Dissecting the peripheral stalk of the mitochondrial ATP synthase of chlorophycean algae.

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Peripheral stalk of algal ATP synthases.

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Abstract

The algae *Chlamydomonas reinhardtii* and *Polytomella* sp., a green and a colorless member of the chlorophycean lineage respectively, exhibit a highly-stable dimeric mitochondrial F_1Fo-ATP synthase (complex V), with a molecular mass of 1600 kDa. The alga *Polytomella*, that lacks chloroplasts and a cell wall, has greatly facilitated the purification of the ATP-synthase. Each monomer of the algal enzyme has 17 polypeptides, eight of which are the conserved, main functional components of the enzyme and nine polypeptides (Asa1 to Asa9) unique to chlorophycean algae. These atypical subunits form the two robust peripheral stalks observed in the highly-stable dimer of the algal ATP synthase in several electron-microscope studies. The topological disposition of the components of the enzyme has been addressed with cross-linking experiments in the isolated complex; generation of sub-complexes by limited dissociation of

complex V; detection of subunit-subunit interactions using recombinant subunits; reconstitution of sub-complexes; silencing of the expression of Asa subunits; and modelling of the overall structural features of the complex by EM image reconstruction. Here, we report that the amphipathic polymer Amphipol A8-35 partially dissociates the enzyme, giving rise to two discrete dimeric sub-complexes, whose compositions were characterized. An updated model for the topological disposition of the 17 polypeptides that constitute the algal enzyme is suggested.

KEY WORDS: F_1F_O-ATP synthase peripheral-stalk; dimeric mitochondrial complex V; Amphipol A8-35; *Chlamydomonas reinhardtii*; *Polytomella* sp.; Asa subunits.

1. The mitochondrial ATP synthase

Mitochondrial F_1Fo-ATP synthase (complex V) is a key participant of oxidative phosphorylation (OXPHOS) and the main ATP producing enzyme in non-photosynthetic eukaryotes. Classic dissociation experiments characterized two main oligomeric domains in the enzyme, an extrinsic moiety (F_1 factor) and the membrane bound sector Fo [1]. F_1Fo-ATP synthase is also a molecular motor [2] in which a central rotor-stalk [γ/δ/ε/*c*-ring] rotates around an axis perpendicular to the plane of the membrane, while the fixed elements (stator components) are subunit *a*, the catalytic core (α_3/β_3), the peripheral stalk (OSCP/b/d/F6), and the dimerization module (A6L/*e*/*f*/*g*) [3,4]. Proton-flux through the two hemi-channels of subunit *a*, formed by membrane-embedded alpha helices, causes the rotary movement of a *c*-subunit ring with a species-dependent variable stoichiometry ($c_{8\text{-}14}$) and concomitantly, the rotation of the central-stalk (γ/δ/ε) [5]. Three sequential 120◦ movements of subunit γ induce conformational changes in the three catalytic β subunits leading to successive substrate binding (ADP + Pi), ATP synthesis, and ATP release [6]. Other subunits play a regulatory role, such as the inhibitory protein IF_1 [7] that prevents futile ATP hydrolysis. The peripheral stator-stalk counteracts the torque generated by the rotation of the central stalk during the function of the enzyme [8]. In the yeast and beef enzymes, the main axis of the peripheral stalk is formed by subunit *b*, that contains two transmembrane stretches near its N-terminal region and extends towards OSCP at the top of the F_1 sector associating also with subunits *d* and F6 (*h* in yeast) [9].

In the inner mitochondrial membrane, the ATP synthase forms oligomeric associations [10] that are responsible for the overall architecture of the mitochondrial cristae [11]. Subunits *b* (Atp4), *d*, F6, A6L, *e*, *f* and *g* are not common to all

mitochondrial ATP synthases, since different 3D-structures or atypical subunit compositions have been observed in the enzymes from ciliates [12], trypanosomatids [13], euglenoids [14], and chlorophycean algae [15].

2. The mitochondrial ATP synthase of chlorophycean algae

2.1. Subunit composition

Early works identified immunochemically the β-subunit of mitochondrial ATP synthase in preparations of thylakoid and mitochondria membranes of *Chlamydomonas reinhardtii* [16]. A fraction enriched in the green algal complex V was obtained after sucrose gradient centrifugation in the presence of Triton X-100 [17]. The cDNA of the β-subunit of *C. reinhardtii* mitochondrial ATP synthase was sequenced and the corresponding protein was found to contain a unique C-terminal extension of 70 residues not present in the β-subunits of plant enzymes [18]. A pure and active mitochondrial ATP synthase preparation from the green alga was obtained by extraction with lauryl maltoside followed by ion exchange and gel permeation chromatography and found to be composed of at least 14 polypeptides [19]. Also, the cDNA encoding the α-subunit of the enzyme was sequenced and the mature subunit was found to exhibit an 18-residues extension not present in other organisms.

Subsequently, a mitochondrial F_1Fo-ATP synthase was isolated by gel permeation chromatography from *Polytomella* sp., which lacks both chloroplasts and cell wall, and thus facilitates the isolation of mitochondria and purification of the OXPHOS components without the interference of thylakoid components [20]. The enzyme from this colorless alga exhibited at least 10 polypeptides with apparent molecular masses that ranged from 6 to 63 kDa [21] and its α and β subunits exhibited high sequence similarities with the ones of *Chlamydomonas* [see also ref. 22]. Here, the *Polytomella* α and β subunits were modelled on the crystallographic structure of the bovine enzyme to visualize the location of these extensions in the F_1 sector. Fig. 1 shows two of the possible conformations for each

of the algal extensions. The α subunit extensions could have a role in stabilizing contacts with the peripheral stalk, while the β subunit extensions were proposed to act as the IF_1 ATPase inhibitor based on sequence similarities [21]. When the extension of the β subunit was overexpressed, partially purified, and added to the algal ATPase at relatively high concentrations, the hydrolytic activity of the enzyme diminished less than 20%, suggesting a null or rather limited inhibitory effect of this extension [Villavicencio-Queijeiro and González-Halphen, unpublished results].

In order to asses if subunit *a* (Atp6) was present in the mitochondrial ATP synthase of *C. reinhardtii*, isolated mitochondria from the green alga were subjected to blue native electrophoresis (BN-PAGE), a technique that uses the charge shift generated by the binding of Coomassie Blue to detergent-solubilized membrane complexes to separate them under native conditions [23]. The first dimensional BN-PAGE gel was then subjected to 2D-denaturing SDS-PAGE to identify the polypeptide composition of complex V [24]. At least 13 different polypeptides were found to be associated to the complex, five of which were unambiguously identified as subunits α, β, γ, δ and *a*. Nevertheless, eight polypeptides of the ATP synthase showed no evident similarity with other ATPase subunits nor with other proteins in the databases, so their identities remained obscure at that time. These results prompted a detailed analysis of mitochondrial protein components of *C. reinhardtii* by BN-PAGE, followed by 2D-SDS-PAGE and Edman degradation of selected bands [25]. Complex V was found to migrate exclusively as a dimer that resolved into at least 13 polypeptides in 2D-SDS-PAGE. In addition, a novel 60-kD protein with no known counterpart in any other organism, named MASAP for mitochondrial ATP synthase-associated protein and later on renamed Asa1, was described and its corresponding cDNA sequence obtained [25]. It was put forth that this novel polypeptide could be responsible for stabilizing the chlorophycean ATP synthase dimer [22, 25].

