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Subunit δ of H^+-ATPases: at the interface between proton flow and ATP synthesis

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Contents

I. Abstract

The ATP synthases in photophosphorylation and respiration are of the F-type with a membrane-bound proton channel, F_0, and an extrinsic catalytic portion, F_1. The properties of one particular subunit, δ (in chloroplasts and *Escherichia coli*) and OSCP (in mitochondria), are reviewed and the role of this subunit at the interface between F_0 and F_1 is discussed. δ and OSCP from the three sources have in common the molecular mass (≈ 20 kDa), an elongated shape (axial ratio in solution about 3 : 1), one high-affinity binding site to F_1 ($K_d \approx 100$ nM) plus probably one or two further low-affinity sites. When isolated δ is added to CF_1-depleted thylakoid membranes, it can block proton flow through exposed CF_0 channels, as do CF_1 or $CF_1(-\delta)+\delta$. This identifies δ as part of the proton conductor or, alternatively, conformational energy transducer between F_0 (proton flow) and F_1 (ATP). Hybrid constructs as $CF_1(-\delta)$ + *E. coli* δ and $EF_1(-\delta)$ + chloroplast δ diminish proton flow through CF_0. $CF_1(-\delta)$ + *E. coli* δ does the same on EF_0. Impairment of proton leaks either through CF_0 or through EF_0 causes 'structural reconstitution' of ATP synthesis by remaining intact F_0F_1. Functional reconstitution (ATP synthesis by fully reconstructed F_0F_1), however, is absolutely dependent on the presence of subunit δ and is therefore observed only with CF_1 or $CF_1(-\delta)$ + chloroplast δ on CF_0 and EF_1 or $EF_1(-\delta)$ + *E. coli* δ on EF_0. The effect of hybrid constructs on F_0 channels is surprising in view of the limited sequence homology between chloroplast and *E. coli* δ (36% conserved residues including conservative replacements). An analysis of the distribution of the conserved residues at present does not allow us to discriminate between the postulated conformational or proton-conductive roles of subunit δ.

Abbreviations: CF_0, chloroplast coupling factor 0 (proton channel); CF_1, chloroplast coupling factor 1 (ATPase); $CF_1(-\delta)$, CF_1 lacking the δ subunit; EF_0, EF_1, MF_0, MF_1, etc. are the respective proteins from *E. coli* and mitochondria; OSCP, oligomycin sensitivity-conferring protein of MF_0MF_1.

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II. Introduction

ATP is synthesized in thylakoids, various microorganisms, and mitochondria by ATP synthases, a class of enzymes which consists of a membrane-embedded part (F_0) conducting protons and a water-soluble, extrinsic part (F_1) containing the nucleotide binding sites. For recent reviews on the enzyme, see refs 97, 101. F_1 consists of five subunits named α to ϵ in order of decreasing molecular mass: $(\alpha\beta)_3\gamma\delta\epsilon$. MF_1-δ is the counterpart of *E. coli* and chloroplast ϵ, MF_1-ϵ so far does not seem to have counterparts in EF_1 or CF_1. The mitochondrial counterpart of *E. coli* and chloroplast δ is OSCP [121]. EF_0 consists of at least three different subunits (ab_2c_{9-12} [43]). CF_0 consists of four subunits [79] (named I–IV; I and II probably are equivalent to *E. coli b*, III is the equivalent of *E. coli c* and IV of *E. coli a*). MF_0 is even more complex, with more than five different subunits [121].

F_1 can be detached from the respective membranes by EDTA (EF_1, CF_1) or chloroform (MF_1, CF_1) treatment, solubilized F_1 then hydrolyses ATP. The membrane-bound H^+-ATP synthase converts the electrochemical potential difference of the proton into useful chemical energy by forming an anhydride bond between ADP and inorganic phosphate. This concept was expressed in the chemiosmotic theory nearly three decades ago by Mitchell [70].

While the coupling between proton translocation and ATP synthesis in principle is generally accepted, the mechanism of coupling is still under debate. There are two sets of hypotheses: (a) Mitchell proposed the channeling of protons into the catalytic headpiece where they directly participate in the chemical reaction with ADP and P_i [71,72] and (b) Boyer suggested an indirect conformational coupling between proton flow through F_0 and ATP liberation in F_1 [14,15]. According to the latter model, ATP forms spontaneously, but remains firmly bound to the F_1 part until it is released after input of energy (Refs. 15, 88 and references therein). Current evidence favors the conformational coupling model (summarized in Refs. 15, 88, see also a critical review on this issue [20]).

Here subunit δ (or OSCP in mitochondrial F_0F_1) gets into focus. As this small subunit of H^+-ATP synthases seems to be located at the interface between F_0 and F_1 [1,9,69,103,115,116,124], it could be, at least in part, responsible for protonic or conformational coupling. δ may be part of the 'stalk' [62,98] which, according to transmission electron microscopic evidence, connects F_0 and F_1 [13,40,49,112]. It could either funnel protons into (the vicinity of) the active site(s) or it could transmit conformational changes [32,99,102,104].

While the role of chloroplast δ was not quite clear for some years, e.g., δ was believed to be responsible for binding of CF_1 to CF_0 [124], but later it was claimed that it is not absolutely required for ATP synthesis [85], we showed that δ can act as a 'stopcock' to CF_0, which prevents proton leakage through exposed CF_0 channels [34,66]. We also showed that functional reconstitution of photophosphorylation is only possible in the presence of δ [35,37]. These results ascribed to δ an important role between F_0 and F_1.

δ from chloroplasts and *E. coli* show only moderate sequence homology and the degree of homology is even lower if mitochondrial OSCP and other eubacterial δ are taken into account. Therefore it came as a surprise that δ from chloroplasts and from *E. coli* could be cross-combined with $EF_1(-\delta)$ or $CF_1(-\delta)$ and the resulting hybrid, $F_1(-\delta)+\delta$, still impaired proton leakage through the respective F_0 channel [36]. We reviewed the literature on δ and OSCP and assessed whether or not a specific function could be deduced from this very extensive mutagenesis experiment performed by nature.

