using the non-ionic detergent digitonin. For reference, analyses were carried out in parallel for the model plant *Arabidopsis thaliana*. The OXPHOS system of *A. thaliana* is well defined (reviewed in Braun 2020). Mitochondrial proteins and protein complexes of both species were subsequently separated by Blue native polyacrylamide gel electrophoresis (BN PAGE). BN PAGE allows the separation of proteins under native conditions; protein complexes remain intact. The molecular masses of the OXPHOS complexes differed considerably between *A. thaliana* and *V. album*. Therefore, second gel dimensions were carried out under non-native conditions for identifying the separated protein complexes based on their subunit compositions.

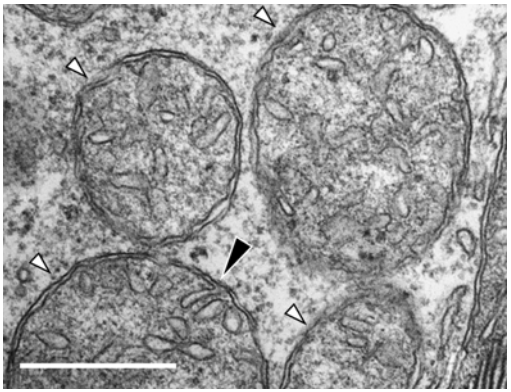

Fig. 1: Transmission electron microscopy (TEM) image of part of a *V. album* leaf cell. White arrowheads, mitochondria; black arrowhead, invagination of the inner mitochondrial membrane. The scale bar corresponds to 0.5 µm. Image taken from Senkler et al. 2018, modified.

The following insights were obtained: In contrast to the OXPHOS system of *A. thaliana*, which consists of the complexes I to V, the OXPHOS system of *V. album* is reduced. On the two-dimensional (2D) gels, only the complexes III and IV are visible. Interestingly, they form a very stable supercomplex, which is not observed in *A. thaliana*. The complexes I, II, V are not visible on the 2D gels. In addition, a supercomplex composed of the complexes I and III, which is present in the mitochondria of *A. thaliana*, is absent in *V. album*. To search for protein complexes of low abundance, proteins visible on the 2D gels were systematically analyzed by mass spectrometry. Based on this experimental approach, subunits of the complexes II, III, IV and V were identified in the mitochondrial fraction of *V. album*. However, the complexes II and V are of comparatively low abundance. No traces of complex I subunits could be detected. To exclude that complex I still has been overseen, a highly sensitive NADH dehydrogenase *in-gel* activity assay has been carried out. In *A. thaliana*, this assay revealed a strong complex I signal. In contrast, no traces of complex I activity could be detected in *V. album*. We conclude that complex I indeed is absent in the mitochondria of *V. album*.

How can cellular respiration function in *V. album* in the absence of complex I? To further investigate mitochondrial functions in *V. album*, protein fractions were analyzed by shot-gun proteome analyses using liquid chromatography coupled to quantitative mass spectrometry (Senkler et al. 2018). More than 400 proteins could be identified. The results gave insights into central mitochondrial metabolism. All enzymes of the citric

acid cycle were identified. Furthermore, so-called alternative respiratory oxidoreductases were identified, like the alternative oxidase (AOX) and alternative NAD(P)H dehydrogenases. These enzymes are part of the ETC system in plants. However, in contrast to the "classical" ETC enzymes, these enzymes do not contribute to the proton gradient across the inner mitochondrial membrane. The alternative oxidoreductases are especially abundant in *V. album*. In the absence of complex I, electron insertion into the ETC therefore can be mediated by alternative NAD(P)H dehydrogenases and complex II. However, based on this OXPHOS mode, ATP formation should be significantly reduced, which was confirmed by oxygen-uptake measurements using isolated *V. album* mitochondria.

In summary, the OXPHOS system of *V. album* is reduced because it surprisingly lacks mitochondrial complex I (Fig. 2).

