Subunits of Mitochondrial Complex I Exist as Part of Matrix- and Membrane-associated Subcomplexes in Living Cells*§

Cindy E. J. Dieteren*,§ Peter H. G. M. Willems†*, Rutger O. Vogel†, Herman G. Swarts†, Jack Fransen§, Ronald Roepman**, Gijs Crienen†, Jan A. M. Smeltink§, Leo G. J. Nijtmans†*, and Werner J. H. Koopman†*

From the Departments of Biochemistry, Cell Biology, and Human Genetics, and the Microscopical Imaging Centre of the Nijmegen Centre for Molecular Life Sciences, the Department of Pediatrics of the Nijmegen Centre for Mitochondrial Disorders, Radboud University Nijmegen Medical Centre, 6500 HB, Nijmegen, The Netherlands

Mitochondrial complex I (CI) is a large assembly of 45 different subunits, and defects in its biogenesis are the most frequent cause of mitochondrial disorders. In vitro evidence suggests a stepwise assembly process involving pre-assembled modules. However, whether these modules also exist in vivo is as yet unresolved. To answer this question, we here applied submitochondrial fluorescence recovery after photobleaching to HEK293 cells expressing 6 GFP-tagged subunits selected on the basis of current CI assembly models. We established that each subunit was partially present in a virtually immobile fraction, possibly representing the holo-enzyme. Four subunits (NDUFV1, NDUFV2, NDUFA2, and NDUFA12) were also present as highly mobile matrix-soluble monomers, whereas, in sharp contrast, the other two subunits (NDUFB6 and NDUFS3) were additionally present in a slowly mobile fraction. In the case of the integral membrane protein NDUFB6, this fraction most likely represented one or more membrane-bound subsassemblies, whereas biochemical evidence suggested that for the NDUFS3 protein this fraction most probably corresponded to a matrix-soluble subassembly. Our results provide first time evidence for the existence of CI subsassemblies in mitochondria of living cells.

The vast majority of cellular ATP is produced by mitochondrial oxidative phosphorylation (OXPHOS). The OXPHOS system consists of five multisubunit complexes of which NADH:ubiquinone oxidoreductase or complex I (CI) is the largest (~1 MDa) and least understood (1). CI liberates electrons from NADH, channels them to ubiquinone, and uses part of their energy to expel protons from the mitochondrial matrix into the intermembrane space. The complex consists of 45 subunits, seven of which are encoded by the mtDNA and the remainder by the nuclear genome (2). Its catalytic core comprises 14 evolutionary conserved subunits (3), which, in humans, are encoded by the nuclear NDUFV1, NDUFV2, NDUFS1, NDUFS2, NDUFS3, NDUFS7, and NDUFS8 genes and the mitochondrial ND1-ND6 and ND4L genes. Once assembled, the complex has an l-shaped structure with one arm protruding into the matrix and the other arm embedded in the inner membrane. The matrix-protruding, or peripheral, arm is involved in electron channeling and contains the flavo-mononucleotide group and eight iron-sulfur clusters. The membrane arm includes all of the mtDNA-encoded subunits and is believed to play a role in proton translocation (3).

At present, it is still unclear how the different subunits are exactly pieced together during CI biogenesis. Understanding this process is also clinically relevant because mutations in CI subunits prevent proper CI assembly, causing a variety of mitochondrial disorders (reviewed in Ref. 4). The current hypothesis is that CI assembly occurs by a stepwise mechanism during which prefabricated modules, or assembly intermediates, are combined (5–9). In agreement with this idea, we recently demonstrated that inhibition of mitochondrial translation caused an accumulation of the NDUFS3 subunit in two distinct subsassemblies (7). Apart from this, recent in vitro import and assembly studies revealed a mechanism involving the replacement of earlier incorporated subunits by newly formed ones (9). It is conceivable that such a mechanism might serve to maintain the functional integrity of CI.

