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Review

Assembly of F_1F_0-ATP synthases☆

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ABSTRACT

F_1F_0-ATP synthases are multimeric protein complexes and common prerequisites for their correct assembly are (i) provision of subunits in appropriate relative amounts, (ii) coordination of membrane insertion and (iii) avoidance of assembly intermediates that uncouple the proton gradient or wastefully hydrolyse ATP. Accessory factors facilitate these goals and assembly occurs in a modular fashion. Subcomplexes common to bacteria and mitochondria, but in part still elusive in chloroplasts, include a soluble F_1 intermediate, a membrane-intrinsic, oligomeric *c*-ring, and a membrane-embedded subcomplex composed of stator subunits and subunit *a*. The final assembly step is thought to involve association of the preformed F_1-c_{10-14} with the ab_2 module (or the *ab8*-stator module in mitochondria) – mediated by binding of subunit δ in bacteria or OSCP in mitochondria, respectively. Despite the common evolutionary origin of F_1F_0-ATP synthases, the set of auxiliary factors required for their assembly in bacteria, mitochondria and chloroplasts shows clear signs of evolutionary divergence. This article is part of a Special Issue entitled: Chloroplast Biogenesis.

1. F_1F_0-ATP synthase function and structure

Adenosine-5′-triphosphate (ATP) is the major energy storage compound in biological systems, and most of it is provided by the membrane-embedded F_1F_0-ATP synthases (in the following designated simply as "ATP synthases") found in bacteria, mitochondria and chloroplasts. These multiprotein complexes couple reversible ATP synthesis to an electrochemical gradient – built up either by protons or sodium ions – across the boundary layer between two cellular compartments. The general 'mushroom-like' structure of ATP synthases is conserved across species and comprises a membrane-spanning subcomplex (F_0) and a soluble component (F_1) that generates or hydrolyses ATP through the action of a rotational mechanism. Bacterial and chloroplast ATP synthases share a similar subunit composition, with $\alpha_3\beta_3\gamma\delta\varepsilon$ constituting F_1 and F_0 comprising ab_2c_{10-15} (Table 1). Subunits γ and ε form a central stalk that non-covalently interacts with the membrane-embedded, hydrophobic *c*-ring. The proteolipidic oligomer rotates against the peripheral stator, which is made up of ab_2 and δ. The stator prevents rotation of the $\alpha_3\beta_3$ hexamer, which harbors six nucleotide-binding sites, each located at an αβ-subunit interface, for reversible ATP synthesis. Ions are translocated across channels at the *a*–*c* ring interface, generating a torque on the central stalk γε. The γ-subunit in turn, which forms an α-helical coiled-coil structure, is embedded in the F_1-hexamer. The torque of γ causes alternating conformational changes in the β-subunits, leading to varying nucleotide binding affinities (open, tight and loose) which finally result in either ATP synthesis or hydrolysis. This mode of action is defined as Boyer's 'binding-change mechanism' [1,2] and is consistent with structural and kinetic analyses (reviewed in [3] and [4]). Mitochondrial ATP synthases (Table 1) possess the bacterial core set of subunits, but contain additional subunits in the F_0 domain (subunits *8*, *f* and *i*/*j*) and in the stator (subunits *d* and *h*) [5,6]. Moreover, a homolog of subunit *b* (subunit *b*′) has been identified as a ninth ATP synthase subunit in cyanobacteria [7] and chloroplasts [8,9]. (See Table 1)

The stoichiometry of the *c*-ring can also vary between species: *Escherichia coli* [11–13] and yeast mitochondria [14] have a c_{10}-oligomer, whereas c_{11}-, c_{13}- and c_{14}-multimers are found in the F_0 domains of *Ilyobacter tartaricus* [15], *Chlamydomonas* [16] and spinach chloroplasts [17,18], respectively. The largest known *c*-ring (in the cyanobacterium *Spirulina platensis*) consists of 15 *c*-subunits [19,20]. The stoichiometry of the *c*-ring has a direct impact on the bioenergetics of the complex, since the number of *c*-subunits defines the number of ions that have to be translocated across the membrane to produce one ATP molecule. Thus, in case of the c_{14}-ring in chloroplasts, 14 protons must be transferred from the lumen into the stroma to drive a complete 360° rotation of $\gamma\varepsilon c_{14}$. Each of the three catalytic sites in the F_1 domain releases one ATP after a complete stalk rotation. Therefore, the calculated H^+ efflux required to generate a single ATP is 4.67 (14 H^+/3 ATP).

A remarkable feature of mitochondrial ATP synthases is their ability to form dimers (reviewed in [21]). This was first demonstrated by separation of solubilized yeast mitochondrial membrane complexes

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Table 1
Subunit composition of F_1F_0-ATP synthase in mitochondria (*S. cerevisiae*), bacteria (*E. coli*) and chloroplasts (*A. thaliana*). Conserved subunits are listed in the same row. Lengths of amino acid sequences (AA) including N-terminal targeting sequences and the genetic compartment in which the gene is located are indicated for all subunits (N, nucleus; M, mitochondrion; C, chloroplast). All protein entries are derived from the KEGG database (http://www.genome.jp/kegg/). The assignment of chloroplast-encoded subunits to the large or small gene cluster is indicated by *atp1* or *atp2*, respectively. Note that in mitochondria Atp16p is structurally related to bacterial and chloroplast ε subunits, Atp15p is not structurally related to bacterial and chloroplast ε subunits and OSCP/Atp5p is structurally related to bacterial and chloroplast δ subunits. Subunits of the inhibitor complex (Inh1p, Stf1p,Stf2p and Sfl2) in yeast mitochondria [5,10] are not listed.

		Mitochondria (*S. cerevisiae*)					Bacteria (*E. coli*)			Chloroplasts (*A. thaliana*)			
	Subunit	Synonym	Gene	Genome/cluster	KEGG entry	AA	Gene	KEGG entry	AA	Gene	Genome/cluster	KEGG entry	AA
F_1	α	Atp1p	*ATP1*	N	YBL099W	545	*atpA*	b3734	513	*atpA*	C, *atp1*	ArthCp007	507
	β	Atp2p	*ATP2*	N	YJR121W	511	*atpD*	b3732	460	*atpB*	C, *atp2*	ArthCp029	498
	$\gamma_{(1)}$	Atp3p	*ATP3*	N	YBR039W	311	*atpG*	b3733	287	*ATPC1*	N	AT4G04640	373
	γ_2	–	–	–	–	–	–	–	–	*ATPC2*	N	AT1G15700	386
	δ	OSCP/Atp5p	*ATP5*	N	YDR298C	212	*atpH*	b3735	177	*ATPD*	N	AT4G09650	234
	ε	δ, Atp16p	*ATP16*	N	YDL004W	160	*atpC*	b3731	139	*atpE*	C, *atp2*	ArthCp028	132
		ε, Atp15p	*ATP15*	N	YPL271W	62							
F_0	*a*	Atp6p	*ATP6*	M	Q0085	259	*atpB*	b3738	271	*atpI*	C, *atp1*	ArthCp010	249
	b	Atp4p	*ATP4*	N	YPL078C	244	*atpF*	b3736	156	*atpF*	C, *atp1*	ArthCp008	184
	b′	–	–	–	–	–	–	–	–	*ATPG*	N	AT4G32260	219
	c	Atp9p	*ATP9*	M	Q0130	76	*atpE*	b3737	79	*atpH*	C, *atp1*	ArthCp009	81
	d	Atp7p	*ATP7*	N	YKL016C	174	–	–	–	–	–	–	–
	f	Atp17p	*ATP17*	N	YDR377W	101	–	–	–	–	–	–	–
	h	Atp14p	*ATP14*	N	YLR295C	124	–	–	–	–	–	–	–
	j/i	Atp18p	*ATP18*	N	YML081C-A	59	–	–	–	–	–	–	–
	8	Atp8p	*ATP8*	M	Q0080	48	–	–	–	–	–	–	–
	e	Atp21p	*ATP21*	N	YDR322C-A	96	–	–	–	–	–	–	–
	g	Atp20p	*ATP20*	N	YPR020W	115	–	–	–	–	–	–	–
	k	Atp19p	*ATP19*	N	YOL077W-A	68	–	–	–	–	–	–	–

