Identification of Mitochondrial Complex I Assembly Intermediates by Tracing Tagged NDUFS3 Demonstrates the Entry Point of Mitochondrial Subunits

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Biogenesis of human mitochondrial complex I (CI) requires the coordinated assembly of 45 subunits derived from both the mitochondrial and nuclear genome. The presence of CI subcomplexes in CI-deficient cells suggests that assembly occurs in distinct steps. However, discriminating between products of assembly or instability is problematic. Using an inducible NDUFS3-green fluorescent protein (GFP) expression system in HEK293 cells, we here provide direct evidence for the stepwise assembly of CI. Upon induction, six distinct NDUFS3-GFP-containing subcomplexes gradually appeared on a blue native Western blot also observed in wild type HEK293 mitochondria. Their stability was demonstrated by differential solubilization and heat incubation, which additionally allowed their distinction from specific products of CI instability and breakdown. Inhibition of mitochondrial translation under conditions of steady state labeling resulted in an accumulation of two of the NDUFS3-GFP-containing subcomplexes (100 and 150 kDa) and concomitant disappearance of the fully assembled complex. Lifting inhibition reversed this effect, demonstrating that these two subcomplexes are true assembly intermediates. Composition analysis showed that this event was accompanied by the incorporation of at least one mitochondrial DNA-encoded subunit, thereby revealing the first entry point of these subunits.

Mitochondrial ATP is produced by the oxidative phosphorylation (OXPHOS) system. This system consists of five complexes, composed of at least 75 nuclear DNA-encoded and 13 mitochondrial DNA (mtDNA)-encoded proteins, and is a prominent example of coordinated assembly. The first four OXPHOS complexes (CI–CIV) constitute the respiratory chain, which transfers electrons from substrates NADH (at CI) and FADH₂ (at CII) to the final electron acceptor molecular oxygen (CIV). Energy released by this electron transport is used to drive proton translocation across the mitochondrial inner membrane at CI, CIII, and CIV. The resulting proton gradient is used to drive the conversion of ADP and inorganic phosphate into ATP by complex V (1).

CI (NADH:ubiquinone oxidoreductase complex; EC 1.6.5.3) constitutes the largest and least understood of the OXPHOS complexes (2, 3). Electron microscopy revealed that CI has an L-shaped structure that consists of a hydrophobic arm embedded in the lipid bilayer of the mitochondrial inner membrane and a hydrophobic peripheral arm exposed to the mitochondrial matrix (4). Using chaotropic salts and the detergent N,N-dimethyldecylamine N-oxide, CI can be fractionated into several fragments (5, 6) that together encompass 45 distinct subunits in bovine CI (7, 8). The recent appearance of the first crystal structure of the hydrophilic domain of CI in Thermus thermophilus is an example of the increasing insight that is gained in this area of research (9).

In contrast, the many steps involved in the assembly of these 45 subunits still remain puzzling. Studies in the fungus Neurospora crassa demonstrated that the membrane and peripheral arms of CI are assembled independently and that the membrane arm, in its turn, is the product of the combination of a small and large assembly intermediate (10–12). Two models are described for the CI assembly pathway in human mitochondria; one is based on the subcomplex distribution in CI-deficient patient cells, and the other is based on the appearance of subcomplexes in a conditional CI assembly system (13, 14). Although both models differ at several points, they agree in that assembly occurs via the combination of large preassembled fragments rather than via sequential addition of individual subunits.

Structural and phylogenetic data strongly suggest that certain CI subunits have co-evolved and are arranged in distinct...
structures, termed modules (15–18). Combination of these modules resulted in the “minimal” CI structure consisting of the 14 most conserved subunits, of which a typical example is *Escherichia coli* CI: NDH-1. The proposed modules are the dehydrogenase module, consisting of the NDUFS2, NDUFS1, and NDUFS1 subunits (homologues of the nuoE, F, and G subunits of bacterial NDH-1); the hydrogenase module, consisting of the NDUFS2, NDUFS3, NDUFS7, NDUFS8, ND1 and ND5 subunits (homologues of the nuoD, C, B, I, H and L subunits of NDH-1); and the proton translocation module, consisting of the ND2, ND3, ND4, ND4L, and ND6 subunits (homologues of the nuoN, A, M, K, and J subunits of NDH-1) (for further details concerning this subject, see Ref. 17). Our assembly model proposes that assembly in part reflects this evolutionary conservation of CI subunits (14). Assembly intermediates were identified by their appearance after the release of doxycycline inhibition of mitochondrial translation (allowing synthesis of the mtDNA-encoded ND subunits to resume). Membrane arm subunits seemed to be assembled in a different intermediate than peripheral arm subunits, and the presence of distinct early subassemblies suggested a link between the assembly process and co-evolution of different CI subunits (14).