Up to now, most insights into the mechanism of CI assembly were obtained from studies using blue native polyacrylamide gel electrophoresis (BN-PAGE) of mitochondrial cell fractions. Here, we extended our investigations into the CI assembly process to living cells. To allow doing so, we generated a collection of human cell lines that expressed AcGFP1-tagged CI subunits at a low level. In these cell lines, the mobility of the tagged subunits was investigated using fluorescence recovery after photobleaching (FRAP) (10–17).
Complex I Assembly in Living Cells

By comparison with matrix-soluble and mitochondrial inner membrane (MIT)-bound versions of AcGFP1 of different size, we could establish the presence of these CI subunits in mobile (matrix-soluble) and/or immobile (membrane-bound) subassemblies. Our findings provide the first evidence that CI assembly occurs in a modular manner in vivo.

EXPERIMENTAL PROCEDURES

Generation of Inducible Cell Lines—cDNA fragments of the following genes were generated by Gateway-adapted PCR procedures according to the manufacturer’s instructions (Invitrogen): the cox8 leader sequence (first 210 basepairs of sequence NM_000040704, NDUF3 (NM_004551), NDUFV1 (NM_007103), NDUF2 (NM_002488), NDUFA12 (NM_018838), NDUF6 (NM_002493; transcript variant 1). Entry clones were generated from the resulting PCR products by recombination with pDONR201 (Invitrogen) using Gateway Clonase II Enzyme Mix (Invitrogen). Entry clones containing NDUFV2 (BC_001632; Clone HsCD00041552) and human ANT1 (BC_008664; Clone HsCD00040509) without stop codons were ordered from The Plasmid Information data base (18). A destination vector was generated by subcloning the BamHI/NotI restriction fragment of pAcGFP1-N1 (Clontech, Westburg, Leusden, The Netherlands) in-frame behind Gateway Reading Frame Cassette B (Invitrogen) in pcDNA5/FRT/TO (Invitrogen). To obtain an inducible vector containing mitochondrial-targeted AcGFP1 and C-terminally AcGFP1-tagged proteins, the entry vectors were recombined with the AcGFP1-Destination vector by using Gateway LR Clonase II Enzyme Mix (Invitrogen). In the same manner, a tandem mitochondrial AcGFP1 expression vector was created by first making an entry vector containing the cox8 leader sequence linked to the N terminus of AcGFP1 (without the stop codon) and then recombining this entry clone with the AcGFP1-Destination vector. Flp-In T-Rex293 cells (Invitrogen) were stably transfected using Superfect Transfection Reagent (Qiagen, Venlo, The Netherlands) according to the manufacturer’s procedures and cultured for selection in the presence of 200 μg/ml hygromycin (Calbiochem, Burlington, MA) and 400 μg/ml of G418 (Gibco). Cells were maintained to 70% confluence. During FRAP measurements, cells were maintained in a colorless HEPES-Tris (HT) solution (132 mM NaCl, 4.2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5.5 mM d-glucose, and 10 mM HEPES, pH 7.4). As a reference, a cox8-AcGFP1-expressing cell line was included in the analysis on each day of experiments. FRAP measurements were carried out at 20 °C to minimize mitochondrial movement (20) using a LEICA TCS SP2 Meta confocal microscope (Leica Microsystems, Wetzlar, Germany). FRAP measurements were acquired at a rate of 10 Hz using a ×63 oil immersion objective (NA 1.4; Carl Zeiss). Routinely, a zoom factor of 4 was used, and pinhole settings were chosen to achieve an optical section thickness of <2 μm. Only single mitochondria that were fully within the focal plane were used for analysis. This was confirmed by an axial (Z) scan through the filament. A FRAP region of 10 × 10 pixels (1.4 × 1.4 μm) was used. After acquiring the base level of fluorescence, the AcGFP1 in the FRAP region was photobleached during 500 ms using 488-nm light delivered by an argon-ion laser set at 100% transmission. Importantly, intense illumination of AcGFP1 is not phototoxic (21). On resuming image acquisition, AcGFP1 fluorescence intensity was reduced by 60–70% (Table 1, F0). Only mitochondria in which FRAP was paralleled by fluorescence loss in photobleaching (FLIP) in a part distal to the FRAP region were considered to possess a continuous mitochondrial matrix and included in the analysis. Following bleaching, fluorescence recovery in the FRAP region was measured using normal 488-nm excitation (0.1% transmission). Individual FRAP curves (F(t)) were calculated as described previously (14) using Equation 1.
Here the fluorescence intensity in the bleached mitochondrial region \((F(t)_{\text{FRAP region}})\) and for the total mitochondrion \((F(t)_{\text{totalmito}})\) is background-corrected \((F(t)_{\text{background}})\) at each time point. Next, the corrected fluorescence signal in the bleached region is divided by the corrected intensity of the total mitochondrion to correct for the loss of fluorescence during the bleach. This is important because in our experiments the bleach area is relatively large compared with the size of the mitochondrion. The data are normalized to the background-corrected pre-bleach intensity \((F_{\text{totalmito}})\) and \((F_{\text{FRAP region}})\) and multiplied by 100 to yield a percentage of initial fluorescence. Mean recovery curves were calculated by averaging individual FRAP curves and fitted by a two-component exponential association model (17) using Equation 2,