III. Published data on *E. coli* and chloroplast δ and mitochondrial OSCP

Kagawa and Racker [59,60] were the first to describe a protein fraction which conferred oligomycin sensitivity to mitochondrial ATPase. The protein was purified and named oligomycin sensitivity-conferring protein (OSCP) by MacLennan and Tzagoloff [69], who pointed out that OSCP might be an element of the stalk that was seen earlier in electron micrographs [40]. The eponymous activity of OSCP is to make the F_1 part of MF_0MF_1 susceptible to inhibition by oligomycin, which itself binds to F_0. This is measurable by, for example, decreased ATP hydrolysis rates in the presence of OSCP and oligomycin due to decreased proton pumping through oligomycin-F_0 or, by, for example, OSCP-improved ATP–P_i exchange activities in MF_1-reconstituted submitochondrial particles [99]. It is noteworthy in this context that an oligomycin-induced change in MF_0 is transmitted to MF_1 where it changes the nucleotide binding affinity [87]. By amino-acid sequence comparison it later became evident that OSCP was the functional counterpart of *E. coli* subunit δ [120] and chloroplast δ [52]. *E. coli* δ was purified and characterized by Smith and Sternweis [103,104,107] and chloroplast δ was first characterized by Racker and coworkers [124].

III-A. Purification and measurement of biological activity

δ from *E. coli* and chloroplasts was first isolated from pyridine-treated F_1 [103,104,124]. Such treatment results in denaturation of the three large subunits α, β, and γ, leaving behind δ and ϵ. Chloroplast δ then was obtained after urea treatment by anion-exchange chro-

matography [124], *E. coli* δ was isolated by gel-filtration [104].

An improved procedure for the preparation of chloroplast δ used a single step, chromatography of CF_1 on hydroxyapatite in the presence of octyl glucoside [3,85].

Pure δ both from EF_1 or from CF_1 was obtained by anion-exchange chromatography of ADP- or ATP-equilibrated F_1 [4,32] in the presence of the nonionic tenside *N*-(D-*gluco*-2,3,4,5,6-pentahydroxyl)-*N*-methylnonanamide (Mega 9) followed by hydrophobic interaction chromatography [33,36,37]. Flow charts showing the various purification procedures are given in the Appendix.

When purified δ was added to partially CF_1-depleted thylakoids it restored photophosphorylation rates [34] by plugging the protonic conductance of CF_0 [64,66]. This activity of δ alone was rapidly lost after storage at subzero temperatures. But such inactive δ still improved the reconstitution of photophosphorylation when added back together with $CF_1(-\delta)$ [34,35]. Obviously, conformationally distorted δ could be induced by the other CF_1 subunits to reassume a functional conformation. Improved reconstitution of (photo)phosphorylation after addition of both, F_1 lacking δ (or OSCP) and δ (or OSCP) in comparison to the reconstitution effects of $F_1(-\delta)$ alone serves as convenient assay in purification protocols.

OSCP was first purified from NaBr-extracted oligomycin-sensitive ATPase complex [69] followed by extractions of the remaining residue with ammonia, ammonium sulfate precipitation and cation-exchange chromatography. The original procedure was improved with respect to purity by starting from submitochondrial particles [98,99]. Further improvement with respect to yield was achieved by substituting the NaBr extractions by alkaline and salt treatments [6].

All three proteins are very susceptible to proteolytic degradation [36,99] and show a tendency for aggregation at low concentrations [35,99,104]. Concentrated solutions of *E. coli* δ and OSCP, however, apparently were monodispers [25,107].

III-B. Stoichiometry

The subunit stoichiometry of *E. coli* F_1 is $\alpha_3\beta_3\gamma\delta\epsilon$ (molecular mass 380 kDa) [97,101] based on in vivo radiolabelling with radioactive precursors [43]. The δ content was estimated also from the finding that about 5% (w/w) of δ were sufficient to fully restore the ability of $EF_1(-\delta)$ to reconstitute depleted membranes [104,107]. In chloroplasts initially an $\alpha_2\beta_2\gamma\delta\epsilon_2$ stoichiometry was reported [79]. Then, in analogy to bacterial and mitochondrial F_1 also a stoichiometry of one δ/CF_0CF_1 was assumed [85]. A higher stoichiometry of three δ/CF_0CF_1 [10] was not confirmed and again only one δ/CF_0CF_1 was found [35]. The apparently higher proportions of δ may have been caused by a tendency of chloroplast δ to aggregate, which leads to its overestimation in protein determinations. It seems to be safe to take a value of one as given for both *E. coli* and chloroplast F_0F_1. It is noteworthy though that there may be two types of binding site for δ in CF_1, one of high and one or two further of low affinity ($K_d \approx 100$ nM and ≈ 2 μM, respectively [119]). A similar behaviour is displayed by subunit ε ($K_d = 0.14$ nM and 60 nM, respectively [2]).

In mitochondrial F_0F_1, either one [24,118], two [89] or three [56] OSCP/MF_1 were determined. Two classes of binding sites with different affinities were found and their total number was 2–3 [27]. The present status seems to be that binding of one OSCP to a high-affinity binding site (K_m in the nanomolar range) is sufficient to induce oligomycin sensitivity, whereas binding to at least one of the additional low-affinity binding sites (K_m in the micromolar range) is required for 2-fold higher rates of ATP synthesis [27,30,90]. That more than one binding site exists for the small subunits may be interpreted as indication for changing attachment sites with different affinities during the catalytic cycle: The small subunits could rotate like a distributor through the $(\alpha\beta)_3$ aggregate with its nearly three-fold symmetry. This is an interesting possibility in view of the postulated rotatory binding change mechanism [50].

