Characterization of Domain Interfaces in Monomeric and Dimeric ATP Synthase*

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We disassembled monomeric and dimeric yeast ATP synthase under mild conditions to identify labile proteins and transiently stable subcomplexes that had not been observed before. Specific removal of subunits α, β, oligomycin sensitivity conferring protein (OSCP), and h disrupted the ATP synthase at the γ-$\alpha_3\beta_3$ rotor-stator interface. Loss of two F_1-parts from dimeric ATP synthase led to the isolation of a dimeric subcomplex containing membrane and peripheral stalk proteins thus identifying the membrane/peripheral stalk sectors immediately as the dimerizing parts of ATP synthase. Almost all subunit a was found associated with a ring of 10 c-subunits in two-dimensional blue native/SDS gels. We therefore postulate that $c_{10}a_1$-complex is a stable structure in resting ATP synthase until the entry of protons induces a breaking of interactions and stepwise rotation of the c-ring relative to the a-subunit in the catalytic mechanism. Dimeric subunit a was identified in SDS gels in association with two c_{10}-rings suggesting that a $c_{10}a_2c_{10}$-complex may constitute an important part of the monomer-monomer interface in dimeric ATP synthase that seems to be further tightened by subunits b, i, e, g, and h. In contrast to the monomer-monomer interface, the interface between dimers in higher oligomeric structures remains largely unknown. However, we could show that the natural inhibitor protein Inh1 is not required for oligomerization. ***Molecular & Cellular Proteomics 7: 995–1004, 2008.***

The possibility to remove a stable hydrophilic F_1-subcomplex, containing subunits α, β, γ, δ, and ε with a $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$ stoichiometry, from mitochondrial ATP synthase historically led to the term F_1F_0-ATP synthase for the holoenzyme (1, 2). Today the discrimination in F_1- and F_0-sectors is less stringent especially because F_1-c subcomplex, an association of F_1-complex and a ring of 10 hydrophobic c-subunits, has been isolated from yeast and crystallized (3). A similar F_1-c complex but with bound natural inhibitor protein IF_1 has also been identified in human mitochondria as an assembly intermediate or dead end product in the biosynthesis of ATP synthase (4). Focusing on functional and mechanistic aspects of ATP synthase, this rotary engine should rather be subdivided in the rotor part, which is an oligomeric ring of c-subunits connected to the central stalk (subunits γ, δ, and ε), and the residual stator part (5). For the yeast enzyme, the stator commonly is further subdivided into the catalytic headpiece $\alpha_3\beta_3$ of the stator that immediately interacts with the rotor subunit γ and also with subunits OSCP and h of the peripheral stalk (6, 7). Two further subunits, the hydrophilic subunit d and the hydrophobic subunit b comprising two transmembrane helices, are also assigned to the peripheral stalk (5). Subunits i, f, e, and g, all containing one transmembrane helix, and possibly subunit k may be regarded as accessory peripheral stalk proteins that reach into the membrane where they presumably stabilize the most hydrophobic subunit, a (or subunit 6 or ATP6), and therefore assist with its stator function. The a-subunit must transiently bind to the oligomeric c-ring, which is an essential part of the rotor. Cross-linking experiments in *Escherichia coli* have shown a close neighborship of subunits c and a (8) but associates of subunits c and a without using cross-linkers could not be verified experimentally so far. One major aim of the present work was to isolate transiently stable subcomplexes that had not been experimentally verified before to obtain further structural information on ATP synthase.

Mitochondrial F_1F_0-ATP synthase from yeast and mammals is commonly isolated as a catalytically functional monomeric complex (9–12), but several lines of evidence suggested that this complex is dimeric in the membrane (13, 14) or even oligomeric (15–17). Two specific proteins, the so-called dimer-specific subunits e and g, have been identified as promotors of dimerization of ATP synthase (13, 18–22) and as essential components for normal mitochondrial crista morphology (23). The term "dimer-specific subunits" originated historically from the presence of subunits e and g in dimeric yeast ATP synthase and a complete lack of these subunits in the monomeric ATP synthase isolated by blue native (BN)[1]-

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[1] The abbreviations used are: BN, blue native; CN, clear native; complex III, ubiquinol:cytochrome *c* reductase; complex IV, cytochrome *c* oxidase; complex V, ATP synthase; DDM, dodecyl β-D-maltoside; dSDS, doubled SDS; M/P, membrane/peripheral stalk; 1-D, first dimension; 2-D, second dimension; 3-D, third dimension; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Su, subunit; WT, wild-type; V_D, dimeric complex V; V_M, monomeric complex V; V_{INT}, intermediate complex V; OSCP, oligomycin sensitivity conferring protein.

PAGE after solubilization by low Triton X-100 concentrations. This term is potentially misleading because low amounts of subunit g and/or subunit e can be isolated with monomeric yeast ATP synthase following solubilization by digitonin and separation by BN-PAGE as described under "Results". The presence of subunits e and g favors dimerization but is not essential for dimerization, suggesting that other F_O-proteins are also involved (24, 25). Cross-linking evidence for the involvement of subunits h, i, and b in supporting the dimerization interface has recently been presented (25–27). A second goal of this work was to molecularly define the interface formed by the dimerizing ATP synthase monomers. Controlled disassembly of dimeric ATP synthase under various mild conditions, as used here, was expected to confirm known components of the interface of two ATP synthase monomers and should reveal novel important protein-protein interactions.

A third focus of the present work was on the higher oligomeric states of ATP synthase. In contrast to a relatively high but still incomplete knowledge on the protein-protein interactions in the monomer-monomer interface, not much is known on the interaction of dimers. These deficits may partly be due to the vanishing amounts of ATP synthase that could be isolated in higher oligomeric forms from yeast mitochondria so far (27). Especially interesting candidates potentially inducing oligomerization were the natural inhibitor protein Inh1 and associated proteins Stf1, Stf2, and Sfl2 that previously have been shown to be not essential for the initial dimerization (28). We can show that this inhibitor and the associated proteins are not essential for oligomerization and do not even favor higher order structures.

EXPERIMENTAL PROCEDURES

Materials—6-Aminohexanoic acid, imidazole, digitonin (catalog number 370006, purity >50%) were obtained from Fluka. Digitonin was used directly without recrystallization. Acrylamide and bisacrylamide (the commercial twice crystallized products), and Coomassie Blue G-250 (Serva Blue G) were purchased from Serva. All other chemicals were from Sigma.

Yeast Strains and Growth Conditions—Yeast strains in this study were W303-1A, Δ*su e*, and Δ*su g* null mutants (13); single gene deletion yeast strains Δ*inh1*, Δ*stf1*, Δ*stf2*, and Δ*sfl2*; and double gene null strains Δ*inh1*/Δ*stf1* and Δ*stf1*/Δ*sfl2* as described previously (28). Strains were grown in yeast extract, peptone-Gal medium (2% galactose) supplemented with 0.5% lactate at 30 °C.