\[
F(t) = F_\infty + A_1(1 - e^{-t/t_{\text{fast}}}) + A_2(1 - e^{-t/t_{\text{slow}}})
\]

with \(t_{\text{fast}}\) and \(t_{\text{slow}}\) being the time constant of the fast and slow recovery phase, respectively and \(A_1 + A_2 + F_\infty\) being the fluorescence intensity \((F_\infty)\) at \(t = t_\infty\).

### Image Processing, Curve Fitting, and Statistics

Image processing and analysis were performed using Image Pro Plus 5.1 (Media Cybernetics, Bethesda, MD). Non-linear curve fitting
Complex I Assembly in Living Cells

(using the Levenberg-Marquardt algorithm) and statistical analysis were performed using Origin Pro 7.5 (Originlabs, Northampton, MA). Values from multiple experiments are expressed as mean ± S.E.

RESULTS

AcGFP1-tagged CI Subunits Are Mitochondrially Localized and Incorporated in CI—For live cell analysis of CI assembly, we used HEK293 cell lines that expressed a monomeric variant of GFP, AcGFP1, fused to the C terminus of a nDNA-encoded CI subunit of interest. Subunits were selected on the basis of current CI assembly models (Fig. 1A and Ref. 8). A total of six cell lines were generated (schematically depicted in Fig. 1B) for inducible expression of subunits of either the dehydrogenase (green, NDUFV1 and NDUFV2), hydrogenase (purple, NDUFS3), or membrane (yellow, NDUFB6) module or accessory subunits (pink, NDUFA2 and NDUFA12). Confocal microscopy showed that all AcGFP1-tagged subunits were readily expressed and mitochondrially localized (supplemental Fig. S1).

Five of the six cell lines (NDUFV1, NDUFV2, NDUFS3, NDUFA2, and NDUFA12) expressed the tagged subunit already under non-induced conditions (leakage). This gave us the opportunity to minimize the risk of overexpression artifacts. An additional advantage is that under non-induced conditions incorporation of tagged subunit is at steady state (7). Fluorescence scanning of native gels of mitochondrial fractions (Fig. 1C, lanes indicated with f) of these five cell lines showed a fluorescent band at the height of holo-CI (closed arrowheads). Their incorporation in CI was confirmed by immunodetection with an anti-EGFP antibody (Fig. 1C, lanes indicated with a). Distinct bands of lower MW, previously identified as CI assembly intermediates (7), were found in NDUFS3 cells (Fig. 1C, open arrowheads 1–6). Other (faint) bands of lower MW, not seen before, were observed in NDUFV1, NDUFV2, and NDUFA2 cells. Both detection methods showed for low-level induced NDUFB6 cells a dim smear at and below the height of the holo-complex, which was in line with the assembly pattern of endogenous NDUFB6 (7). Further

![Figure 2](image-url)
induction of the tagged subunits increased their incorporation into CI without altering the CI amount and in-gel activity (supplemental Fig. S2). This indicates that the AcGFP1 tag did not interfere with proper assembly and functioning of the holo-complex.