III-C. Topology

Early functional studies indicated an involvement of δ and OSCP in the binding of F_0 to F_1 [9,44,69,77,104, 124]. It could not be decided though, whether δ or OSCP were necessary and sufficient for this task. For EF_0EF_1 it was shown that subunit ε also is required for functional binding of EF_0 to EF_1 [107,108]. In mitochondria, MF_1 can bind to MF_0 in the absence of OSCP [30,57,90,94,115]. Later it was demonstrated that at least two proteins, F_6 and OSCP seem to 'improve' binding independently but only their concerted action ensures proper function of the entire MF_0MF_1 complex [38,54,55,117]. In chloroplasts, subunit δ clearly is not needed for binding [3,35].

A location of δ and OSCP *between* the two sectors is suggested by the fact that, depending on the dissociation procedure, the proteins either can be separated along with solubilized F_1 or not. In the latter case they may remain bound to F_0 (although direct experimental evidence for this is difficult to obtain). The case is illustrated by examples from *E. coli* [44,78] chloroplasts [32,58,64,66], and mitochondria [42,69,118].

It is unknown to which F_0 subunits *E. coli* and chloroplast δ bind. OSCP interacts at least with a 24 kDa protein of MF_0 [6,110]. With respect to F_1, there are several reports demonstrating an interaction with the α subunit(s). The amino-terminal portion of EF_1-α

is required for binding EF_1-δ [22]. A very similar finding was published later for OSCP [55]. An α-δ intersubunit disulfide bridge is generated by column centrifugation of EF_1 [111]. However, OSCP can be crosslinked to both α and β subunits [28], and photolabeling studies [26,30] showed that, depending on the experimental conditions, either MF_1-β (photoirradiation of azido-OSCP in the presence of submitochondrial particles that were largely depleted of MF_1 and OSCP) or MF_1-α (azido-OSCP + MF_1) were preferentially labeled. For CF_1 also an interaction of δ with both, α and β subunits was shown [8]. Tryptic degradation experiments [76] revealed a rapid degradation of subunit α as reported for MF_1 and EF_1. Subunit δ was degraded even more quickly, however, and therefore the ability of cleaved α to bind δ was not investigated. In this context it is also noteworthy that crude preparations of *E. coli* δ usually contained subunit α [36], whereas chloroplast δ in early stages of preparation was contaminated with β [33]. In addition, a β–δ interaction in CF_1 was suggested by the reconstitutive activity of a $\beta\delta$ complex [32].

By immunodecoration it was shown that OSCP is accessible to monoclonal antibodies even in membrane-bound and intact MF_0MF_1 [5]. However, investigation of the functional consequences of trypsination of MF_0 and MF_0MF_1 demonstrated that OSCP is shielded by F_1 in the membrane [57]. *E. coli* δ was cleaved rather slowly by trypsin if present in intact EF_0EF_1, it was cleaved rapidly after dissociation of the complex by detergent addition [47]. Polyclonal antisera against *E. coli* δ affected ATPase-dependent energy transduction by detaching EF_1 from the membrane [105] and monoclonal antibodies recognized δ within EF_0EF_1 [23]. Although this indicated some accessible sites on *E. coli* δ even in intact EF_0EF_1, a strong reaction with solubilized EF_1 pointed towards exposure of δ after detachment of EF_1 from the membrane [105]. Similar data were obtained from experiments with chloroplast membranes and CF_1 with polyclonal antisera directed against δ: The epitopes were recognized best with denatured membranes and with soluble CF_1, indicating that in intact CF_0CF_1 the major part of chloroplast δ is inaccessible to polyclonal antibodies [12,35]. This was further corroborated by experiments aiming at proteolytic digestion of chloroplast δ either in CF_0CF_1, in CF_1, or in isolated form [12]. The results were comparable to those obtained with *E. coli* [47] and mitochondria [57].

In summary, despite sound evidence for subunit α constituting the 'major' (F_1-)counterpart of subunit δ or OSCP, subunit β also seems to be involved. The other contact site between δ and F_0 is established simply by its consequence, the block of the proton leak [34,66]. Subunit δ and OSCP are shielded to quite some extent from solute in intact F_0F_1 complexes, although some epitopes of the subunit may be accessible to some

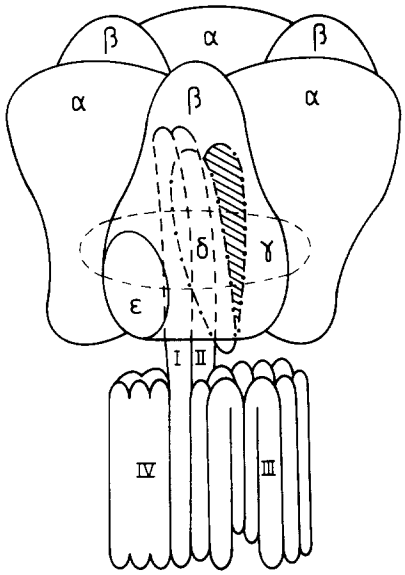

Fig. 1. A sketch of CF_0CF_1 based on electron microscopic [13,109] and resonance energy transfer data [73,93].

antibodies. Preliminary data would seem to suggest that only 10% of the amino acids of chloroplast δ are accessible in CF_0CF_1 [12]. Considering the stalk that was observed in electron microscopy [13,49], it might be that it contains just four to five α-helices, due to its rather small diameter [49]. These helices, subunits *b* in *E. coli* or I and II in chloroplasts, might well be sufficient to contribute (*E. coli b* is predicted to be highly helical in the large stretch which extends from the membrane [100]). In this case, δ would probably be located very much inside the $\alpha\beta$ hexagon, at the upper end of the stalk. Alternatively, δ might be shielded by subunits *b* or I and II. Fig. 1 shows a sketch of the integral ATP synthase, taking into account several binding sites for subunit δ on F_1.

III-D. Structure

The shape of subunits δ and OSCP in aqueous buffer is rather elongated. For *E. coli* δ this has been inferred from gel-filtration experiments [107], for chloroplast δ on small-angle X-ray scattering [96] and on hydrodynamic behaviour [119], and for OSCP on small-angle neutron scattering [25]. The elongated shape (axial ratio 10 : 2.8 nm [119]) may be interpreted as indication that subunits δ and OSCP are part of the connecting 'stalk' that was observed in electron microscopy.