Isolation of Mitochondria—Yeast cells were harvested by centrifugation at 1800 × *g* and washed with sucrose buffer (250 mM sucrose, 5 mM 6-aminohexanoic acid, 1 mM EDTA, and 10 mM sodium phosphate, pH 7.0). Five grams of sedimented cells, 5 ml of glass beads (0.25–0.5 mm), and 5 ml of sucrose buffer were vortexed for 10 min in a 50-ml tube. Following dilution with 10 ml of sucrose buffer, the sedimented glass beads were removed, and the supernatant was centrifuged for 20 min at 1250 × *g*. Mitochondrial membranes were collected by 30-min centrifugation at 18,000 × *g* and stored in sucrose buffer at −80 °C.

Solubilization of Mitochondrial Complexes for Native Electrophoresis—Mitochondrial membranes were thawed shortly before BN- or clear native (CN)-PAGE. Aliquots containing 400 μg of protein or multiples thereof were then sedimented by 15-min centrifugation at 20,000 × *g* and solubilized for BN-PAGE and CN-PAGE as described previously (29). Two different detergents, digitonin and Triton X-100, were used for isolation of dimeric ATP synthase: general solubilization of mitochondrial complexes and supercomplexes for first dimension (1-D) BN-PAGE (see Figs. 1*A* and 2*A*) was achieved using a digitonin/protein ratio of 3 (g/g). Low digitonin/protein ratios (0.75–3 g/g) were applied to solubilize higher oligomers of ATP synthase for CN-PAGE (see Figs. 3 and 5). Dimeric ATP synthase was solubilized from mitochondrial membranes using low Triton X-100/protein ratios (0.5–0.6 g/g as described in Ref. 13), separated by preparative BN gels, and extracted by native electroelution (29) for Tricine-SDS-PAGE (30) and doubled SDS (dSDS)-PAGE analysis (31).

Electrophoretic and Associated Techniques—1-D CN-PAGE (17), 1-D BN-PAGE, native protein extraction, native second dimension (2-D) BN/BN-PAGE, 2-D BN/SDS-PAGE (29), dSDS-PAGE, and silver staining (31) were performed as described previously. In-gel ATP hydrolysis assays followed the protocol of Zerbetto *et al.* (32) as modified in Ref. 33. A novel technique for semidry electroblotting of hydrophobic proteins from high percentage acrylamide gels was used (30). Polyclonal antibodies against subunits e, g, a, b, c, i, and h were raised in rabbits and used for immunological detection of the subunits on PVDF membranes as described previously (34).

RESULTS

Selective Dissociation of Stator Subunits α, β, h, and OSCP from ATP Synthase—To identify the most labile subunits and domains of yeast ATP synthase, the multiprotein complex was isolated as a dimer by 1-D BN-PAGE (dimeric complex V (V_D); Fig. 1*A*) and subsequently disassembled under very mild conditions namely by 2-D BN-PAGE using the common cathode buffer for BN-PAGE but supplemented with 0.02% dodecyl β-D-maltoside (DDM). Mixed micelles of the neutral detergent DDM and the anionic dye (Coomassie Blue G-250) that were formed in this cathode buffer were able to dissociate the respiratory supercomplexes (Fig. 1*A*, S_1 and S_2) into the constituent complexes III and IV (Fig. 1*B*, *III* and *IV*). Similarly V_D (Fig. 1*A*) dissociated almost completely into monomeric complex V (V_M; Fig. 1*B*) thereby losing subunits e, g, and k, which are the most detergent-labile subunits, as shown previously (13). In addition to the prominent band of monomeric complex V, two poorly staining subcomplexes of complex V (assigned as V_X and V_Y; Fig. 1*B*) were hardly detectable in the 2-D BN gel. However, after cutting out the gel strip from the 2-D BN gel *boxed* in Fig. 1*B*, both subcomplexes were clearly identified and characterized by 3-D SDS-PAGE (Fig. 1*C*). The larger subcomplex V_Y lacked subunits α and β, the headpiece of the stator, and peripheral stalk subunits h and OSCP. Subunit ε was weakly stained and was hardly detectable in Fig. 2*C*, but it could be identified in the original gel, and the band shape was comparable to that of other subunits. The normal band shape suggested that no significant loss had occurred and that binding is stoichiometric in subcomplex V_Y. No information on the presence or absence of subunit 8 could be obtained because this subunit was poorly silver-stained, and no specific antibody was available. Subunit a, also named subunit 6 or ATP6, was retained in subcomplex V_Y as shown by Western blotting (Fig. 1*D*). Surprisingly subunit a-specific an-