Both assembly studies for human CI have used disturbed assembly systems, such as patient cell lines or cells treated with inhibitors of mitochondrial translation (13, 14). Thus far, however, no subcomplexes have been identified in undisturbed systems. A useful strategy to trace assembly without disturbing its dynamics is by tagging a CI subunit, provided that the tag does not interfere with its biological function. This was previously done by Scheffler and co-workers (19, 20), who used inducible tagged versions of the human homologues of the MWFE and ESS subunits of CI to study their incorporation and function in assembly by complementation of CI-deficient Chinese hamster cell lines. However, assembly intermediates were not studied.

In this study, we have used leakage expression of an inducible HEK293 cell line expressing monomeric green fluorescent protein (AcGFP1)-tagged NDUFS3 subunit. This strategy allowed detection of the NDUFS3-containing subcomplexes under steady state labeling conditions and at relatively low expression levels on native one-dimensional gels. Moreover, it allowed the analysis of accumulation or disappearance of individual subcomplexes upon interference with the assembly process. The NDUFS3 subunit was selected for labeling given the apparent ease with which its eYFP-His-tagged *Yarrowia lipolytica* homologue can be used to isolate CI, demonstrating that the C-terminal tag does not disturb assembly (21). Additionally, it has been argued that this core subunit is incorporated at an early stage of assembly (13, 14), so that tagging will provide more insight into onset and subsequent CI assembly steps. We demonstrate the existence of at least six distinct NDUFS3-containing intermediates and distinguish these stable intermediates from products of CI instability and breakdown by differential solubilization and heat incubation. Furthermore, inhibition of mitochondrial translation reveals an essential step in which two NDUFS3-containing subcomplexes of 100 and 150 kDa require mtDNA-encoded proteins for progression in assembly. Determination of the constitution of all subcomplexes by two-dimensional SDS immunodetection demonstrates that mtDNA-encoded subunit ND1 makes its first appearance in this key step, indicating that this is the first step in the assembly process that requires the availability of mtDNA-encoded proteins.

**EXPERIMENTAL PROCEDURES**

**Generation of an Inducible NDUFS3-AcGFP1 Stable Cell Line**

The NDUFS3 open reading frame sequence (NM_004551; without stop codon) flanked by Gateway® AttB sites (Invitrogen) was created by PCR following the manufacturer’s instructions and cloned into pDONR201 by using Gateway® BP Clonase II enzyme mix (Invitrogen). A Gateway® destination vector was produced by subcloning the BamHI/NotI restriction fragment of pAcGFP1-N1 (Clontech) in frame behind Gateway® reading frame cassette B (Invitrogen) in pcDNA5/FRT/TO (Invitrogen). To obtain an inducible vector containing C-terminally GFP-tagged NDUFS3 (NDUFS3-GFP), the pDONR201-NDUFS3 vector was recombined with the AcGFP1 destination vector using the Gateway® LR Clonase II enzyme mix (Invitrogen). Flip-In T-Rex293 cells (Invitrogen) were stably transfected using Superfect® Transfection Reagent (Qiagen) following the manufacturer’s protocols. Clones with low leakage levels (non-induced expression of the transgene) were selected to obtain steady state NDUFS3-GFP labeling. To induce NDUFS3-AcGFP1 overexpression in these cell lines, doxycycline (Sigma) was added to the medium at a final concentration of 0.1 μg/ml. To reversibly block mitochondrial translation chloramphenicol (Sigma) was used at a final concentration of 40 μg/ml. For blue native analysis, the cells were harvested at the indicated time points.

**Cell Culture and Mitochondria Preparation**

Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco’s modified Eagle’s medium (Biowhittaker) supplemented with 10% fetal calf serum (v/v) and 1% penicillin/streptomycin (v/v) (Invitrogen). HEK293 cells were harvested and washed twice in cold phosphate-buffered saline (Invitrogen). For mitochondrial protein localization, the cells were solubilized (common solubilizations are performed using 1.6 g/g DDM) by 10 min of incubation on ice using 0.3, 0.6, and 1 g of DDM (Sigma-Aldrich)/g of protein in solubilization buffer (1.75 mM 6-aminoacaproic acid; Fluka), 75 mM bis-Tris-HCl (pH 7.0; Fluka). After centrifugation (30 min, 10,000 × g, 4 °C), the supernatant containing solubilized mitochondrial proteins was used for blue native analysis.