Mitochondrial F_1Fo-ATP synthases of *Chlamydomonas reinhardtii* and *Polytomella* sp. migrated in BN-PAGE in the presence of lauryl-maltoside as stable dimers of 1,600 kDa [25, 26] while the monomeric F_1Fo or free F_1 moieties could not be detected. This suggested the presence of a highly stable, detergent-resistant

dimeric enzyme and contrasted starkly with the behavior of other complex V from different sources, including mammals, fungi and higher plants that usually migrate in BN-PAGE in the presence of lauryl-maltoside as monomers of 550–600 kDa. Also, conventional enzymes tend to partially dissociate during electrophoresis, releasing the F_1 sector [27]. In order to obtain dimers from these latter biological sources, milder solubilization conditions, usually with digitonin, are required [28].

The completion of the *C. reinhardtii* genome [29] allowed the identification of the ATP synthase subunits whose N-terminal sequences had been obtained previously [24]. Thus, the mitochondrial ATP synthase from *C. reinhardtii* was now found to contain 14 subunits of 7 to 60 kDa [30, 31]. Seven polypeptides were identified as the classical subunits α, β, γ, δ, *a* (ATP6), *c* (ATP9), and OSCP. Besides Asa1, several other polypeptides with no counterparts in the databases were identified and named Asa2 to Asa7 (this last one previously known as Nuop6) in accordance with the *C. reinhardtii* genome project nomenclature. In addition, no homologs of the ε, *b*, *d*, *e*, *f*, *g*, IF_1, A6L, and F6 subunits were found encoded in the algal genome. This reinforced the idea that unique proteins were associated with chlorophycean mitochondrial ATP synthases [30] and pin-pointed the Asa subunits as the main constituents of the peripheral stalk of the enzyme. The mitochondrial ATP synthase of *Polytomella* sp. was isolated, its polypeptide composition characterized, and found to be similar to the one of the *C. reinhardtii* enzyme [15, 22]. Furthermore, small-angle X-ray scattering analysis estimated a molecular mass of the complex of 1696 kDa [32]. In addition, the presence of two additional low molecular mass subunits, ASA8 and ASA9, was described.

Chlorophycean algae originated approximately 600 million years ago [33]. Some characteristics of this lineage are the drastic reduction of its mitochondrial genomes in size and in gene content [34], the fragmentation of the mitochondrial *cox2* gene and its migration to the nucleus [35], and the appearance of nucleus-encoded atypical subunits of ATP synthase (Asa subunits) [30, 36]. Genes encoding homologs of Asa subunits were identified in several other chlorophycean algae including *Volvox carteri*, *Scenedesmus obliquus* and *Chloroccocum ellipsoideum* [36]. The list can be updated to include the green unicellular algae *Chlamydomonas*

chlamydogama, *Chlamydomonas leiostraca*, *Chlamydomonas* sp. and *Dunaliella tertiolecta* and the colorless algae *Polytomella capuana*, *Polytomella magna*, *Polytomella piriformis* and *Polytomella parva* [37]. It seems that in the chlorophycean lineage, all the orthodox structural components of the peripheral arm were substituted by polypeptides from a complete different origin, probably proteins that already had a structural role in some other cell compartment.

2.2. Hydrolytic activity of the purified algal enzyme

When isolated, the *Polytomella* sp. enzyme exhibits a very low ATPase activity that increases either with heat treatment (due to the release of the F_1 sector) or when being unmasked by the presence of non-ionic detergents in an activity assay [38]. The algal dimer hydrolyzes ATP on a wide range of pH's and temperatures and shows sensitivity to the classical inhibitors oligomycin and DCCD [39]. The dimeric ATPase exhibited and apparent Km for Mg-ATP of 0.19 mM and a Vmax of 0.065 U/mg in the absence of detergent. The Vmax increased 60-fold in the presence of detergent (Km of 0.24 mM and a Vmax of 3.77 U/mg). Oligomycin seems to bind loosely to the enzyme, probably due to structural differences in the chlorophycean algae subunit *c* [39] that may affect the antibiotic binding site [40]. It is therefore necessary both to pre-incubate the enzyme in the presence of the inhibitor and to add an equal concentration of oligomycin in the assay medium. Overall, the enzyme does not exhibit strong differences in its hydrolytic activity as compared with the enzymes obtained from many other sources.

2.3. Three dimensional structure of the enzyme

The stable dimeric nature of the algal ATP synthase prompted a series of electron-microscope studies. The *Polytomella* enzyme, obtained by digitonin solubilization of mitochondria and sucrose gradient centrifugation, was subjected to electron microscopy and single particle analysis. Projection maps with a resolution

of about 17 Å showed a dimeric enzyme stabilized by interactions between the membrane-bound Fo sectors that formed an angle of 70° [26]. This implied that the enzyme could induce a strong local bending of the inner mitochondrial membrane [11] that may favor effective ATP synthesis under proton-limited conditions [41]. Also, the peripheral stalks of the algal dimeric enzyme were noted to be much more robust than their counterparts in other F-type ATPases, including the ones from bovine [42], yeast [43], spinach chloroplasts [44], and *Escherichia coli* [45].

Polytomella sp. mitochondria were subjected to ultrathin sectioning, and cristae membranes were found to fold into lamellae and tubuli. The ATP synthase oligomers were found to make helical arrangements along these tubular membranes, confirming the role of complex V in determining the shape of the inner mitochondrial membrane [46]. This has been further substantiated by RNA interference silencing of the *Atp*2 gene encoding subunit β of the *C. reinhardtii* ATP synthase: ATP synthesis was fully impaired, the enzyme failed to assemble, and the algal mitochondria were deprived of cristae [47].

Dual-axis cryo-electron tomography confirmed a supramolecular organization of dimeric ATP synthase in the cristae membranes of *Polytomella* sp. mitochondria that exhibited rows of dimers at 12 nm intervals [48]. In addition, averaged 3D sub-volumes of the algal oligomeric enzyme were obtained at 5.7 nm resolution. These 3D tomography data were the first indicators that contacts existed between the peripheral stalks of the monomers [48] suggesting their very rigid architecture. An additional EM analysis of the *Polytomella* sp. mitochondrial ATP synthase complex confirmed the dimeric nature of the enzyme and presence of robust peripheral stalks [32].

More recently, an electron cryo-microscopy map at 6.2 Å resolution of the *Polytomella* sp. dimeric ATP synthase was obtained [49]. Fig. 2 shows the electron density map of the dimer and the principal domains that constitute the oligomeric complex. The model has several salient features: *i*) Subunit *a* exhibits horizontally membrane-embedded alfa-helices that seem to embrace the *c*-ring and that form the two proton-translocating hemi-channels. The presence of these horizontal helices has been subsequently observed in the bacterial [50] and in the beef heart

mitochondrial enzymes [51]. *ii)* The robust peripheral stalks are constituted by several entwined alpha-helices that form a very solid scaffold. This is in accordance with the high propensity of several Asa subunits (Asa1, Asa2, Asa4 and Asa7) to form coiled-coils [52]. *iii)* Local resolution estimates indicated that the peripheral stalk is more ordered that the catalytic F_1 sector, furthermore, this peripheral stalks are united in their middle region by protein-protein contacts. The Asa subunit responsible for forming this extra-membranous bridge between the monomers is unknown, but could be either Asa4 as suggested previously [22] or Asa7 (see below). *iv)* The peripheral stalk, besides its substantial mass, seems to make several contacts with the F_1 sector. These contacts may be, among others, the interactions Asa2-α [52] and Asa1-OSCP [37]; *v)* the *c*-ring is formed by 10 monomers. This *c*-ring seems to be SDS-resistant [37], as those observed in yeast [53], in Na^+-dependent ATP synthases of bacteria [54] and in the A_1Ao-ATP synthases of archaea [55].

2.4. RNA-mediated expression silencing of subunits

The Asa7 subunit of the green algae *C. reinhardtii* was knocked-down using RNA interference. The absence of this polypeptide neither affected growth nor the OXPHOS of the alga [36]. Nevertheless, attempts to purify the ATP synthase from the Asa7-silenced mutant invariably failed, because lauryl maltoside solubilization dissociated the complex and released its F_1 sector. Subunit Asa7 is known to bind to at least three other subunits (Asa1, Asa2 and Asa4); it therefore must play a pivotal role in stabilizing the overall architecture of the peripheral stalk.