using Blue-native gel electrophoresis [22]. The three subunits *e*, *g* and *k* (Table 1) were found to be associated with dimers; of these subunits, *e* and *g* are essential for dimer formation and important for cristae morphology but not for ATP synthase activity [23]. In addition, the *b*-subunit is also involved in dimerization [24]. Subunit *b* in mitochondria consists of two membrane-spanning segments, of which the first from the N-terminus is unique to mitochondrial ATP synthases. Cross-linking studies with truncated forms of subunit *b* and analyses of mutants showed that this N-terminal transmembrane helix interacts with the dimer-specific subunit *g*, but is not necessary for ATP synthase activity. Electron cryotomography of mitochondrial membranes from yeast has yielded a structural model of the ATP synthase dimer at 3.7 nm resolution, which reveals its V-like shape with the long axes of the two monomers separated by an angle of 86° [25]. The interface in the membrane could be localized to the base of the peripheral stalks of two adjacent monomers. Cryotomographic studies showed that chloroplast ATP synthases are found on stroma lamellae and nonstacked grana end membranes [26], but are excluded from grana stacks [27] which can be explained by the fact that the bulky CF_1 subcomplex with a size of about 16 nm protrudes into the stroma and prevents migration into the densely packed grana structures. Evidence for chloroplast ATP synthase dimerization in *Chlamydomonas reinhardtii* comes from blue-native polyacrylamide gel electrophoresis analyses of solubilized thylakoid membranes [28]. In contrast to mitochondrial ATP synthase dimers, these putative dimers dissociated upon treatment of thylakoid membranes with vanadate or phosphate. Since no homologs of the mitochondrial subunits *e*, *g* and *k* are present in chloroplasts, a different dimerization mechanism must be postulated for chloroplast ATP synthases, with a contact site on the hydrophilic F_1 domain. However, cryoelectron tomography analyses of thylakoid membranes of spinach

Table 2
F_1F_0-ATP synthase auxiliary factors in mitochondria (*S. cerevisiae*), bacteria (*E. coli*) and chloroplasts (*A. thaliana*). The length of amino acid sequences (AA) including N-terminal targeting sequences are provided for all factors. Protein entries are derived from the KEGG database (http://www.genome.jp/kegg/). Note that the ALB4 homologs in bacteria (YidC) and yeast (Oxa1p) appear to have different functions from their plant counterpart.

	Mitochondria (*S. cerevisiae*)				Bacteria (*E. coli*)				Chloroplasts (*A. thaliana*)			
	Name	KEGG entry	AA	Reference	Name	KEGG entry	AA	Reference	Name	KEGG entry	AA	Reference
Translation	Aep1p	YMR064W	518	[121]					ATP4/SVR7	AT4G16390	702	[122–124]
	Aep2p	YMR282C	580	[121,125]					PPR10[a]	AT2G18940	802	[102]
	Atp22p	YDR350C	684	[126]					TDA1[b]	FR850045[c]	1266	[127]
	Atp25p	YMR098C	612	[128]								
F_1 assembly	Atp11p	YNL315C	318	[129,130]								
	Atp12p	YJL180C	325	[129,131]								
	Fmc1p	YIL098C	155	[132]								
	Hsp90	YPL240C	709	[133]								
		YMR186W	705									
F_0 assembly	Atp25p	YMR098C	612	[128]	YidC	b3705	548	[134]				
	Atp23p	YNR020C	270	[135]	Atp1/ UncI	b3739	126	[136,137]	CGL160	AT2G31040	350	[120]
	Mia40	YKL195W	403	[138]								
	Oxa1p	YER154W	402	[139]								
	Atp10p	YLR393W	279	[140]								
Stator and F_1-F_0 assembly	Ina17	YPL099C	182	[141]					ALB4	AT1G24490	462	[142]
	Ina22	YIR024C	216	[141]								

[a] *Arabidopsis* ortholog.
[b] *Chlamydomonas reinhardtii* TDA1, no ortholog found in *Arabidopsis*.
[c] EMBL accession.

and pea showed that only a small fraction of chloroplast ATP synthase complexes assemble into higher-order complexes, and that this is a random process [26]. This finding casts doubt on the idea that dedicated mechanisms exist to drive chloroplast ATP synthase dimerization.

2. Regulation of F_1F_0-ATP synthase activity

Regulation of ATP synthase activity in mitochondria and bacteria has been described in previous reviews [29–34] and is not dealt with in the following. ATP synthase activity in chloroplasts is regulated by several, concerted mechanisms which control the switch from an active into a resting state in the dark, as well as enabling fine-tuning of the enzymatic activity under various environmental conditions. Reversible, enzymatic inactivation of hydrolysis is especially important for chloroplast ATP synthases, since excessive hydrolytic depletion of the ATP pool during the night, when the proton motive force (*pmf*) is low, must be prevented. It has been demonstrated that the inactivity of chloroplast ATP synthases at low *pmf* is mediated by the binding of one Mg^{2+}-ADP molecule to one of the three catalytic sites, which switches the complex into a resting state [35]. Conversely, activation requires an increase in the *pmf* to displace the tightly bound Mg^{2+}-ADP [36,37]. Following prolonged periods (about 20 min) in darkness, higher *pmf* levels are required to restart ATP synthase activity in chloroplasts [38]. This phenomenon can be attributed to the thioredoxin-dependent thiol modulation of the γ-subunit, and constitutes a regulatory mechanism which is specific to plants [39] and green algae [40], and is not found in diatoms [41] or cyanobacteria [7]. A disulfide bridge between two cysteine residues in the γ-subunit is under the redox control of thioredoxin-*f* [42], which in turn is reduced by PSI via the ferredoxin:thioredoxin oxidoreductase in the light. Thiol modification of the γ-subunit modulates the *pmf* amplitude necessary for chloroplast ATP synthase activation. Thus, the *pmf* required for activation is reduced to ~50 mV in the active state compared to ~100 mV in the resting state [38].

In bacteria and chloroplasts, ATP synthase activity is also regulated by inhibition of ATP hydrolysis mediated by subunit ε [43–46], whose structure is conserved between bacterial and chloroplast enzymes. It comprises an N-terminal β-sandwich domain (ε-NTD) and a C-terminal helix-turn-helix domain (ε-CTD) [47,48], with the latter being responsible for its inhibitory activity [49,50]. Two different ε confirmations have been described [51–53]. Tsunoda and coworkers showed that hydrolysis, but not ATP synthesis, is inhibited in the 'up state' or the 'extended conformation', i.e. when ε-CTD is facing the F_1 domain [53], and reactivated when ε-CTD returns to the 'down state' or the 'retracted conformation' i.e. is nearer the F_0 domain [53]. When ε-CTD interacts in its extended form with the DELSEED motif of the β-subunit, ATP hydrolysis is inhibited [54]. The conformational changes of the ε-subunit are influenced by the ATP/ADP ratio: bacterial ε-subunits can bind ATP [55–57] and in the presence of ATP and an increased *pmf*, the extended conformation of the ε-subunit is converted into the down-state conformation. Conversely, addition of ADP reverses this transition [58]. Several studies indicate that chloroplast ATP synthase subunit ε undergoes conformational changes similar to those seen in the bacterial enzyme [49,59–61]. Remarkably, the chloroplast subunit seems to have a more pronounced effect on ATP hydrolysis than its bacterial counterpart [45,50,62] (reviewed in [63]). Moreover, the conformational transitions of ε-CTD are induced by the rise in *pmf* upon illumination [64], but are independent of ATP [45]. This points to a distinct molecular mechanism, which is probably based on a structural interplay of subunits ε, γ and thiol modifications [65,66].