The amino-acid sequences of OSCP, of *E. coli* δ and of spinach chloroplast δ are known [48,52,68,80,82,122]. All three proteins contain about 180–190 amino acids, their molecular masses are about 20 kDa. The isoelectric points of *E. coli* and chloroplast δ lie in the acidic range, whereas OSCP is a basic protein [69]. The latter contains an internal sequence homology [51,83] indicative of gene duplication, which is not obvious in the two

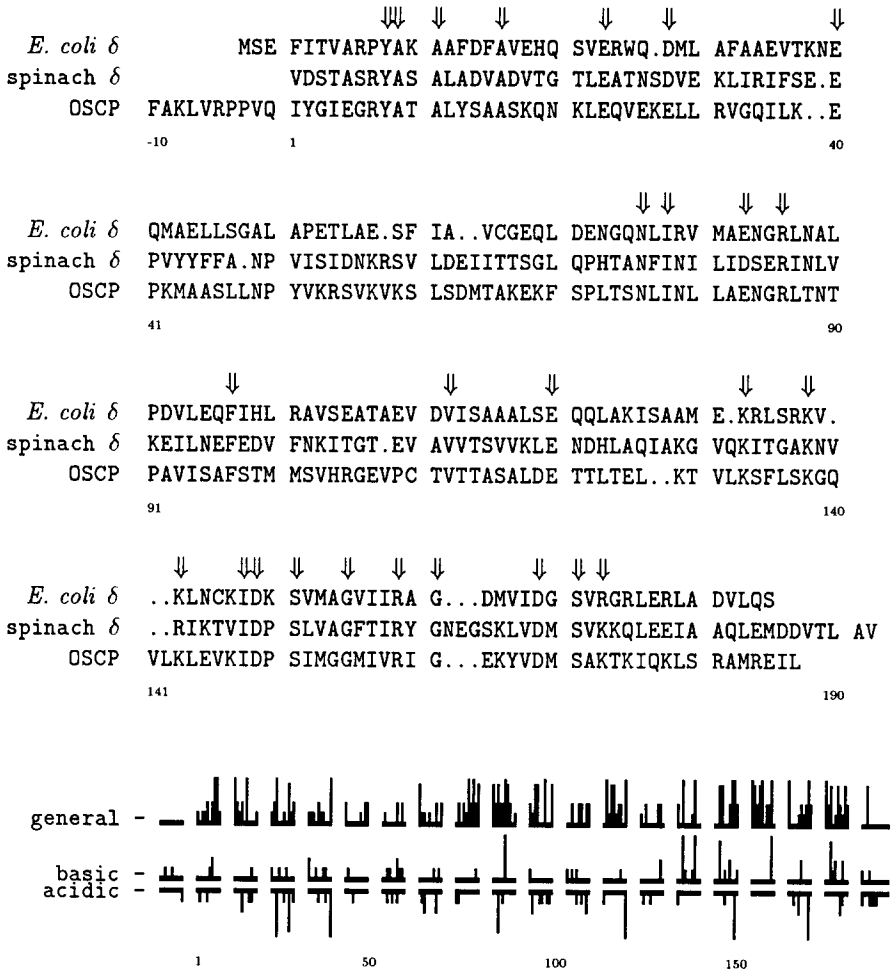

Fig. 2. Alignment of the amino-acid sequences of spinach and *E. coli* δ and of OSCP. The sequences [48,52,122] were pairwise aligned by the University of Wisconsin GCG alignment program GAP (gap weight 5.0, length weight 0.3). Further alignment was done manually starting from the spinach δ – *E. coli* δ and spinach δ – OSCP alignments. The result is shown in the upper part of the figure with the arrows pointing to identities and, in positions 28, 83, 143 and 173, respectively, to conservative replacements of acidic and basic amino acids. The lower part of the figure shows the distribution of identical or similar, basic, and acidic amino acids throughout the sequence. Each horizontal bar represents 10 amino acids, the height indicates no hits (1 unit), two conservatively replaced amino acids (3 units), two identical amino acids (5 units), three conservatively replaced amino acids (9 units) or three identical amino acids (10 units) in the scheme named 'general'. Charged amino acids in the two other schemes are weighted in the following way: 1 charged amino acid, 3 units, two charged amino acids, 5 units, three conservatively replaced charged amino acids, 9 units, and three identical amino acids, 10 units. There are eight positions in OSCP where the data based upon protein sequencing by the Soviet-Swedish group [82,83] differ from the DNA sequence-deduced amino-acid sequence published by Walker's group [122]. The above alignment is based on the latter one, differences may be neglected except for positions 28 (E → Q) and 177 (Q → E), i.e., the first exchange would destroy a conserved amino-acid position and the second would generate one.

other proteins. The N-terminal half of OSCP also shows some sequence homology (including conservative replacements) with *E. coli* F_0 subunit *b* (32%) and with the mitochondrial ADP/ATP carrier (33%) [51,83]. Data following from the published amino-acid sequences and an alignment are summarized in Table I and Fig. 2; data were processed by the University of Geneva PC/Gene and University of Wisconsin GCG/GAP programs.

