Evolution of photochemical reaction centres: more twists?

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Abstract

One of the earliest events in the molecular evolution of photosynthesis is the structural and functional specialisation of Type I (ferredoxin-reducing) and Type II (quinone-reducing) reaction centres. In this opinion article we point out that the homodimeric Type I reaction centre of Heliobacteria has a calcium-binding site with striking structural similarities to the Mn_4CaO_5 cluster of Photosystem II. These similarities indicate that most of the structural elements required to evolve water oxidation chemistry were present in the earliest reaction centres. We suggest that the divergence of Type I and Type II reaction centres was made possible by a drastic structural shift linked to a change in redox properties that coincided with or facilitated the origin of photosynthetic water oxidation.

Evolution of Photosystem II

The origin of oxygenic photosynthesis was one of the most transformative evolutionary innovations in the history of life. However, there is still no consensus on when and how oxygenic photosynthesis originated as both the timing and the evolutionary mechanisms involved are disputed [1]. It is our opinion that a complete scenario for the evolution of oxygenic photosynthesis should first explain how and when water oxidation to oxygen originated at the level of the **photochemical reaction centre**. That is, how and when Photosystem II (PSII), the

water oxidising enzyme of oxygenic photosynthesis, evolved the **Mn_4CaO_5 cluster** and the oxidising photochemistry required to split water (Figure 1).

The Mn_4CaO_5 cluster is a unique feature of PSII [2] and even though it is usually assumed that Photosystem II originated from an anoxyenic photosystem, no clear precursor or transitional stage to the cluster seems to have been preserved within any of the well-characterised photosystems of anoxygenic photosynthesis. This situation might have now changed with the release of the crystal structure of the first homodimeric Type I reaction centre [3].

At a structural level, the Mn_4CaO_5 cluster is coordinated by the carboxylic C-terminus of the core subunit D1, which acts as a bidentate ligand that bridges the Ca and a Mn atom [4], numbered Mn2 in Figure 2. Several Mn ligands, D342, E333, and H332 are also provided from the C-terminal domain of D1. Furthermore, E354 from the antenna subunit CP43 provides a bidentate ligand bridging Mn2 and Mn3, and R357 provides a hydrogen-bond to O4 and is within 4.2 Å of the Ca. These two residues are located in an extrinsic protein domain between the 5th and 6th transmembrane helices of CP43 that reaches into the electron donor-side of D1.

After **charge separation** the oxidised chlorophyll (P_{D1}^+) extracts an electron from the redox active tyrosine, D1-Y161, known as Y_Z, forming the neutral tyrosyl radical, which in turn oxidises the Mn_4CaO_5 cluster. Y_Z is hydrogen-bonded to H190 and the electron transfer step to P_{D1}^+ is coupled to a movement of the hydroxyl proton to H190. There is no Mn_4CaO_5 cluster in D2 (Figure 2), however the tyrosine-histidine pair is conserved in a symmetrical position in this subunit too (D2-Y160 and D2-H189) giving rise to another redox active tyrosine, Y_D [5, 6]. The presence of strictly conserved redox active tyrosine residues in both D1 and D2 led to the suggestion that water oxidation originated in a homodimeric ancestral photosystem with primordial Mn clusters on each side of the reaction centre [7]. It was also predicted that structural evidence for the vestiges of a metal binding site on D2 would exist [7]. This prediction was confirmed once refined structures of PSII became available [2, 4] (Figure 2) and its

implications for the evolution and timing of the origin of oxygenic photosynthesis were discussed in a detailed study recently [8].

In the vicinity of Y_D in D2 there is no metal cluster and instead of ligands, several "space filling" phenylalanine residues are found (Figure 2). Like CP43, the CP47 subunit also has an extrinsic domain that reaches into D2, but instead of ligands, phenylalanine residues are also found, one of them located just 3.4 Å from Y_D. These structural observations indicate that water oxidation could have originated in a homodimeric system before the duplication of the protein ancestral to D1 and D2, and that D2 evolved a unique hydrophobic space-filling plug to prevent the access of Mn and bulk water to Y_D, thereby eliminating catalysis on the D2 side of PSII.