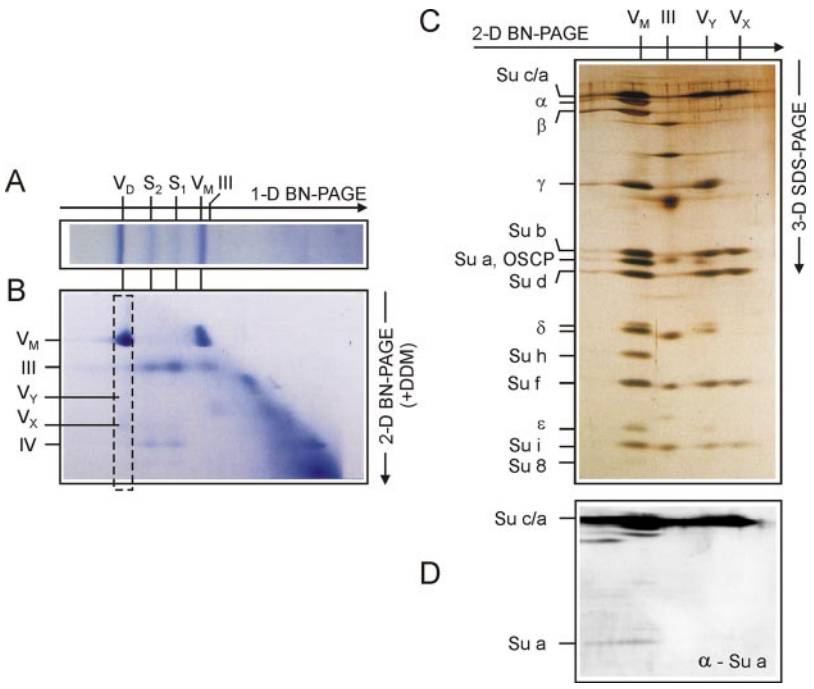

FIG. 1. **Dodecyl maltoside/Coomassie Blue G-250 micelles can dissociate the γ-$\alpha_3\beta_3$ rotor-stator interface.** S_2 and S_1, respiratory supercomplexes containing dimeric complex III (*III*) and two (S_2) or one (S_1) copy of complex IV (*IV*), respectively. *A*, yeast mitochondrial complexes were solubilized by digitonin (3 g/g of protein) and separated by 1-D BN-PAGE. *B*, V_D from 1-D BN-PAGE was dissociated into V_M by the detergent DDM (+*DDM*) that was added to the cathode buffer for 2-D BN-PAGE. Further fragments of dimeric complex V within the *boxed area* were the hardly detectable subcomplexes V_Y and V_X. *C*, a gel strip from the 2-D BN-gel (*boxed area* in *B*) was further analyzed by 3-D Tricine-SDS-PAGE using 14% acrylamide gels with high cross-linker (6% C) and 6 M urea. Subunits are assigned on the *left side*. The larger subcomplex V_Y lacked the stator subunits α and β that interact with subunit γ of the rotor part and subunits h and OSCP that connect the $\alpha_3\beta_3$-headpiece of the F_1-part to the peripheral stalk. Subcomplex V_X also lacked the rotor subunits γ, δ, and ε. No information on the presence or absence of subunit 8 could be obtained. *D*, Western blot of the upper half of a gel similar to that in *C*. A specific antibody for subunit a (α-*Su a*) recognized only very small amounts of individual subunit a because almost all subunit a migrated as an associate with oligomeric subunit c (*Su c/a*).

tibody identified almost all a-subunit as a stable Su c-a complex and very small amounts as free subunit a (Fig. 1*D*). The smaller subcomplex V_X was similar to subcomplex V_Y but also lacked three further subunits of the rotor part (subunits γ, δ, and ε). Another part of the rotor was preserved, namely the ring of c-subunits, which was associated with Su a (Fig. 1*D*). Verification of the Su c-a complex that was dissociated from subcomplexes V_X and V_Y and identification of the separated subunits (c and a) was performed by dSDS-PAGE (data not shown).

Removal of subunits α, β, OSCP, and h disrupted the ATP synthase at the γ-$\alpha_3\beta_3$ rotor-stator interface and indicated a certain lability of this interface that had not been directly shown before. Comparing subcomplexes V_X and V_Y suggested that dissociation of the $\alpha_3\beta_3$ stator part destabilizes the rotor part of the ATP synthase thereby leading to loss of rotor subunits γ, δ, and ε. Subunits h and OSCP that bind to the $\alpha_3\beta_3$ stator part and to peripheral stalk subunits b and d as well (6) seem to stabilize the $\alpha_3\beta_3$ stator part, which in turn stabilizes the rotor.

Isolation of a Dimeric Membrane/Peripheral Stalk Sector—Solubilization of *Saccharomyces cerevisiae* mitochondria by digitonin and separation by BN-PAGE commonly results in band patterns similar to those in Fig. 1*A*. Occasionally an additional "intermediate" complex V (V_{INT}) between V_D and V_M was observed (Fig. 2*A*). Because V_{INT} contained all subunits of complex V, it seemed possible that one of the two F_1-sectors dissociated from the dimeric complex. We therefore asked whether it is possible to dissociate two F_1-sectors and to isolate a dimeric membrane/peripheral stalk (M/P) sector. This would immediately identify the M/P-sector as the dimerization interface.

A protocol to prepare the intermediate complex, V_{INT}, and dimeric M/P-sector reproducibly and on a larger scale could be elaborated as shown in Fig. 2*B*. Bands of monomeric and dimeric complex V were excised from preparative BN gels (analogous to Fig. 2*A*) and extracted by native electroelution. The extracts were then reapplied to gels for 2-D BN-PAGE (Fig. 2*B*, V_D and V_M). In contrast to the 2-D BN-PAGE described above using DDM addition to the cathode buffer (Fig. 1*B*), no detergent was added here to the cathode buffer for the second BN-PAGE (Fig. 2*B*). Therefore, complex V dissociated in a different way. The dissociated fragments from monomeric and dimeric complex V (as observed in Fig. 2*B*) were then identified by 3-D SDS-PAGE (Fig. 2, *C* and *D*).