The sequence alignment (Fig. 2) shows that 23% of the amino-acid residues are strictly conserved in pairwise comparison, 12% are identical in all three proteins (spinach chloroplast and *E. coli* δ, bovine OSCP). If conservative replacements are included, these numbers are raised to 36% conserved residues (pairwise comparison) and 21% conserved residues (comparison of all three proteins). Inspection of the plot in the lower part of Fig. 2 reveals that the conserved residues are scattered rather evenly throughout the sequence. Only the N- and C-terminal ten or so amino acids do not show any homology. Further low-homology regions are found between positions 40 and 60, 100 and 110, and 120 and 130. The high predicted content of α helix in *E. coli* δ (cf. Table I) probably is related to its low number of proline residues. It is noteworthy that all prolines in chloroplast δ have a counterpart at the same position in OSCP [41,50,72,150], but only one proline is conserved between *E. coli* δ and OSCP (position 91). If proline

TABLE I

Summary of data on primary and secondary structure of E. coli δ, chloroplast δ and OSCP

	E. coli δ	Chloroplast δ	OSCP
Number of residues	177	187	190
Molecular mass	19332	20486	20969
Theoretical isoel. point	4.71	4.41	10.66
Amino-acid composition			
hydrophobic	87	90	84
hydrophilic	31	43	43
acidic	26	28	15
basic	21	20	31
Cys	2	0	1
Met	7	2	8
Pro	3	4	8
Predicted % [a] of			
helical conformation	82	50	62
extended conformation	15	39	33
turns and coils	3	11	6
Position of conserved acidic residues [b]	(23), 28, (40), (83), 120, 149, 169		

[a] Prediction by the method of Garnier [46]. Circular dichroism measurements indicated 55–70% α-helical structure for *E. coli* δ [107] and 43% α-helix content of OSCP [25]. The circular dichroism spectrum from chloroplast δ is nearly indistinguishable from its *E. coli* counterpart (Engelbrecht, S., unpublished data).

[b] Numbering according to Fig. 2. Numbers in brackets refer to conserved acidic residues which are found in the given alignment of chloroplast and *E. coli* δ and OSCP but *not* in the respective positions of *Synechococcus* 6301 [19], *Rhodopseudomonas blastica* [39], *Rhodospirillum rubrum* [114], and *Bacillus megaterium* [18].

were to be helix-breaking, the molecule would be divided in roughly four parts: The N-terminal part extending to approximately position 41, the next stretch from approximate position 50 to position 72 (chloroplast δ and OSCP) or 91 (*E. coli* δ), another stretch extending to position 150 (chloroplast δ and OSCP) or to the C-terminus (*E. coli* δ). In OSCP there is one additional proline at position 109. These four regions roughly match the domains with highly conserved residues: The N-terminal part up to position 40, the center part from position 70 to 100, a short segment from position 110 to 120 and the C-terminal part starting at position 130. It must be kept in mind though that in hydrophobic environment prolines can be accommodated in helices, as was shown recently for α-helices of the reaction center of *Rhodopseudomonas viridis* [21].

With one exception (position 86), the conserved basic amino acids are found at the C-terminal part, whereas the conserved acidic residues are evenly distributed throughout the sequence. OSCP is rich in lysines, which often substitute for hydrophilic or even acidic residues at the respective positions in the two δ subunits. It might be that the difference in the isoelectric points is caused simply by differences in the surface charge of the proteins which are related neither to attachment sites nor to function *. The finding that both *E. coli* and chloroplast δ can be detached from (immobilized) F_1 by washing with surfactant-containing buffer [3,33,36,85] points towards hydrophobic interactions as being mainly responsible for their attachment. OSCP is bound to MF_1 by both ionic and hydrophobic interactions [118].

III-E. Function

III-E.1. E. coli δ

In *E. coli* F_1, subunit δ is required for binding of EF_1 to EF_0 as evidenced by the lack of $EF_1(-\delta)$ to restore ATP-coupled transhydrogenase activity in depleted membranes [44,103,104,107,113]. In addition, it probably can block proton conduction through EF_0. This is suggested by the recent finding that *E. coli* δ partially can fulfill the function of chloroplast δ in CF_0CF_1 [36] and also by earlier studies with mutants defective in the δ subunit [53,81]. It remains unclear, however, whether EF_0 was assembled correctly in these mutants. A report that detachment of EF_1 from the membrane in addition to the usual low-ionic-strength/EDTA wash requires proteolytic cleavage of the δ subunit [16] has not been substantiated since.

Interestingly, genetic studies suggested that EF_0 can be synthesized and probably assembled in non-conducting form from plasmid-borne genes. A conducting channel was obtained by expression of a gene containing also EF_1-α and EF_1-δ [17,106]. If in addition the EF_1-γ gene was expressed, again a non-conducting EF_0 was obtained [84]. In the defective mutant the nonconducting channel became conducting after rebinding and subsequent detachment of EF_1. This indicated that a transient interaction with F_1 left behind a changed F_0 (Brusilow, W.S.A., personal communication).

For TF_0TF_1, the protonmotive ATPase from thermophilic bacterium PS3, it was shown several years ago that passive proton conduction through TF_0 could be blocked *only* by the *concerted* action of the TF_1 subunits γ, δ and ε; therefore, these three subunits were called a proton gate [61,123].

III-E-2. Chloroplast δ

Isolated chloroplast δ, when added to CF_1-depleted thylakoids, can block proton conduction through exposed CF_0 [34]. Conversely there is evidence that CF_1 extraction by EDTA treatment may leave δ behind on CF_0, which then is nonconducting [32,58,64,66]. One may argue that the effect of δ on CF_0 is accidental and

* Preliminary data from reconstitution experiments with OSCP and $CF_1(-\delta)$ indicate though no improved photophosphorylation by $CF_1(-\delta)$ in the presence of OSCP.

does not reflect the role of δ in photophosphorylation. The specific action of δ, however, rather points the other way, namely that its plugging action *does* indicate the involvement of δ in photophosphorylation. This is supported by further observations: $CF_1(-\delta)$, when added to CF_1-depleted thylakoids, also lowers the proton conductance of CF_0, but without fully blocking it, as added δ, CF_1, or $CF_1(-\delta)+\delta$ do [37]. In studies aiming at a discrimination between structural (leak plugging) and functional reconstitution (restoration of functional CF_0CF_1 from CF_0 and added CF_1) of photophosphorylation, we found that functional reconstitution obligatorily required the presence of one copy of δ per CF_1 [35,37]. $CF_1(-\delta)$ effectively competed with CF_1 for binding to CF_0. Obviously, one copy of δ is required for the function of CF_0CF_1 but not for binding of CF_1 to CF_0. The leak-plugging action of δ in conjunction with $CF_1(-\delta)$ is shared to some extent by the hybrids, $EF_1(-\delta)$ + chloroplast δ and $CF_1(-\delta)$ + *E. coli* δ. This does not hold for EF_1-depleted vesicles from *E. coli*, where plugging only was observed with EF_1 and, to lesser extent, with CF_1 and $CF_1(-\delta)$ + *E. coli* δ [36]. The different behaviour in these experiments of CF_0 as compared to EF_0 might reflect a more essential role of *E. coli* δ in binding EF_1 to EF_0 [104,107,113]. These data leave little doubt about a role of subunit δ at the interface between 'proton and ATP'.