All scenarios for the origin of oxygenic photosynthesis *assume* that Type II reaction centres were first anoxygenic and that they did not have enough oxidising power to split water to oxygen. The reasons behind this assumption are more historical than evidence-based [1]. Paradoxically, it is PSII that seems to retain a greater number of ancestral traits. This is visually evident in Figure 1 and it is consistent with a number of recent observations. For example, PSII core subunits are the slowest evolving of all reaction centre proteins [8] which means that PSII not only retains more symmetry at the core, but also more structural similarity with Type I reaction centres [9]. In particular, PSII has retained a Type I-like core antenna system, the CP43 and CP47 subunits. Their current role in water oxidation and in space-filling close to Y_D suggest an early function in cluster binding in the homodimeric forerunner of PSII. When taken with the fact that an *in series* Type II-Type I arrangement is exclusively found in oxygenic photosynthesis, these considerations led to the proposal that the origin of two types of reaction centres was linked to the origin of oxygenic photosynthesis [1, 9]. Here we provide additional evidence supporting the premise that water oxidation originated at, or soon after, the divergence of Type I and Type II reaction centres. That is, at one of the earliest stages in the evolution of photosynthesis.

A calcium-binding site in homodimeric Type I reaction centres

The recent structure of the homodimeric Type I reaction centre [3] from an anoxygenic photoheterotrophic firmicute, *Heliobacterium modesticaldum*, revealed a previously unknown Ca-binding site with a number of intriguing structural similarities to the Mn_4CaO_5 cluster of PSII (Figure 3). Firstly, this Ca-binding site is positioned at the electron donor side of each monomer of the reaction centre, in a location corresponding to that of the redox tyrosine-histidine pair in D1 and D2, in the immediate vicinity of the Mn_4CaO_5 cluster (Figure 3A). Secondly, the Ca-binding site is connected to the C-terminus of the reaction centre protein, PshA, by L605 and V608. L605 coordinates the Ca via the backbone carbonyl and V608 via an oxygen from the C-terminal carboxylic group. This carboxylic acid ligand was not modelled in the structure [3], but can be seen in the electron density map (Figure 3E). In a similar way, the C-terminus of D1 provides direct ligands to the Mn_4CaO_5 cluster, i.e. D342 and A344 (Figure 3G). The A344 is a ligand not only to Mn, but also to the Ca via the carboxylic C-terminus. Thirdly, the Ca-binding site is linked to the antenna domain of PshA, via N263, which is located in the extrinsic domain between the 5th and 6th transmembrane helices. N263 connects to the Ca via two water molecules. In PSII, as described above, the CP43 residues E354 and R357, which are located in a large extrinsic domain between the 5th and 6th helices, connect the antenna to the Mn_4CaO_5 cluster. Fourthly, PshA residue D468, located at a position that overlaps with that of Y_Z, provides a hydrogen bond to a tyrosine (Y513) found just 4 Å from the Ca. This resembles the Y_Z-H190 pair, although PshA-Y513 and D1-H190 do not occupy identical structural positions (Figure 3C). In PSII, Y_Z is 4.7 Å from the Ca. The Y513-D468 hydrogen-bonded pair in the homodimeric reaction centre is present in the PsaB subunit of cyanobacterial PSI as Y629-H601 (Figure 3D), both of which are phenylalanine residues in PsaA.

In *H. modesticaldum*, it is not known whether this is a true Ca-binding site or the result of non-specific binding. Nothing like it had been seen in any other reaction centres and its biochemical role has not been studied yet. Nevertheless, none of the buffers used for purification

of the complex [10, 11] or crystallisation conditions explicitly included Ca salts [3], which may indicate that it is a binding site with significant affinity for Ca. Sequence comparisons suggests that a similar site might exist in homodimeric Type I reaction centres from the Chlorobi (**Box 1** and online supplemental information Figure S1). The presence of a divalent metal site in homodimeric Type I reaction centres so distant from PSII, yet in a manner so similar to the Mn_4CaO_5 cluster, is puzzling and potentially significant from an evolutionary perspective.