Submitochondrial FRAP Discriminates between Matrix-soluble and MIM-associated Proteins—Before FRAP analysis of the AcGFP1-tagged CI subunits was performed, we first established that submitochondrial FRAP analysis was able to distinguish matrix-soluble from MIM-associated proteins.

Given the fact that the mobility of matrix-soluble proteins might depend on their size (23), we first assessed the relationship between protein size and mobility in the matrix. To this end, we generated two inducible HEK293 cell lines expressing either an N-terminal fusion from AcGFP1 to the mitochondrial leader sequence of cox8 or a mitochondria-targeted tandem fusion of AcGFP1 (Fig. 2A, cell lines designated GFP and GFP\textsuperscript{2}, respectively). Fluorescence scanning (Fig. 2B, lanes indicated f) and anti-EGFP immunostaining of Western blots (Fig. 2B, lanes indicated a) revealed a specific band of the expected size on SDS-PAGE gels. Fig. 2C depicts a typical FRAP experiment with GFP cells. Following the bleach pulse, the fluorescence signal in the FRAP region (square) rapidly recovered to pre-bleach values. Recovery kinetics was appropriately fitted by a two-component model. Averaged FRAP curves are shown in Fig. 2D. Calculation of the mobile fraction (F\textsubscript{m}) revealed values of 1.01 and 0.99 for GFP and GFP\textsuperscript{2} cells, respectively (Table 1), demonstrating that both unconjugated matrix proteins were fully mobile. In GFP cells, fluorescence recovered with time constants of 0.216 s (t\textsubscript{fast}) and 1.12 s (t\textsubscript{slow}). For GFP\textsuperscript{2} cells, both time constants (0.442 s and 2.42 s for t\textsubscript{fast} and t\textsubscript{slow}, respectively) were 2-fold slower, indicating that the mobility of matrix-soluble GFP decreases with size.

Next, we verified whether submitochondrial FRAP could distinguish between GFP, GFP\textsuperscript{2}, and AcGFP1 fused to an integral protein of the MIM. To this end, an inducible HEK293 cell line was generated that expressed an N-terminal fusion from AcGFP1 to mitochondrial adenine nucleotide translocase 1 (Fig. 2A, cell line designated ANT). Fluorescence scanning (Fig. 2B, lanes indicated f) and anti-EGFP immunostaining of Western blots (Fig. 2B, lanes indicated a) confirmed the presence of a specific product of the correct size after SDS-PAGE. The averaged ANT recovery curve is shown in Fig. 2D. Calculation of F\textsubscript{m} revealed a value of 0.63 for ANT cells (Table 1). In contrast to GFP and GFP\textsuperscript{2}, which were fully mobile, 37% of AcGFP1-tagged ANT was virtually immobile. The remaining fraction of AcGFP1-tagged ANT displayed a much slower mobility than GFP and GFP\textsuperscript{2} (Fig. 2D). This is reflected by t\textsubscript{slow}, which was much larger for ANT cells (8.04 s) than for GFP (1.12 s) and GFP\textsuperscript{2} (2.42 s) cells. Proper calculation of t\textsubscript{fast} was not feasible for ANT cells given the limited number of data points constituting the fast phase of the recovery curve (Fig. 2D). Taken together, these results demonstrate that matrix-soluble and MIM-associated proteins display different submitochondrial FRAP kinetics. This property can be used to study CI assembly, which is thought to occur in the mitochondrial matrix and MIM (Fig. 1A).
Evidence for Coexistence of Mobile and Immobile CI Assemblies within the Mitochondrion—After having established the FRAP characteristics of matrix-soluble and MIM-bound proteins, we next applied this technique to the cell lines expressing the tagged CI subunits (Fig. 3, A–F, red curve). Again, recovery kinetics was adequately described with a two-component model. Calculation of $F_m$ revealed that in all six cell lines significant amounts of the AcGFP1-tagged subunit resided in the immobile fraction (Table 1).