3. Addressing the topology of the components of the peripheral arm of algal mitochondrial ATP synthase

The neighboring interactions of Asa subunits in the ATP synthase of the colorless chlorophycean alga *Polytomella* sp. have been addressed using a variety of

experimental approaches: defining near-neighbor relationships of subunits after treating the whole enzyme with cross-linking agents; characterizing protein-protein interactions *in vitro* employing recombinant subunits; and dissociating the enzyme into sub-complexes by heat or high detergent concentrations. Thus, models for the topological disposition of the Asa polypeptides in the peripheral arm of the enzyme have been successively refined [15, 22, 32, 52, 37].

3.1. Neighboring interactions between Asa subunits as revealed by cross-linking agents

To assess neighboring interactions between subunits in the algal ATP synthase subunits, cross-linking experiments with several water-soluble and water-insoluble homo-bifunctional and heterobifunctional reagents were carried out. Besides some expected cross-link products between the orthodox subunits (i.e., α-β or α-OSCP), the cross-link products that were reproducibly obtained with the atypical subunits were (Asa1-Asa4), (Asa1-Asa7), (Asa2-Asa4), (Asa2-Asa7), (Asa3-Asa8), (Asa6-Asa6) and the triple cross-link product (Asa2-Asa4-Asa7) [32]. The inferred close vicinities between polypeptides were used to predict an overall topology of the algal ATP synthase.

3.2. Overexpression of subunits

In order to gain more insights on how Asa proteins interact, recombinant subunits were over-expressed and purified and their interactions *in vitro* explored. It was shown that Asa2, Asa4 and Asa7 interact, and furthermore, that the interaction Asa4-Asa7 is mediated by the C-terminal halves of both proteins [52]. Asa2 can bind Asa7 and the C-terminal half of Asa4. An interaction Asa2-α was also observed, suggesting the proximity of Asa2 with the catalytic head of the enzyme. In addition, subunits Asa2, Asa4 and Asa7 formed a subcomplex with a 1:1:1 stoichiometry that could be reconstituted *in vitro*. This subcomplex was proposed to establish additional contacts with Asa1 and with OSCP.

Subunit Asa1 was shown to be a membrane-extrinsic subunit using sodium carbonate treatment on mitochondrial membranes. It was proposed to represent the bulky structure observed near the F_1 sector in EM studies, and therefore to interact with OSCP [22]. Indeed, subunit Asa1 seems to be one of the main components of the peripheral stalk of the algal enzyme, probably forming the main column that unites the extrinsic subunit OSCP with other components of the enzyme in close contact with the membrane (Asa3, Asa5, Asa8, *a* and c_{10}-ring). Thus, Asa1 may be the main support of the peripheral stalk and may have a scaffolding role similar to the one of subunit *b* in orthodox enzymes [37]. The OSCP subunits of chlorophycean algae differ in its amino acid sequences from the classical plant and algal OSCP subunits, these differences may be important for binding of the algal OSCP to Asa1 instead of the *b* subunit.

3.3.Dissociation of the enzyme into subcomplexes

Work carried out with the *Polytomella* ATP synthase established other subunit-subunit interactions, mainly through the identification of sub-complexes formed by heat dissociation [15, 38]. Upon heat treatment, the ATPase is dissociated into its monomers and immediately afterwards it releases its F_1 sector and disassembles [15, 22]. Subunits Asa2, Asa4 and Asa7 and OSCP seem to dissociate concomitantly with the liberation of the F_1 sector. In addition, a sub-complex that contained subunits Asa1/Asa3/Asa5/Asa8/*a*/*c* appeared transiently [15]. A stable Asa1/Asa3/Asa5/Asa8/*a*/c_{10} subcomplex could be obtained by treating the enzyme with relatively high lauryl maltoside concentrations [37]. Furthermore, heat denaturation or tauro-deoxy-cholate treatment of the dimeric enzyme leads to a monomeric form with sub-stoichiometric amounts of ASA6 and ASA9 subunits [38], thus these two proteins seem to promote enzyme dimerization. In addition, the monomeric form of the complex is less active than the dimer, exhibits diminished oligomycin sensitivity, and is more labile to heat treatment, high hydrostatic pressures, and protease digestion.

Amphipols, the short amphipathic polymers that can substitute detergents, help the folding and stability of several integral membrane proteins [56]. To find whether Amphipol A8-35 would also help stabilize the algal ATP synthase, the purified enzyme was incubated with the polymer. As judged by BN-PAGE, in the presence of increasing concentrations of Amphipol A8-35 the dimeric enzyme (V_2) partially dissociated and released some free F_1 sector. A band, with a slightly faster migration than the dimer, was named subcomplex SC1 (Fig 3A). Another sample of the purified enzyme was incubated with 3.5 % Amphipol A8-35 for 40 min (Fig. 3B, lane 1) and subjected to 2D-SDS-PAGE, where the polypeptides of subcomplex SC1 were resolved (Fig 3C). Subunit OSCP was found to readily dissociate, migrating towards the front of the BN-PAGE gel. In parallel, a second sample of the purified enzyme was incubated with 3.5 % Amphipol A8-35 for 24 hours (Fig. 3B, lane 2) and then resolved in 2D-SDS-PAGE. In these conditions, a second subcomplex (SC2), with faster mobility in BN-PAGE than SC1, was formed (Fig 3D). The population of free F_1 was much smaller, and the dissociated subunits α, β, γ, δ, ε, Asa2, Asa4 and OSCP were found to migrate towards the front of the BN-PAGE polypeptide pattern. The subunit composition of the two Amphipol A8-35-generated sub-complexes SC1 and SC2 are shown in Fig 3E. Their identities were assigned by their apparent molecular masses as Asa1 (66.1 kDa), *c*-ring (45.5 kDa), Asa2 (45.3 kDa), Asa3 (32.9 kDa), subunit *a* (25.1 kDa), Asa7 (19.0 kDa), Asa5 (13.9 kDa), Asa6 (13.1 kDa), Asa8 (9.9 kDa) and Asa9 (11.0 kDa). The apparent molecular masses of the SC1 and SC2 sub-complexes of 681 and 528 kDa respectively, suggesting that these sub-complexes are dimeric. Upon Amphipol A8-35 treatment, the dimeric enzyme liberates OSCP and its F_1 sectors to form the SC1 subcomplex (illustrated in Fig. 4), while longer polymer treatment additionally releases the Asa2 and Asa4 subunits and forms the SC2 subcomplex (not shown). Thus, it seems that Amphipol A8-35 does indeed stabilize the membrane-embedded subunits of complex V, but destabilizes other extrinsic components, mainly OSCP, Asa2 and Asa4. It is notable that subunit Asa7 remains bound to the dimeric SC2 subcomplex even after the dissociation of the Asa2 and Asa4 subunits, most probably attaching to Asa1. It is therefore tempting to speculate