**Sample Preparation**

**DDM Solubilizations**—Mitochondrial protein was mildly solubilized (common solubilizations are performed using 1.6 g/g DDM) by 10 min of incubation on ice using 0.3, 0.6, and 1 g of DDM (Sigma-Aldrich)/g of protein in solubilization buffer (1.75 mM 6-aminoacaproic acid; Fluka), 75 mM bis-Tris-HCl (pH 7.0; Fluka). After centrifugation (30 min, 10,000 × g, 4 °C), the supernatant containing solubilized mitochondrial proteins was used for blue native analysis.

**Digitonin Solubilizations**—Mitochondrial protein was solubilized by 30 min of incubation on ice with 4, 8, and 16 g of
digitonin/g of protein in solubilization buffer (30 mM HEPES, pH 7.4, 150 mM potassium acetate (Merck), 10% (w/v) glycerol (Sigma-Aldrich), and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich)). After gentle centrifugation (2 min, 600 × g, 4 °C) the supernatant containing solubilized mitochondrial proteins was used for blue native analysis.

37 °C Incubations—For temperature incubation experiments, mitochondrial protein was mildly solubilized using 1 g of DDM (Sigma-Aldrich)/g of protein in solubilization buffer (1.75 mM 6-aminocaproic acid; Fluka), 75 mM bis-Tris-HCl (pH 7.0; Fluka). After centrifugation (30 min, 10,000 × g, 4 °C) the supernatant containing solubilized mitochondrial proteins was divided into three samples. One sample was kept on ice for 60 min, one was kept at 37 °C for 10 min and on ice for 50 min, and another was kept at 37 °C for 60 min. After this period, mitochondrial lysates were used for blue native analysis.

Blue Native Electrophoresis and In-gel Activity Assays

Blue native gradient gels (5–15%) were cast as described previously (23) and run with 40 or 80 μg of solubilized mitochondrial protein. After electrophoresis, the gels were further processed for in-gel activity assays, Western blotting, or second dimension 10% SDS-PAGE as described in Ref. 23. In addition to CI, in-gel activity assay typically resulted in a previously described smaller band at the bottom half of the gel, which was not shown (19). The proteins were transferred to a PROTRAN® nitrocellulose membrane (Schleicher & Schuell).

SDS-PAGE Analysis

Mitochondrial lysates were prepared as described under “Cell Culture and Mitochondria Preparation” and “DDM Solubilizations.” Subsequently the supernatant was mixed with an equal volume of Tricine sample buffer (Bio-Rad) containing 2% (v/v) 2-mercaptoethanol. The mixture was incubated at room temperature for 60 min. Protein (20 μg/lane) was separated on 10% polyacrylamide gel. The proteins were blotted to PROTRAN® nitrocellulose membrane (Schleicher & Schuell).

Antibodies and ECL Detection

Protein immunodetection was performed using the following primary antibodies directed against EGFP (a gift from Dr. Frank van Kuppeveld, Nijmegen, The Netherlands), NDUFS2 (a gift from Professor Brian Robinson, Toronto, Canada), NDUFA9 (Invitrogen), NDUFS3 (Invitrogen), ND1 (a gift from Dr. Anne Lombes, Paris, France), NDUFB6 (Mitosciences), NDUFA13 (Mitosciences), NDUFA6 (Mitosciences), NDUFA1 (a gift from Professor Immo Scheffler, San Diego, CA), the CII 70-kDa subunit (SDHA; Invitrogen), the CIII Core2 subunit (UQRCR2; Invitrogen), CIV coxII subunit (COXII; Invitrogen), and CV ATPase α (ATPA1; Invitrogen). For generation of the NDUFA2-specific antibody, the rabbits were immunized with peptide CDQVTRALENVLSGKA that was keyhole limpet hemocyanin-coupled using the Imject® maleimide-activated mCKLH kit (Pierce). The obtained antisera was found suitable for specific NDUFA2 detection on Western blots (data not shown). Secondary antibodies used peroxidase-conjugated anti-mouse or anti-rabbit IgGs (Invitrogen). The signal was generated using ECL® Plus (Amersham Biosciences). The sizes of the observed subcomplexes were determined by their relative migration compared with the OXPHOS complexes (CII, 150 kDa; CIII, 600 kDa (dimer); CIV, 240 kDa; CV, 750 kDa), previously described CI subcomplexes (14) and to each other on the same two-dimensional blot.

