Role of cryo-ET in membrane bioenergetics research

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Abstract
To truly understand bioenergetic processes such as ATP synthesis, membrane-bound substrate transport or flagellar rotation, systems need to be analysed in a cellular context. Cryo-ET (cryo-electron tomography) is an essential part of this process, as it is currently the only technique which can directly determine the spatial organization of proteins at the level of both the cell and the individual protein complexes. The need to assess bioenergetic processes at a cellular level is becoming more and more apparent with the increasing interest in mitochondrial diseases. In recent years, cryo-ET has contributed significantly to our understanding of the molecular organization of mitochondria and chloroplasts. The present mini-review first describes the technique of cryo-ET and then discusses its role in membrane bioenergetics specifically in chloroplasts and mitochondrial research.

Introduction
Membrane bioenergetics is the study of energy-converting processes involving biological membranes, including ATP production, active transport of substrates and flagellar rotation. Key to these processes is the use of electrochemical gradients to drive energy-demanding reactions. The sources of these gradients are often distinct from the consumer and their effect on reaction rates is a fundamental question in bioenergetics research.

ATP is the universal energy currency of all living organisms. Its synthesis is catalysed primarily by the membrane-bound enzyme F1Fo-ATP synthase. In eukaryotes, this enzyme is located in the inner membrane of mitochondria and in the thylakoid membranes of plant chloroplasts. The F1Fo-ATP synthase of these organelles uses the energy stored in an electrochemical gradient of protons to power the conversion of ADP and Pi into ATP by rotary catalysis [1]. The proton gradients are formed by either oxidative phosphorylation (mitochondria) or photosynthesis (chloroplasts) using proteins distinct from the F1F0-ATP synthase. These proteins either pump protons across membranes while transferring electrons along a redox pathway (NADH dehydrogenase, cytochrome c reductase and cytochrome c oxidase in mitochondria, and cytochrome b6f in chloroplasts) or generate protons by catalysing the oxidation of water [PSII (Photosystem II) in chloroplasts] in ATP synthase (Figure 1).

As early as the 1970s with the advent of freeze–fracture, low-angle shadowing techniques in EM, it became evident that the protein complexes involved in ATP synthesis by photosynthesis were spatially separated [2,3]. Membrane fracture planes that showed both stacked and unstacked thylakoid membranes of chloroplasts revealed a non-random distribution of protein complexes. Proteins on the unstacked membrane surfaces were larger and more dispersed than those on the stacked membranes. Through a combination of immunolabelling and biochemical methods, it was concluded that PSII was located primarily on the stacked membranes, whereas PSI (Photosystem I), along with ATP synthase, were located on the unstacked membranes [4,5].

Similar experiments to determine the distribution of proteins involved in ATP synthesis by oxidative phosphorylation were not reported until 1989 when Allen et al. [6] used the technique of freeze–fracture deep-etch EM to analyse protein distribution in mitochondria from the protist Paramecium multimicronucleatum. Here two rows of interdigitating particles forming a ‘zipper-like’ arrangement were seen along the outer edge of helical cristae membranes and a single row of 13-nm-wide particles along the inner edge. These particles were thought to be ATP synthase and NADH dehydrogenase (Complex I) on the basis of their size and shape, but no further proof was obtained. Similar experiments with mitochondria from other species were unsuccessful, but proteomic studies [7,8] and 2D class averaging by single-particle EM [9,10] have shown that both ATP synthase and the respiratory chain complexes do form higher-order assemblies in the cristae membranes of various species.

Progress in assessing the effect of protein distribution on the rates of enzyme reactions that involve electrochemical gradients requires a technique which can directly determine protein identity and distribution at nanometre resolution in an unperturbed cellular context. Although fluorescence microscopy has recently broken the theoretical resolution limit of light microscopy (STED (stimulated emission depletion), PALM (photo-activated localization microscopy), etc. [11]), the currently obtainable resolution (20–100 nm) is still below that required for assessing the distribution
Figure 1 | ATP synthesis in mitochondria and chloroplasts