The structural similarities between the homodimeric Type I reaction centre and PSII could have radical implications for the evolution of photosynthesis. Notably, these similarities could indicate that the **most recent common ancestor** of PSII and homodimeric Type I reaction centres had a site that was readily accessible to divalent cations at the electron donor side and near the photochemical pigments (P). The most recent common ancestor of homodimeric Type I reaction centres and PSII is the ancestral photosystem existing before the divergence of Type I and Type II reaction centres (**Box 2**). This would mean that several of the structural elements needed for the origin of a water-oxidising cluster were already in place at this point in time. These elements include the location itself, at least two ligands available from the C-terminus, at least one ligand available from the antenna domain, Ca, and even potentially the tyrosine. These similarities provide a structural blueprint to explain the evolutionary origin, location and ligand sphere of the Mn_4CaO_5 cluster. They indicate a more ancient origin for the involvement of Type I-like antenna proteins in the binding of the cluster than has been considered until now [12, 13], but as predicted in [9]. From this perspective, it seems quite likely that the CP43 and CP47 subunits were retained in PSII, at least in part, because of their interaction with the electron donor site and their role in binding the cluster in the ancestral homodimer.

The divergence of Type I and Type II reaction centres

The biggest structural changes that occurred in early reaction centre evolution are those needed for the functional specialisation that produced the two known types and those that were needed for the evolution of water oxidation chemistry. Again, given that two reaction centres *in series* is a unique trait of oxygenic photosynthesis and that PSII seems to retain a greater number of ancestral traits that, as we have seen above, can extend to the electron donor side, it is valid to ask whether these two major structural changes were actually one and the same. In this section we provide a structural and functional rationale for the divergence of Type I and Type II reaction centres and show how this divergence can be linked to the emergence of a photosystem ready to catalyse the oxidation of water to oxygen.

Structural changes in ancestral reaction centres

Structural comparisons suggest that major modifications had to occur at the divergence between the two types (Figure 4). Of importance are the changes that determined the position of the ligand to P, the nature of the electron acceptor, and the nature of the electron donor. The ancestral states of each of these elements before the divergence of Type I and Type II are not known [14] (see outstanding questions). Available phylogenetic and structural data are inconclusive. Here, to illustrate the structural changes, we start with a scenario in which the ancestor was more "Type I-like" with an F_X-like Fe_4S_4 cluster connecting the homodimeric core subunits. We will also briefly consider what would happen if the ancestral reaction centre was more "Type II-like" or somewhat in-between.

One of the major distinctions between the reaction centre types is the position of the ligand to P. In Type I reaction centres the histidine ligand is in the middle of the transmembrane helix (Figure 4B to D and H, red arrows), while in Type II reaction centres it is at the bottom of the helix. Despite this difference in the location of the liganding histidine residues, the P chlorophylls are at the same positions relative to the membrane plane, with the histidine in Type I reaching downwards, while the histidine in Type II is at the same level as the central Mg. This

difference indicates that a change in the position of the helix took place. It required an upward movement of about 12 Å relative to a Type I reaction centre and a swap of histidine ligands, while the position of P remained unchanged. The change in position of the helix would have altered the interconnecting domains between the 9th and 10th helices and between the 10th and 11th helices (Figure 4E to H), which make part of the donor and acceptor side, respectively. Now, if the redox cofactors are overlapped and those of Type II are placed on a Type I protein scaffold, the top of the helix overlaps the quinones (Figure 4F, Q_A and Q_B), and the middle of the helix overlaps the monomeric chlorophylls (Chl_{D1} and Chl_{D2} in PSII; Bch_L and Bch_M in anoxygenic Type II reaction centres). If the reverse is done instead, the last two transmembrane helices of the Type II core protein overlaps the F_X cluster.

Such a structural rearrangement within the membrane would have altered the electrostatic environment of the photochemical pigments and likely affected any existing tuning of the redox potentials. In today's reaction centres, this difference in the position of P relative to the transmembrane helix results in an upshift in the E_m of P^+/P of at least +140 mV in Type II reaction centres relative to Type I [15]. Additionally, the "upward" shift would have completely disrupted the F_X-binding site, it would have changed groups on the electron acceptor end of the same helix (Figure 4H, blue arrows), and it probably caused the rotated position of the monomeric chlorophylls relative to Type I, a conserved trait of Type II reaction centres (Figure 4C and D). It seems plausible that a histidine, now positioned near the top of the 10th helix, could have become the ligand for the non-heme Fe^{2+}. This transition should have favoured the selection of the second histidine from the 11th helix (5th in Type II) to provide a strong stable central symmetrical Fe^{2+} coordination sphere, leaving two coordination positions in the Fe^{2+} empty to be filled by exchangeable ligands like for example, bicarbonate, as still exists in PSII today. Notably, in anoxygenic Type II reaction centres the non-heme Fe^{2+} is asymmetrically coordinated by a glutamate from the M subunit, indicating that this is unlikely to be the ancestral state. In contrast, PSII retains a highly symmetric bicarbonate binding site involving both D1

and D2 [1, 16]. The newly formed His-Fe^{2+}-His motif could have provided hydrogen-bonds to the distal carbonyl of available quinones, making their O-O axes near parallel with the membrane, and providing the quinone-to-quinone electron transfer pathway that is characteristic of Type II reaction centres.