Besides the dark–light switch, chloroplast ATP synthase activity is subject to fine-tuning during steady-state photosynthesis, which is referred to as 'metabolism-related' chloroplast ATP synthase regulation. These adjustments are observed under changing CO_2 or O_2 concentrations [67], drought [68], altered Calvin–Benson cycle capacities or altered starch synthesis [69,70]. The mechanisms underlying metabolism-related regulation have not yet been resolved, but several processes may contribute to it, including (i) substrate binding of ADP and P_i [71–74], (ii) posttranslational subunit phosphorylation [75–79], (iii) posttranslational subunit acetylation [80,81], and (iv) reactive oxygen species [82–84]. In vivo spectroscopic analyses of *Arabidopsis* mutant lines lacking thiol-based modulation of the γ-subunit imply that metabolism-related and dark–light regulations are mechanistically distinct [85].

3. Genetic organization of F_1F_0-ATP synthase genes

In bacteria, ATP synthase genes are generally organized in a single transcription unit (*atpIBEFHAGDC*) [86,87], while in cyanobacteria the ATP synthase genes are organized into two operons [7,88,89]. In bacteria, the *atp1/uncI* gene precedes the structural genes of the large operon and encodes a small, hydrophobic protein. Early studies showed that Atp1/UncI can be copurified with bacterial ATP synthases [90] but is present in substoichiometric amounts relative to subunits of the ATP synthases [91]. Genes encoding subunits of the F_0 (*atpBEF* for subunit *a*, *c* and *b*) and the F_1 sector (*atpHAGDC* for subunit δ, α, γ, β and ε) are arranged in two clusters, but are all translated from a single polycistronic mRNA. In order to ensure balanced subunit stoichiometry, expression is regulated by a variety of translational mechanisms which involve translational coupling (interdependency of the translation of two genes) and control of translation initiation by mRNA secondary structures [92–94].

The biogenesis of eukaryotic ATP synthase subunits requires more sophisticated controls, since ATP synthase genes are encoded in both nuclear and organellar genomes. Consequently, ATP synthase genes have to be expressed in different compartments and the products of nuclear genes must be imported into the appropriate organelle and coordinately assembled into functional complexes. Seven of nine chloroplast ATP synthase subunits are encoded by two chloroplast gene clusters [95]. As in cyanobacteria, subunits β and ε are encoded in a small gene cluster (*atpB/E*) designated as *atp2*, whereas genes for *a*, *c*, *b* and α are arranged in a large cluster (*atpIHFA*) referred to as *atp1* (reviewed in [96]). Genes for subunits γ (*atpC*), δ (*atpD*) and *b′* (*atpG*) form part of the large gene cluster *atp1* in cyanobacteria (*atpIHGFDAC*), but their plant counterparts reside in the nucleus. For subunit γ, two nuclear genes (*ATPC1* and *ATPC2*) have been identified in *Arabidopsis thaliana* [97] and other subgroups of Viridiplantae [98]. *ATPC1* is predominantly expressed in photosynthetic tissues and *ATPC2* in roots, and Kohzuma et al. [98] proposed that the γ_2-containing ATP synthase in non-photosynthetic plastids maintains an ATP-hydrolysis driven, basal proton gradient required for non-photosynthetic processes (e.g. protein translocation or ion transport). Several promoters are located in the plastome-encoded *atp1* cluster [99], as well as a group II intron in *atpF* [100], which give rise to polycistronic precursors that are processed and spliced to generate a complex set of transcripts whose number varies among plant species [99,101,102]. Similarly, the two genes in the small *atp2* cluster are transcribed from several start sites to generate bicistronic *atpB/E* transcripts. In addition, *atpE* is transcribed from an *atpB*-internal promoter as a monocistronic mRNA [103]. Remarkably, the start codon of *atpE* overlaps with the stop codon of *atpB* [104], suggesting that *atpE* is translationally coupled to *atpB* [105]. However, in vitro studies in tobacco chloroplasts and a newly developed ribosome footprint profiling approach provided evidence that *atpE* translation from bicistronic mRNAs is independent of *atpB* translation [106,107] and that *atpE* translation is under the control of its own *cis*-acting elements in the *atpB* coding region [106].

In the majority of eukaryotes, most of the genes for mitochondrial F_1-subunits are found in the nucleus, and only the three hydrophobic F_0-subunits *a* (Atp6p), *c* (Atp9p) and *8* (Atp8p) (Table 1) are encoded by mitochondrial genes. However, some exceptions exist. Plants still retain *ATP1* (codes for the α-subunit) in their mitochondrial genomes

[108], subunit *c* in animals is the product of a nuclear gene [109] and the entire set of genes for the mitochondrial ATP synthase was transferred to the nuclear genome in algae [110]. Furthermore, a gene duplication of *ATP9* (codes for subunit *c*) with one copy in the mitochondrial and a second copy in the nuclear genome has been identified in the filamentous fungi *Neurospora crassa* [111] and *Aspergillus nidulans* [112]. In yeast, the mitochondrially encoded *ATP9* is co-transcribed with the serine-specific tRNA ($tRNA^{Ser}$) and the *VAR1* gene (*ATP9*/$tRNA^{Ser}$/*VAR1* cluster) [113,114]. The two other mitochondrial genes, *ATP6* (codes for subunit *a*) and *ATP8*, form a transcriptional unit with *COX1* and – in some yeast strains – with the *ENS2* gene [115], which code for cytochrome oxidase 1 and a DNA endonuclease, respectively (*COX1*/*ATP8*/*ATP6*/*ENS2* cluster) [113,114]. The *COX1*/*ATP8*/*ATP6*/*ENS2* cluster is transcribed from one and the *ATP9*/$tRNA^{Ser}$/*VAR1* cluster from two initiation sites [116]. In both cases, polycistronic precursors are processed at multiple, internal cleavage sites, generating a whole set of mRNAs.

4. Assembly of the mitochondrial F_1F_0-ATP synthase

Because most of F_1F_0-ATP synthase assembly is known in mitochondria, we discuss research on this process in the order of mitochondria, bacteria and chloroplast. This design allows us to refer to already described mitochondrial factors or assembly steps in subsequent chapters.

Recent studies indicate that mitochondrial ATP synthases are built up from several pre-assembled subcomplexes and that the pore-forming complex, consisting of the *c*-ring and subunit *a*, is assembled last, in order to avoid a futile proton efflux concomitant with the dissipation of the mitochondrial membrane potential. Translation and the modular assembly process are guided by several auxiliary factors (Table 2), most of which have been identified by screening for respiration-defective yeast mutants [143]. Factors necessary for expression of mitochondrial-encoded subunits have been described in detail in previous reviews [144,145] and are not discussed in the following.