Upper panel: in mitochondria, ATP is generated by oxidative phosphorylation. Electrons are transferred from the electron donors NADH$^+$ and FADH$^+$ to O$_2$ via NADH dehydrogenase (Complex I, blue), ubiquinone (UQ), cytochrome c reductase (Complex III, orange), cytochrome c (black), and cytochrome c oxidase (Complex IV, green). During electron transfer, Complexes I, III and IV pump protons across the inner mitochondrial membrane generating the electrochemical proton gradient used by ATP synthase (Complex V, cream) to produce ATP. Additional electrons from succinate oxidation enter the electron-transfer pathway via succinate dehydrogenase (Complex II, pink), which reduces ubiquinone, but does not pump protons. Lower panel: in chloroplasts, ATP is generated by photosynthesis. Electrons derived from water oxidation are transferred to NADP$^+$ via PSI (blue), plastoquinone (PQ), cytochrome $b_6f$ (orange), plastocyanin (pink), PSI (green), ferredoxin (Fd, brown) and ferredoxin-NADP reductase (FNR, deep red). Light energy captured by the light-harvesting complexes (LHC1, LHCII, cyan, and minor LHC, lilac) and transmitted to PSI or PSII promotes electron transfer by exciting electrons to a higher-energy state. During electron transfer, cytochrome $b_6f$ pumps protons across the thylakoid membrane and, together with water oxidation, catalysed by PSII, forms the electrochemical proton gradient used by ATP synthase (cream) to produce ATP. Membrane bilayers are represented by blue bars. Figure created with Chimera using the PDB codes 3M9S, 1NTK, 3AEF, 2Y69, 2001, 1Q90, 1A70, 1GJR, 2BHW, 3BQV, 3ARC and 3PL9. Protein density maps (except ATP synthase) were created in CCP4 by calculating MZ files from PDB files with a resolution cut-off of 30 Å ($1\text{ Å} = 0.1\text{ nm}$). The ATP synthase density map was obtained from the EM database (emd-1357) and fitted with PDB codes 3V3C and 1FX0 or 4B2Q.

Cryo-ET

Cryo-ET is a relatively new technique that can image cellular features in three dimensions as well as the distribution and, in favourable cases, the structure of protein complexes in situ. The technique has been used to investigate biological structures ranging from isolated protein and viral particles to whole cells and organelles [16–19]. The principles of electron
tomography were worked out some time ago [20–22], but its routine application to cryo-specimens has depended on the development of suitable preparation techniques, cryo-capable electron microscopes, sufficiently large digital cameras and automated data collection software [23–26].

Cryo-ET combines the principles of 3D volumetric imaging (tomography) with cryo-preservation of samples. The process of collecting a tomogram works in a similar way to 3D medical imaging in CAT (computerized axial tomography) or MRI. The sample is placed inside an appropriate instrument and a series of projection images are acquired at different angles. These images are then aligned relative to each other in the computer and re-projected along the acquisition angle to generate a 3D volume [19].

Sample preparation
To image protein structures, samples have to be prepared without the use of destructive stains or fixatives, protected from dehydration in the high vacuum of the electron microscope and imaged with low electron doses to limit radiation damage. For cryo-EM and cryo-ET, samples are rapidly cooled to liquid nitrogen temperature at freezing rates greater than $10^5$ K s$^{-1}$ to avoid the formation of ice crystals [23]. Samples that can be thinly spread on to EM grids, such as purified proteins, organelles, virus suspensions or small bacteria, are usually plunge-frozen. Solutions are applied to EM grids covered with a 100–200-nm-thick holey or small bacteria, are usually plunge-frozen. Solutions are applied to EM grids covered with a 100–200-nm-thick holey carbon support film, blotted to remove excess liquid and applied to EM grids covered with a 100–200-nm-thick holey carbon support film, blotted to remove excess liquid and immediately plunged into liquid ethane using a guillotine device [27]. Larger cells, which produce thin protrusions of interest, e.g. axons, synapses or filopodia, can also be frozen using this method [28–30].

Thicker samples, such as tissues, whole cells or large cellular compartments such as chloroplasts, have to be frozen at high pressure to obtain good freezing rates to depths of a few hundred microns. Samples are placed in protective metal carriers and exposed to high-pressure jets of liquid nitrogen [31,32]. The frozen samples are then sliced with a cryo-microtome into 50–250-nm-thick sections and transferred to an EM grid, all at liquid-nitrogen temperature [33].

Identification of proteins in tomograms
The greatest difference between conventional room-temperature EM of plastic sections and cryo-ET is the level of observable detail. Figures 2(A) and 2(B) show plastic sections of mitochondria and a chloroplast. In both images, the membranes show up clearly, but no molecular detail is visible. By contrast, slices through tomographic volumes of plunge frozen mitochondria (Figure 2C) or high-pressure frozen chloroplasts (Figure 2D) show large protein complexes such as ATP synthase (yellow arrowheads) and PSII (red arrowheads) in the membranes.

Protein densities in tomograms can be identified by various techniques. Large protein complexes with characteristic shapes such as ATP synthase, PSII and ribosomes can usually be detected by eye (Figures 2C and 2D), but for unbiased identification, template matching is often employed. For this approach, atomic models of known structure are filtered to a resolution of 40–60 nm and used as a template to search entire tomographic volumes [34]. This technique has been used to identify large protein complexes such as ribosomes in whole cells [35–37]. Success is highly dependent on sample thickness, contrast and information content, as well as the size and abundance of the target protein. The accuracy of template matching falls not only with protein size and abundance, but also with increasing molecular crowding [34,37]. Therefore this method is currently not appropriate for the analysis of protein-dense organelles such as mitochondria or chloroplasts.