So far, blocking of proton conduction through F_0 by one single isolated F_1 subunit only was observed with chloroplast δ [34]. Attempts to achieve this blocking effect with *E. coli* δ on EF_0, or crosswise reaction between CF_0 and *E. coli* δ and EF_0 and chloroplast δ were not successful ([36] and Engelbrecht, S., unpublished data). In contrast, the work of Kagawa's group demonstrated that proton conduction through TF_0 could be blocked only by the concerted action of TF_1-γ, -δ and -ϵ (Ref. 123, reviewed in Ref. 61). In mitochondria, OSCP modified NADH-dependent proton flux only in the presence of MF_1 [90]. The question arises, how the difference might be explained between action of either δ alone or only in the presence of (all) other F_1 subunits. As it was (and is) experimentally difficult to observe such blocking of proton conduction by isolated δ, the answer to this question might be the requirement not only for a fresh and 'conformationally intact' preparation of δ [33,34] but also (and possibly to even greater extent) a F_0 conformation, which does react with δ. Unfortunately, whereas we obtained many hints for the existence of very different CF_0 'states' [37,63,65,67], to date we cannot isolate defined populations of these CF_0 'states'.

The finding that, depending on the nucleotide content of the CF_1 which was used as starting material, either δ or a $\beta\delta$ complex was obtained and the probable location of the active sites on β subunits led to us to the working hypothesis that δ in CF_0CF_1 might transduce conformational changes into β, thereby promoting catalytic events within that subunit [32,34,36].

III-E.3. OSCP

The current model for the interaction of OSCP with MF_1 and MF_0 suggests a direct interaction between MF_0 and MF_1 and, in addition, a second link which consists of F_6 [41] and OSCP in such a manner that OSCP interacts with MF_1 and F_6 and the latter interacts with all, MF_1, MF_0 and OSCP [38]. So far, F_6 seems to be unique for the mitochondrial system. This model accounts for the fact that, under proper conditions, MF_1 can rebind to MF_1/OSCP/F_6-depleted membranes [95] and that F_6 is required for proper anchorage of OSCP. OSCP seems to be related not only to EF_1-δ but also to EF_0-*b* [83].

OSCP has been derivatised by various fluorescent probes without apparent loss of biological activity [29,31]. The data suggest a conformational change upon binding to MF_1 and a location of the probes in a hydrophobic pocket of OSCP. This is in line with the location of the derivatised residue Cys-118 in the amino-acid sequence. The relative exposal of the probes upon binding of MF_1 to OSCP, which was reversed upon further addition of membranes, suggests a shielding of OSCP in MF_0MF_1 [31]. Treatment with oligomycin or *N,N'*-dicyclohexylcarbodiimide (DCCD) yielded no significant fluorescence changes, indicating that at least the region around Cys-118 (position 110 in Fig. 2) did not sense binding of oligomycin or DCCD to MF_0 [31].

The efficient reconstitution of energy transfer reactions from depleted membranes and MF_1 required two OSCP/MF_0MF_1 [90]. Although both OSCP and MF_1 rebound independently, the best way to achieve maximal restoration of biological functions was to rebind first MF_1, *followed by* addition of OSCP. Upon binding of OSCP to MF_1-treated, previously depleted membranes, ATP-driven proton flux was restored and ATPase activity became uncoupler-sensitive. Proton efflux could not be blocked by either MF_1 or OSCP alone, only both together were effective. It was concluded that OSCP is required for proper fit of MF_1 to MF_0 thus allowing for a correct channelling of protons [90]. These studies therefore would suggest accessibility of OSCP to its binding site(s) in (partially assembled?) MF_0MF_1.

From trypsination studies of submitochondrial particles it was concluded [57] that in the intact complex both, OSCP and F_6 are shielded by MF_1, upon removal of the latter OSCP becomes accessible and after extensive degradation of OSCP F_6 is affected. As such treatment did not impair the passive proton conductance of MF_0, it was concluded that neither OSCP nor F_6 are required for proton conduction. In contrast, the rate of passive proton conductance was increased 4-fold, a finding which was paralleled by similar experiments

with EF_0-*b* [91] and which might be interpreted in terms of an unblocking of the channel upon digestion of OSCP or subunit *b*.

III-F. Conclusions and perspectives

The functional similarity between δ and OSCP is not paralleled by a pronounced similarity at the level of primary structure. The three proteins share only 13% of their amino-acid residues and, while chloroplast and *E. coli* δ both are acidic proteins, OSCP is basic. All three proteins seem to be located somewhat secluded between F_0 and F_1. All of them may facilitate binding F_1 to F_0, but to very different extent. All these properties fit the concept of δ and OSCP forming part of a functional link between F_0 and F_1. Such a link, according to current thinking about the coupling mechanism, either should transmit conformational changes from F_0 into F_1 or should form part of a proton pathway which extends from F_0 into F_1.