Concerning the mobilities of GFP-tagged subunits, the results obtained for GFP and GFP$^2$ cells show that these can only be compared if the size of the subunits is taken into account. Thus, a plot of $t_{fast}$ versus MW shows that the values for fully mobile GFP and GFP$^2$ lie on a line through the origin, whereas those for the AcGFP1-tagged subunits are below this line (Fig. 4A). This suggests that our cell lines contained AcGFP1-tagged product(s) that move faster than predicted on the basis of the MW of the intact fusion protein. NDUFA2 and NDUFA12 cells displayed similar $t_{fast}$ values as observed in GFP cells, demonstrating the presence of fully mobile AcGFP1 in these two cell lines. To enquire the presence of fluorescent breakdown products, we analyzed mitochondrial enriched fractions by SDS-PAGE. The fluorograms (Fig. 4B, lanes indicated f) show that most cell lines displayed, in addition to a clear band of correct size (closed arrowheads), bands of lower MW. The relative intensity of these latter bands was low in NDUFS3 and NDUFA2 cells, but high in NDUFA12 cells. Because GFP fluorescence is extremely sensitive to truncations (24), the lower MW breakdown products most probably consist of full-length AcGFP1 alone or fused to partially truncated subunit. Together, these results support the conclusion that the fast component of the FRAP signal reflects the movement of lower MW breakdown products demonstrated to be present in these cell lines. This is substantiated by the fact that the fast component of the FRAP signal was virtually absent in ANT and NDUFB6 cells, which do not contain lower MW breakdown products (see Figs. 2B and 4B).

Also in a plot of $t_{slow}$ versus MW, the values for GFP and GFP$^2$ lie on a line through the origin (Fig. 4C). In this case, however, the values for the AcGFP1-tagged CI subunits are either on this...
DISCUSSION

The biogenesis of CI is complicated by its large size and its regulation by two genomes, with seven subunits encoded by the mtDNA and the remainder by nuclear genes. Knowledge of this process might provide important insights into the pathogenesis of many hitherto unexplained OXPHOS disorders. Previous in vitro studies strongly support the idea that the 45 subunits assemble via different routes to form the functional enzyme. Proper understanding of how macromolecular assemblies such as CI are built up requires investigation of protein interactions in the dynamic environment of the living cell. Here, we reasoned that if CI assembly occurs through pre-assembled modules it should be possible to demonstrate their existence by submitocondrial FRAP in living cells. Indeed, FRAP analysis revealed that the NDUFS3 subunit, proposed to be incorporated at an early stage during the assembly of CI, were either immobile or present as highly mobile matrix-soluble subunits. Using differential extraction, we confirmed the presence of this subunit in a matrix-soluble assembly, probably representing the slowly mobile fraction, and several membrane-bound assemblies including the holo-enzyme, probably representing the immobile fraction. In contrast to the NDUFS3 subunit, the NDUV1, NDUFV2, NDUFA2, and NDUFA12 subunits, proposed to be incorporated at a late stage during the assembly of CI, were either immobile or present as highly mobile matrix-soluble monomers. Consistent with its possession of a transmembrane domain, NDUFB6 was either immobile or part of one or more slowly diffusing membrane-bound subassemblies. Together, these results show for the first time that CI subassemblies exist in vivo and that they might be present in both the matrix and the MIM.

To allow proper interpretation of the FRAP data, we first determined the mobilities of a matrix-targeted version of AcGFP1 (GFP), a matrix-targeted tandem version of AcGFP1 (GFP2), and an MIM-targeted version of AcGFP1 (ANT). The FRAP mobility data obtained with GFP and GFP2 demonstrate that a small change in size of a freely diffusing and non-interacting protein influences its mobility in the mitochondrial matrix. GFP and GFP2 were fully mobile and displayed a biphasic FRAP curve. Importantly, both the fast and slow time-constant of this curve (t_fast and t_slow) showed a linear relationship with the MW. These findings are compatible with the idea that mitochondrial structures (e.g. cristae) can temporarily trap freely moving and non-interacting proteins. In this context, evidence has been presented in the literature indicating that mitochondrial cristae are connected to the periphery of the MIM by narrow tubular junctions (cristae junctions, (27)), which might be responsible for the observed molecular sieving effect. Additionally, we showed that binding of AcGFP1 to the
Complex I Assembly in Living Cells