Smaller proteins or proteins of unknown or uncharacteristic shape have to be identified by labelling. Electron-dense tags such as colloidal gold or quantum dots conjugated to primary or secondary antibodies are easily visible in cryo-tomograms. Protein densities within a ∼23 nm radius of these tags are likely to be the protein of interest. Using this method, we have identified NADH dehydrogenase in mitochondrial membranes [13] and have determined the orientation and subunit topology of the pre-protein translocase in the chloroplast outer membrane (TOC) [38]. Electron-dense fusion tags, akin to GFP for light microscopy, are currently being developed for cryo-ET. One promising candidate is metallothionein, a 6 kDa heavy-metal-binding protein, which appears as a 2 nm black density in tomograms. This tag has been used to identify proteins associated with microtubules and intermediate filaments [39].

Protein structure determination and organization
Subtomogram averaging is a technique which can both identify known proteins and determine previously unknown protein structures within tomograms [14,16–18,40] (Figure 2E). Individual subvolumes containing the protein of interest are extracted from the tomogram, aligned with each other and averaged together using specialized software. By subvolume averaging, characteristic features of a particular protein are amplified, whereas random features around it are averaged out increasing the signal-to-noise ratio and hence observable structural detail. During the alignment procedure, a list of co-ordinates and rotation angles are produced describing how the final average aligns with each extracted subvolume. The resulting average or fitted atomic model can then be positioned back into the tomographic volume to provide information about the spatial organization of the target protein within the cell or organelle. Using this method, we have determined the arrangement of ATP synthase dimers in mitochondrial cristae and PSII in thylakoid membranes [12,14] (Figures 2G and 2H).

Cryo-ET and membrane bioenergetics
Using cryo-ET, our laboratory has uncovered the structure and distribution of proteins involved in ATP synthesis in both mitochondria and chloroplasts. One of our most
Figure 2 | EM of mitochondria and chloroplasts
Image of (A) mitochondria from the yeast *Pichia pastoris*, and (B) a chloroplast from *Marchantia*, prepared by conventional EM on resin-embedded samples. Membranes are clearly visible in both images, but molecular information is lacking. (C) Slice through a tomogram of a cryo-preserved mitochondrion from the fungus *Podospora anserina*, and (D) a chloroplast from spinach. Membranes and protein densities such as ATP synthase (yellow arrowheads) and PSII (red arrowheads) are clearly visible. (E) Subtomogram average of ATP synthase dimers picked from mitochondrial membranes of the fungus *P. anserina* (left) and fitted with the atomic X-ray model PDB code 4B2Q (right). (F) Comparison of the protein
striking findings was the long rows of dimeric ATP synthase, which were observed in cristae membranes from mammals, fungi and plants [13,15], but not in chloroplasts [12] or the plasma membranes of bacteria or archaea (B. Daum and A. Mühleip, unpublished work). Although rows of mitochondrial ATP synthase dimers were first reported by Allen et al. [6] over two decades ago, cryo-ET has provided the first direct 3D visualization of these rows and has shown by subtomogram averaging that the particles are, in fact, ATP synthase [13,14]. In addition, we have shown directly that ATP synthase dimers introduce sharp local membrane curvature in the cristae membranes [13,14].

ATP synthase dimer rows are mostly found on the tightly curved ridges of lamellar cristae. Disruption of the ATP synthase dimers in Saccharomyces cerevisiae, through the deletion of the ATP synthase subunits \(e\) or \(g\), led to a profound change in cristae morphology in which sharp local membrane curvature was no longer observed [14]. Instead of the lamellar cristae typical for wild-type yeast strains, mitochondria of these mutants contained a number of inner membrane vesicles, which occasionally formed balloon-shaped protrusions similar to those predicted by mathematical modelling [41]. In these mutants, ATP synthase monomers were randomly distributed over the entire inner membrane surfaces, but no dimers were observed. Yeast mutants lacking subunits \(e\) and \(g\) have longer generation times, a lower membrane potential and decreased decoupling rates compared with wild-type mitochondria, but no alteration in the functionality of the ATP synthase complex [42,43]. The reduced viability of these mutants is therefore most likely to be due to the disruption of the ATP synthase organization in the membrane rather than a loss of enzyme functionality.