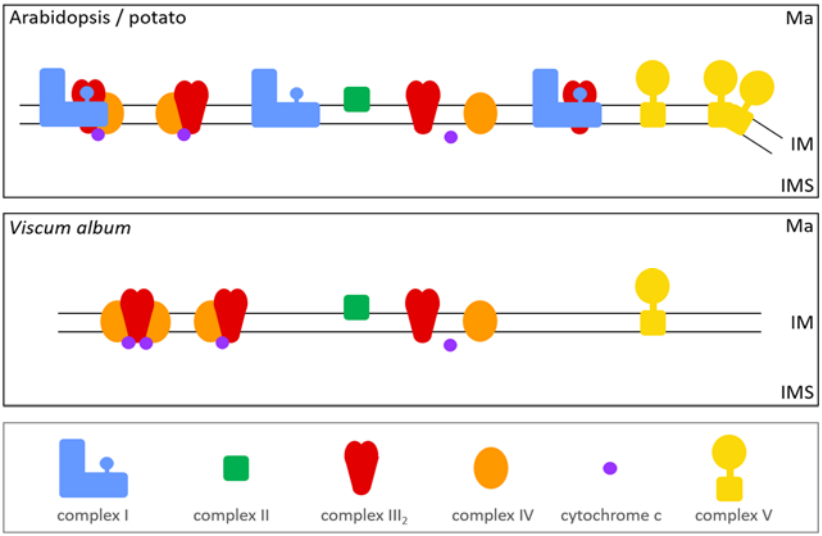

Fig. 2: The protein complexes and protein supercomplexes of the Oxidative Phosphorylation system in the model plants Arabidopsis thaliana and potato (top) and Viscum album (bottom). The identities of the complexes are given below the two boxes. Ma, mitochondrial matrix; IM, inner mitochondrial membrane; IMS, mitochondrial intermembrane space. Alternative oxidoreductases are also part of the OXPHOS system in all three species but are omitted from the figure.

This is the first example of a multicellular species that can live in the absence of this prominent OXPHOS complex. Still, OXPHOS is functional in *V. album*, which is based on several rearrangements of the OXPHOS system: The complexes III and IV form a remarkably stable supercomplex, which should allow especially efficient electron transport between these two protein complexes. Furthermore, alternative oxidoreductases are abundant in *V. album*. Finally, cristae formation is reduced in *V. album*. This correlates with comparatively low abundance of the ATP synthase complex (complex V), which is known to form dimers that contribute to bending the inner mitochondrial membrane and thereby induce cristae formation. Indeed, ATP synthase dimers were not observed in *V. album* (Senkler et al. 2018).

Discussion

V. album has a very special life cycle. It differs in many respects from the life cycles of other flowering plants. Also, on the molecular level, *V. album* has remarkable features. Its mitochondrial OXPHOS system is rearranged. Complex I is not synthesized in *V. album*, which saves a significant amount of energy, since its biosynthesis is expensive. It is by far the largest protein complex of the ETC. About 50 subunits form part of this protein complex, which are encoded by nuclear and mitochondrial genes in all other multicellular species. However, absence of complex I comes with a price: mitochondrial ATP synthesis is less efficient in *V. album*. How can *V. album* survive with less mitochondrial ATP? This currently is not known. Results of Senkler et al. (2018) have been nicely confirmed and complemented by another study (Maclean et al. 2018) and commented by Busch (2018) and Da Fonseca-Pereira et al. (2018). *V. album* might need less ATP, because it has a comparatively slow growth rate and reduced sink organs. Furthermore, energy-rich compounds might be provided by the host tree. However, absence of complex I has not been found in any other parasitic or hemi-parasitic plant so far (Petersen et al. 2020). Finally, *V. album* might compensate reduced mitochondrial ATP formation by increased formation of ATP in other cellular compartments.

Indeed, glycolysis was found to be enhanced in *V. album* (Maclean et al. 2018). Furthermore, the chloroplast ATP synthase complex is very abundant in *V. album* (Senkler et al. 2018; see also recent results by Schröder et al. 2020, this volume). The energy biology of V. album might hold further surprises and should be further investigated.

Conflict of Interest

We hereby confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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