4.1. Assembly of mitochondrial F_1

The soluble F_1 module with its $\alpha_3\beta_3$ hexamer and the central stalk $\gamma\delta\varepsilon$ is assembled independently of both F_0 and the stator module [146,147], and with the assistance of the nucleus-encoded but matrix-localized chaperones Atp11p and Atp12p [129,148] (Fig. 1A). In the absence of either chaperone, the hexamer fails to form and α- and β-subunits aggregate into insoluble, high-molecular-weight complexes [149]. Protein–protein interaction studies have revealed that Atp12p and Atp11p bind α- and β-subunits respectively, yielding the heterodimers Atp12p-α and Atp11p-β [150,151] and it has been suggested that both act as decoys to prevent unwanted homomeric α–α or β–β complexes. Recently, 3-D structures of Atp11p from *Candida glabrata* and Atp12p from the Gram-negative bacterium *Paracoccus denitrificans* were reported [152]. These reveal that the C-terminal region of Atp12p and the coiled-coil tail of the γ-subunit are structurally similar, prompting the hypothesis that the binding of γ to the Atp12p-α complex initiates the release of Atp12p to allow the correct formation of αβ complexes. Other chaperones, Fmc1p and Hsp90, are required for proper F_1 assembly under conditions of heat stress [132,133]. Interestingly, the absence of Fmc1p can be compensated by increasing levels of Atp12p. This observation points to a function for Fmc1p in Atp12p folding or stabilization [132].

4.2. Assembly of mitochondrial F_0

Construction of the *c*-ring is probably the first step in F_0 assembly, which occurs independently of F_1 (Fig. 1A). The accessory factors Aep1p, Aep2p and Atp25p are necessary for efficient expression of subunit *c* [121,125,128,153]. Remarkably, Atp25p is posttranslationally cleaved into two fragments which exhibit two distinct functions; the 35-kD C-terminal half stabilizes the *ATP9* mRNA coding for the *c*-subunit, whereas the N-terminal half promotes *c*-ring oligomerization [128]. Cotranslational insertion of several membrane proteins in mitochondria – like subunit 2 of the cytochrome oxidase [154] – depends on the Oxa1p translocase. Oxa1p is not required for membrane insertion of subunit *c* [155–157], but it does play a role in the association of the *c*-ring with the *a*-subunit [156]. In the absence of Oxa1p, c_{10}-oligomerization still occurs, but the assembly with the membrane-integrated *a*-subunit is perturbed, implying that Oxa1p stabilizes a competent c_{10}-containing assembly intermediate, which is capable of interacting with subunit *a* [156]. Once the *c*-ring module is assembled, the soluble F_1 module is thought to bind independently of other F_0-subunits to the c_{10} oligomer via the stalk subunits to form a larger ATP synthase intermediate [117] (Fig. 1A).

The two integral membrane F_0-subunits *a* and *8* constitute the core of a precursor complex that forms independently of other mitochondrial ATP synthase intermediates [117] and represents the third module in the assembly process (Fig. 1A). Subunits *a* and *8* are mitochondrially encoded and are each translated from distinct bicistronic mRNAs. Translation of mRNAs for *a*- and *8*-subunits depends on direct activation by an F_1 assembly intermediate via an as yet unknown mechanism [158]. Mutants that fail to assemble F_1 transcribe normal levels of the bicistronic mRNAs (for *a*- and *8*-subunits), but synthesize neither subunit *8* nor *a*. Those observations support the existence of a mechanism, which ensures that production of mitochondrially encoded subunits of F_0 is dependent on the presence of an assembly intermediate composed exclusively of imported, nucleus-encoded subunits. Accordingly, the F_1-dependent control of F_0-subunit translation ensures a balance between mitochondrially encoded F_0-subunits and the imported F_1-subunits. Surprisingly, the translational defect was partially rescued when *ATP22*, which codes for a component of the mitochondrial inner membrane, was overexpressed in F_1-defective mutants. In fact, Atp22p promotes synthesis of subunit *a* and interacts with the 5′-UTR of the mRNA for subunit *a* [126,159], but levels of mitochondrial transcripts encoding F_0-subunits are not altered in the absence of Atp22p. The effect of Atp22p on *a*-subunit synthesis is specific, since *8*- and *c*-subunits were still synthesized in *atp22* yeast mutants [159]. The posttranslational fate of the *a*-subunit is also highly regulated [160]. After translocation across the inner membrane, the first ten amino acids at the N-terminus are cleaved off by the metalloprotease Atp23p [135,161]. Atp23p is conserved from yeast to humans and is localized to the mitochondrial inner membrane. Folding of Atp23p and correct formation of intramolecular disulfide bonds are mediated by the oxidoreductase Mia40, which prevents aggregation of Atp23p [138]. Deletion of the *ATP23* gene results in abnormal F_0 assembly in yeast. However, yeast strains that express Atp23p variants defective only in the N-terminal processing of subunit *a* are still able to assemble functional ATP synthase complexes [135], implying that Atp23p has an additional function in the assembly process besides its proteolytic activity.

The function of the N-terminal pre-sequence of subunit *a* was further examined by constructing mutants carrying leaderless subunit *a* variants [162]. These deletions reduced the efficiency of ATP synthase assembly by 50%, with concomitant accumulation of an *a8*-containing intermediate. These findings indicate that the pre-sequence is involved in the targeting of subunit *a* to the *c*-ring or in promoting insertion of the subunit *a* precursor into a micro-compartment of the membrane for efficient interaction with the *c*-ring.

After membrane translocation and maturation, subunit *a* associates with subunit *8* and appears to form a transient complex with at least two stator subunits (*b*, *d* and probably *h*) and the chaperone Atp10p, but not with the aforementioned chaperone Atp23p [117]. Atp10p is a component of the mitochondrial inner membrane that binds subunit *a* and facilitates the subsequent incorporation of the *a8*-containing intermediate with the *c*-ring-containing module [117,163].

4.3. Linkage of mitochondrial F_1 and F_0

Late steps in the assembly of the mitochondrial ATP synthase are mediated by the inner membrane assembly complex INAC [141]. INAC

is composed of the two transmembrane proteins Ina22 and Ina17, which are found exclusively in the fungal kingdom. INAC associates with several F_1 and peripheral stator subunits, but is not a component of the fully assembled ATP synthase. In the absence of either Ina22 or Ina17, a larger fraction of the F_1 portion remains unconnected to the F_0 portion and the peripheral stalk subunits OSCP (related to bacterial δ) and *b* fail to assemble properly [141]. Nevertheless, *ina22* and *ina17* strains were still capable of forming fully assembled ATP synthases. These results together imply that INAC mediates stator assembly and promotes the linkage of F_1 and F_0. Indeed, the discovery of INAC suggests the existence of an alternative stator subunit incorporation pathway: in contrast to the recently discovered *a8*-Atp10p-stator intermediate [117], Lytovchenko et al. (2014) [141] postulate the existence of a precursor comprising the complete F_1 module and the stator subunits.

In spite of uncertainties concerning the order of late assembly steps, the intermediates involved and the role of subunit OSCP (related to bacterial δ), it is generally accepted that the pore-forming subunits *c* and *a* come together in the last step of ATP synthase assembly (Fig. 1A), thus avoiding the risk of dissipation of the *pmf* without ATP generation.

Fully assembled monomeric mitochondrial ATP synthases dimerize via the interaction of the first transmembrane helix of two adjacent *b*-subunits and the participation of subunits *e* and *g* [25]. This stepwise assembly process is aided by the two small subunits *i* and *k*, which facilitate incorporation of subunits *e* and *g* into the monomer and promote stabilization of the dimer, respectively [164]. Dimers, in turn, can assemble into rows, which account for the formation of highly curved ridges in mitochondrial cristae [25,165,166].