In contrast with ATP synthase, most other proteins involved in mitochondrial ATP synthesis are either too small (Complex III and Complex IV) or cannot easily be identified in tomographic volumes (Complex I). Nevertheless, using antibody labelling, we were able to identify Complex I in cristae membranes [13]. This complex appeared to be randomly distributed, at either side of the rows of ATP synthase dimers, rather than forming respiratory strings or patches predicted by proteomics and EM on detergent-solubilized protein complexes [7–9]. Complex I, however, does form supercomplexes with Complex III and IV in some species [44], but higher-order associations, e.g. Complex I dimers in the membrane, have not been found. The occurrence of supercomplexes appears to be related to the increased energy requirements of certain tissues or organisms, but the random distribution of Complex I to ATP synthase is a common feature of all organisms studied. A consequence of this organization is that the proton pumps (Complex I) are physically separated from the proton sinks (ATP synthase) (Figure 3A). The effect of this separation on ATP synthesis rates is unknown, but its disruption e.g. by \(\Delta e\) and \(\Delta g\) mutants described above, does appear to reduce the cell’s fitness [14,42,43].

In chloroplasts, our cryo-ET findings fully support the lateral heterogeneity of photosynthetic membrane protein complexes described previously using freeze–fracture EM [12,45]. In accordance with these studies, we found that the chloroplast ATP synthase was located solely on the stroma-exposed thylakoid membranes and PSII in the stacked grana membranes, where paracrystalline PSII arrays were occasionally observed [12] (Figures 2D and 2H). In contrast with the mitochondrial ATP synthase, the chloroplast ATP synthase is entirely monomeric and randomly distributed in the flat stroma-exposed membranes. No ATP synthase complexes were found in the highly curved margins of the thylakoid membranes [12]. The separation of ATP synthase from PSII again leads to the segregation of proton sources and sinks as observed in mitochondrial cristae.

Previous electron tomography of cryo-sections [12] or serial sections of plastic-embedded chloroplasts [46] has shown that the unstacked stroma thylakoids wind around the stacked membranes in a helical fashion and fuse with successive stacked grana thylakoids by small tubular or lamellar protrusions [46,47]. The size of these openings and the number of membranes in a stack appear to be highly variable [46,48]. As the proton sources (PSII) and proton sinks (ATP synthase) of the thylakoids are located in
**Figure 3** Distribution of proton sources and sinks in mitochondria and chloroplasts

(A) In mitochondria, ATP synthases (yellow) form rows of dimers along highly curved edges of lamellar cristae, whereas the respiratory chain complexes (green) are distributed randomly in the flat membrane regions. This distribution segregates proton sources and sinks, and is thought to result in a directional flow of protons (red spheres) towards the cristae edges (red arrows). Reproduced with kind permission from Davies, K.M., Strauss, M., Daum, B., Kief, J.H., Osiewacz, H.D., Rylovksa, A., Zickermann, V., Kühbrandt, W. (2011) Macromolecular organization of ATP synthase and complex I in whole mitochondria. Proc. Natl. Acad. Sci. U.S.A. **108**(34): 14121–14126. (B) In chloroplasts, the segregation of proton sources and sinks is even more extreme. ATP synthases (proton sinks, yellow) are located on the stroma-exposed thylakoid membranes and PSIs (proton source, blue) are located in the stacked grana membranes. These two membrane compartments are separated by narrow openings, which are likely to restrict protein and ion movement. Protons (red spheres) generated by water oxidation activity of PSII must exit the grana membranes via these connections in order to find their sinks (ATP synthase), which are located up to a few microns away.

different subcompartments of the thylakoid membranes, the diameter of these putatively dynamic membrane connections is likely to affect the rate of ATP synthesis under different environmental conditions by controlling the flow of ions and proteins between the two compartments [46] (Figure 3B). To test this hypothesis, ATP synthesis rates and membrane protein distribution under different environmental conditions needs to be investigated in parallel. This is only possible through the combination of membrane bioenergetics and cryo-ET.

**Conclusion**

We have used cryo-ET to show that proton sources and proton sinks are physically segregated in the energy-converting membranes of both mitochondria and chloroplasts.
chloroplasts. This separation is more extreme in chloroplasts than in mitochondria, but the common principle indicates a fundamental underlying energetic advantage to this arrangement. We have already seen that disruption of this protein distribution in mitochondria through the deletion of the dimer-specific subunits of ATP synthase results in decreased cell viability, suggesting that the separation of proton sources and sinks in mitochondria is required for high rates of ATP synthesis [15,42,43]. This is likely to pertain also to chloroplast thylakoids, where the membrane proteins of grana and stroma membranes intermix randomly when membrane stacking is abolished [49,50]. At present, the underlying membrane organization can be visualized only by cryo-ET. Therefore, to truly understand energy-converting processes at the level of the cell, bioenergetic measurements must be combined with cryo-ET. These two techniques, when used in combination, are likely to become a powerful tool especially when investigating the molecular causes of mitochondria-related human diseases and aging.

Acknowledgements

We thank Werner Kühbrandt for helpful comments on the paper, Friedricke Joos for providing images for Figures 2(A) and 2(B), and Paolo Lastriko for preparing Figure 3.

Funding

This work was funded by the Max Planck Society (to K.M.D. and B.D.).

References