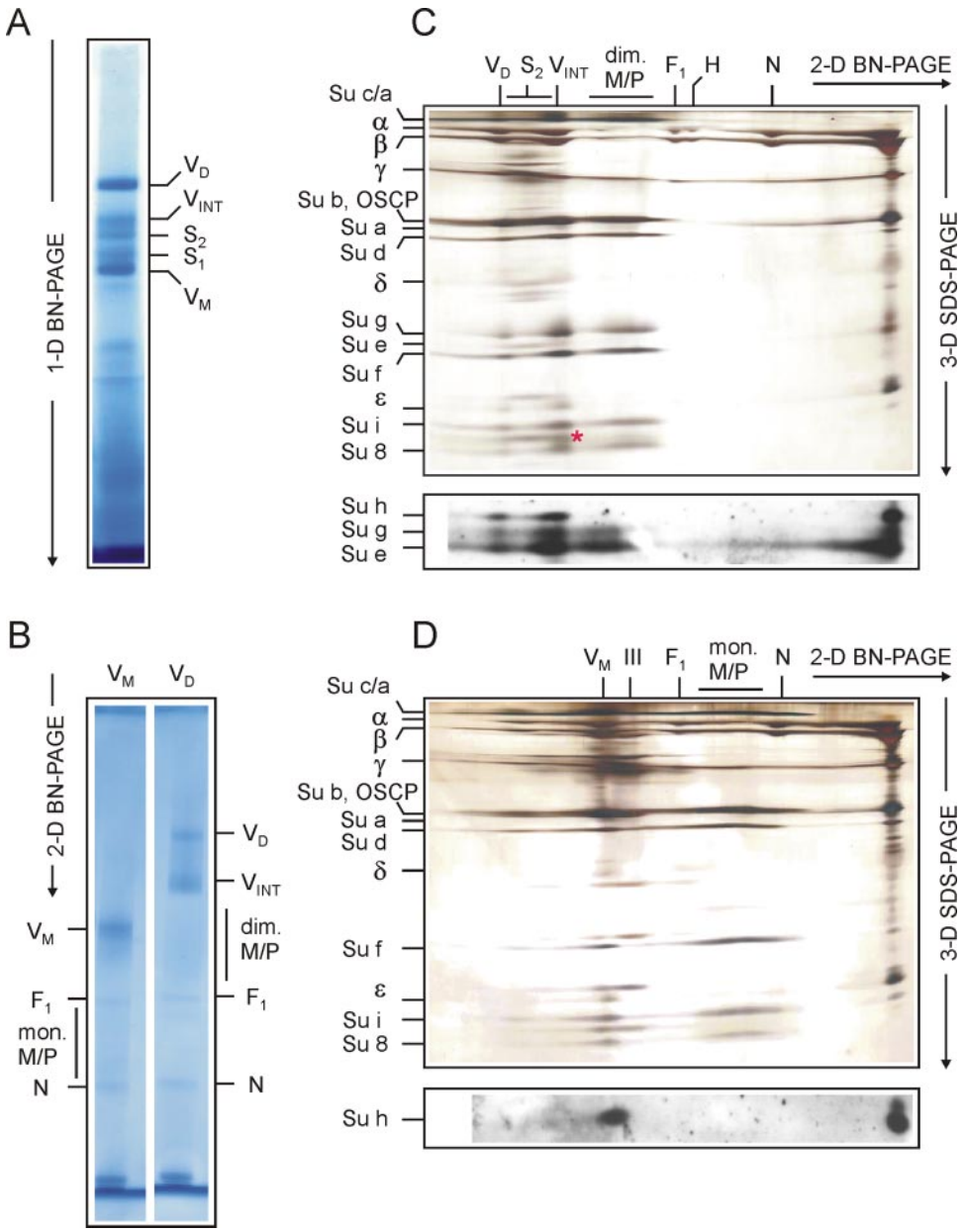

FIG. 2. **Isolation of a dimeric associate of membrane and peripheral stalk proteins from dimeric ATP synthase.** *A*, digitonin-solubilized V_M and V_D, V_{INT}, and respiratory supercomplexes (S_1 and S_2) were isolated by 1-D BN-PAGE. *B*, V_M and V_D extracts from 1-D BN-gels (*A*) were reapplied to a 2-D BN gel. Detection of lower mass bands indicated fragmentation. The identity of these fragments was then verified by 3-D SDS-PAGE using lanes of dimeric complex V (*C*) and of monomeric complex V (*D*). Assignment of subunits of complex V are on the *left side*. A protein band (marked * in *C*) migrating between Su i and Su 8 in lanes for both S_2 and V_{INT} presumably is a spillover from the adjacent S_2 lane. Comparison with previous protein identification (35) suggests that this band potentially is a composite band containing Qcr9 subunit of complex III and cox7 and -9 subunits of complex IV. F_1, F_1-subcomplex; *N*, presumed α-β heterodimer; *H*, presumed $\alpha_3\beta_3$-headpiece of F_1. Western blots (*lower panels* of *C* and *D*) were used to identify subunits e, g, and h. Monomeric (*mon.*) M/P differed from dimeric (*dim.*) M/P by a considerable reduction of subunits e and g (see "Results").

Analysis of the fragments of dimeric complex V (Fig. 2*C*) revealed that a small fraction of the applied sample was recovered in intact form (V_D). The next largest complex (V_{INT}) showed intermediate mass between V_D and V_M and seemed to contain all subunits of dimeric complex V as detected in silver stain (Fig. 2*C*). Two alternative explanations seemed possible: loss of one of the two F_1-parts or loss of one M/P-part. We find it more likely that one F_1-part was dissociated from the dimeric complex because no monomeric M/P-sector was identified in Fig. 2*C*. Further dissociation of monomeric M/P-sector can be excluded because monomeric M/P-sector is stable as shown in Fig. 2*D*. Dissociated F_1-subcomplex (F_1)

and further fragments H and N (also containing α- and β-subunits) were correspondingly detected. Band H was slightly smaller than the F_1-subcomplex and therefore tentatively assigned as $\alpha_3\beta_3$-headpiece with an apparent mass around 300–350 kDa. Band N with apparent mass around 100 kDa presumably constituted a stable α-β heterodimer. Dissociation of the second F_1-sector from V_{INT} or of two F_1-sectors directly from the complex V dimer generated dimeric M/P-sector migrating as a broad band in the 400–1000-kDa range. Other theoretical fragments, like monomeric complex V and monomeric M/P-sector, could not be identified, underlining the stability of the dimeric M/P-sector under the conditions used. Dimeric M/P-sector contained the Su c-a complex described above, subunits b, d, f, and i, and subunits e and g. It should be noted that subunit h was not found in the dimeric M/P-sector (Fig. 2*C*, Western blot). Minor amounts of other proteins like subunit γ (detected in silver stain) were considered as substoichiometric contaminants.

Using similar dissociation conditions for monomeric complex V isolated by digitonin solubilization and BN-PAGE yielded monomeric M/P-sector that contained very low amounts of subunit g. This was suggested by a missing band in the Su g range (Fig. 2*D*) and by a low signal intensity on Western blots compared with dimeric M/P-sector (Western blots not shown). Subunit e, if present at all in the monomeric M/P-sector, was hardly detectable in the Western blots. This means that substoichiometric amounts of subunit g are bound to digitonin-solubilized monomeric ATP synthase following BN-PAGE as was observed before (36), and subunit e amounts were close to the detection limit. In contrast, subunits e and g are completely lacking with Triton X-100-solubilized monomeric ATP synthase following BN-PAGE (13).

We conclude that the membrane/peripheral stalk sectors are immediately involved in the dimerization of yeast ATP synthase as evidenced before by cross-link studies in yeast (*e.g.* Refs. 26 and 27) and by electron microscopic studies of dimeric ATP synthase from *Saccharomyces* and *Polytomella* mitochondria (37, 38). However, the dimer interface of bovine ATP synthase was formed by contacts of both the F_0- and the F_1-domains (39).

Subunits e and g Are Important but Not Essential for the Formation of Dimeric and Oligomeric Complex V—In the first report on the isolation of dimeric ATP synthase from yeast by BN-PAGE (13), subunits e and g were isolated with dimeric but not with monomeric ATP synthase. This does not mean that subunits e and g were essential for the formation of dimers in the membrane (24, 25). It just means that dimeric/oligomeric ATP synthase is stabilized by subunits e and g so that dimeric/oligomeric ATP synthase can be isolated under the conditions of BN-PAGE. We now asked whether CN-PAGE (17), which is milder than BN-PAGE, can preserve dimeric or higher oligomeric structures even in the absence of these two stabilizing proteins. Furthermore the commonly used in-gel ATP hydrolysis assay (or lead phosphate precipitation assay), as applied for Fig. 3, is relatively fast (≥1 h) and fully oligomycin-sensitive in CN gels (14). Using very low digitonin/protein ratios for solubilization of mitochondrial membranes (around 1.0 g/g), various oligomeric states of complex V could be observed for wild-type (WT) yeast, ranging from the monomeric to the octameric state (Fig. 3*A*, *M* and *O*). Tetrameric, hexameric, and octameric (Fig. 3*A*, *T*, *H*, and *O*) states were assigned using the monomeric and dimeric forms for calibration as exemplified in