Quantitative Data Analysis

After Western blotting, luminescent signals were quantified using Image Pro Plus 5.1 (Media Cybernetics, San Diego, CA). The integrated optical density of each band was determined and corrected for background. The resulting numerical values were expressed relative to the CII-SDHA signal to correct for loading differences.

RESULTS

Induction of NDUFS3-GFP Expression Results in GFP Labeling of CI and Six Distinct Subcomplexes—To trace the appearance of CI subcomplexes, we have made stable HEK293 clones containing a tetracycline inducible vector for the NDUFS3 subunit with C-terminal monomeric GFP (AcGFP1). To exclude that the GFP tag disturbs CI assembly, we first investigated the effects of induction on CI assembly and activity (Fig. 1). After 4 h of induction, the NDUFS3-GFP protein was clearly immunodetectable on an SDS Western blot of mitochondrial lysates stained with an anti-NDUFS3 antibody and increased thereafter (Fig. 1A, top panel). Coinciding with the increase of induced protein, endogenous NDUFS3 decreased in time, possibly because of competition, often observed for inducible expression systems (20, 24). Blue native (BN) PAGE followed by Western blot analysis with anti-NDUFA9 antibody and measure-
FIGURE 2. Subcomplexes 2 and 3 accumulate upon inhibition of mitochondrial translation and require mtDNA-encoded subunits for progression in assembly. A, inhibition of mitochondrial translation results in the accumulation of subcomplex 2 and 3. Mitochondrial lysates of inducible NDUFS3-GFP cells were analyzed by blue native gel electrophoresis after inhibition of mitochondrial translation using 40 μg/ml chloramphenicol for time periods of 0, 24, 36, and 60 h under leakage expression of NDUFS3-GFP. After Western blotting, immunodetection was performed for CI (CI-SDHA), CI (CI-NDUFA9), and GFP. Furthermore, CI in-gel activity was assayed (CI-IGA). Complexes 2, 3, and 7 are indicated on the right. B, redistribution of subcomplexes after the release of inhibition of mitochondrial translation. Mitochondrial lysates of inducible cells were analyzed by blue native gel electrophoresis. The cells were treated with 40 μg/ml chloramphenicol for 60 h (0-h time point), followed by washing and growth without chloramphenicol for 4, 8, and 18 h. Shown from top to bottom are GFP-labeled complexes on one-dimensional BN Western blot (incubated anti-GFP), CI in-gel activity (CI-IGA), CI on one-dimensional BN Western blot (incubated anti-NDUFA9), CI on one-dimensional BN Western blot (incubated anti-CI-SDHA). GFP-tagged complexes (1–7) and the position of monomeric NDUFS3-GFP (m) are indicated on the right. Furthermore, the bottom two panels show minute NDUFS3-GFP expression on SDS Western blot (anti-GFP) and endogenous NDUFS3 expression on SDS Western blot (anti-NDUFS3). Minute NDUFS3-GFP expression is also detected using the NDUFS3 antibody and is indicated with an asterisk. C and D, quantitative analysis of the GFP-tagged subcomplexes in A and B, respectively. The integrated optical density (I.O.D.) of the anti-EGFP signals was corrected for background and normalized to the integrated optical density of the CI-SDHA signal. For presentation purposes, the obtained integrated optical density values were divided by a factor of 1000.

with the finding that neither the amount nor the activity of CI is altered, this indicates that the labeled subunit is gradually incorporated into the six subcomplexes and the fully assembled complex without disturbing this process or the activity of the holocomplex.

mtDNA-encoded Proteins Are Required for the Formation of Subcomplexes Larger than Subcomplex 3—To highlight the role of mtDNA-encoded CI subunits (ND subunits) in the appearance of the subcomplexes, production of these subunits was blocked by specifically inhibiting mitochondrial translation with chloramphenicol. Using translation inhibitors such as chloramphenicol or doxycycline has previously been shown to result in strong reduction of mitochondrial translation and thus ND subunit synthesis (14). In the absence of ND subunits, CI assembly will seize at the point at which incorporation of these subunits is essential for formation of the next assembly intermediate. To obtain steady state labeling of NDUFS3-GFP, we specifically selected a clone with low leakage level (noninduced expression) resulting in steady state labeling. Cell lines were incubated with chloramphenicol for 24, 36, or 60 h (Fig. 2A). Prolonged chloramphenicol incubations induced the specific accumulation of NDUFS3-GFP-labeled subcomplexes 2 and 3 and the disappearance of subcomplex 6 and the holocomplex. This accumulation was paralleled by a decrease in the total amount of CI (as revealed using the CI-NDUFA9 antibody) and a reduced in-gel NADH-NBT oxidoreductase activity (CI-IGA) on a one-dimensional BN PAGE gel. Expression of the nuclear DNA-encoded SDHA subunit of CII was not affected by chloramphenicol treatment (CI-SDHA). These results support the conclusion that CI assembly cannot proceed beyond the formation of subcomplexes 2 and 3 in the absence of ND subunits. Subsequent quantification of the ECL signals of Fig. 2A in Fig. 2C demonstrates that whereas subcomplexes 2 and 3 accumulate 10–20-fold, fully assembled CI decreases only 5-fold. This strongly suggests that subcomplexes 2 and 3 predominantly accumulate because of new synthesis. However, we cannot exclude the possibility that subcomplexes 2 and 3 to some
Tracing Complex I Assembly Using Tagged NDUFS3

