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Supercomplexes and subcomplexes of mitochondrial oxidative phosphorylation

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Abstract

Dimerization or oligomerization of ATP synthase has been proposed to play an important role for mitochondrial cristae formation and to be involved in regulating ATP synthase activity. We found comparable oligomycin-sensitive ATPase activity for monomeric and oligomeric ATP synthase suggesting that oligomerization/monomerization dynamics are not directly involved in regulating ATP synthase activity. Binding of the natural IF_1 inhibitor protein has been shown to induce dimerization of F_1-subcomplexes. This suggested that binding of IF_1 might also dimerize holo ATP synthase, and possibly link dimerization and inhibition. Analyzing mitochondria of human rho zero cells that contain mitochondria but lack mitochondrial DNA, we identified three subcomplexes of ATP synthase: (i) F_1 catalytic domain, (ii) F_1-domain with bound IF_1, and (iii) F_1-c subcomplex with bound IF_1 and a ring of subunits c. Since both IF_1 containing subcomplexes were present in monomeric state and exhibited considerably reduced ATPase activity as compared to the third subcomplex lacking IF_1, we postulate that inhibition and induction of dimerization of F_1-subcomplexes by IF_1 are independent events. F_1-subcomplexes were also found in mitochondria of patients with specific mitochondrial disorders, and turned out to be useful for the clinical differentiation between various types of mitochondrial biosynthesis disorders. Supramolecular associations of respiratory complexes, the "respirasomes", seem not to be the largest assemblies in the structural organization of the respiratory chain, as suggested by differential solubilization of mitochondria and electron microscopic analyses of whole mitochondria. We present a model for a higher supramolecular association of respirasomes into a "respiratory string".

Keywords: Dimeric ATP synthase; Oligomeric ATP synthase; Supercomplex; Supramolecular organization; Respiratory chain; Respirasome

1. Introduction

F_1F_O-ATP synthase, also named mitochondrial complex V, often is described as an assembly of two major building blocks, a hydrophobic F_O part and a hydrophilic F_1 subcomplex where synthesis of ATP from ADP and phosphate takes place. Pioneering electron microscopic analyses of *Paramecium multimicronucleatum* mitochondria performed in 1989 by Richard D. Allen et al. [1] identified in tubular cristae of the inner mitochondrial membrane "F_1 complexes that are arranged as a double row of particles". In the light of present day knowledge this supramolecular structure can be interpreted as a helix formed from dimeric ATP synthase building blocks. Some years later, Allen [2] presented a model for membrane tubulation by supramolecular association of proton pumps (F- and V-ATPases) starting with a lateral association of single complexes, i.e. with formation of dimeric ATP synthase. Further association of dimeric ATP synthase into tilted helical structures would then form a rigid arc that protrudes from the planar membrane surface carrying the membrane with it and thus would initiate formation of tubular cristae. If this nucleation process of ATP synthase association was under the control of some unknown inducing factor, cristae formation of the mitochondrial inner membrane might in turn be used for regulating metabolic activity of mitochondria. Because oxidative phosphorylation relies on rapid diffusion of ions and substrates to sites of transport or reaction in the inner mitochondrial membrane, the number and shape of the contacts of the cristae with the intermembrane space (cristae junctions), could regulate ATP synthesis [2,3].

Some years later, in 1998, when dimeric yeast mitochondrial ATP synthase containing three dimer-specific subunits was

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isolated [4], the majority of researchers in the field of mitochondrial bioenergetics and ATP synthase apparently were not aware of the observations by Allen and coworkers [1,2], as reflected in the initial skepticism about the dimeric state of ATP synthase. Therefore, important issues relating to the supramolecular organization of ATP synthase have not been addressed or answered so far: Is yeast and mammalian ATP synthase organized in higher oligomeric structures like in *Paramecium* mitochondria? Which proteins can be found in the monomer/monomer and dimer/dimer interfaces of dimeric and oligomeric ATP synthase, respectively? Can dimerization/oligomerization of ATP synthase immediately affect the catalytic activity of ATP synthase or do these structural changes indirectly affect the metabolic state of cells, e.g. by controlling cristae formation or by conferring stability to the ATP synthase enzyme? Does binding of the natural inhibitor proteins of ATP synthase, INH1 in yeast and the homologous IF_1 in mammalian mitochondria, induce dimerization of holo ATP synthase, similar to the experimentally verified dimerization of bovine F_1-subcomplexes by IF_1? Does inhibition correlate with dimerization of F_1-subcomplexes and ATP synthase?