5. Assembly of the bacterial F_1F_0-ATP synthase

In bacteria, as in mitochondria, a modular ATP synthase assembly process involving formation of several defined intermediates (Fig. 1B) and the intervention of auxiliary factors is thought to take place [118]. However, fewer accessory factors have been identified in bacteria, perhaps because less are required (See Table 2). This hypothesis is supported by the fact that bacterial ATP synthase genes are all clustered in a single, chromosomal operon, obviating the need to coordinate nuclear with organellar gene expression, and import proteins synthesized elsewhere. Furthermore, bacterial ATP synthases are made up of only eight subunits (nine in cyanobacteria), whereas their mitochondrial counterparts harbor additional stator and dimer-specific components (Table 1). Thus, fewer intermediate steps can be expected for bacteria, and would be compatible with a smaller set of assembly factors.

5.1. Assembly of bacterial F_1

Like its mitochondrial equivalent, the bacterial F_1 module $\alpha_3\beta_3\gamma\varepsilon$ accumulates independently of F_0 in the cytosol [118], but the precise course of its assembly is unclear. In fact, F_1 can be reconstituted in-vitro from purified F_1 components [167,168]. Moreover, a γε-complex has been purified from *E. coli* [52] and formation of an active $\alpha_3\beta_3\gamma$ cytosolic complex in an *E. coli* strain over-expressing the respective subunits has been reported recently [169]. Accumulation of such catalytic F_1 intermediates has to be prevented during assembly since wasteful ATPase activity leads to a lowered intracellular ATP/ADP ratio concomitant with slower growth rates. The F_1-specific chaperone Atp11p found in mitochondria is absent in bacteria and Atp12p homologs have been identified only in Proteobacteria [170], but not in *E. coli*. Despite the availability of a high-resolution crystal structure from the proteobacterium *P. denitrificans* [152], a thorough functional characterization of (proteo)bacterial Atp12p is still lacking.

5.2. Assembly of bacterial F_0

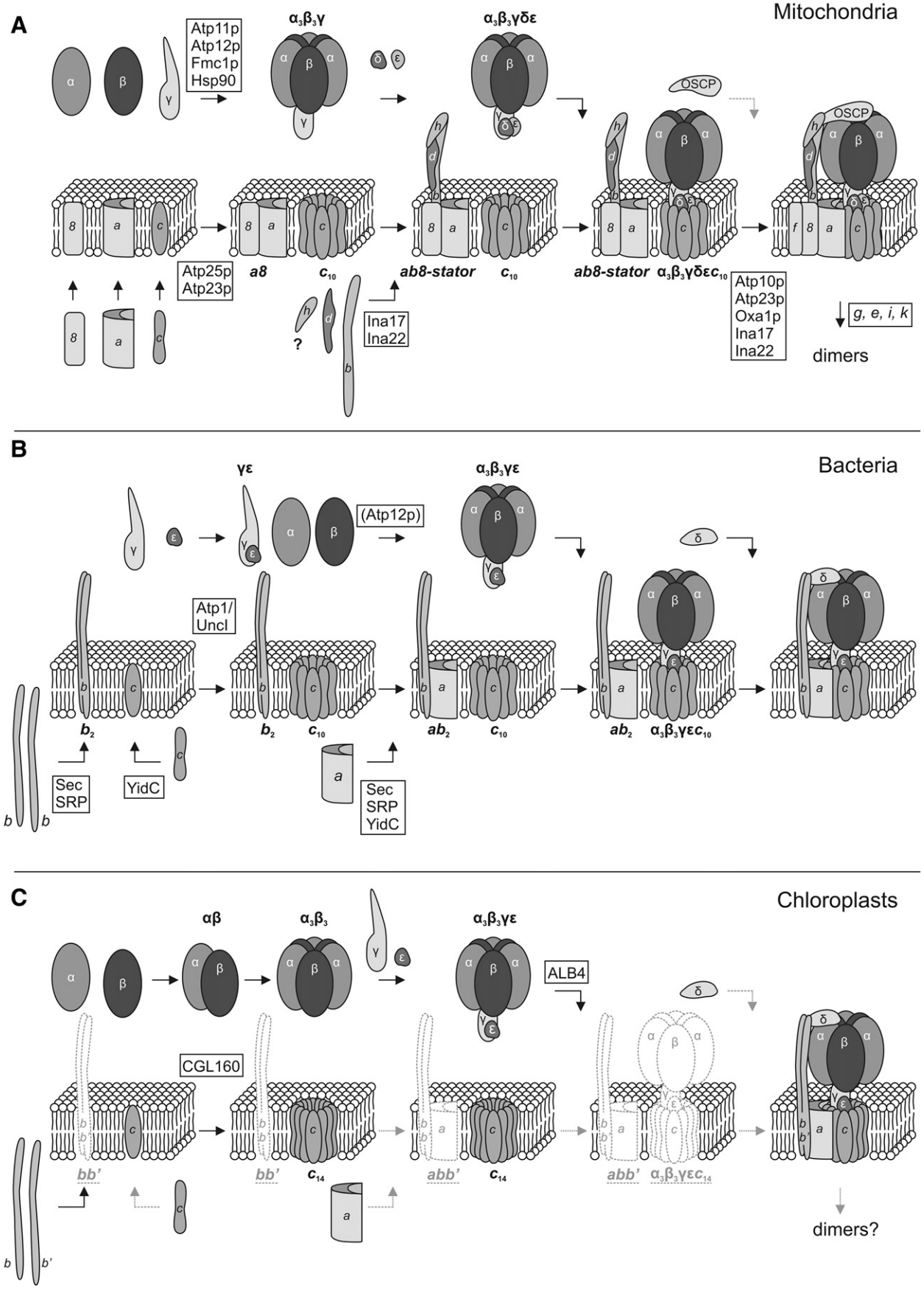
Membrane insertion of F_0-subunits is dependent on the SecYEG translocon, the signal recognition particle pathway (SRP) and YidC, which belongs to the Oxa1 protein family. The SecYEG translocon and the SRP pathway are essential for co-translational insertion of both subunits *a* and *b* [171], but efficient insertion of *a* requires YidC as well [172,173]. Conversely, insertion of the hydrophobic subunit *c* is mediated by YidC alone [134]. Subunits *b* and *c* can be cotranslationally inserted into the membrane independently of *a* [174], whereas stable integration of *a*-subunits strictly depends on the presence of *b* and *c* [174]. Moreover, the free subunit *a* is subject to rapid degradation by the protease FtsH [175]. In contrast to the observed cotranslational SecYEG/SRP/YidC-dependent membrane integration of *a*, it has been reported that membrane-embedded, monomeric *c*-subunits are sufficient to trigger the insertion of subunit *a* [176].