356 that Asa7 is the extrinsic protein bridge that unites the peripheral stalks of the algal
357 model observed in the 3-D model of the *Polytomella* sp. ATP synthase [49].
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359 **3.4.The dimerization domain of the algal mitochondrial ATP synthase**
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361 It is notable that only small-molecular mass subunits of the algal ATP
362 synthase seem to be embedded in the membrane. Only subunits Asa6, Asa8 and
363 Asa9 are predicted to have transmembrane stretches (TMS), two, one and one,
364 respectively [22]
365 The stoichiometry of all the Asa subunits in the algal ATP synthase has
366 not been established yet. An estimated stoichiometry based on cysteine-labelling
367 fluorescent probes revealed a 1:1:1 stoichiometry for subunits Asa3:Asa4:Asa5
368 per monomer [32], although this assessment was limited to those subunits that
369 contain at least one cysteine residue. Cross-sections through the membrane
370 domains of the electron cryo-microscopy map of the algal enzyme shows the four
371 horizontal-embedded helices of subunit *a*, the c_{10}-ring and six additional
372 transmembrane helices per monomer [49]. These six TMS could be ascribed, in
373 principle, to the fifth vertical alpha helix of subunit *a* [51], the two reentrant
374 helices and one TMS of subunit Asa6, one TMS of Asa8, and one TMS of Asa9
375 (Fig 5). Asa8 may be instrumental for the dimerization of the enzyme, since it
376 contains a GxxxG domain, which is a weak predictor of membrane protein
377 dimerization [57]. The presence of GxxxG domains in subunits *e* and *g* are known
378 to play an important role in dimerizing the yeast ATP synthase [58-60].
379 All the above mentioned data can be summarized in an updated model for
380 the topological disposition of the 17 polypeptides that constitute the algal enzyme
381 (Fig. 6).
382
383 **4. Perspectives**
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385 A regulatory protein that may control the ATPase activity of the algal
386 enzyme has not been characterized. Nevertheless, one of its subunits is expected to

have a regulatory role similar to subunit IF_1 in mitochondrial enzymes [61], to the gamma-proteobacterial ε subunit [62] or to the alpha-proteobacterial ζ subunit [63].

Many biochemical data have been obtained on the ASA subunits arrangement in the peripheral stalk, but to elaborate a more precise model of organization and to validate or invalidate the model in Fig 6, high resolution structural data is needed. In the model available to date [49], it is still not possible to pin-point unambiguously the individual Asa subunits.

All the subunits of the chlorophycean mitochondrial ATP synthases are nucleus-encoded, since no gene encoding any complex V polypeptide has been found in the mitochondrial genomes of these algae [64, 65]. It is of interest to learn how all the nucleus-encoded subunits, especially the Asa subunits, are imported into mitochondria and how they assemble to form the very large, 1600 kDa algal complex V.

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FIGURE LEGENDS

FIGURE 1. Model of the *Polytomella* sp. ATP synthase subunits α and β and their corresponding extensions. The *Polytomella* sp. sequences of the mature α subunit with its C-terminal extension of 67 residues, and the β subunit with its N-terminal extension of 21 residues were modeled *in silico* using the I-TASSER server [66]. Two of the obtained models that showed no steric hindrances were selected for each subunit. The algal F_1 sector was modelled on the bovine crystallographic structure (PDB Id: 2WSS) [67]. Subunits in the complex are colored: α subunits (dark green), β subunits (light green), γ subunit (gray), algal α subunit extensions (one possible conformation in red, the other in magenta), algal β subunit extensions (one possible conformation in orange, the other in dark blue).

FIGURE 2. Electron cryo-microscopy map of the algal *Polytomella* sp. ATP synthase.

The model shown corresponds to EMDB accession number EMD-2852 [49]. The main domains of the complex were visualized and colored with the UCSF Chimera package [68]: rotor in yellow (subunits γ/δ/ε/*c*-ring), subunit *a* in red, the catalytic domain (α_3/β_3) in orange, OSCP in violet, and the peripheral stalk (Asa1 to 5 and Asa7) plus the membrane dimerization domain (Asa6, Asa8 and Asa9) in cyan.

FIGURE 3. Amphipol A8-35 dissociates the dimeric algal ATP synthase into discrete subcomplexes.

A) BN-PAGE of purified ATP synthase samples [15] incubated for 40 min at 4 °C under mild agitation in the presence of Amphipol A8-35 (Anatrace) at the indicated increasing concentrations (% w/v). 100 μg of protein were loaded in each lane. The control lane in the absence of the detergent is labelled 0. Dimer (V_2), F_1 sector (F_1), and subcomplex (SC1) are indicated. V_2' denotes an isoform of the algal ATP synthase with a different electrophoretic migration, whose subunit composition is identical to the one of V_2. B) BN-PAGE of purified ATP synthase samples (50 μg of protein in each lane) incubated with or without Amphipol A8-35. Lane 1: sample incubated in the presence of 3.5% Amphipol A8-35 for 40 minutes; lane 2: sample incubated in the presence of 3.5% Amphipol A8-35 for 24 hours; lane 3: control ATP synthase without treatment. Dimer (V_2), F_1 sector (F_1), and sub-complexes SC1 and SC2 are indicated. C) Silver-stained 2D gel of an ATP synthase sample incubated for 40 min in the presence of 3.5 % Amphipol A8-35. D) Silver-stained 2D gel of an ATP synthase sample incubated for 24 hours in the presence of 3.5 % Amphipol A8-35. E) Identity of the subunits that form the SC1 and SC2 sub-complexes.

FIGURE 4. Model showing the dissociation of the ATP synthase induced by Amphipol A8-35. The model illustrates the dissociation of the algal ATP

774 synthase by Amphipol A8-35 (40 min incubation) to form the dimeric SC1
775 subcomplex after the dissociation of the F_1 sector and subunit OSCP.
776
777 **FIGURE 5. Hydrophobicity-based models of the individual subunits involved**
778 **in the membrane-embedded dimerization domain.** Several algorithms predict
779 the Asa8 and Asa9 subunits with a single TMS. The GxxxG motif in the Asa8
780 subunit could prompt its dimerization. The predicted models for Asa6 vary with
781 the different programs used: one TMS, two TMS, or one TMS with a reentrant
782 helix (depicted).
783
784 **FIGURE 6. Arrangement of subunits in the algal mitochondrial ATP**
785 **synthase.** The model is consistent with the data summarized in this manuscript
786 and illustrates the dimeric enzyme with a colored subunit composition. Letters
787 denote the conserved ATP synthase subunits, while numbers refer to Asa subunits
788 (Asa1-Asa9). Subunits Asa6, 8 and 9 form the dimerization module, while the rest
789 of them are constituents of the peripheral stalks.
790