Finally, the pioneering work of Allen et al. [1] can stimulate further research on the supramolecular organization of respiratory chain complexes, since two different types of helical bands or strings winding around tubular cristae have been observed. The first helical band clearly contains a double row of F_1-particles as described above but what is the identity of the considerably larger particles seen in the second helical string that might contain dimeric complex I as suggested by Allen? Recent evidence supports the view that the second helical string, the "respiratory string", contains respiratory supercomplexes in regular intervals.

2. Dimeric and oligomeric mitochondrial ATP synthase

2.1. Isolation of dimeric and oligomeric ATP synthase

Dimeric ATP synthase has been isolated from various sources including mitochondria from mammalia, algae, higher plants [4–8], and also from chloroplast membranes of *Chlamydomonas reinhardtii* [9]. ATP synthase from *Chlamydomonas reinhardtii* mitochondria is especially detergent-stable. It has been isolated as a stable dimer in the presence of the detergent dodecylmaltoside [8]. No monomeric form has been identified. In contrast, the associate of two monomers is rather detergent-sensitive in most other species but often the dimeric state could be retained by using one of the mildest detergents for membrane solubilization, namely digitonin [5], or by applying low amounts of other mild detergents like Triton X-100 [4]. In principle, these solubilization conditions can be used in combination with conventional protein separation techniques like size exclusion chromatography or ultracentrifugation but the resolution of these separation techniques is poor for large membrane protein complexes. Therefore, blue-native PAGE (BN-PAGE [10,11]), a high resolution electrophoretic technique, has been preferentially applied. This technique uses negatively charged Coomassie-dye which binds to hydrophobic surface areas of solubilized membrane proteins. The imposed negative charge pulls the proteins to the anode during electrophoresis irrespective of the proteins' intrinsic isoelectric point. In some cases, minor amounts of ATP synthase forms larger than the dimer have been identified in yeast mitochondria using BN-PAGE, [7,12] but also in mammalian mitochondria where minor bands of oligomeric ATP synthase often were detectable but not explicitly mentioned. Since the combination of neutral detergent and negatively charged Coomassie-dye in BN-PAGE can potentially dissociate oligomeric ATP synthase into dimeric and monomeric forms, we applied clear-native PAGE (CN-PAGE [13]) for separation of membrane protein complexes. This technique uses the same cathode and anode buffers as BN-PAGE except that no Coomassie-dye is added to samples and buffers. CN-PAGE is a very useful separation technique whenever Coomassie-dye interferes with techniques required to further analyze the native complexes, e.g., determination of in-gel catalytic activities [13,14] or efficient microscale separation of membrane protein complexes for fluorescence resonance energy transfer (FRET) analyses [15]. As demonstrated in Fig. 1, in particular, the combination of digitonin and CN-PAGE can better retain labile supramolecular assemblies of membrane protein complexes that usually are dissociated under the conditions of BN-PAGE. Rat heart mitochondrial sediments were dissolved with digitonin and identical samples were then applied to BN-PAGE (Fig. 1a) and CN-PAGE (Fig. 1b). Direct comparison of the two gels revealed that the resolution of BN-PAGE was considerably higher compared to CN-PAGE, as judged from the sharpness of bands for individual respiratory complexes and supercomplexes (assigned 0, 1, 2 in Fig. 1a). However, tetrameric and hexameric complex V (V_T and V_H) were hardly detectable in BN-gels (for molecular mass calibration and assignment of oligomeric states of complex V see ref. [13]). The observation of higher amounts of tetrameric and hexameric complex V in CN-gels (V_T and V_H in Fig. 1b) seemed no strong argument in favor of a physiological oligomeric state of complex V, since protein complexes that are applied as dilute samples for CN-PAGE become highly concentrated protein bands within the sample wells and may aggregate [13]. In contrast to BN-PAGE, no annulus of negative Coomassie-dye charges around the complexes can protect against protein aggregation. Therefore, artificial association of membrane protein complexes during CN-PAGE cannot be excluded [13]. Reducing the digitonin amounts for membrane solubilization to 25% of the amounts used for Fig. 1a, b had no severe effect on the amount of solubilized complex V (Fig. 1c, d). Under these low digitonin conditions oligomeric complex V was also observed following BN-PAGE which supports the view that ATP synthase forms oligomeric structures in the membrane. According to our recent analysis of masses and oligomeric states of complex V [13], only even numbered complex V oligomers exist in reasonable amounts. This is in contrast to another analysis of mammalian tissues that assigns a trimeric state to a highly abundant form of complex V [16].