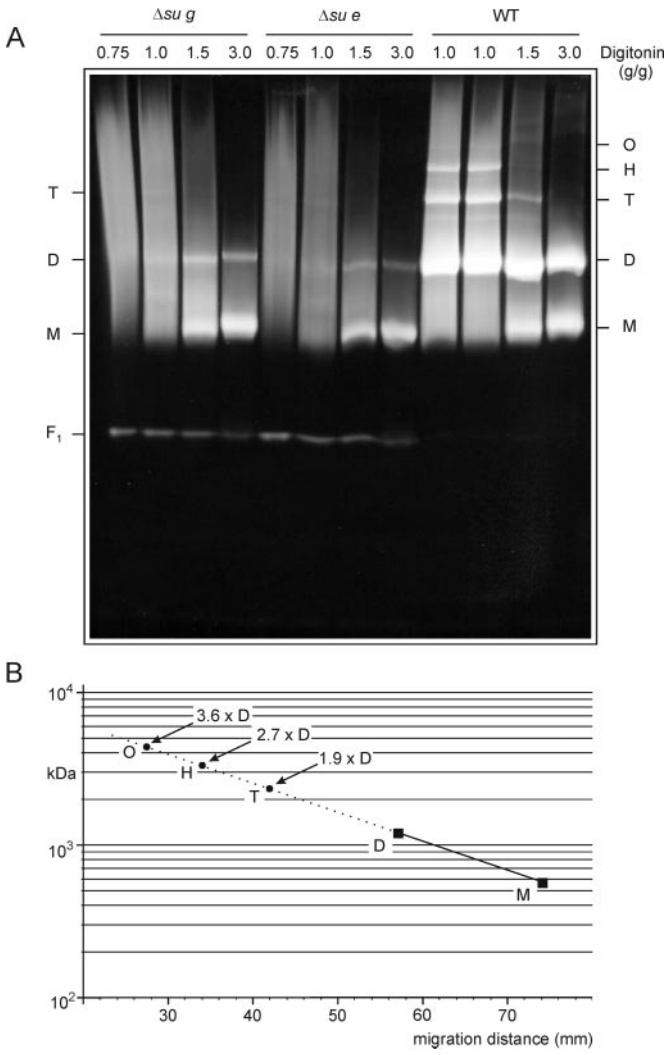

FIG. 3. **Analysis of the supramolecular organization of complex V from wild-type yeast and null mutants of subunits e and g.** Mitochondrial membranes from WT yeast and from null mutants of subunit e (Δ*su e*) and subunit g (Δ*su g*) were solubilized by digitonin using the digitonin/protein ratios indicated (0.75–3.0 g/g), and the solubilized complexes were separated by CN-PAGE. A digitonin ratio of 1.0 g/g was erroneously used twice for WT yeast. *A*, complex V monomers (*M*), dimers (*D*), tetramers (*T*), hexamers (*H*), octamers (*O*), and F_1-subcomplex (F_1) were identified by the lead phosphate precipitates formed during the in-gel ATP hydrolysis assay. *B*, the monomeric and dimeric forms with known masses (*black squares*; monomeric (*M*), 573,067 kDa; dimeric (*D*), 1,208,616 kDa) were used for mass calibration. The higher oligomeric forms showed 1.9, 2.7, and 3.6 times (1.9 × *D*, 2.7 × *D*, and 3.6 × *D*) the masses of the dimer and were therefore assigned as tetramers (*T*), hexamers (*H*), and octamers (*O*).