MIM through its fusion with ANT1 slowed down the recovery kinetics by a factor 4 compared with matrix-soluble GFP. This result agrees with previous data on ANT diffusion (10). About 37% of AcGFP1-tagged ANT1 was immobile. Most probably this represents the fraction of molecules bound to the mitochondrial permeability transition pore complex (28).

All AcGFP1-tagged subunits displayed an immobile fraction. Previous estimates of holo-CI diffusion in mitoplasts revealed a diffusion constant of 0.0046 μm²/s (29, 30). Assuming that this value is similar to the mobility of holo-CI in living cells and comparing it to the diffusion constant of matrix-soluble EYFP in living cells (25 μm²/s (22)), holo-CI can be expected to move ~5100 times slower than unconjugated AcGFP1. Based on these considerations, it is reasonable to conclude that the virtually immobile fraction observed with all subunits tested consists of holo-CI. This conclusion is supported by earlier work of Bush et al. (31) who observed a speckled distribution of GFP- and RFP-tagged CI during ongoing mitochondrial fusion and fission.

Although we used low expression levels of the AcGFP1-tagged subunits, inspection of the $t_{\text{fast}}$ values revealed that all five cell lines displayed a fast component with a mobility that corresponded to or was faster than expected on the basis of the MW of the AcGFP1-tagged subunit. Fluorograms of SDS-PAGE gels confirmed that these faster mobilities were caused by a decrease in the MW of the AcGFP1-tagged subunit. Fluorograms of SDS-PAGE gels confirmed that these faster mobilities were caused by a decrease in the MW of the AcGFP1-tagged subunit. Previous estimates of holo-CI diffusion in mitoplasts revealed a diffusion constant of 0.0046 μm²/s (29, 30). Assuming that this value is similar to the mobility of holo-CI in living cells and comparing it to the diffusion constant of matrix-soluble EYFP in living cells (25 μm²/s (22)), holo-CI can be expected to move ~5100 times slower than unconjugated AcGFP1. Based on these considerations, it is reasonable to conclude that the virtually immobile fraction observed with all subunits tested consists of holo-CI. This conclusion is supported by earlier work of Bush et al. (31) who observed a speckled distribution of GFP- and RFP-tagged CI during ongoing mitochondrial fusion and fission.

Although we used low expression levels of the AcGFP1-tagged subunits, inspection of the $t_{\text{fast}}$ values revealed that all five cell lines displayed a fast component with a mobility that corresponded to or was faster than expected on the basis of the MW of the AcGFP1-tagged subunit. Fluorograms of SDS-PAGE gels confirmed that these faster mobilities were caused by a decrease in the MW of the AcGFP1-tagged subunit. Previous estimates of holo-CI diffusion in mitoplasts revealed a diffusion constant of 0.0046 μm²/s (29, 30). Assuming that this value is similar to the mobility of holo-CI in living cells and comparing it to the diffusion constant of matrix-soluble EYFP in living cells (25 μm²/s (22)), holo-CI can be expected to move ~5100 times slower than unconjugated AcGFP1. Based on these considerations, it is reasonable to conclude that the virtually immobile fraction observed with all subunits tested consists of holo-CI. This conclusion is supported by earlier work of Bush et al. (31) who observed a speckled distribution of GFP- and RFP-tagged CI during ongoing mitochondrial fusion and fission.