2.2. Catalytic activity of various oligomeric states of ATP synthase

Using chromatographic techniques and mild non-ionic detergents like dodecylmaltoside or Triton X-100, the mitochondrial

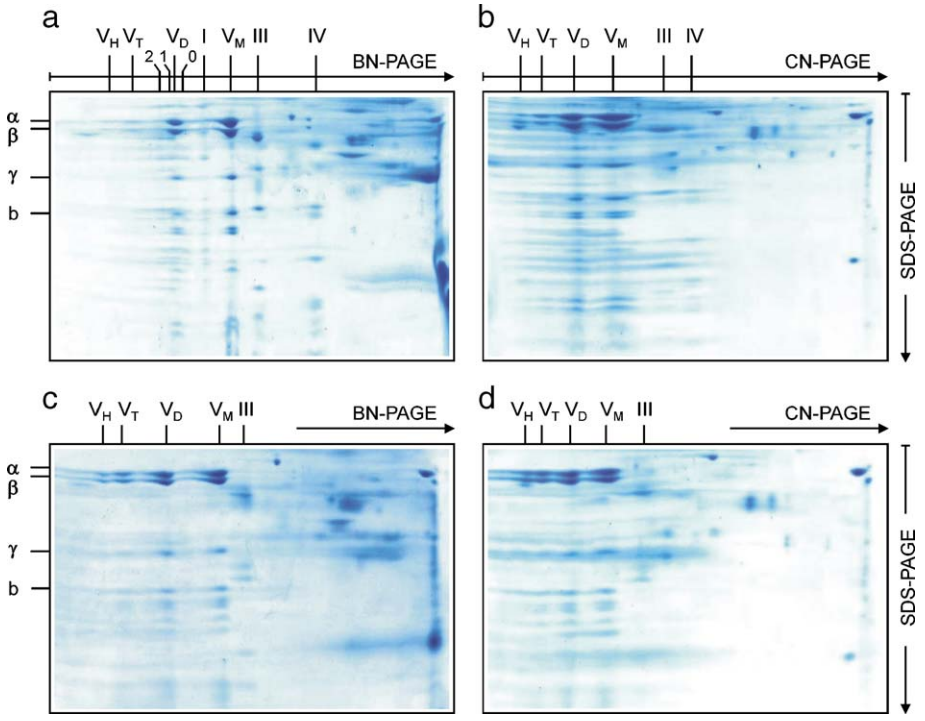

Fig. 1. Two-dimensional separation of oligomeric ATP synthase from rat heart mitochondria using 1-D native electrophoretic techniques and 2-D SDS-PAGE. (a) Solubilization by standard digitonin amounts and separation by BN-PAGE. (b) Solubilization by standard digitonin amounts and separation by CN-PAGE. (c and d) Similar to a and b, but using low digitonin (25% of the standard amounts used in a and b). I, III, IV, respiratory complexes I, III, and IV; V_M, V_D, V_T, V_H, monomeric, dimeric, tetrameric, and hexameric complex V, respectively; (0, 1, 2), respiratory supercomplexes containing monomeric complex I, dimeric complex III, and zero (0), or one (1), or two (2) copies of complex IV. Subunit b (b) and the α, β, and γ subunits of ATP synthase (complex V) are assigned.