extent also originate from partial CI breakdown or recycling of its constituents.

Next, we analyzed the effects of releasing chloramphenicol inhibition on the distribution of NDUFS3-GFP-containing subcomplexes. Contrary to the induction pattern shown in Fig. 1, these conditions do not disturb endogenous NDUFS3 expression (Fig. 2B, bottom panel) and prevent possible saturation of the assembly process because no monomeric NDUFS3-GFP accumulates (Fig. 2B, BN PAGE NDUFS3-GFP signal). Following 60 h of chloramphenicol treatment, the cells were washed and chased for 0, 4, 8, and 18 h (Fig. 2B, time points 0, 4, 8, and 18 h). Reinitiation of mitochondrial translation resulted in a gradual return of CI amount and activity (Fig. 2B, panels CI-NDUFA9 and CI-IGA). Remarkably, subcomplex 1 appeared 4 h after chloramphenicol removal, whereas it was absent from Fig. 2A. In addition, a shift in the NDUFS3-GFP signal occurred from subcomplexes 2 and 3 toward subcomplexes 4–6 and CI. Because subcomplexes 2 and 3 decrease, whereas larger intermediates appear, subcomplexes 2 and 3 must represent products of assembly and not instability. Furthermore, these findings imply that ND subunits are required for the formation of subcomplexes 4–6 and fully assembled CI. Quantification of the ECL signals of Fig. 2B in Fig. 2D demonstrates that, directly after translation progresses, the levels of subcomplexes 2 and 3 rapidly decrease and then reach a rather constant level. This suggests that the accumulated subcomplexes 2 and 3 are rapidly incorporated into higher molecular weight complexes upon active translation of mitochondrial DNA-encoded subunits. The comparable kinetics and amounts of the two subcomplexes in Fig. 2 (C and D) suggest that subcomplex 2 reaches a rapid equilibrium with subcomplex 3, which underlines the dynamic nature of the assembly process and allows the possibility that subcomplex 2 originates from subcomplex 3 and vice versa.

Composition of CI Subcomplexes—To confirm that the subcomplexes identified using NDUFS3-GFP are also present in wild type HEK293 cells and to determine the composition of these subcomplexes, we used two-dimensional blue native SDS-PAGE followed by Western blotting and specific immunodetection of CI subunits. Use of two-dimensional SDS-PAGE was essential because this method, in contrast to one-dimensional SDS-PAGE analysis of wild type HEK293 mitochondrial lysates, allowed detection of wild type NDUFS3 protein using a commercially available antibody, likely because the NDUFS3 antibody binds more efficiently to the unfolded protein. Fig. 3A shows that the subcomplexes identified by GFP tagging of the NDUFS3 subunit (Fig. 2B) are also present in two independent isolations of wild type HEK293 mitochondria (see also Table 1). To analyze the composition of these complexes, we performed immunodetection of nine CI subunits (Fig. 3A). Subcomplex 1 is poorly visible, but subcomplexes 2–6 and CI (indicated with 7) can be clearly discriminated. Alignment between the different subunits shows that subcomplexes 2–6 and CI contain the NDUFS2 and NDUFS3 subunits with the addition of ND1 in subcomplexes 4–7 and the addition of NDUFA13 in subcomplex 5–7. Close inspection furthermore reveals the “appearance” of an additional subcomplex termed a1 (appearing subcomplex 1) that only contains membrane subunits ND1, NDUFB6, NDUFA13, NDUFA6, and NDUFA1. Furthermore, the level of CI-NDUFA9 and CI-NDUFA6, both absent from Fig. 2B, time points 0, 4, and 8 h, appeared 4 h after chloramphenicol removal, whereas it was absent from Fig. 2A. In addition, a shift in the NDUFS3-GFP signal occurred from subcomplexes 2 and 3 toward subcomplexes 4–6 and CI. Because subcomplexes 2 and 3 decrease, whereas larger intermediates appear, subcomplexes 2 and 3 must represent products of assembly and not instability. Furthermore, these findings imply that ND subunits are required for the formation of subcomplexes 4–6 and fully assembled CI. Quantification of the ECL signals of Fig. 2B in Fig. 2D demonstrates that, directly after translation progresses, the levels of subcomplexes 2 and 3 rapidly decrease and then reach a rather constant level. This suggests that the accumulated subcomplexes 2 and 3 are rapidly incorporated into higher molecular weight complexes upon active translation of mitochondrial DNA-encoded subunits. The comparable kinetics and amounts of the two subcomplexes in Fig. 2 (C and D) suggest that subcomplex 2 reaches a rapid equilibrium with subcomplex 3, which underlines the dynamic nature of the assembly process and allows the possibility that subcomplex 2 originates from subcomplex 3 and vice versa.