After membrane insertion, monomeric *c*-subunits are assembled into a c_{10}-ring, a process, which is thought to involve the chaperone Atp1/UncI (Fig. 1B). However, based on in vitro analyses it was also suggested that *c*-subunit oligomerization can occur spontaneously and that the ability to form rings is determined by the primary structure of subunit *c* [177]. Atp1/UncI are small, hydrophobic proteins (14 kDa in *E. coli*) and are generally encoded by the gene that precedes the structural genes in the large bacterial ATP synthase operon [87,178]. Deletion of *uncI* results in only slightly reduced growth yields in *E. coli*, implying that Atp1/UncI is not essential for ATP synthase assembly [179]. In the alkaliphilic *Bacillus pseudofirmus* OF4 also, Atp1/UncI is not essential for ATP synthase function, and a *B. pseudofirmus* strain deleted for *atp1* could still grow nonfermentatively [180]. Recently, a hybrid ATP synthase with F_1 from *Bacillus* PS3 and F_0 from *Propionigenium modestum* was generated in *E. coli* [136]. Atp1/UncI from *P. modestum* was found to be indispensable for *c*-ring formation and for the ATP synthase activity of this hybrid complex. Similarly, functional production of the sodium-driven F_1F_0-ATP synthase from *Acetobacterium woodii* in *E. coli* requires the *A. woodii atp1/uncI* gene for proper assembly [181]. Free *c*-monomers, as well as assembled *c*-rings, can be copurified together with *P. modestum* UncI/Atp1 [136]. Moreover, the oligomerization of *P. modestum* *c*-subunits into c_{11}-rings is mediated by Atp1/UncI in vitro [137], indicating that Atp1/UncI plays a role in *c*-ring assembly.

A combination of nearest-neighbor analyses based on substituted cysteine pairs for disulfide cross-linking and His-tag affinity purification of membrane-bound subcomplexes in different mutant strain backgrounds has identified potential intermediates in the assembly process ([12,182,183] summarized in [118]). F_0 intermediates can act as uncouplers of the *pmf* in bacteria [184,185], thus formation of the membranous ATP synthase subcomplex requires also tight regulation and a coordinated assembly process. The *c*-ring is assumed to be the starter complex in the assembly process and is formed independently of other ATP synthase subunits, since it could be detected in an *atp* operon-deficient strain expressing only the *atpE* gene (for subunit *c*) [12]. Similarly, dimers of subunit *b* accumulate in the absence of other ATP synthase subunits [183]. However, b_2c_{10} intermediates could not be identified in cross-linking studies when both subunits were synthesized simultaneously in an *atp* operon-deficient *E. coli* strain [183], which is incompatible with recently published assembly models for bacterial ATP synthases in which the formation of a b_2c_{10} intermediate was suggested [186,187]. Instead, a second F_0 module made up of subunit *a* and the b_2 dimer was identified (Fig. 1B). This ab_2 intermediate does not interact with α-, β- or *c*-subunits in the absence of subunit δ [183].

5.3. Linkage of bacterial F_0 and F_1

The integration of F_1 into the ATP synthase complex is thought to occur through the binding of $\alpha_3\beta_3\gamma\varepsilon$ to the *c*-ring module, and the presence of an $\alpha_3\beta_3\gamma\varepsilon c_{10}$ subcomplex in addition to an ab_2 intermediate could be demonstrated in the Δδ mutant background [183] (Fig. 1B). Both complexes appear to be true assembly intermediates as demonstrated by the use of a delayed expression system in which subunit δ synthesis was induced in the Δδ mutant background. In a similar


A
Mitochondria
Atp11p
Atp12p
Fmc1p
Hsp90
α3β3γ
α3β3γδε
OSCP
Atp25p
Atp23p
a8
c10
ab8-stator
c10
ab8-stator α3β3γδεc10
Atp10p
Atp23p
Oxa1p
Ina17
Ina22
g, e, i, k
dimers
Ina17
Ina22
?
B
Bacteria
γε
α3β3γε
(Atp12p)
Atp1/
UncI
b2
b2 c10
ab2
c10
ab2 α3β3γεc10
Sec
SRP
YidC
a
Sec
SRP
YidC
C
Chloroplasts
αβ
α3β3
α3β3γε
ALB4
CGL160
bb'
bb' c14
abb'
c14
abb' α3β3γεc14
dimers?

approach, *a*-subunits synthesized after a delay could be functionally integrated into a preformed $\alpha_3\beta_3\gamma\varepsilon\delta b_2c_{10}$ complex in Δ*a* mutants [182]. A comparable result was obtained with the thermophilic bacterium *Bacillus* PS3, which is able to assemble an ATP synthase complex lacking only the *a*-subunit. Such purified or in vitro translated *a*-less complexes could be functionally reconstituted by supplying purified *a*-subunits [188,189]. Moreover, an ATP synthase lacking only subunits *a* and A6L has been identified in human ρ^0 cells [190].

Compelling experimental evidence for the final steps in the bacterial ATP synthase assembly process is currently lacking, but by analogy to the mitochondrial ATP synthase assembly model, it has been suggested that the ab_2 stator constitutes a distinct module, which is then incorporated into the complex with the help of the δ-subunit [118,183] (Fig. 1B). Whether δ binds first to ab_2 to form an $ab_2\delta$ pre-complex or associates with the N-terminal region of the α-subunit in F_1F_0 core complexes [191,192] needs to be clarified in future experiments.

6. Assembly of the chloroplast F_1F_0-ATP synthase

Compared to mitochondrial ATP synthase assembly, relatively little is known about ATP synthase assembly in chloroplasts, and only a few auxiliary factors have been characterized (See Table 2). Although its subunit composition resembles that of the bacterial enzyme (Table 1), an additional level of complexity arises from the fact that the structural genes encoding the constituents of chloroplast F_1 (CF_1) and F_0 (CF_0) are distributed between nuclear (subunits γ, δ and *b′*) and plastid (subunits α, β, ε, *a*, *b* and *c*) genomes. Therefore, gene expression in both compartments has to be tightly controlled to avoid accumulation of pre-complexes and ensure the coordinated assembly of widely different numbers of the various subunits of CF_1 ($\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$) and CF_0 ($a_1b_1b'_1c_{14}$).

6.1. Auxiliary factors necessary for chloroplast F_1F_0-ATP synthase subunit expression

Some nucleus-encoded proteins involved in the expression of chloroplast genes for ATP synthase subunits have been identified and characterized (Table 2). ATP4 is a pentatricopeptide repeat (PPR) protein necessary for translation of the chloroplast *atpB* open reading frame in maize [122]. It associates in vivo with sequences near the 5′ end of the unusually long 5′ UTR of the *atpB/E* mRNA and facilitates ribosome binding. ATP4 is multifunctional, because it also enhances *atpA* translation and is required for the accumulation of specific processed *atpF* and *psaJ* transcripts. ATP4 is orthologous to the Arabidopsis protein SVR7, but their mutant phenotypes differ. Whereas knockout of *ATP4* causes pale-green phenotypes and seedling lethality, mutations in *SVR7* result in slightly pale green and developmentally retarded but viable and fertile plants, most probably because levels of chloroplast ATP synthase are less drastically affected in *svr7* than in *atp4* [123,124]. Unlike *atp4* mutants, *svr7* plants are defective in *rbcL* translation. Another PPR protein in maize (PPR10) stabilizes two sets of chloroplast transcripts: RNAs with a 5′- or 3′-end that lies in either the *atpI–atpH* or *psaJ–rpl33* intergenic region [102]. Thus, PPR10 is essential for the stability of specific processed *atpH* mRNAs and enhances the translation of *atpH*. TDA1 is an octotricopeptide repeat (OPR) protein specifically required for translation of the chloroplast *atpA* transcript in *Chlamydomonas* [127]. TDA1 has a dual function: in addition to activating translation of *atpA* transcripts, it diverts a subset of untranslated *atpA* transcripts into non-polysomic complexes.