ATP synthase from yeast and mammalian tissues is always isolated in monomeric form. Using digitonin for solubilization, dimeric or higher oligomeric ATP synthase seemed to be preserved in the first purification steps but all attempts to further purify dimeric or oligomeric ATP synthase failed (Schägger, H., unpublished). An isolation technique that separates various oligomeric forms like BN-PAGE therefore was highly welcome. Unfortunately, the in-gel ATP hydrolysis activity of complex V in BN-gels was so low [14,17] that overnight incubation in the slightly alkaline assay buffer was required to visualize the ATPase activity of complex V by the appearance of a white lead phosphate precipitate. Another important disadvantage of the ATPase assay in BN-gels was the insensitivity towards the inhibitor oligomycin so that it remained unclear whether the observed low rate of ATPase activity corresponded to complex V activity or rather reflected ATPase activity of free F_1-domains that continuously dissociate from holo-complex V in the presence of Coomassie-dye at slightly alkaline pH. Regarding these disadvantages of BN-PAGE, it was a major progress when digitonin and CN-PAGE could be used for in-gel separation of monomeric and dimeric yeast ATP synthase, since the ATPase assay in CN-gels was at least 10 times faster than in BN-gels and fully oligomycin-sensitive, indicating that only holo-ATPase and not dissociated F_1-domains was analyzed [14]. Using digitonin and CN-PAGE, the oligomycin-sensitive catalytic activities of monomeric and dimeric ATP synthase were comparable [13]. This strongly suggested that the physiological ATP synthase activity is not regulated by monomer/dimer dynamics of ATP synthase. Furthermore, formation of the yeast ATP synthase dimer did not require the ATPase inhibitor protein INH1. This has been demonstrated using BN-PAGE for the separation of complex V monomers and dimers and ATPase activity measurement in isolated mitochondria [18]. How can these data suggesting a permanent inhibitor protein independent dimeric state be reconciled with the data of Cabezon et al. [19,20] who showed that the natural IF_1 inhibitor protein of bovine ATP synthase can induce dimerization of monomeric F_1-domains? Of course, it seems tempting to speculate that IF_1, a homologue of yeast INH1, might be involved also in dimerization of holo-ATP synthase thereby potentially regulating catalytic activity. Are there major structural and functional differences between yeast and mammalian ATP synthase? To our knowledge, there is only one report suggesting that there might be in fact a major functional difference between yeast and bovine ATP synthase. Tomasetig et al. [21] reported that, in contrast to the monomeric state, dimeric complex V from bovine mitochondria is inactive, and that this inactive dimeric state is independent of the degree of IF_1 binding. However, our functional analyses of various oligomeric states of rat heart complex V indicated that the specific activities of monomeric, dimeric, tetrameric, hexameric and octameric complex V were comparable and oligomycin-sensitive [13]. Yeast INH1 and mammalian IF_1 inhibitor proteins usually are partly removed from their binding sites on monomeric and dimeric ATP synthase during BN-PAGE [18,22]. However, IF_1-binding to higher oligomeric states of mammalian ATP synthase has not yet been analyzed. Substoichiometric binding of inhibitor protein to these oligomers exhibiting uniform catalytic activities would suggest that formation of large supramolecular structures from ATP

synthase dimers is not immediately involved in regulating ATP synthase activity. However, in spite of that, the cells' metabolic state, generation and loss of mitochondrial cristae, and shrinking and swelling of mitochondria may correlate with formation and disintegration of supramolecular ATP synthase structures.