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Stability of CI Subcomplexes—In principle, BN analysis reveals native CI subcomplexes after detergent-mediated isolation. To determine whether the observed subcomplexes originate from partial CI solubilization, we investigated their presence in wild type HEK293 mitochondria under different, increasingly stringent, concentrations of DDM and digitonin, as shown for the OXPHOS proteins indicated at the top of the panels. CI is indicated with the thick arrow (also with 7), additionally appearing subcomplexes are indicated with a1 and a2.

The occurrence and composition of subcomplexes in each of the three conditions (Fig. 4, A and B) is summarized in Table 2.

DISCUSSION

A better understanding of how nuclear- and mtDNA-encoded subunits of human mitochondrial CI are combined not only aids elucidation of how macromolecular assemblies are formed in the mitochondrial inner membrane but also helps to clarify the molecular background of many unexplained CI deficiencies. Although the finding of subassemblies in CI-deficient patients hints toward the existence of distinct steps in the assembly pathway, proper interpretation of such data requires detailed insight into assembly under normal conditions. Especially the dynamics, rate-limiting steps, and specific assembly proteins involved in this process might deliver important clues.

In this study, we have used an inducible NDUFS3-GFP HEK293 cell line to investigate the CI assembly process. We show that upon induction six GFP-labeled subcomplexes appear in addition to GFP-labeled CI itself. Importantly, this labeling procedure did not alter expression and/or activity of the fully assembled complex. The same subcomplexes were detected in mitochondria of wild type HEK293 cells, excluding the possibility that they were labeling artifacts. We furthermore show that inhibition of mitochondrial translation under condi-
TIONS OF STEADY STATE “LEAKAGE” LABELING RESULTED IN ACCUMULATION OF THE SMALLER SUBCOMPLEXES 2 AND 3, BUT NOT 1, AND THE DISAPPEARANCE OF SUBCOMPLEX 6 AND CI. THIS FINDING UNAMBIGUOUSLY DEMONSTRATES THE REQUIREMENT OF MITOCHONDRIAL TRANSLATION PRODUCTS FOR THE ASSEMBLY PROCESS TO PROCEED BEYOND SUBCOMPLEXES 2 AND 3. BECAUSE RIGOROUS TESTING DEMONSTRATED THAT IT IS HIGHLY UNLIKELY THAT SUBCOMPLEXES 2 AND 3 ARE ISOLATION ARTIFACTS, THEIR ACCUMULATION DURING INHIBITION OF MITOCHONDRIAL TRANSLATION SHOWS THAT THESE TWO SUBCOMPLEXES ARE TRUE ASSEMBLY INTERMEDIATES.

The existence of CI subassemblies in the absence of mtDNA-encoded CI subunits has been described previously (19, 26–29). In our previous assembly study, we analyzed 143B cells that were depleted of mitochondria DNA (rho-0 cells) and found that NDUFS3 was present in three subcomplexes very similar to subcomplexes 1, 2, and 3 described in the present study (14). We termed these subcomplexes H, G, and F. We now learn that subcomplex H is not monomeric NDUFS3 but already includes the NDUFS2 subunit. Intriguingly, this subcomplex appears not only during induction (Fig. 1B) but also after degradation (Fig. 4B) of CI, which makes it difficult to determine its exact origin. Because it does not accumulate after chloramphenicol inhibition (Fig. 2A), it most likely does not originate directly from subcomplexes 2 and 3. It may originate from larger assemblies, but as yet its exact origin remains enigmatic.