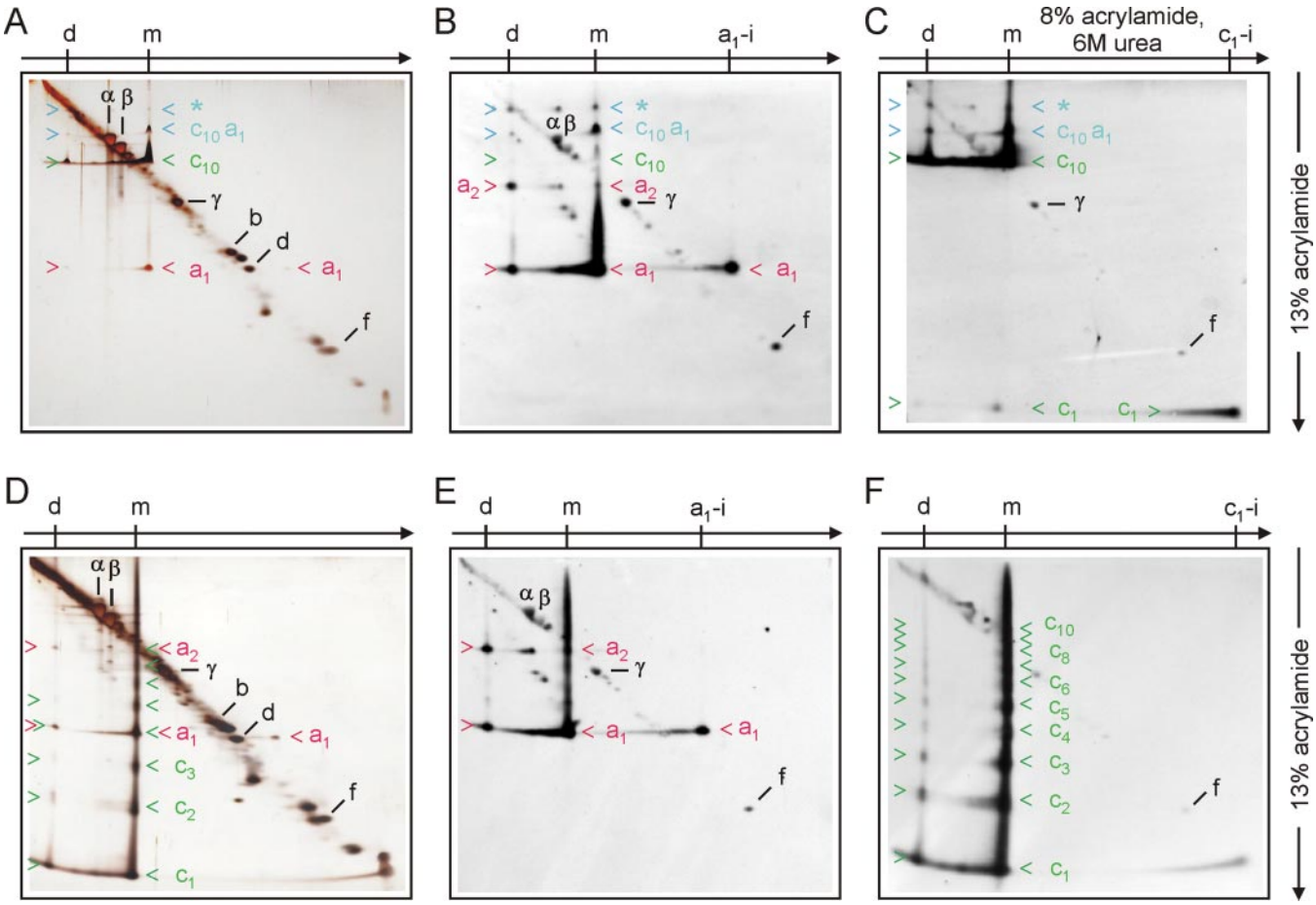

FIG. 4. **Subunit analysis of dimeric ATP synthase by doubled SDS-PAGE reveals dimeric a-subunit associated with two c-rings.** 1-D Tricine-SDS-PAGE of ATP synthase (from *left* to *right*) on 8% acrylamide gels containing 6 M urea was identical for all six gels (*A–F*). Three 1-D gel strips (for *A–C*) were then incubated in acidic solution (see "Experimental Procedures") for 5 min and three strips (for *D–F*) were incubated for 15 min prior to 2-D separation on 13% acrylamide gels (from *top* to *bottom)*. dSDS gels were then silver-stained (*A* and *D*) or electroblotted for immunological detection of subunit a (*B* and *E*) and subunit c (*C* and *F*). The migration in 1-D gels of individual subunit a (a_1-*i*), of individual subunit c (c_1-*i*), and of the monomeric (*m*) and presumed dimeric (*d*) complexes of subunit a and c-ring are indicated. Fragments of subcomplexes (m and d) were observed after 2-D resolution and assigned to monomeric and dimeric subunit a (a_1 and a_2, respectively; marked *red*), complete and dissociated c-rings (c_{10}–c_1, marked *green*), and residual subcomplex (m) ($c_{10}a_1$, marked *blue*). * marks minor amounts of an artificial subcomplex (presumably $c_{10}a_2$; see "Results"). Subunits marked *black* are characteristic individual subunits of ATP synthase. Some of these subunits (α, γ, and f) were faintly identified by antibodies against subunits a and c.

Fig. 3*B*. No odd numbered oligomeric states were identified in significant amounts. This supported the view that dimeric complex V is the building block for oligomeric structures. Using null mutants of subunits g and e, dimeric complex V (Fig. 3*A*) was clearly identified, and even tetramers were just detectable at least by direct inspection of the original gels.

We conclude that subunits e and g stabilize the dimers and higher oligomers of complex V, but the two subunits are not essential for the formation of dimers and oligomers. Dimers and oligomers are at least partially preserved in CN-PAGE, and higher amounts are expected to exist in the mitochondrial membrane.

Isolation of Subunit c-a Complexes and Evidence for Dimeric Subunit a Associated with Two c-rings in Dimeric ATP Synthase—We next addressed whether subunits a and c can directly contribute to the dimeric organization of the ATP synthase complexes. Both subunits were separated here for the first time as SDS-resistant subcomplexes. We therefore analyzed whether evidence for dimeric a-c subcomplexes could be obtained by dissociating the peripheral F_O-subunits from the c-a subcomplexes from isolated ATP synthase. To do so, the band corresponding to the dimeric ATP synthase was extracted from BN-PAGE and used for dSDS-PAGE and silver staining (Fig. 4, *A* and *D*), immunological detection of subunit a (Fig. 4, *B* and *E*), and immunological detection of subunit c (Fig. 4, *C* and *F*). First dimension SDS-PAGE resolved individual subunits, for example the individual monomeric subunits a and c (assigned as a_1-*i* and c_1-*i* in Fig. 4, *B* and *E* and *C* and *F*, respectively), and also the monomeric (m) and dimeric (d) forms of a subcomplex containing c-ring and subunit a. Iden-

tical gel strips from 1-D SDS-PAGE were used for all second dimension SDS gels (Fig. 4, *A–F*), but gel strips in *A–C* were treated only briefly (5 min) with dissociating acidic solution (100 mM Tris, 150 mM HCl, pH 1.6) to retain the c-ring largely intact, whereas gel strips in *D–F* were incubated for 15 min to partly dissociate the c-ring. It is important to note that almost all subunit c from dimeric complex V was found associated with subunit a in 1-D SDS-PAGE as deduced from the band intensities of individual subunits c and a in 2-D SDS-PAGE (Fig. 4): compared with the subunit c and a amounts dissociated from c-a complexes (*spots below* subcomplexes (d) and (m)) the intensities of individual subunits c and a (*below* c_1*-i* and a_1*-i*) were very low.

Comparing the silver-stained gel (Fig. 4*A*) with Western blots for detection of subunits a and c (Fig. 4, *B* and *C*) suggested that subcomplex (m) contained subunits c and a with a $c_{10}a_1$ stoichiometry and presumably no other proteins because at least the larger protein subunits of ATP synthase would have been detected in silver stain (Fig. 4*A*). Subunit c was dissociated during 2-D SDS-PAGE as an intact c_{10}-ring (the copy number of c_{10} according to Ref. 3 is also apparent from Fig. 4*F*), and the a-subunit was mostly dissociated as monomer (assigned as a_1) from subcomplex (m). Considerably smaller amounts of both subunits (a and c) were also identified as residual non-dissociated subcomplex (assigned as $c_{10}a_1$) that was about 20–30 kDa larger than c_{10}. Another very faint spot located a further 20–30 kDa above (assigned as *) most likely is a $c_{10}a_2$-complex that seems to be artificially generated during SDS-PAGE for several reasons. (i) The band of subcomplex (m) in first dimension SDS-PAGE was very sharp indicating a homogeneous band with strict stoichiometry. Any protein in addition to subunits a and c must therefore be stoichiometrically bound to the subcomplex; however, no extra protein in addition to subunits c and a was identified in silver stain. (ii) Specific antibodies against subunits d and b with masses in the 20–30-kDa range did not identify this artificial spot (immunoblots not shown). Thus dimeric a-subunit came into consideration, although band (m) was too sharp to accommodate two different components, $c_{10}a_1$ and $c_{10}a_2$, in the same band. (iii) A small fraction of subunit a in the column of subunits of subcomplex (m) in Fig. 4*B* was identified as a dimer (assigned as a_2). Because there is no evidence that band (m) consisted of $c_{10}a_2$- instead of $c_{10}a_1$-complexes, the observation of small amounts of dimeric subunit a (a_2) points to some artificial dimerization of the a-subunit during SDS-PAGE because of the highly hydrophobic properties of this protein and the considerable protein concentration by the stacking effect of discontinuous electrophoresis systems. Similarly, spot * may represent minimal amounts of an artificial $c_{10}a_2$-complex pointing to a general tendency of subunit a to dimerize.