The emergence of a highly oxidising photosystem

The consequences of this structural transition become clear when the energetics of the reaction centres are compared. In today's PSII the E_m of $P680^+$/P680, is estimated to be about +1200 mV [17, 18] and in PSI, the E_m of $P700^+$/P700 is estimated to be about +450 mV [19, 20]. The E_m of chlorophyll *a* in dichloromethane is +800 mV [15, 21], therefore PSII is 400 mV more oxidising and PSI is 350 mV less oxidising than chlorophyll *a* in an organic solvent. These differences indicate that the protein environment strongly modulates the E_m of the photochemical pigments in both directions and in both types of reaction centre relative to that of the isolated pigment [15]. In fact, Ishikita *et al.* [15] calculated that in the absence of any protein charges or specific electrostatic effects the E_m of P in PSI and PSII would be closer to that of chlorophyll *a* in an organic solvent, about +720 mV for both systems. This value is noteworthy because the E_m for water oxidation to oxygen is +820 mV at pH 7 [22]. However, the concentration of oxygen in the Archean atmosphere was likely well below 10^{-5} of the present level [23, 24], which translates to a concentration of dissolved oxygen in water below 2 nM [25]. At 2 nM, the E_m of water oxidation to oxygen is +735 mV [25], below that of the intrinsic chlorophyll *a* potential. Now, if we assume that the ancestral reaction centre was Type I-like, the massive movement of the helix could have disrupted all the specific electrostatic effects responsible for down-shifting the E_m of P^+/P relative to the intrinsic or unmodulated chlorophyll *a* potential (+720-800 mV). That would have been enough to bring the potential of P^+/P up to a level similar to that required for water oxidation in the Archean world.

Previously, much of the discussion on the evolution of PSII focused on the changes that were responsible for making a reaction centre capable of oxidising water [15, 26, 27]. While the energetic and mechanistic considerations are valid, this was done under the assumption that the ancestral Type II reaction centres were like those found in phototrophic Protoebacteria and that it did not have enough driving force to split water, an assumption that, as we have seen above, needs to be revisited in the light of new data [1]. Part of the difference in the potential of P^+/P between PSII and the anoxygenic Type II reaction centres is found in that the latter use bacteriochlorophyll *a*, which is less oxidising (+640 mV in dichloromethane [15]) than chlorophyll *a*. It is worth noting that the synthesis of bacteriochlorophyll *a* requires three additional enzymatic modifications of the tetrapyrrole ring to be made from a chlorophyll *a* precursor [28].

There has been much debate on the type of pigments that were used by the earliest photosystems. Recent discussions have favoured scenarios in which chlorophyll *a* is indeed the ancestral pigment [29, 30]. In agreement with these discussions, the evolution of 3-vinyl-bacteriochlorophyll hydratase, an enzyme strictly required to make bacteriochlorophyll *a,* indicated that the late steps needed to make bacteriochlorophyll *a* were added to the synthesis pathway only after the divergence of Type I and Type II reaction centres [31]. Moreover, we showed recently, that the duplication leading to L and M occurred well after the duplication leading to D1 and D2, and therefore, after the origin of water oxidation [8], reinforcing the observation that a bacteriochlorophyll *a*-containing Type II reaction centre is a relatively late evolutionary innovation and represents an inadequate model for ancestral Type II reaction centres. Accordingly, if the ancestral Type II reaction centre bound chlorophyll *a* then this was likely a highly oxidising photosystem.