Based on their composition and size and their accumulation in the absence of mitochondrial translation, we propose that subcomplexes 2 and 3 represent early stages in the assembly process. It is conceivable that assembly reflects the conserved evolutionary structural relationship of CI subunits via the formation of distinct modules (14, 30). The evolutionary conserved relation between the NDUFS2 and NDUFS3 as fused proteins in several bacterial species and as part of the hydrogenase module of CI (31, 32) corresponds well with their early association during the assembly process. The hydrogenase module is important early in assembly, as illustrated by the finding that the overexpressed dehydrogenase module of E. coli NDH-1 is only incorporated when the homologues of the NDUFS3, NDUFS2, and NDUFS7 subunits are also overexpressed (31). Based on their size of ~100–150 kDa, subcomplexes 2 and 3 likely include additional subunits, such as NDUFS7 and NDUFS8. Prommeenate and co-workers (33) describe the existence of distinct subcomplexes containing the homologues of NDUFS2, NDUFS3, and NDUFS7 subunits in cyanobacteria. In addition, Bourges and co-workers (34) have co-immunoprecipitated subcomplexes containing iron-sulfur fraction subunits NDUFS2, NDUFS3, and NDUFS7 using anti-NDUFS3 antibody in wild type and rho-0 143B osteosarcoma cells and in cell lines devoid of ND4 and ND5.

The accumulation of subcomplexes 2 and 3 after inhibition of mitochondrial translation and the specific appearance of larger subcomplexes after releasing this inhibition suggest that mtDNA-encoded subunits are incorporated after subcomplex 3 is formed. The additional presence of an ND subunit in subcomplexes 4 and 5 (Fig. 3B), which reappear after assembly has resumed (Fig. 2B), may represent expansion of the hydrogenase module and anchoring to the mitochondrial inner membrane. Unfortunately, because of the lack of proper antibodies, we were not able to investigate the presence of other ND subunits in subcomplexes 4 and 5 except ND1. Membrane subunit ND1 is one of the first subunits clearly expressed after release of inhibition of mitochondrial translation in 143B osteosarcoma

TABLE 2

Overview of the distribution of the NDUFS3 subcomplexes per solubilization condition

| Subcomplex 1 | Subcomplex 2 | Subcomplex 3 | Subcomplex 4 | Subcomplex 5 | a2 | a1 | Subcomplex 6 | Subcomplex 7 (CI) | S1/S2 |
|--------------|--------------|--------------|--------------|--------------|----|----|--------------|-------------------|-------|
| DDM solubilization | | | | | | | | | |
| NDUFS2 | A/D | X | X | X | X | X | X | X | D |
| NDUFA9 | | X | X | X | X | X | X | X | X |
| NDUFS3 | A/D | X | X | X | X | X | X | X | X |
| ND1 | | X | X | A | A | X | X | X | X |
| NDUFB6 | | X | X | X | X | X | X | X | X |
| NDUFA13 | | X | X | A | A | X | X | X | X |
| NDUFA6 | | X | X | A | A | X | X | X | X |
| NDUFA2 | | X | X | A | A | X | X | X | X |
| NDUFA1 | | X | X | A | A | X | X | X | X |
| Digitonin solubilization | | | | | | | | | |
| NDUFS2 | A/D | X | X | X | X | X | X | X | X |
| NDUFA9 | | X | X | X | X | X | X | X | X |
| NDUFS3 | A/D | X | X | X | X | X | X | X | X |
| ND1 | | X | X | A | A | X | X | X | X |
| NDUFB6 | | X | X | X | X | X | X | X | X |
| NDUFA13 | | X | X | A | A | X | X | X | X |
| NDUFA6 | | X | X | A | A | X | X | X | X |
| NDUFA2 | | X | X | A | A | X | X | X | X |
| NDUFA1 | | X | X | A | A | X | X | X | X |
| 37 °C solubilization | | | | | | | | | |
| NDUFS2 | A | X | X | D | D | A/D | D | D | D |
| NDUFA9 | | X | X | D | D | A/D | D | D | D |
| NDUFS3 | A | X | X | D | D | A/D | D | D | D |
| ND1 | | D | D | A | A | X | X | X | X |
| NDUFB6 | | D | D | A | A | X | X | X | X |
| NDUFA13 | | D | D | A | A | X | X | X | X |
| NDUFA6 | | D | D | A | A | X | X | X | X |
| NDUFA2 | | D | D | A | A | X | X | X | X |
| NDUFA1 | | D | D | A | A | X | X | X | X |
cells (14), and absence of ND1 and ND6 results in severe assembly disturbances (29, 35, 36). In contrast, lack of ND4 or ND5 does not have such a “null” effect on assembly. For example, in Chlamydomonas CI, loss of ND4 or ND4/ND5 still allows formation of substantial portion of CI (36). Also in humans, absence of ND4 or ND5 still allows formation of nuclear DNA-encoded subcomplexes (34), possibly even displaying activity (27–29). ND3 mutation and absence of ND5 seem to have an effect on activity rather than assembly (28, 37). Therefore, as opposed to ND3, ND4, and ND5, it appears that ND1 and ND6 are incorporated early in assembly and that the appearance of ND1 in subcomplex 4 may represent the first incorporation of mtDNA-encoded CI subunits, in line with what is observed after the chloramphenicol inhibition experiments (Fig. 2, A and B).