The $t_{\text{slow}}$ values of the cells lines expressing AcGFP1-tagged NDUFA2, NDUFA12, NDUVF2, or NDUVF1 were in good agreement with the predicted values. Thus, these four subunits were present either as part of the virtually immobile holo-complex (see above) or in their free form, i.e. not bound to any other larger-sized complex. This demonstrates that these four subunits are incorporated at a relatively late stage during in vivo CI assembly. In a recent study we described a patient with a mutation in the NDUFA2 subunit of CI (32). Interestingly, this patient accumulated a CI subassembly of ~830 kDa, indicating that the NDUFA2 subunit is indeed a late subunit. A similar ~830-kDa subassembly was observed in a patient with a mutation in the NDUVF1 subunit (33). Experimental evidence for this subunit being a late subunit comes from metabolic labeling studies showing that the top of matrix-protruding part of the holo-enzyme, which contains at least the NDUVF1 and NDUVF2 subunits, is added at the very end of the CI assembly process (9). It has been suggested that the NDUFA12 subunit, similar to the NDUVF1 and NDUVF2 subunits, is added after the formation of the ~830-kDa subassembly (34). Our results provide first in vivo evidence that the NDUFA12 subunit indeed is a late subunit.

NDUFS3-expressing cells displayed a slow component with a mobility that was slower than expected on the basis of the MW of the AcGFP1-tagged subunit, but faster than expected when linked to a membrane-bound protein. This result is indicative for the presence of a matrix-soluble subassembly. Evidence in support of this idea was provided by a differential extraction experiment, revealing not only a matrix-soluble NDUFS3-AcGFP1 containing subassembly (subassembly 1) but also two loosely membrane-associated subassemblies (subassemblies 2 and 3) and four strongly membrane-bound assemblies (subassemblies 4 to 6 and holo-CI) containing this fusion protein. We previously demonstrated that subassemblies 4 to 6 and holo-CI contain the MIM spanning ND1 subunit (7). In this respect, the membrane-associated characteristics of subassemblies 2 and 3 are unexpected because, so far, only soluble proteins have been identified in these subcomplexes (6, 7, 9). In contrast to the current assembly models, these results support the conclusion that subassemblies 2 and 3 are assembled at the MIM. It is tempting to speculate that linkage of these two subassemblies to the MIM requires a, currently unknown, membrane-anchoring protein. Further analysis of the composition of these subassemblies is required to provide clarity on this issue.

In summary, this study provides first time evidence that CI subassemblies exist in vivo in the matrix and the inner membrane. This indicates that a modular CI assembly pathway is operational in different mitochondrial compartments in the living cell.

Acknowledgment—We thank E. Leijen for optimization of the fractionation protocol.