Figure01

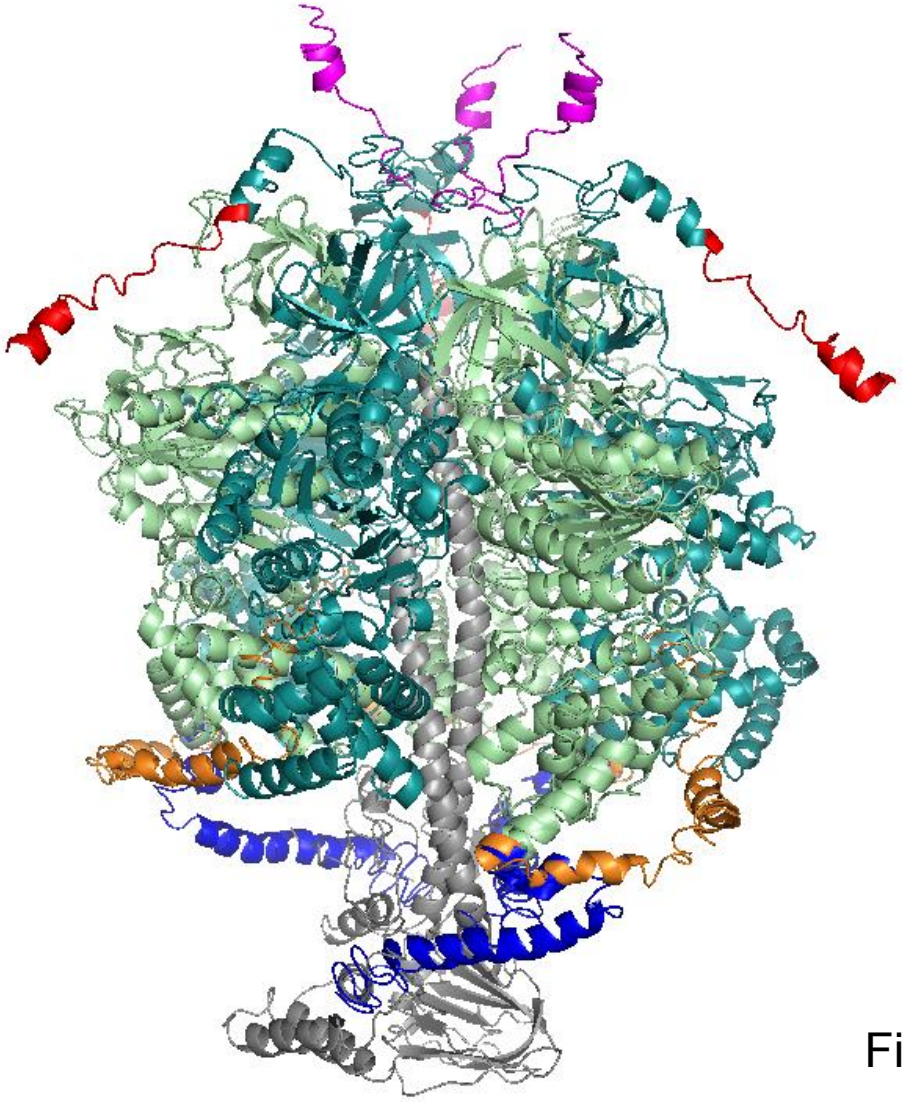

Figure 1

Figure02

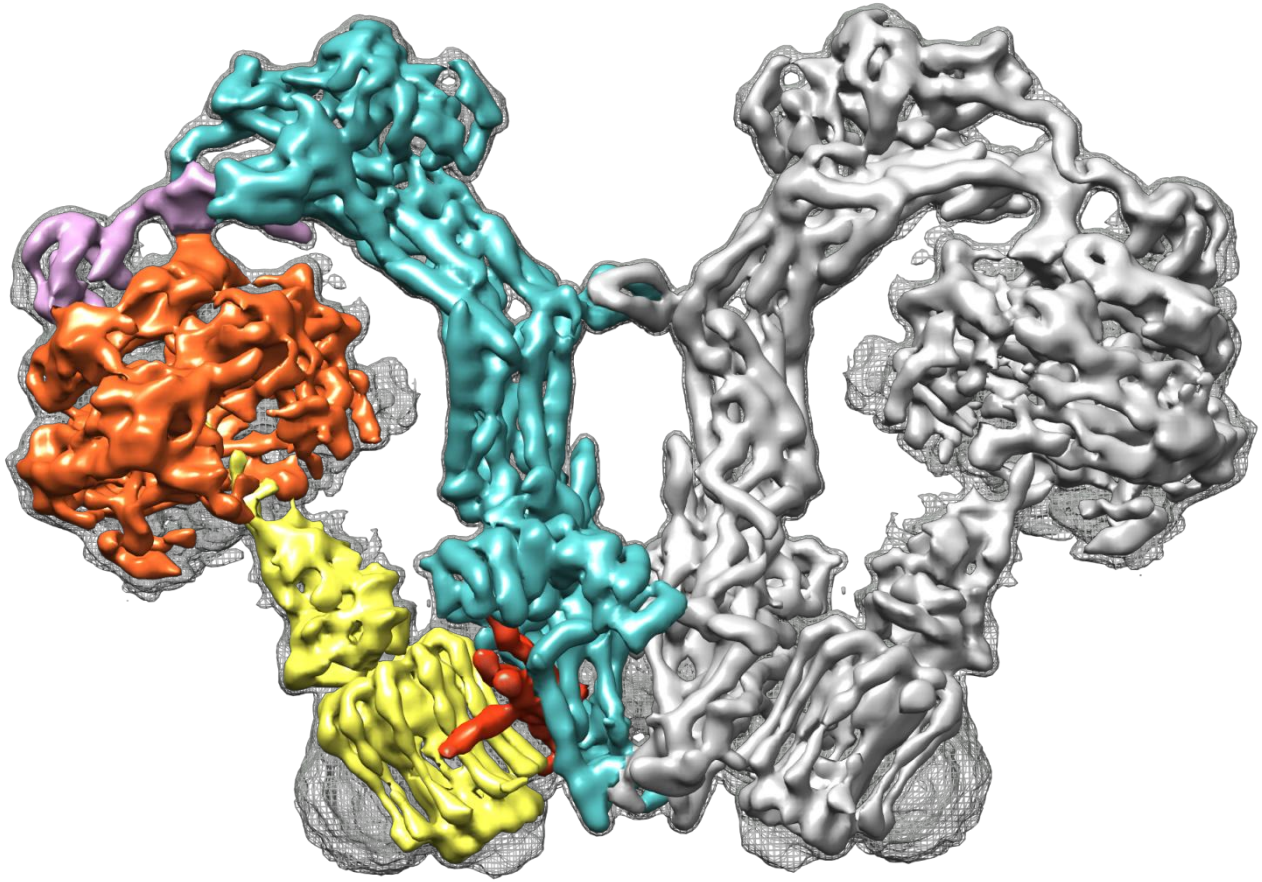

Figure 2

Figure03AB