Along the same lines, PSII has in common with Type I reaction centres not only the presence of antenna and the core peripheral chlorophylls ($ChlZ_{D1}/ChlZ_{D2}$), but also the fact that the monomeric chlorophylls (A_{-1} in Type I, Chl_{D1}/Chl_{D2} in PSII) are coordinated by water

molecules (Figure 4D and E). In anoxygenic Type II reaction centres, the Mg of the equivalent bacteriochlorophylls (Bch_L/Bch_M) are coordinated by histidine ligands, indicating that the absence of these histidine ligands is the likely ancestral state. The lack of histidine ligands to Chl_{D1} and Chl_{D2} accounts for an upshift in their E_m of +135 mV relative to the anoxygenic Type II reaction centres [15]. Mutations of the histidine ligands to the monomeric bacteriochlorophylls do not affect electron transfer, but instead destabilise the complex [30]. This also indicates that chlorophyll *a*, the inherently more oxidising pigment, was the likely ancestral pigment of Type II reaction centres and that the histidine residues evolved in the anoxygenic system, to stabilise bacteriochlorophyll *a*, at a later stage [30]. Additionally, Ishikita *et al.* [15] also calculated that the presence of the antenna subunits, the likely ancestral trait, can contribute over 100 mV to the oxidising power of PSII. When taken together, these comparisons suggest that the structural shift resulted in an ancestral Type II reaction centre that was more oxidising than Type I and the Type II reaction centres of the anoxygenic phototrophs.

There are also major differences at the acceptor side between Type I and Type II. The potential of F_X in Type I reaction centres is about -700 mV [32]. The potential of non-heme Fe^{2+}/ Fe^{3+} in Type II reaction centre is over +400 mV [33], although this is not directly involved in electron transfer reactions. At the level of the quinone there is a more than 500 mV difference in the potential of the A_1 phylloquinone (about -800mV) in PSI [32] compared to that of Q_A (-150 mV) in PSII [34], with most of the difference (about 500 mV) attributed to the electrostatic effect of the net negative charge on F_X compared to the net positive charge on the non-heme Fe^{2+} [35, 36]. At the level of the primary acceptors there is also a similar difference in the potential between A_0 (over -1000 mV) and the equivalent pheophytin pigments (about -0.5 V) in Type II reaction centres [36-38]. In this case, part of the difference is attributed to the S-Mg coordination of A_0 in PSI [39]. The other Type I reaction centres use bacteriochlorophyll *g* or *a* in Heliobacteria and Chlorobi respectively, as the primary photochemical donors: A_0 remains

chlorophyll *a* however, likely to maintain the strong reductant after the acquisition of bacteriochlorophyll pigments.

An oxidative jump at the origin of two types of reaction centres

The idea that PSII could have achieved its high oxidising power by only a limited number of mutations, an *oxidative jump*, rather than a very gradual process to a higher potential, was proposed when it was realised that this jump likely occurred in homodimeric reaction centres, since a change in the single gene could have a double effect in the homodimer [7, 27]. However, the problem with the oxidative jump in the context of the Type I and Type II divergence is that an upshift in the oxidising potential of the donor-side must be accompanied by a matching upshift in the potential of the acceptor-side. Without this, the reducing power of the excited state P* could not reduce the very low potential electron acceptors, typical of Type I reaction centres; as both A_0, A_1, and F_X would be out of reach. The lack of a matching increase in the potential of the electron acceptors would have resulted in the loss of charge separation or at least a marked decrease in the quantum yield. This is what happened when anoxygenic Type II reaction centres were engineered to increase the oxidising power of P^+/P without increasing the potential of the electron acceptors [40, 41]. Consequently, increasing the potential of the P^+/P will only be feasible when the acceptor side is already oxidising. This then requires the homodimeric ancestor to have evolved a high potential acceptor side without any obvious evolutionary advantage or selection pressure. A solution to this problem would be that the acceptor side also underwent an oxidative jump at the same time as the donor side. Just such a dual effect might have resulted from the structural shift described above.

All in all, the structural changes required to explain the divergence of Type I and Type II reaction centres may have led to the evolution of a photosystem with an oxidising potential close to that required for the oxidation of water in Archean conditions. If this ancestral reaction centre was also capable of stabilising a primordial Mn/Ca cluster at the donor side, this would have led

to a further increase in the E_m of P thus raising the driving force for water oxidation to oxygen. In PSII the Mn_4CaO_5 cluster is calculated to contribute +200 mV [15] to the E_m of P_{D1}, which is compensated by an asymmetric downshift of -135 mV resulting from electrostatic effects from nearby residues, indicating that the heart of PSII can be more oxidising than it is.