In either case, rather high sterical restrictions are to be expected and the whole assembly during evolution should have been subject to little change. The expected high sequence homology, however, is found only for subunit β of the F_1 sector [97,101], which contains the active site, and to lesser extent for α, which also contains a nucleotide-binding site. The amino-acid sequences of the remaining subunits including δ and OSCP all are not so well conserved [48,52,68,80,82,122]. This indicates that the function of these subunits more likely requires just few amino acids at certain fixed locations. The problem how to connect these 'fixed points in space' then allowed for many solutions during evolution. As demonstrated by the recently published three-dimensional structure of *N*-(5′-phosphoribosyl)anthranilate isomerase-indole-3-glycerol-phosphate synthase from *E. coli* [92], a homologous primary sequence is *not* an absolute requirement for very similar three-dimensional structures.

Considering the relevance of acidic residues for proton conduction (see the recent work on bacteriorhodopsin [75]), a total number of 4 conserved acidic amino acids (between chloroplast δ, *E. coli* δ, and OSCP) distributed throughout the entire sequence might be sufficient for a 'proton wire' to be constructed, not to mention the role of possibly bound water molecules. Involvement of tyrosines in proton conduction by subunit δ [11] seems less probable [74]. However, the available data at present do not allow for a clearcut decision between 'direct coupling' and 'conformational coupling' models.

Nevertheless, conformational coupling seems to be favored in view of several independent observations. The conversion of F_1-bound ADP and P_i into F_1-bound ATP takes place without appreciable energy input [86]. When MF_0 is covalently modified by *N*,*N*′-dicyclohexylcarbodiimide or noncovalently by oligomycin, the affinity for nucleotide binding in the F_1 part is altered [87]. *E. coli* δ improves the reconstitution of photophosphorylation by $CF_1(-\delta)$ [36] despite a low sequence homology. In a multi-plasmid mutant, nonconducting EF_0 is converted into conducting EF_0 after a transient exposure to EF_1 (Brusilow, W.S.A., see above). Reconstitution of photophosphorylation by addition of CF_1 (which had been stored for some time but did not show a decrease in ATPase activity) could be improved by prolonged incubation (60 min instead of the usual 10 min) [37] and the impairment of proton leakage through CF_0 by $CF_1(-\delta)$ [37] also all point to a strong conformational interaction between the F_0- and F_1-portions.

The question remains how the apparent differences between the three proteins with respect to stoichiometry and net charge fit the concept of a functional link. MF_0MF_1 additionally contains further subunits which also seem to be part of the connection between MF_0 and MF_1, but which are lacking in bacterial and chloroplast F_0F_1. As discussed above, the different net charge of OSCP as compared to the two δ's might simply result from unimportant patches on the surface of the molecule. Alternatively, if electrostatic interactions are important (as for the interplay between cytochrome *c* and cytochrome *c* oxidase [45]) still there exists the possibility of concerted changes on both proteins, which results in an inversion of the charge distribution without changing the overall coulombic interaction.

How can blocking of proton flow through CF_0 by subunit δ be explained? One possibility would be the following. Proton influx into F_0 occurs until (nearly) all proton saturatable groups are protonated, then a conformational change needed for efflux of protons takes place. The latter may be inhibited by subunit δ. This inhibitory action of δ must be relieved in the intact F_0F_1 complex during ATP synthesis or ATP hydrolysis-linked proton pumping. One might think of a contractory movement of δ, which upon movement towards F_0 opens one of three clefts in F_1 thus allowing for ATP liberation. After dissociation of one ATP molecule the entire system returns (relaxes?) to its original state. In this model there is no problem in accommodating three alternating active sites. An interesting feature is the fact that the opening of clefts on F_1 by a contractory motion of δ (and possibly other subunits of F_0F_1) would necessitate at least a second contact site between F_0 and F_1, a dolly which would keep part of F_1 in place. Clearly these other contact sites are present and sufficient in CF_0CF_1 to allow for competitive binding of $CF_1(-\delta)$ and CF_1 to CF_0 [35]. For EF_0EF_1, a close proximity between subunits α and *b* of the membrane sector and EF_1-β was demonstrated [7]. It might be that the stalk observed between F_0 and F_1 in electron micrographs shows just one possibility out of several 'conformational positions'. Parts of F_1 might change their distance from

the membrane surface during the catalytic cycle. This is a hypothetical model of the manner in which δ might participate in 'conformational coupling'. Another interesting explanation for the blocking action of δ either in the absence of other CF_1 subunits or in the presence of *N,N'*-dicyclohexylcarbodiimide derivatized CF_1 (i.e., inhibited CF_1) [37] is that in intact F_0F_1 δ blocks proton conduction until 'enough' protons have accumulated. ATP synthesis and/or liberation and proton 'discharge' then go hand in hand.