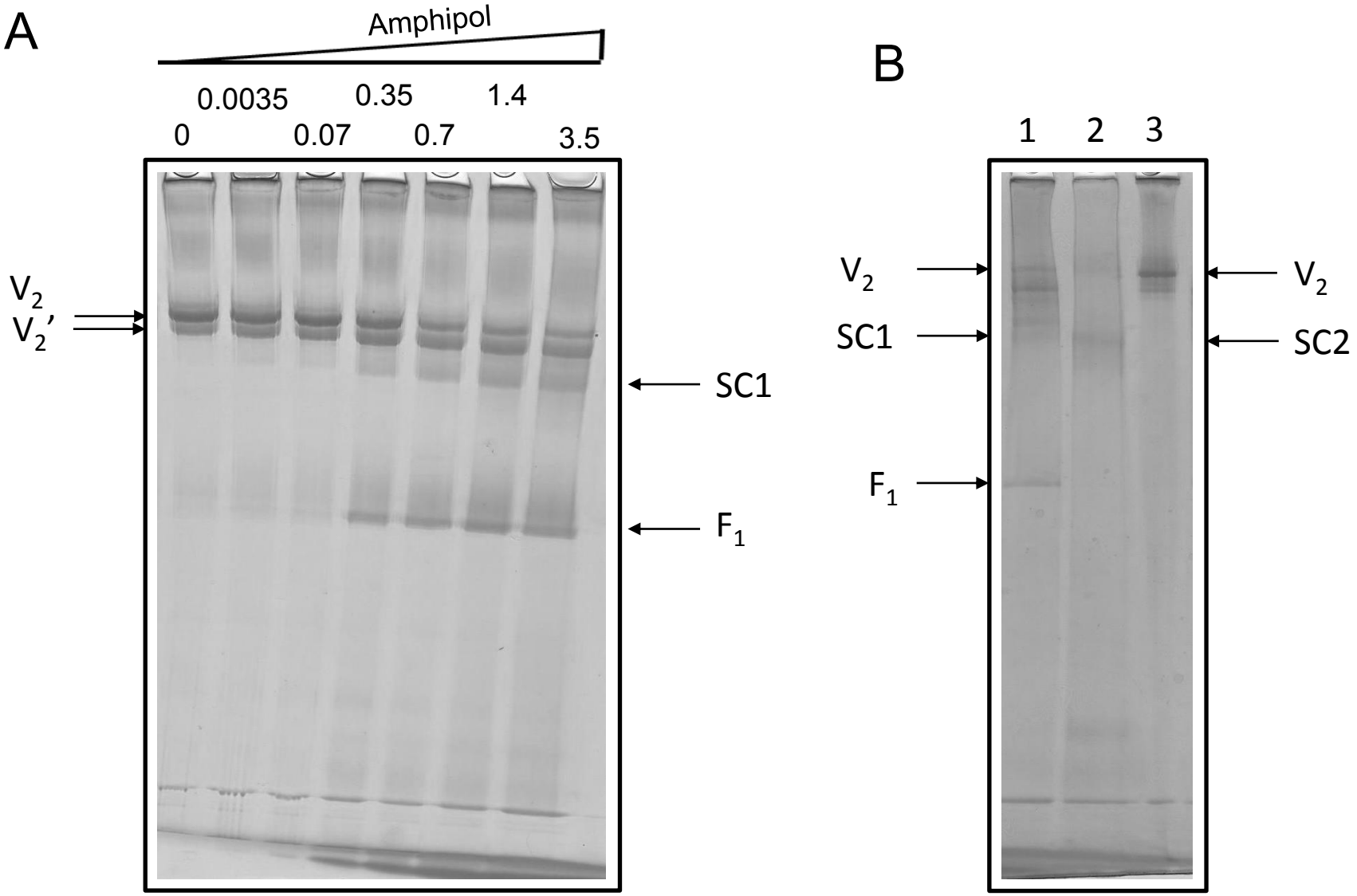

Figure 3

Figure03C

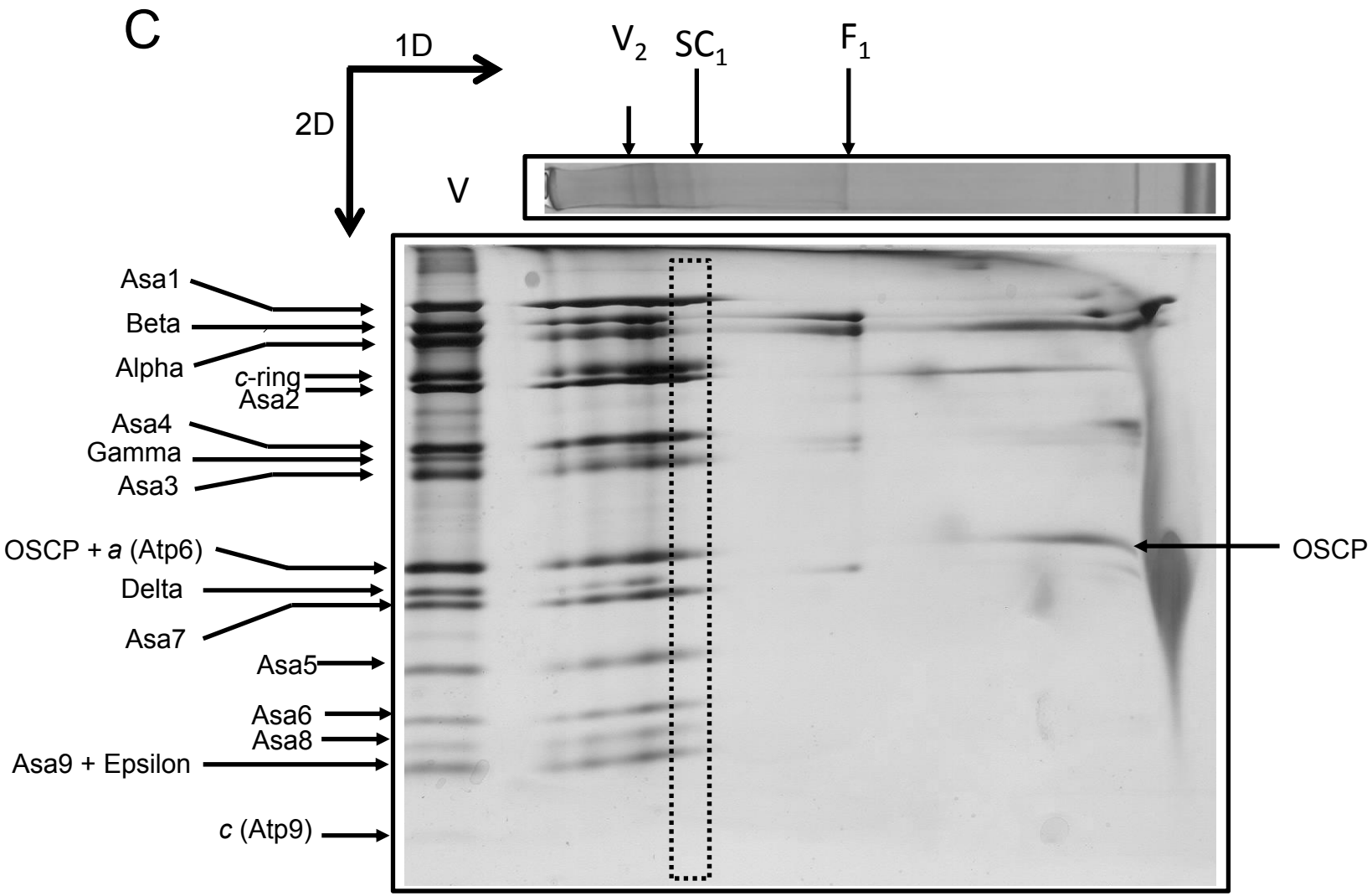

Figure 3 (continued)