3. Monomer/monomer and dimer/dimer interfaces

Two subunits of yeast ATP synthase, subunits e and g, have initially been suggested to be essential for the formation of dimeric ATP synthase, since the dimer could not be detected following BN-PAGE upon deletion of either one of the two genes for subunits g and e [4]. Later, some experimental evidence suggested that subunits g and e might not be essential for formation but for stabilization of the dimer so that it can be isolated by BN-PAGE using either low Triton X-100 or relatively high digitonin for solubilization [4,5]. Subunits g and e have also been proposed to be important for the generation of cristae morphology [7,12] although lack of these subunits does still allow close neighborship of two ATP synthase monomers in the membrane. This became apparent from FRET analyses using subunit b-GFP and subunit b-BFP fusions to demonstrate direct physical interactions of two monomers in membranes of subunit e deletion mutants [23]. Direct physical interactions of two monomers via subunit b have also been shown by crosslinking of two ATP synthase monomers in the membranes of the parental yeast strain [24] and subunit e or subunit g deletion strains [7]. Also, crosslinking of two ATP synthase monomers via subunit i has been observed in yeast mitochondrial membranes [25].

Subunits e and g have clearly been identified as the major dimer-stabilizing subunits in the monomer/monomer interface [4,12,26–28]. Since not only dimeric but also oligomeric states of yeast ATP synthase have been observed [7,12,13,16], it has been suggested that crosslinks involving subunit b and/or i might not play a role for the monomer/monomer interactions in the initial dimer but for the dimer/dimer interactions in oligomeric ATP synthase [7,23–25]. Although the number and identity of all protein subunits involved in the monomer/monomer interface is not exactly known, it seems clear that at least subunits e and g are directly involved in linking the membrane domains of the two monomers together [4,12,26–28]. Both subunits cooperate to stabilize the detergent-sensitive interaction of two membrane domains under the conditions of BN-PAGE using digitonin or low Triton X-100. The view that the hydrophobic domains are involved in the monomer/monomer interface is also supported by two recent electron microscopic single particle analyses of dimeric ATP synthase from bovine heart mitochondria and from mitochondria of the alga *Polytomella* [29,30]. In both cases, the ATP synthase monomers were linked via F_O-domains that joined at an angle of about 40° [29] or about 70 ° [30], respectively, to form conical structures. Surprisingly, a second stalk was not identified in the bovine structure showing closely opposed F_1-domains, whereas it was clearly observed in the *Polytomella* structure where the F_1-domains were completely separated. May this indicate that the two structures are views from different sides? Is the interface seen in the *Polytomella* structure the one that we called the monomer/monomer interface in the initial dimer? Does the bovine structure, in contrast, exhibit the dimer/dimer interface? It should be noted here that the bovine and the algal ATP synthase as well, have not been analyzed following native extraction of the complexes from BN-gels, as the presentation of BN-gels might suggest [29,30], but immediately following the isolation with density gradients, i.e., in the presence of detergents but in the absence of Coomassie-dye. Therefore, it seems possible that dimeric structures with different interfaces, abbreviated as monomer/monomer and dimer/dimer interfaces, have been isolated by the density gradients and analyzed by electron microscopy. Control BN-gels that indicate dimers in both papers [29,30] presumably cannot discern how the two monomers are associated, since all experience with BN-PAGE suggests that BN-PAGE does not dissociate supramolecular structures if samples are applied with detergent concentrations close to the critical micelle concentration.

The interface between F_1-domains in the bovine structure seems especially interesting, since the IF_1 inhibitor protein is potentially bridging the two hydrophilic domains [29]. Other candidates for the dimer/dimer interface are subunits b and i. Instead, these two proteins may reside in the vicinity of subunits e and g thus contributing to the monomer/monomer interaction.

4. Subcomplexes of ATP synthase

Following discussion of oligomeric and dimeric ATP synthase and their potential interfaces we will now focus on selected aspects of assembly and disassembly of monomeric ATP synthase.