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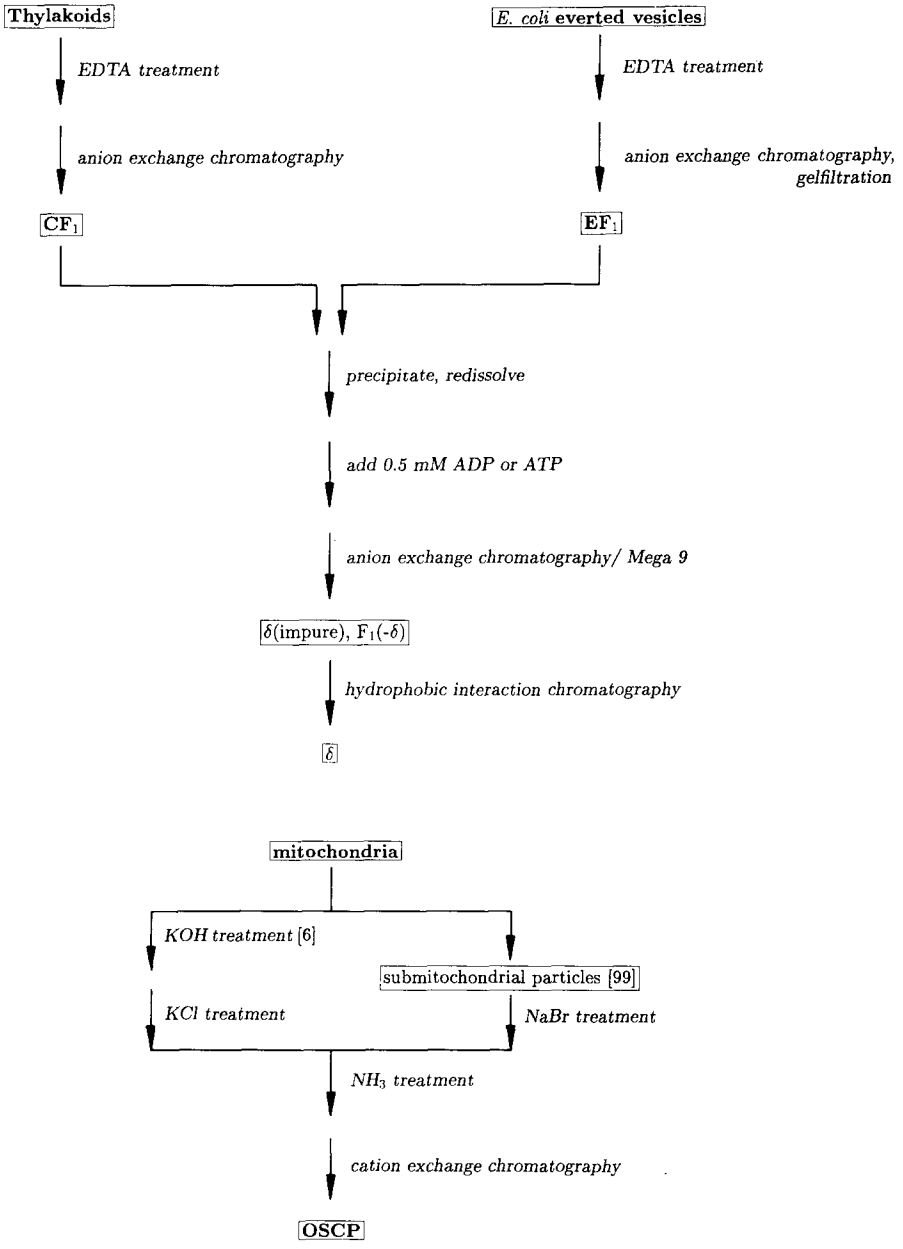
V. Appendix

The critical step of the preparation procedure for chloroplast δ is the preincubation of CF_1 with either ADP or ATP. If $(NH_4)_2SO_4$-precipitated CF_1 (which retains one tightly bound ADP) is subjected to anion-exchange chromatography in the presence of nonionic



Scheme I.

detergent, a $\beta\delta$ complex is obtained [32]. If the same CF_1 is loaded with at least three nucleotides *, essentially pure δ is obtained presumably by 'washing off' the subunit from the oligomer [33]. In the hydrophobic interaction chromatography δ subunit from either source (*E. coli* or chloroplast) eluted with Tris-HCl in the absence of ammonium sulfate [36,37]. This protocol emerged from the earlier procedure involving repeated anion-exchange chromatography in the presence of Mega 9 [33,35]. With increasing Mega 9 concentrations during the wash both $CF_1(-\delta)$ and subunit ϵ increasingly were lost. A brief preincubation of CF_1 with 1 mM ADP or ATP and 20–22 mM Mega 9 followed by rapid elution from the anion exchange column in the presence of 22 mM Mega 9 was optimal. Still, the resulting $CF_1(-\delta)$ contained about 14% δ, which could be removed only by repetition of the procedure (Engelbrecht, S., unpublished data).

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* Figures of six nucleotides/CF_1 in Ref. 33 might be erroneous because suboptimal NaCl concentrations were used for removal of loosely bound nucleotide during gel filtration: McCarty, R.E., personal communication.

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Note added in proof (Received 15 December 1989)

During the processing of the manuscript the following information has appeared.

In one report the binding abilities and specificities of CF_1 and $CF_1(-\delta)$ to CF_1-depleted thylakoid vesicles

were investigated (Xiao, J. and McCarty, R.E. (1989) Biochim. Biophys. Acta 976, 203–209). It was found that both CF_1 and $CF_1(-\delta)$ bound specifically and competitively to thylakoid membranes, i.e., to CF_0. However, there were at least two kinds of CF_0 present in NaBr- and EDTA-treated thylakoid vesicles, a functional and a damaged population. Whereas CF_1 bound to both populations (and, in case it hit a functional CF_0, restored photophosphorylation), for unknown reasons $CF_1(-\delta)$ rebound only to the damaged CF_0 and therefore it did not restore photophosphorylation. It should be noted that these results are partially in accordance and partially at variance with data from our laboratory [35,37].

By cryoelectron microscopy, Capaldi's group investigated EF_1 and EF_1 immunodecorated with monoclonal antibodies directed against the individual subunits (Gogol, E.P., Lücken,, U., Bork, T. and Capaldi, R.A. (1989) Biochemistry 28, 4709–4716 and Gogol, E.P., Aggeler, R., Sagermann, M. and Capaldi, R.A. (1989) Biochemistry 28, 4717–4724). They observed six elongated protein densities in a hexagonal 'barrel' arrangement with alternating α and β subunits and an aqueous cavity extending nearly or entirely through this structure. A seventh compact protein density was found to be located at one end of the barrel. This asymmetrically positioned mass was primarily linked to a β subunit. Trypsination released the δ and ϵ subunits and reduced this seventh mass, but did not eliminate it. Thus, this mass is at least partially composed of the γ subunit. In addition, there were accessible epitopes to the γ, δ, and ϵ subunits located at the periphery of the barrel, near the β subunits. Simultaneous labeling with the respective Fab's revealed a wide separation of approx. 4 nm of some epitopes on the γ and δ subunits. It may be relevant here to note that a small-angle neutron-scattering study did not reveal any secluded water spaces within CF_1 (Ibel, K., Engelbrecht, S., Wagner, R., Andreo, C.S. and Junge, W. (1989) FEBS Lett. 250, 580–584).