In the green alga *Chlamydomonas*, a feedback control mechanism links the synthesis of CF_1 subunits [193]. Synthesis of the nucleus-encoded γ-subunit is required for sustained translation of the chloroplast-encoded β-subunit, which in turn transactivates the translation of chloroplast-encoded α-subunit. Translational down-regulation of β- or α-subunits, in the presence of unassembled copies, involves the untranslated regions (UTRs) of their own mRNAs, indicating that control occurs at the level of translation initiation. In addition, a negative feedback loop exerted by αβ assembly intermediates on the translation of β-subunit can be released when γ-subunit attaches to $\alpha_3\beta_3$ hexamers. Consequently, those intertwined, translational regulatory loops result in the excess accumulation of α and β subunits relative to the γ-subunit, which is an important requirement for the assembly of CF_1 in the correct $\alpha_3\beta_3\gamma_1$ stoichiometry. Thus, γ represents a nucleus-encoded component which controls the translation of its chloroplast-encoded interaction partners in *Chlamydomonas*. However, this feedback control mechanism is not found in land plants. Thus the *Arabidopsis* mutant *dpa1*, which lacks γ, translates α- and β-subunits at normal rates [194], in sharp contrast to its *Chlamydomonas* counterpart [193]. In addition, profiling of ribosome footprints from maize mutants that fail to translate *atpB* suggests that *atpA* translation and ribosome occupancy of *atpA* are not markedly altered [107]. This discrepancy might be explained by the fact that chloroplast ATP synthase genes in chloroplasts of *Chlamydomonas* and higher plants are organized differently. Plastid-encoded subunits are

Fig. 1. Schematic representation of F_1F_0-ATP synthase complex assembly in mitochondria, bacteria and chloroplasts. (A) The mitochondrial ATP synthase assembly process is mainly adapted from Rak et al. 2011 [117]. The soluble F_1 module ($\alpha_3\beta_3\gamma\delta\varepsilon$) assembles independently of both the F_0 and the stator module, and requires several matrix-localized chaperones. The *c*-ring (c_{10}) represents a second assembly module and is probably the first intermediate in F_0 assembly to be completed. The third precursor module is composed of the two membrane-integrated F_0 subunits *a* and *8* (*a8*), is also assembled separately from other F_0 compounds and appears to form a transient complex with at least two stator subunits (*ab8-stator*). Note that the association of subunit *h* with the *ab8-stator* module at this stage in the process is conjectural (hence the question-mark) [117]. The F_1 module is thought to bind via the stalk subunits to the c_{10}-oligomer, independently of other F_0 subunits, to form a larger ATP synthase intermediate ($\alpha_3\beta_3\gamma\delta\varepsilon c_{10}$). Despite uncertainties concerning the order of late assembly steps, the intermediates involved, and the role of the subunit OSCP, it is assumed that subunits *a* and *c* come together to form the proton-translocating ring in the final step of monomeric ATP synthase assembly. Dimerization of fully assembled monomeric ATP synthases is mediated by subunits *e*, *g* and the first transmembrane helix of two adjacent *b*-subunits. Note that *f*-subunit assembly is not shown, but that *f* is part of the fully assembled ATP synthase monomer. Inhibitory factors are omitted for clarity and the dimer is not shown. (B) The bacterial ATP synthase assembly model is mainly adapted from Deckers-Hebestreit 2013 [118]. As in mitochondria, the F_1 module ($\alpha_3\beta_3\gamma\varepsilon$) accumulates independently of F_0 in the cytosol. Homologs of the F_1 chaperone Atp12p have been identified only in proteobacteria and are absent in *E. coli* (indicated by parentheses). The occurrence of a γε intermediate is inferred from the successful purification of such a complex following overexpression of the respective subunits in *E. coli* [52]. Membrane insertion of F_0 subunits depends on the SecYEG translocon (Sec), the signal recognition particle pathway (SRP) and YidC. After YidC-dependent membrane insertion, monomeric *c*-subunits are assembled into a c_{10}-ring, probably with the aid of the chaperone Atp1/UncI. The c_{10}-ring is presumed to be the starter complex in the F_0 assembly process and forms independently of other ATP synthase subunits. Dimers of subunit *b* accumulate in the absence of other ATP synthase subunits and associate with subunit *a* to yield a second F_0 module (ab_2). Integration of F_1 into the ATP synthase complex is thought to occur via the binding of $\alpha_3\beta_3\gamma\varepsilon$ to the *c*-ring module. In the final stage of bacterial ATP synthase assembly, the ab_2 module is incorporated into the complex by binding of the δ-subunit. (C) In contrast to the case in mitochondria and bacteria, CF_0 does not accumulate independently of CF_1. Homologs of the two mitochondrial chaperones Atp11p and Atp12p are absent in chloroplasts, and other auxiliary factors specifically involved in CF_1 assembly have not been characterized. In in-vitro reconstitution experiments, subunits α and β form dimers in a chaperone-dependent process [119]. Three αβ dimers are then assembled into the hexamer. In the final step, folded γ associates with the hexamer to yield an active CF_1 core. The precise nature of the actual intermediates (indicated by white, dashed drawings and by underlined characters) and the sequence of steps in CF_0 assembly (indicated by gray, dashed arrows) remain unclear and are inferred from the bacterial assembly process. Monomeric *c* subunits are assembled into a c_{14}-ring by CGL160, which is similar to its bacterial counterpart Atp1/UncI. A *c*-subunit-containing subcomplex, which does not comigrate with other chloroplast ATP synthase subunits in 2D gel electrophoresis, might represent a true c_{14} oligomer intermediate in CF_0 assembly [120]. CF_1–CF_0-containing intermediates have not been characterized in vivo, but the integral thylakoid membrane protein ALB4 has been proposed to stabilize or promote the assembly of CF_1 during its attachment to the CF_0 portion. No studies of late assembly steps in chloroplast ATP synthase formation and of the role of subunit δ have yet been conducted. Here, we assume, based on the mitochondrial and bacterial assembly models, that the proton-translocating complex, which is made up of a c_{14}- and a subunit *a*-containing subcomplex, assembles in the last step of the chloroplast ATP synthase assembly process. Auxiliary factors required for ATP synthase assembly are highlighted by black boxes. Identified ATP synthase intermediates are indicated in bold and intermediates inferred by association in gray, underlined characters, respectively.

part of two conserved gene clusters in higher plants, whereas they are distributed over the chloroplast chromosome in *Chlamydomonas* [195,196]. Thus, the specialized mechanisms of translational regulation might have evolved in response to gene rearrangements in the plastome of *Chlamydomonas*.

6.2. Import and membrane insertion of chloroplast F_1F_0-ATP synthase subunits

After translation on cytosolic ribosomes, nucleus-encoded subunits γ, δ and *b′* must be imported into the chloroplast and processed into their mature forms. Transit peptides were identified in import assays using isolated pea chloroplasts and in-vitro translated precursors of γ and δ subunits from tobacco [197,198]. The N-terminal presequences of tobacco γ and δ are 55 and 66 residues long and processing results in mature proteins of 36 kD and 20 kD, respectively. In its mature form, nucleus-encoded *b′* is anchored in the thylakoid membrane by a single N-terminal hydrophobic domain, whereas the hydrophilic, C-terminal portion protrudes into the stroma. Subunit *b* is functionally and structurally related to *b′*, but is encoded in the plastid genome. Targeting of *b′* is guided by a long, bipartite transit peptide, which is functionally equivalent to transit peptides found in hydrophilic, lumenal proteins capable of crossing the thylakoid membrane [8]. This peptide comprises a stroma-targeting sequence, a hydrophobic stretch and a recognition motif for the thylakoid processing peptidase. Due to the presence of three negatively charged amino acids near the N-terminal end of the mature protein, membrane insertion of subunit *b′* strictly depends on the hydrophobic targeting signal [199]. Remarkably, thylakoid membrane integration of *b′* occurs independently of all known targeting machineries, and is completed by N-terminal cleavage mediated by the thylakoid processing peptidase [200].