4.1. Biochemical analysis of F_1-subcomplexes

We used human rho zero cells [31] that contain mitochondria but lack mitochondrial DNA to search for the largest subcomplex of ATP synthase that is assembled in the absence of the two genes for the mitochondrially encoded subunits ATP6 and ATP8 that are also named subunits a and A6L [22]. Using BN-PAGE we identified three types of F_1-subcomplexes: (1) F_1 catalytic domain (F_1 x; mass around 370 kDa) containing subunits α, β, γ, δ, and ε, (2) F_1 catalytic domain with bound inhibitor protein IF_1 (F_1 y; mass around 400 kDa), and (3) F_1-c subcomplex, i.e., F_1 catalytic domain with bound ring of c-subunits and bound inhibitor protein IF_1 (F_1 z; mass around 470 kDa). We conclude that F_1-c subcomplex is a rather stable assembly intermediate or dead-end result of aborted complex V assembly in mammalian mitochondria [22]. In fact, subunit c had been identified as the first F_O-subunit that associates with the F_1-domain during assembly of yeast and *Escherichia coli* ATP synthase [32–34]. This interaction between an oligomeric ring of subunits c and soluble F_1-domain generates a rather stable F_1-c subcomplex. In yeast, this subcomplex was isolated by dissociation of other subunits from holo-ATP synthase and crystallized [35].

In order to compare specific ATP hydrolysis activities, we used in-gel ATPase assays and estimated complex V protein amounts by Coomassie-staining or Western blots [22]. The smallest F_1 subcomplex (F_1 x) not containing bound IF_1 inhibitor showed highest specific ATPase activity. Corresponding lead phosphate precipitates for the smallest F_1 subcomplex were detectable after 1 h incubation in assay buffer whereas overnight extension of the assay

was required for the two other F_1-bands. The detection of some ATPase activity for F_1 subcomplexes y and z, in spite of the presence of bound inhibitor protein, is explained by the extended overnight incubation in slightly alkaline buffer and a presumed continuous dissociation of IF_1-inhibitor protein [22]. We conclude that IF_1 inhibitor protein (in unknown state of oligomerization) can inhibit monomeric F_1-subcomplex and does not require dimerization of F_1-domains. Thus, dimerization of F_1-domains and inhibition of F_1-domains by IF_1 inhibitor protein are independent effects.

4.2. Subcomplexes of human ATP synthase mark mitochondrial biosynthesis disorders

To analyze whether deficiency of mtDNA encoded subunits had similar effects in human mitochondrial disorders, we used biopsy samples of patients with mtDNA depletion syndrome (MDS [36–40]) which is a phenotypically heterogeneous group of disorders characterized by reduced but, in contrast to rho zero cells, not completely missing mtDNA. Furthermore, we used biopsy samples of patients with other mitochondrial biosynthesis disorders such as neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) and maternally inherited Leigh's syndrome (MILS) that are associated with mutations in one of the two mitochondrial genes of ATP synthase, namely, the ATP6 gene coding for subunit 6, also named subunit a [41–43].

Not surprisingly, we observed large amounts of three types of F_1-subcomplexes also in MDS and NARP/MILS patients, and in biopsy samples of patients with unknown genetic defects, as shown in Fig. 2, but not in patients with a variety of different mitochondrial tRNA mutations. By quantifying the F_1-subcomplexes and also the other oxidative phosphorylation complexes in parallel, we were able to discriminate different classes of defects of mitochondrial biosynthesis, as described in detail [22]. Briefly: Class 1 comprises NARP/MILS syndromes that are characterized by accumulating F_1-subcomplexes, whereas the other mitochondrial complexes are normal. Class 2 comprises MDS syndromes that are associated with accumulating F_1-subcomplexes and reduced amounts of complexes I and IV. Class 3 comprises tRNA mutations like mitochondrial encephalomyopathy with lactic acidosis and stroke-like symptoms (MELAS) or myoclonus epilepsy with ragged red fibers (MERRF) that are characterized by reduced amounts of complexes I and IV, as with MDS. However, the amounts of F_1-subcomplexes are negligible. Class 4 comprises EFG1 deficiency and deficiencies of other factors involved in mitochondrial translation. The patterns of oxidative phosphorylation complexes in EFG1 deficiency seem to be similar to MDS but complex III is also reduced. This has been deduced from one-dimensional BN-gels in the work of Coenen et al. [44] but a two-dimensional electrophoretic analysis is still missing. Other so far unknown genetic alterations, potentially in one of the numerous factors involved in mitochondrial translation [45], are expected to produce similar two-dimensional protein patterns of oxidative phosphorylation complexes, as summarized above for EFG1 deficiency or MDS. An example for one patient who had normal mtDNA content and no genetic alteration of EFG1 is shown in Fig. 2. This type of mitochondrial biosynthesis disorder seems not to be rare, since we observed similar two-dimensional protein patterns in about 5% of all patients with mitochondrial encephalomyopathies that we had analyzed. Despite of some unsolved problems, the suggested electrophoretic approach currently seems to be the most straightforward approach to localize established and novel defects in genes for oxidative phosphorylation complexes and factors required for mitochondrial biosynthesis.