As opposed to the detergent-stable intermediates 1–6, differential detergent solubilization results in subcomplex a1 as a product of CI instability (Fig. 3A). That this intermediate most likely does not represent an assembly intermediate is supported by its composition in relation to subcomplexes 4 and 5. Subcomplexes 4 and 5 contain at least the NDUFS2, NDUFS3, and ND1 subunits (Table 2). Subcomplex a1, which migrates at a higher molecular weight, consists primarily of membrane arm CI subunits, including ND1 but not NDUFS2 and NDUFS3 (Table 2). This means that subcomplex a1 has either specifically “lost” two of its subunits or is of different origin altogether. This discrepancy illustrates that subcomplex a1 is not simply a successive intermediate of CI assembly and that different subcomplexes seem to have different origins, possibly in assembly but possibly also in instability and breakdown, as also demonstrated for cyanobacterial CI (33).

Additional heat incubation at 37 °C shows that membrane arm subcomplex a1 also results from breakdown and is stable, possibly protected from proteases because of its lipid environment. It appears in conjunction with subcomplex a2, which consists of peripheral arm subunits and is rapidly broken down in time. Judging from their size and composition, observation of subcomplexes a1 and a2 points toward fractionation of CI into its membrane and peripheral arms. The disappearance of subcomplexes 4 and 5 and intensification of subcomplexes 1–3 after incubation at 37 °C remains puzzling (Fig. 4C). This finding suggests that subcomplexes 1–3, under certain conditions, can originate from subcomplexes 4 and 5 or a2.

Somewhat to our surprise, the NDUFA9 subunit is not detected in any of the observed subcomplexes 1–6, which may illustrate its late assembly into CI. This is supported by the absence of this subunit in accumulated intermediates of nearly the size of CI in NDUFS6 and B17.2L patients (38, 39). The presence of the NDUFA1 subunit (homologue of the bovine MWFE subunit) in CI subcomplexes was not investigated previously. In Chinese hamster cells, this subunit is speculated to serve as a membrane anchor to which membrane arm subunits attach during CI assembly (20). Although NDUFA1 is detected in a membrane arm fraction of CI (subcomplex a1), it is consistently not detected in subcomplexes 4 and 5. It therefore seems that the hydrogenase core subcomplex has already acquired at least one membrane arm subunit (ND1) prior to the addition of NDUFA1. Two other previously unstudied subunits in relation to their presence in subassemblies are NDUFA2 and NDUFA13. The co-migration of the NDUFA2 subunit with peripheral arm subunits NDUFS2, NDUFS3, and NDUFA9 in subcomplex a2 (that appears during breakdown) is in line with its fractionation with the 1A fragment (40). The NDUFA13, or GRIM19, subunit of CI co-migrates with membrane arm subunits and, in addition to its presence in subcomplex a1, shows a particular presence in subcomplex 5 (consisting of the NDUFS2/NDUFS3/ND1/NDUFA13 subunits). It seems that a subunit that is considered “accessory” and involved in regulation of cell death, is already
present in a smaller structure containing the conserved “basic” core subunits of CI (41).

In conclusion, subcomplexes 1–6 represent equilibria in the assembly pathway of CI and highlight important steps in the process. It is likely that more assembly intermediates exist but were not detected using our set of antibodies or have escaped detection because incorporation during assembly occurs too rapidly. The existence of these particular intermediates could be demonstrated in CI-deficient patient cell lines, when assembly disturbance leads to accumulation of an assembly step. We have updated our previous assembly model to incorporate our findings, which supports modular CI assembly on the basis of evolutionary conservation (Fig. 5). Future analysis of the exact composition of each subcomplex will allow further verification and refinement of existing CI assembly models that, in turn, will aid the understanding of many yet unexplained CI assembly disturbances.

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