In addition to monomeric subcomplex (m), small amounts of a larger subcomplex (d), presumed to represent the dimeric form of subcomplex (m), were separated by 1-D SDS-PAGE. The band of subcomplex (d) seemed too sharp to accommodate more than one complex with stoichiometric protein composition because two complexes with 5% mass difference, *e.g.* generated by partial dissociation of a protein subunit, would appear as double bands. Similarly the presence of multiple subcomplexes generated by nonspecific hydrophobic interactions can be excluded because such associations would appear as broad or smearing bands. The important question therefore was whether subcomplex (d) contained monomeric or dimeric a-subunit in addition to two c-rings. Resolution by 2-D SDS-PAGE dissociated comparable amounts of dimeric and monomeric a-subunit (a_2 and a_1) from subcomplex (d). This is in contrast to a large excess of a_1 over a_2 that was observed upon dissociation of the monomeric subcomplex (m) in the same gel (Fig. 4*B*). Therefore, the relatively high a_2 amounts in the SDS gel that dissociated from subcomplex (d) cannot be explained by the same type of artificial dimerization as described for subcomplex (m). We conclude that subcomplex (d) most likely represents the $(c_{10})_2a_2$- and not a $(c_{10})_2a_1$-subcomplex. Using harsher conditions for the transition to 2-D SDS-PAGE (Fig. 4, *D–F*) showed essentially the same spot intensity of dimeric subunit a (Fig. 4*E*, a_2) relative to monomeric subunit a in the SDS gel, but the c-ring was largely dissociated. As a side effect, the known copy number of 10 c-subunits in the c-ring (3) could be reassessed as indicated by the ladder of c-ring fragments (Fig. 4*F*). Minor cross-reactions of the antibodies against subunits a and c with the α- and β-subunits (Fig. 4, *E* and *F*) helped to locate the largest component of the ladder (assigned as c_{10}) just below the α- and β-subunits, which is the position of the intact c_{10}-ring (compare with silver-stained gel in Fig. 4*A*).

Very hydrophobic proteins show anomalous migration behavior in SDS-PAGE (31), meaning that the apparent masses in SDS gels often are considerably below the calculated masses. This anomalous migration strongly depends on the acrylamide concentration of the gels used. Apparent masses are closer to the calculated masses with high percent acrylamide gels but can fall below 50% of the actual masses with low percent acrylamide gels. The anomalous migration can be quantified for each individual protein as described below so that mass estimations are still valid. This phenomenon was also observed here with 8% acrylamide gels containing 6 M urea. Using subunits α, γ, d, and f (55, 31, 20, and 10 kDa) for mass calibration, the apparent mass for the individual subunit a (a_1) was 15 kDa compared with the actual mass of 28 kDa. Similarly apparent masses of 45 and 90 kDa were determined for the $c_{10}a_1$-subcomplex (m) and its presumed dimer (d) with calculated masses of 105 and 210 kDa. Subtracting the apparent mass of the a-subunit, the apparent mass of the c_{10}-ring in an 8% acrylamide gel was around 30 kDa compared with a calculated mass of 76 kDa.

Using a higher percentage of acrylamide (13% acrylamide), as used for the second dimension SDS-PAGE, the apparent

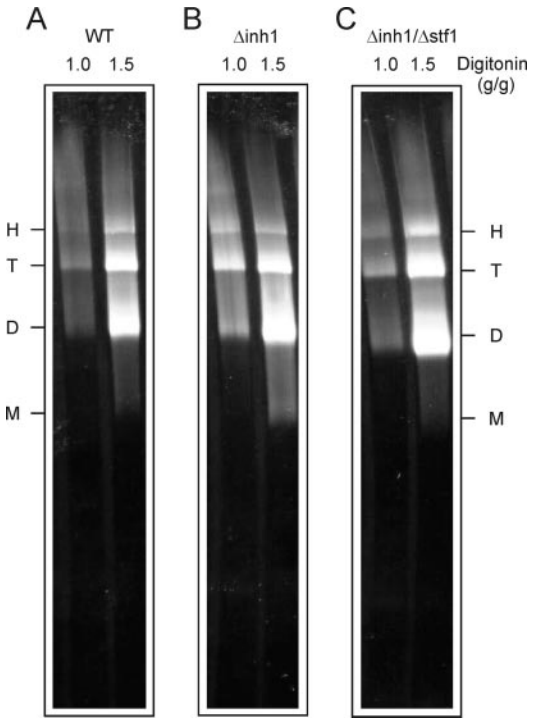

FIG. 5. **Null mutants of the inhibitor protein Inh1 and its known accessory protein Stf1 can form oligomeric complex V.** Mitochondrial membranes were solubilized using the digitonin/protein ratios indicated (1.0 and 1.5 g/g). The solubilized complexes were then separated by CN-PAGE, and various oligomeric forms of complex V were identified by in-gel ATP hydrolysis assay. *A*, WT yeast. *B*, null mutant deficient in the inhibitor protein Inh1 (Δ*inh1*). *C*, null mutant deficient in two proteins, Inh1 and accessory protein Stf1 (Δ*inh1*/Δ*Stf1*). Monomeric (*M*), dimeric (*D*), tetrameric (*T*), and hexameric (*H*) complex V was similarly identified (not shown) in all null mutants of Inh1 and its known accessory proteins Stf1 and Stf2 listed under "Experimental Procedures."

mass of the a-subunit was 5 kDa larger (around 20 kDa), thus approaching the actual mass of 28 kDa. Similarly the apparent mass of the $c_{10}a_1$-complex (around 57 kDa) was 12 kDa closer to the actual mass of 105 kDa as expected.

We conclude that the c-ring is almost quantitatively associated with the a-subunit in isolated ATP synthase. The essentially quantitative aspect of this association is surprising because subunit a must dissociate from the c-ring during the catalytic cycle of the rotating ATP synthase (see "Discussion"). We next asked how dimeric ATP synthase can interact with neighboring dimers to form oligomeric structures.

Formation of Oligomeric Complex V Does Not Require the Inhibitor Protein Inh1—It is not known at present whether oligomerization is based on in-membrane interactions of dimers, similar to the in-membrane interactions involved in dimerization of complex V, or on extramembranous interactions. Presumably the most interesting candidate protein for a potential extramembranous interaction of dimers is the natural inhibitor protein Inh1 because the bovine homologue IF_1 has been shown to promote the dimerization of purified bovine F_1-sectors at slightly acidic pH (40, 41).