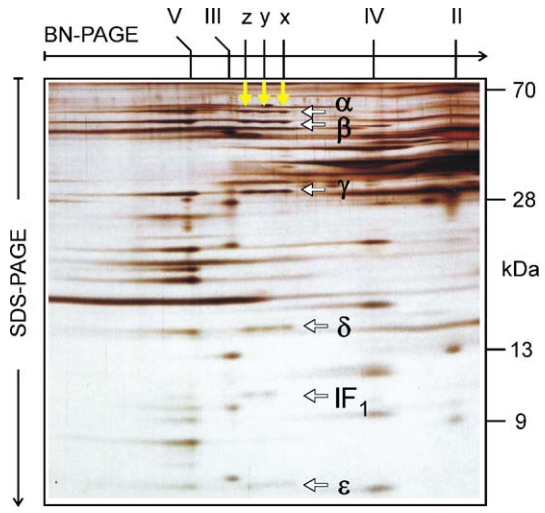

Fig. 2. Identification of three different F_1-subcomplexes of mitochondrial ATP synthase in a mitochondrial encephalomyopathy patient with unknown genetic defect. MDS and EFG1 deficiency have been excluded. Ten-milligram skeletal muscle of the patient was used to separate the native oxidative phosphorylation complexes by 1-D BN-PAGE and the subunits by 2-D Tricine-SDS-PAGE. II, III, IV, V, complexes II, III, IV, and V. The masses of complex II subunits are assigned. Indicators for a mitochondrial disorder are: (i) the absence of detectable amounts of complex I, and (ii) the accumulation of three types of F_1-subcomplexes (z, y, x, yellow arrows). All three F_1-subcomplexes contain F_1-subunits α, β, γ, δ, and ε, as indicated. F_1-subcomplexes y and z contain also bound inhibitor protein (IF_1). F_1-subcomplex z contains a ring of c-subunits in addition (immunoblot not shown). This F_1-subcomplex (470 kDa) is almost as large as the neighboring complex III (500 kDa).

5. Respiratory string model

In the pioneering electron microscopic analyses of Allen et al. [1], not only 9-nm projections from the inner mitochondrial membrane, the F_1 headpieces of ATP synthase, had been observed but also larger 13 × 22-nm projections that tentatively were assigned as complex I dimers, since complex I is the only oxidative phosphorylation complex that is larger than ATP synthase. These large particles were clearly viewed at intervals of 26–30 nm, and seem to wind as a helical band around the tubular cristae, similar to the helical F_1 band. In many cases, the large projections appeared to be subdivided by a cleft suggesting that each elongated projection is composed of two monomers. Also the ratio of the 13 × 22-nm and 9-nm projections, the presumed representatives of complex I dimers and ATP synthase, is compatible with the expected ratio of the two complexes. Comparing the number of the 13 × 22-nm and 9-nm projections, a ratio of 18 complex I dimers per 75 ATP synthase dimers was estimated which is in the same order as the 1:3 ratio of complexes I and V determined for bovine heart mitochondria [46]. However, an explanation for the regular