Figure03D

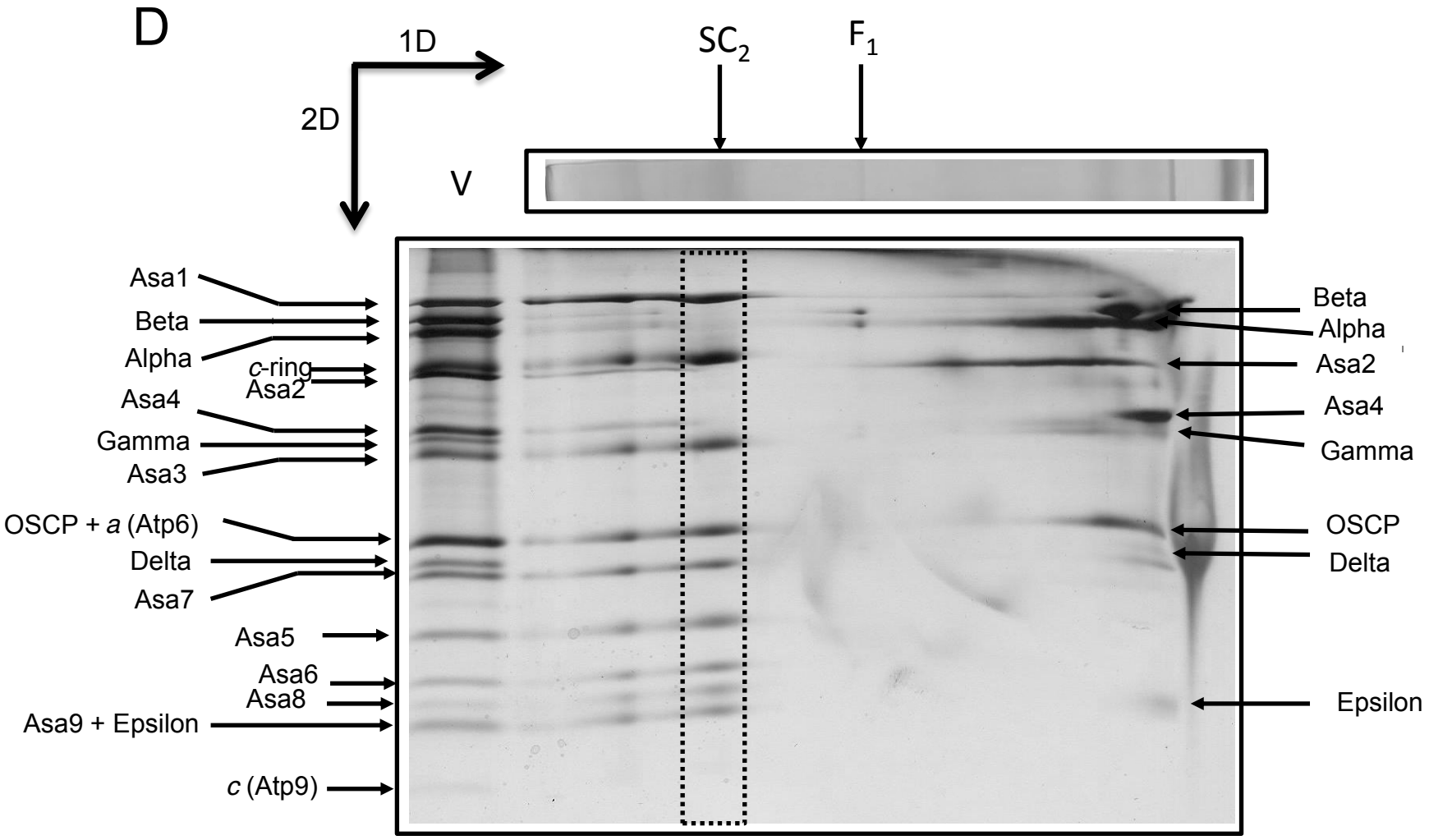

Figure 3 (continued)

Figure03E

E

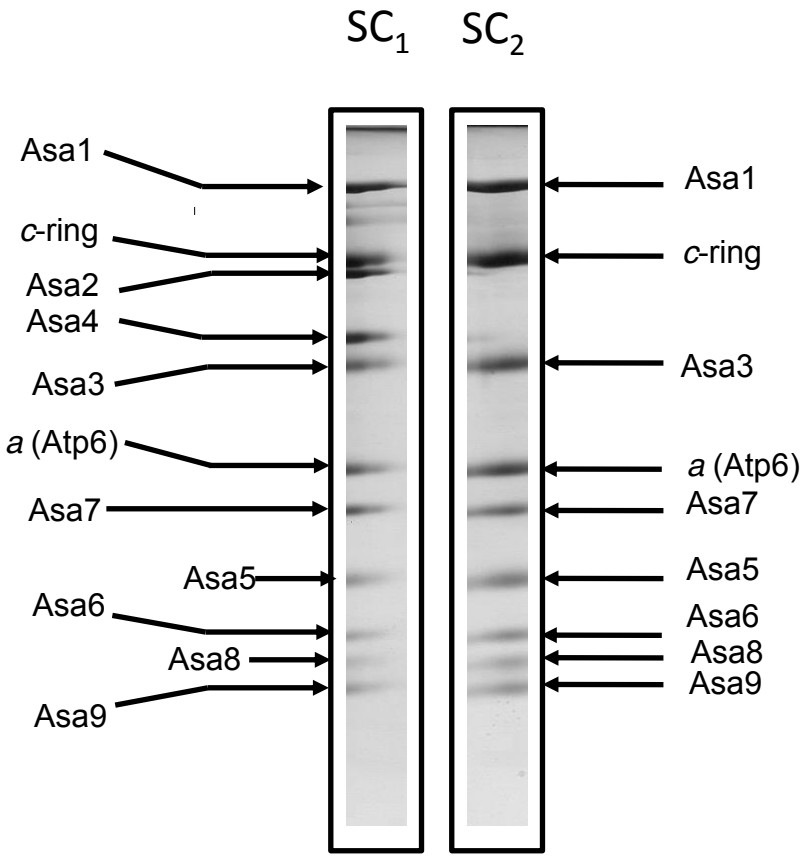

Figure 3 (continued)