Here we used mitochondria from null mutants deficient in Inh1, Stf1, and Stf2 (and further mutants listed under "Experimental Procedures") to analyze potential effects of the natural inhibitory proteins on the oligomerization of complex V (Fig. 5). All mutant and wild-type strains had been grown and processed for native electrophoresis under identical conditions to avoid unintended experimental variations. Analysis by CN-PAGE and in-gel ATPase assay revealed almost identical activity patterns and oligomeric states for all null mutants compared with the wild-type yeast as exemplified for the null mutant deficient in Inh1 (Δ*inh1*) and for a double mutant deficient in Inh1 and Stf1 (Δ*inh1*/Δ*stf1*). The complex V protein amounts corresponding to the different oligomeric states were densitometrically quantified on Coomassie-stained 2-D gels (not shown) and used to calculate specific ATP hydrolysis activities. Specific activities for the bands of dimeric and tetrameric complex V were comparable with wild-type yeast and mutant strains when activity assays were kept short (75 min) to avoid saturation effects of lead phosphate precipitation.

We conclude that oligomerization of complex V is not affected by the inhibitory protein Inh1 and its known accessory proteins Stf1 and Stf2 (not shown). Assuming that the oligomeric states are preserved during 75-min incubation in the assay buffer at pH 7.8–8.0, which, however, cannot be controlled at present, oligomerization does not influence the ATP hydrolysis activity of complex V.

DISCUSSION

The rotor-stator interface is a well defined part of ATP synthase especially because the structure of the F_1-ATPase has been resolved (42), and the subunit rotation in F_1-ATPase has been experimentally verified (43, 44). Even transient loosening of interactions between the γ-subunit of the rotor part and the $\alpha_3\beta_3$-headpiece of the stator part that must occur during the catalytic cycle did not lead to dissociation of the two parts. Here we show for the first time that yeast ATP synthase can dissociate at the presumed rotor-stator interface, *i.e.* the $\alpha_3\beta_3$-headpiece dissociated from the γ-subunit that was still assembled in a very large residual complex. The concomitant removal of subunits h and OSCP with $\alpha_3\beta_3$ suggested that these four subunits confer stability to the $\alpha_3\beta_3$-headpiece on the one side and to interact with subunits b and d on the other side as suggested by the structure of a subcomplex crystallized from recombinantly expressed proteins (6).

Subunits e and g are neighboring subunits in monomeric bovine ATP synthase (11). In yeast, they stabilize the dimeric form of ATP synthase so that dimers can be isolated under the conditions of BN-PAGE (13, 15, 18–22), but the two proteins were postulated to be not essential for dimerization in the membrane because fluorescence resonance energy transfer analyses (24) and cross-linkage of subunits h (25) using subunits g and e null mutants suggested interactions of ATP synthases in the membrane also in the absence of subunits e

and g. Here we identified dimeric and also minimal amounts of tetrameric ATP synthase in null mutants of subunits e and g. This supports previous postulations that subunits e and g favor supramolecular structures of ATP synthase but are not essential for the formation of dimers/oligomers (24, 25) by a completely different and direct CN-PAGE approach.

In the membrane-embedded ATP synthase, the c-ring rotates against the laterally orientated a-subunit that in turn is bound to subunit b in a bacterial ATP synthase (45) and to further membrane/peripheral stalk subunits in the mammalian complex. The mechanism of proton translocation must involve sophisticated interactions between subunit a and the rotating c-ring to grant stability of the subunit c-a complex and at the same time to allow for almost frictionless rotation of the c-ring against subunit a (46, 47). This seemed to explain why subunit c-a complexes could not be isolated so far. Unexpectedly subunit c-a complexes could be isolated here by SDS-PAGE, and even more surprising the monomeric c-a complex was not isolated as a minor by-product, but both subunits (c and a) were almost quantitatively isolated in this associated form. This suggested that the mechanism of ATP synthase includes a resting position with stable subunit c-a association.

We asked what is special in our SDS gels allowing isolation of the subunit c-a complex because we and others had previously separated the c-ring from the a-subunit following normal SDS incubation of isolated ATP synthase (9). Here we used 1-D native gels first to separate the native complexes. Two different protocols were then used for the transition from BN-PAGE to 2-D SDS-PAGE. Either native gel strips were wetted for 30 min with 1% SDS before starting the SDS-PAGE (Figs. 1, *C* and *D*, and 2, *C* and *D*), a common protocol for 2-D BN/SDS-PAGE previously not known to favor artificial hydrophobic interactions, or the gel strips were just wetted with water (Fig. 4, *A–F*). The SDS concentration in the 2-D SDS gel (in the latter case solely originating from the 0.1% SDS-containing cathode buffer) was sufficiently low to retain the subunit c-a complex in both situations. We think that we have demonstrated more than just a situation where subunits c and a are more stable in association with one another than in the surrounding medium because (i) no other proteins except subunits c and a could be identified with the monomeric c-a subcomplex, (ii) the stoichiometry was uniform ($c_{10}a_1$), and (iii) the recovery was almost quantitative. SDS-PAGE from BN gel strips also revealed some percentage of a larger complex with a minimal $(c_{10})_2a_2$ composition (further protein constituents cannot be excluded because of low protein amount) from which c_{10}-ring and a_2 dimer could be dissociated in SDS gels. Because no hints for an immediate association of two c-rings via c-c interfaces were obtained from the 2-D gels and this interface would not be compatible with rotation, we postulate that subcomplex (d) contained two c-rings linked by dimeric subunit a. Assuming that native subunit interactions existing in mitochondrial membranes are maintained through BN-PAGE and also through further and successive steps of electrophoresis, it follows that subunit a dimers can link two c_{10}-rings in dimeric ATP synthase. Subunit a possesses more transmembrane helices than every other subunit of the complex (five and seven transmembrane helices predicted by TMpred and HMMTOP, respectively) suggesting dimeric a-subunit as the central component of a basic c-a_2-c monomer-monomer interface that can be tightened by subunits e, g, b, i, and h.

Natural inhibitor Inh1 and associated proteins Stf1, Stf2, and Sfl2 have been excluded here as important linkers of dimers. Other candidates that must be considered as potential linkers are the carriers for phosphate and ADP/ATP that have been described to form a supercomplex with the ATP synthase, the ATP synthasome (48), and F_0-subunits f, ATP8, and subunit a (involving transmembrane helices not engaged in monomer-monomer interactions). Based on the postulations that the metabolic state of a cell correlates with mitochondrial crista morphology (49) and that establishment of the normal crista membrane architecture requires the presence of Su e and Su g, which support the dimerization and oligomerization of ATP synthase (18, 23), the elucidation of the ATP synthase dimer-dimer interface represents an important future task.

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