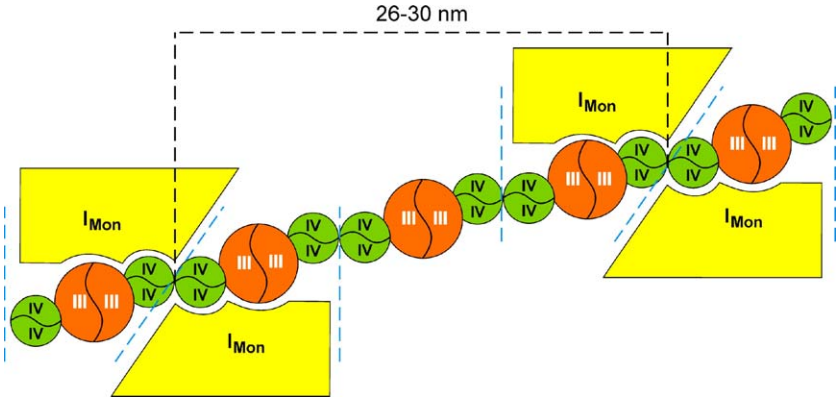

Fig. 3. Model for a linear association of respiratory chain supercomplexes in mammalian mitochondria. Solubilization properties of yeast and bovine mitochondria using low Triton X-100 suggested that respiratory chain supercomplexes interact to form larger supramolecular structures. This "respiratory string" model can explain the regular 26–30 nm intervals (dashed black lines) of the large 13 × 22-nm projections and the cleft dissecting the 13 × 22-nm particles as observed with *Paramecium* mitochondria [1]. It is in accordance with (i) the isolation of large $I_1III_2IV_4$ and smaller III_2IV_4 respiratory supercomplexes (separated by dashed blue lines), (ii) the determined ratio of respiratory complexes I:III:IV that is 1:3:6 in bovine heart [46], and (iii) the observation of tetrameric bovine complex IV. Flavoprotein-dependent dehydrogenases may interact directly with the central III_2IV_4 supercomplex or indirectly via the quinone pool.

intervals of the 13 × 22-nm projections has not yet been given in this pioneering work.

In the light of new evidence for the existence of respiratory chain supercomplexes or respirasomes [5,47], it is tempting to speculate that the 13 × 22-nm projections do not represent complex I dimers but represent respirasomes containing complex I. The regular intervals could originate from linearly associated complexes III and IV to which complex I is bound at specific sites as depicted in Fig. 3. This model for a linear association of respiratory chain supercomplexes is supported by two other previously reported lines of evidence: (1) Respiratory complex II (succinate:ubiquinone reductase) and mitochondrial complex V (ATP synthase) can be selectively and almost quantitatively solubilized from yeast or bovine heart mitochondria using low amounts of Triton X-100 [4,48]. This indicates that a certain detergent/protein ratio is sufficient to solubilize lipid areas between mitochondrial complexes, thereby dissolving complexes II and V almost quantitatively, and even dissociating dimeric bovine ATP synthase into the monomers. In contrast, respiratory supercomplexes are not dissolved suggesting that the supercomplexes remain associated as huge structures that are only broken and solubilized if increasing amounts or more aggressive detergents are used. (2) Bovine complex IV can tetramerize under certain conditions. When we used preparative BN-PAGE to further purify complex IV from bovine heart that was isolated chromatographically using Triton X-100, we could observe a major band of tetrameric complex IV in addition to minor bands of monomeric and dimeric complex IV (Schägger, H., unpublished data). We therefore suggest that respirasomes associate specifically by tetramerization of complex IV thus generating "respiratory strings", i.e. linear assemblies of respiratory supercomplexes. The association of two "complete" respirasomes, i.e., $I_1III_2IV_4$ supercomplexes containing monomeric complex I, dimeric complex III, and two copies of dimeric complex IV, as shown in Fig. 3, is consistent with the cleft dissecting the 13 × 22-nm particles that has been observed in electron microscopic studies [1]. In accordance with the determined ratio of respiratory complexes I:III:IV which is 1:3:6 in bovine heart mitochondria, we postulated that the respiratory chain consists of interconnected $I_1III_2IV_4$ and III_2IV_4 supercomplexes in a 2:1 ratio [46]. Alternating connection of one copy of the III_2IV_4 supercomplex and dimeric $I_1III_2IV_4$ supercomplex as depicted in Fig. 3 can explain the regular 26–30-nm intervals of the 13 × 22-nm projections.

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