Figure04

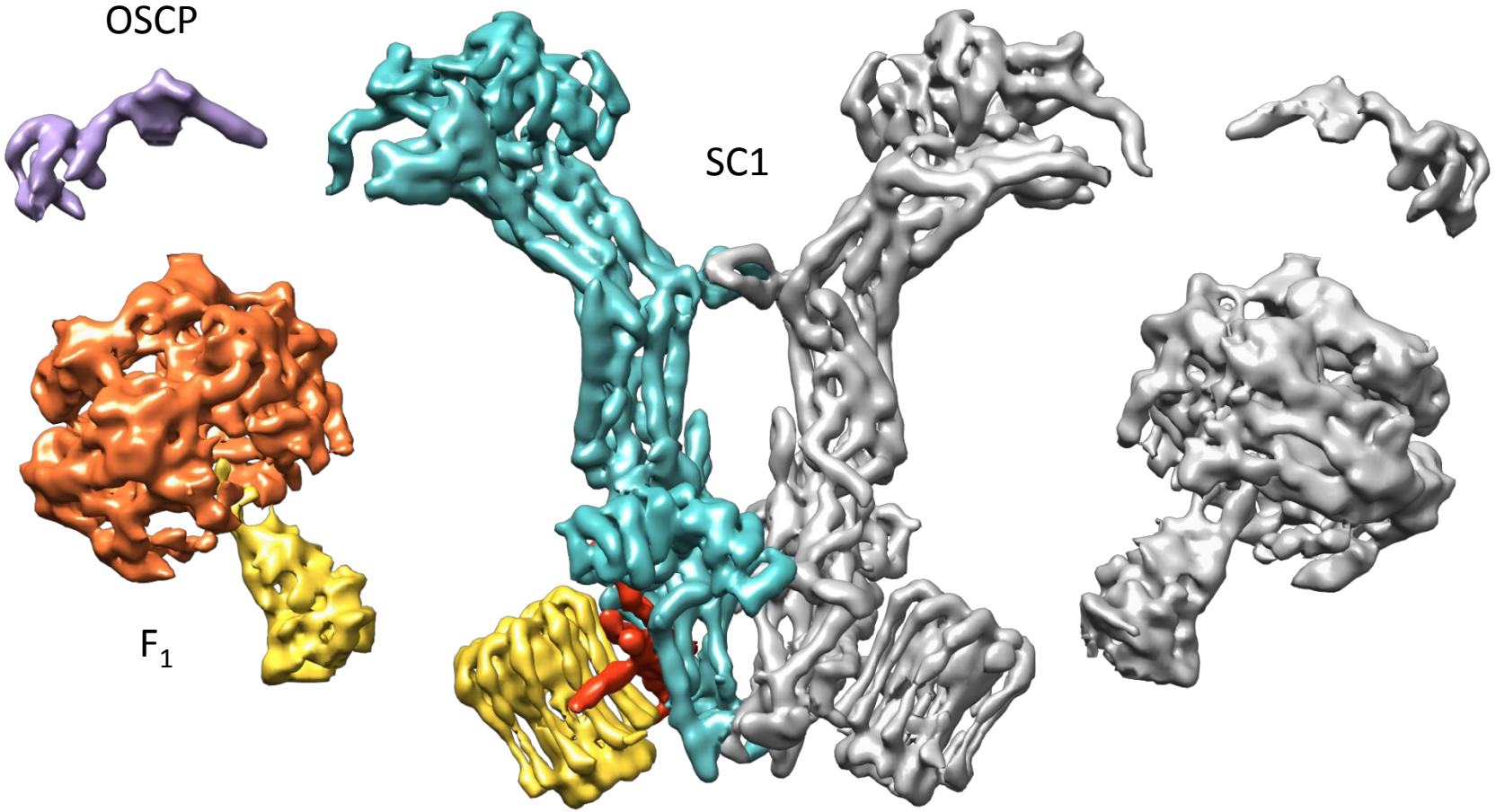

Figure 4

Figure05

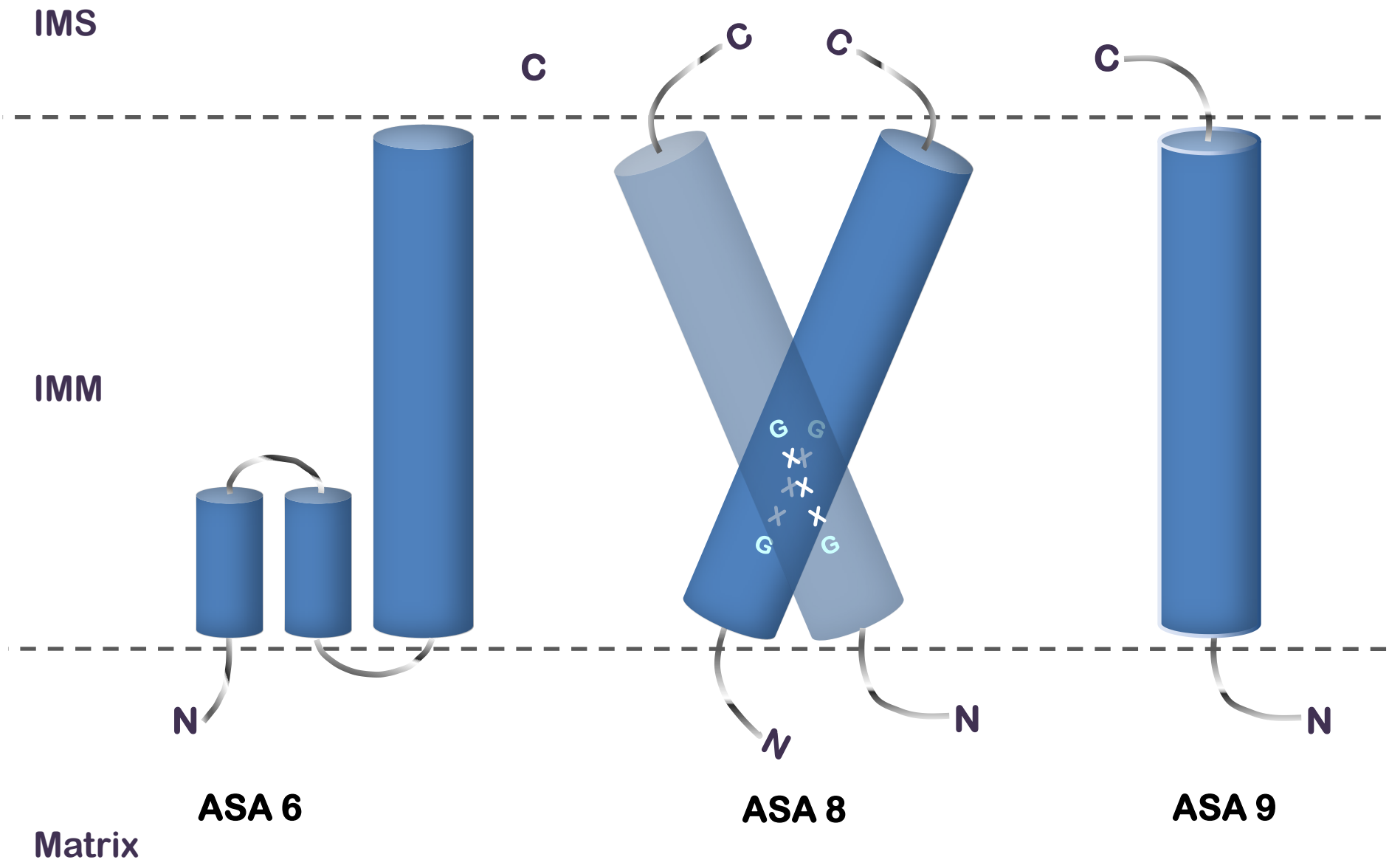

Figure 5

Figure06

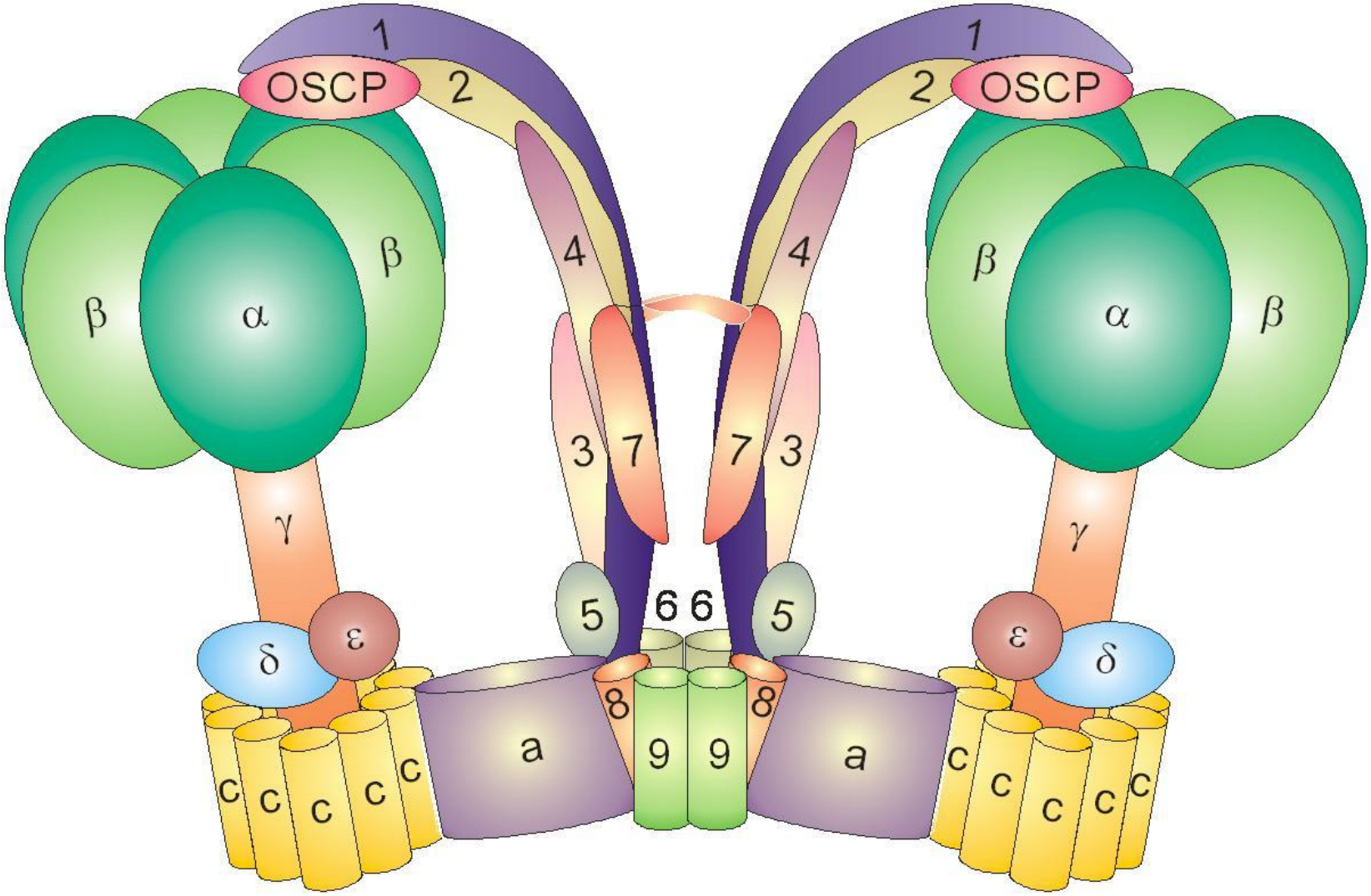

Figure 6