Posttranslational, N-terminal processing has also been observed for the chloroplast-encoded subunits *a* and *b*. Isolation of native *a* from spinach and N-terminal amino acid sequencing revealed that the first 18 amino acids are missing from the mature subunit [201]. In a similar approach, mature *b* from spinach was shown to be 17 residues shorter than the deduced amino acid sequence [100]. However, the function of the processed N-terminal sequences of both subunits in the biogenesis of chloroplast ATP synthase requires clarification.

No studies on membrane integration of subunit *c* have yet been carried out. Split-ubiquitin assays indicate that *c* interacts with ALB3, a factor which is required for the insertion of light-harvesting chlorophyll-binding proteins into the thylakoid membrane [202]. However, ALB3 depletion does not affect chloroplast ATP synthase accumulation in *C. reinhardtii* [203].

6.3. Assembly of the chloroplast F_1

Most information on chloroplast ATP synthase assembly comes from in vitro reconstitution experiments and studies with *Chlamydomonas* and *Arabidopsis* mutants defective for structural subunits. One major divergence from mitochondria and bacteria is that CF_0 does not accumulate independently of CF_1 [204]. Conversely, only residual amounts of CF_1 were detectable in CF_0 mutants of *C. reinhardtii*. Similarly, the lack of the nucleus-encoded subunits γ and δ in *Arabidopsis* mutants dramatically affects not only the accumulation of CF_1 but also of CF_0 subunits [194,205]. Homologs of the mitochondrial chaperones Atp11p and Atp12p are absent in chloroplasts, and auxiliary factors specifically involved in CF_1 assembly have not been characterized. Interestingly, in vitro reconstitution of active $\alpha_3\beta_3\gamma$ from purified tobacco subunits synthesized in *E. coli* has been achieved in the presence of MgATP, K^+ and stromal extract containing a mixture of chloroplast chaperones [119]. When chaperonin 60 was eliminated from stromal extract by a 300-kDa molecular mass cut-off filter, no functional $\alpha_3\beta_3\gamma$ could be assembled in reconstitution assays. However, addition of purified chaperonin 60 reversed this effect. Based on the results of activity assays, in which different subunit combinations of α, β and γ were treated with the chaperone-containing stromal extract prior to reconstitution experiments, the authors proposed an assembly model for CF_1. In the first step, α and β form dimers in a chaperone-dependent process (Fig. 1C). Then, three αβ-dimers are assembled into the hexamer. In the final step, folded γ associates with the hexamer, resulting in an active CF_1 core. Remarkably, when α or β alone was incubated with the chaperone extract before combining all the reaction components together, no active CF_1 was formed, which indicates that folding of α and β is irreversible [119]. Supporting evidence for an early interaction of α and β has been provided by studies on the *Chlamydomonas* strain *fud16*, in which the α polypeptide chain carries a mutation close to its nucleotide binding site [206]. In the *fud16* mutant, subunits α and β accumulate to form stromal inclusion bodies. Strikingly, in yeast mutants that are unable to assemble the $\alpha_3\beta_3$ subcomplex, α and β similarly aggregate into inclusion bodies in the mitochondrial matrix [149]. Once formed, the chloroplast $\alpha_3\beta_3$ hexamer is further stabilized by association of γ, and this in turn is a prerequisite for ε binding [119,207].

6.4. Assembly of the chloroplast F_0

CF_0 assembly in *Arabidopsis* is known to depend on the thylakoid-membrane protein AtCGL160 [120]. AtCGL160 contains four transmembrane-spanning α-helices near its C-terminus. This membrane domain is related to stretches of prokaryotic Atp1/UncI proteins, and the relative positions of the four predicted transmembrane-spanning α-helices coincide. This suggests that the secondary structure of this domain is conserved between eukaryotic CGL160 and bacterial Atp1/UncI proteins. Plants lacking AtCGL160 are defective in chloroplast ATP synthase accumulation and display a photosynthetic phenotype, similar to the one found in mutants expressing aberrant amounts of chloroplast ATP synthase [194,205,208]. Interaction of AtCGL160 with *b* and the ring-forming subunit *c* was demonstrated by split-ubiquitin assays. Moreover, monomeric *c*-subunits accumulate in *atcgl160* thylakoid membranes, indicating that AtCGL160 is involved in *c*-ring formation [120]. In consequence, those findings argue in favor of AtCGL160-assisted *c*-ring assembly, in agreement with the assumed role of its bacterial counterpart Atp1/UncI [136,137]. The nature of the intermediates involved and the order of subsequent CF_0 assembly steps remain elusive (Fig. 1C). A regulatory feedback mechanism, in which the presence of *c*-subunits stimulates the translation of *a*, has been proposed based on studies of *c* mutants of *Chlamydomonas* [193,204]. However, studies in maize failed to provide evidence for such a mechanism in land plants, as ribosome footprint analyses of mutants that failed to synthesize subunit *b* or *c* revealed only minor effects on the translation of other chloroplast ATP synthase mRNAs [107].

6.5. Linkage of chloroplast F_0 and F_1

Like CF_0 subcomplexes, CF_1–CF_0-containing intermediates have yet to be characterized in vivo, but the thylakoid-membrane-embedded protein ALB4 has been proposed to stabilize or promote the assembly of CF_1 during its attachment to CF_0 [142] (Fig. 1C). ALB4 is a paralog of the ALB3 translocase [209], which has been implicated in the biogenesis of several thylakoid multiprotein complexes (reviewed in [210]). ALB4 is known to play a role in chloroplast ATP synthase biogenesis, as it interacts physically with β and *b′*. Moreover, plants lacking ALB4 are characterized by low levels of chloroplast ATP synthase [142]. ALB3 and ALB4 are related to YidC from *E. coli* and Oxa1p from yeast mitochondria. Therefore, ALB4 represents an ATP synthase assembly factor that is conserved between prokaryotes, yeast and plants. No studies on late assembly steps in chloroplast ATP synthase formation and on the involvement of δ have yet been conducted but, based on mitochondrial and bacterial assembly models, we assume that

formation of the proton-translocating unit is the final step in the chloroplast ATP synthase assembly process (Fig. 1C).

7. Conclusion

Despite conservation of their overall structure and function, F_1F_0 ATP synthases display remarkable plasticity in their assembly depending on the organelle/organism studied. Phylogenomic studies have demonstrated that mitochondrial ATP synthase assembly factors are not conserved among eukaryotic species [170], and some of them are even absent from bacteria or chloroplasts (e.g. Atp10p, Atp11p). In addition, several lineage-specific assembly factors have evolved. For instance, the Ina17/Ina22 complex and Fmc1p are only found in fungi [141,170]. Moreover, ALB4 seems to have functionally diversified from a preexisting factor (ALB3) after a gene duplication event, to be recruited in the assembly of the chloroplast ATP synthase [142]. In this context, it is interesting to note that, besides a conserved C-terminal membrane domain (~140 aa), CGL160 harbors an N-terminal (~180 aas) stretch of sequence found in moss, algae and land plants but not in bacteria [120]. Thus, CGL160 represents another ATP synthase assembly factor that seems to have diversified during evolution.

Sequence and structural comparisons have provided conclusive evidence for the notion that F_1 evolved from an ATP-dependent helicase [211] and F_0 derives from an ion channel [212]. The modular nature of the ATP synthase assembly process, which is characterized by independently assembled F_1 and F_0 intermediates and the occurrence of related sub-complexes in mitochondria and bacteria, support this notion [117]. Future studies on the assembly mechanisms in mitochondria, bacteria and chloroplasts will provide further insights into the evolution of ATP synthases.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

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