REFERENCES

1. Shoubridge, E. A. (2001) Hum. Mol. Genet. 10, 2277–2284
2. Carroll, J., Fearnley, I. M., Skehel, J. M., Shannon, R. J., Hirst, J., and Walker, J. E. (2006) J. Biol. Chem. 281, 31724–31727
3. Brandt, U. (2006) Cytometry A 69, 1–12
4. Janssen, R. J., Nijtmans, L. G., van den Heuvel, L. P., and Smeitink, J. A. (2006) J. Inherit. Metab Dis. 29, 499–515
5. Antonicka, H., Olgivie, I., Taivassalo, T., Anitori, R. P., Haller, R. G., Vissing, J., Kennaway, N. G., and Shoubridge, E. A. (2003) J. Biol. Chem. 278, 43081–43088
6. Ugalde, C., Vogel, R., Huijben, R., Van Den, H. B., Smeitink, J., and Nijtmans, L. (2004) Hum. Mol. Genet. 13, 2461–2472
7. Vogel, R. O., Dieteren, C. E., van den Heuvel, L. P., Willems, P. H., Smeitink, J. A., Koopman, W. J., and Nijtmans, L. G. (2007) J. Biol. Chem. 282, 7582–7590
8. Vogel, R. O., Smeitink, J. A., and Nijtmans, L. G. (2007) Biochim. Biophys. Acta. 1767, 1215–1227
9. Lazarou, M., McKenzie, M., Ohtake, A., Thorburn, D. R., and Ryan, M. T. (2007) Mol. Cell. Biol. 27, 4228–4237
10. Güpte, S. S., Chazotte, B., Leesnitzer, M. A., and Hackenbrock, C. R. (1991) Biochim. Biophys. Acta 1069, 131–138
11. Sekse, O., Biwersi, J., and Verkman, A. S. (1997) J. Cell Biol. 138, 131–142
12. Haggie, P. M., and Verkman, A. S. (2002) J. Biol. Chem. 277, 40782–40788
13. Lippincott-Schwartz, J., and Bonnet, N., and Patterson, G. H. (2003) Nat. Cell Biol. (suppl.), S7–S14
14. Goodwin, J. S., and Kenworthy, A. K. (2005) Methods 37, 154–164
15. Roy, S., Plowman, S., Rotblat, B., Prior, I. A., Muncke, C., Grainger, S., Parton, R. G., Henis, Y. I., Kloo, Y., and Hancock, J. F. (2005) Mol. Cell. Biol. 25, 6672–6733
16. Chen, Y., Lagerholm, B. C., Yang, B., and Jacobson, K. (2006) Methods 39, 147–153
17. Picard, D., Suslova, E., and Briand, P. A. (2006) Exp. Cell Res. 312, 3949–3958
18. Zuo, D., Mohr, S. E., Hu, Y., Taycher, E., Rolfs, A., Kramer, J., Williamson, J., and LaBae, J. (2007) Nucleic Acids Res. 35, D680–D684
19. Nijtmans, L. G., Henderson, N. S., and Holt, I. J. (2002) Methods 26, 327–334
20. Koopman, W. J., Visch, H. J., Smeitink, J. A., and Willems, P. H. (2006) Cytometry A 69, 1–12
21. Bulina, M. E., Chudakov, D. M., Britanova, O. V., Yanushevich, Y. G., Staroverov, D. B., Chepurnykh, T. V., Merzlyak, E. M., Shkrob, M. A., Lukyanov, S., and Lukyanov, K. A. (2006) Nat. Biotechnol. 24, 95–99
22. Koopman, W. J., Hink, M. A., Verkaart, S., Visch, H. J., Smeitink, J. A., and Willems, P. H. (2007) Biochim. Biophys. Acta. 1767, 940–947
23. Sprague, B. L., and McNally, J. G. (2005) Trends Cell Biol. 15, 84–91
24. Li, X., Zhang, G., Ngo, N., Zhao, X., Kain, S. R., and Huang, C. C. (1997) J. Biol. Chem. 272, 28545–28549
25. Cardol, P., Boutaffala, L., Memmi, S., Devreese, B., Matagne, R. F., and Remacle, C. (2008) Biochim. Biophys. Acta. 1777, 388–396
26. Olvezcky, B. P., and Verkman, A. S. (1998) Biophys. J. 74, 2722–2730
27. Mannella, C. A. (2006) Biochim. Biophys. Acta. 1763, 542–548
28. Kroemer, G., Galluzzi, L., and Brenner, C. (2007) Physiol. Rev. 87, 99–163
29. Chazotte, B., and Hackenbrock, C. R. (1991) J. Biol. Chem. 266, 5973–5979
30. Lenaz, G., and Genova, M. L. (2007) Am. J. Physiol. Cell Physiol. 292, C1221–C1239
31. Busch, K. B., Bereiter-Hahn, J., Wittig, I., Schagger, H., and Jendrach, M. (2006) Mol. Membr. Biol. 23, 509–520
32. Hoefs, S. J., Dieteren, C. E., Distelmaier, F., Janssen, R. J., Epplen, A., Swarts, H. G., Forkink, M., Rodenburg, R. J., Nijtmans, L. G., Willems, P. H., Smeitink, J. A., and van den Heuvel, L. P. (2008) Am. J. Hum. Genet. 82, 1306–1315
33. Vogel, R. O., van den Brand, M. A., Rodenburg, R. J., van den Heuvel, L. P., Tsuneoka, M., Smeitink, J. A., and Nijtmans, L. G. (2007) Mol. Genet. Metab. 91, 176–182
34. Lazarou, M., Thorburn, D. R., Ryan, M. T., and McKenzie, M. (2008) Biochim. Biophys. Acta, in press