After the structural shift, a tyrosine, hydrogen-bonded to a histidine and nearby a Ca-binding site, could have become oxidised upon charge separation. This Tyr-His pair could have initially occupied a position similar to D468-Y513 in PshA. The newly formed tyrosyl radical could have oxidised several aqueous Mn^{2+}. Oxidised Mn can be immediately stabilised by the Ca, a ligand from the antenna domain, and the carboxylic C-terminus, already present in the ancestral reaction centre as demonstrated here. Oxidation of Mn is followed by the ejection of a proton from any of the bound waters leading to the favourable formation of μ-oxo-bridges between adjacent Mn cations [22]. This process could have occurred in a manner very similar to the photoactivation of the Mn_4CaO_5 cluster in PSII, which can occur without the aid of chaperons or any specialised assembly factors [42-44].

What was the possible nature of the ancestral reaction centre?

At the moment, there are no conclusive ways to determine or measure the exact properties of the ancestral reaction centre before the Type I and Type II divergence. Another possibility is that the ancestral reaction centre was bifunctional. That is, able to reduce F_X, and under certain conditions, exchangeable quinones occupying a position similar to that of A_1. Experimental evidence for such a dual function has been reported for Heliobacteria recently [45]. However, the role of quinones in homodimeric Type I reaction centres is still unresolved. They are not needed for electron transfer to F_X, but they have been detected in reaction centres in several different ways [10, 45-48] and no bound quinones were observed in the structure [3]. Kashey *et al.* [45] reported that the heliobacterial reaction centre oxidises F_X in low light and menaquinone in high light. It was shown that menaquinone reduction is inhibited by terbutryn, a Type II

reaction centre inhibitor, which would indicate the presence of a well-defined binding site [49]. If so, it would likely be under the electrostatic influence of F_X, even if only transiently, but the comparable binding-site in PSI seems to be blocked by an arginine sidechain [3]. Do these seemingly contradictory observations hint at structural shifts when switching from F_X to menaquinone reduction? A vestige of early days?

Nevertheless, Orf, Gisriel and Redding [12] recently concluded based on a structural and functional rationale that every major structural difference between PSI and the lineage leading to the homodimeric Type I reaction centres can be explained in the context of avoidance of reactive oxygen species after the origin of water oxidation (see Box 2, Figure I, stage **3**). It was argued that PSI obtained protective adaptations before the duplication leading to PsaA and PsaB (Box 2, Figure I, stage **5**): one of these adaptations was the evolution of tightly bound quinones as opposed to exchangeable quinones. For that to be true, it requires that water oxidation originated before the divergence of the homodimeric PSI ancestor and the remaining homodimeric Type I reaction centres: *because it is oxygen what triggered their structural divergence at a homodimeric stage*. In other words, it requires that water oxidation had originated before the most recent common ancestor of all Type I reaction centres. That is, at or soon after the divergence of Type I and Type II reaction centres.

A final possibility is that the ancestral reaction centre before the divergence was more like Type II than like Type I. The consequence of this scenario is that the ancestral reaction centres was in many ways like Photosystem II, but homodimeric. That is to say, that under this perspective the earliest reaction centres were naturally more oxidising than the reaction centres of known anoxygenic phototrophs or PSI. Regardless of the direction in which the structural changes occurred, the selecting factor that allowed these drastic structural changes to provide an increase in fitness, was perhaps not just the functional specialisation in quinone or ferredoxin reduction, but also the simultaneous establishment of a linear electron transfer chain from Mn and water to ferredoxin. When this did not occur, the result was competition for electron

donors, redundancy, and eventual loss of one reaction centre or the other. This explains the paradoxical fact that no anoxygenic phototroph has ever acquired a second anoxygenic reaction centre "of the other type" even when horizontal gene transfer is thought to be a common driving force in the diversification of photosynthesis.

Concluding remarks

The structural characteristics of the heliobacterial reaction centre and Photosystem II are hard to reconcile with traditional evolutionary scenarios in which oxygenic photosynthesis represents a late evolutionary innovation emerging hundreds of millions of years, if not a billion years, after the origin of anoxygenic photosynthesis. We challenge this view by showing how an early origin of photosynthetic water oxidation has more explanatory power than traditional perspectives. It not only explains in precise detail why the Mn_4CaO_5 cluster is the way it is, but also it provides a good rationale to account for the structural changes at the origin of the two distinct reaction centres of photosynthesis. We believe the observations and scenarios presented here merit further consideration and encourage a critical rethink of what we take for granted about the evolution of photosynthesis.

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Supplemental information

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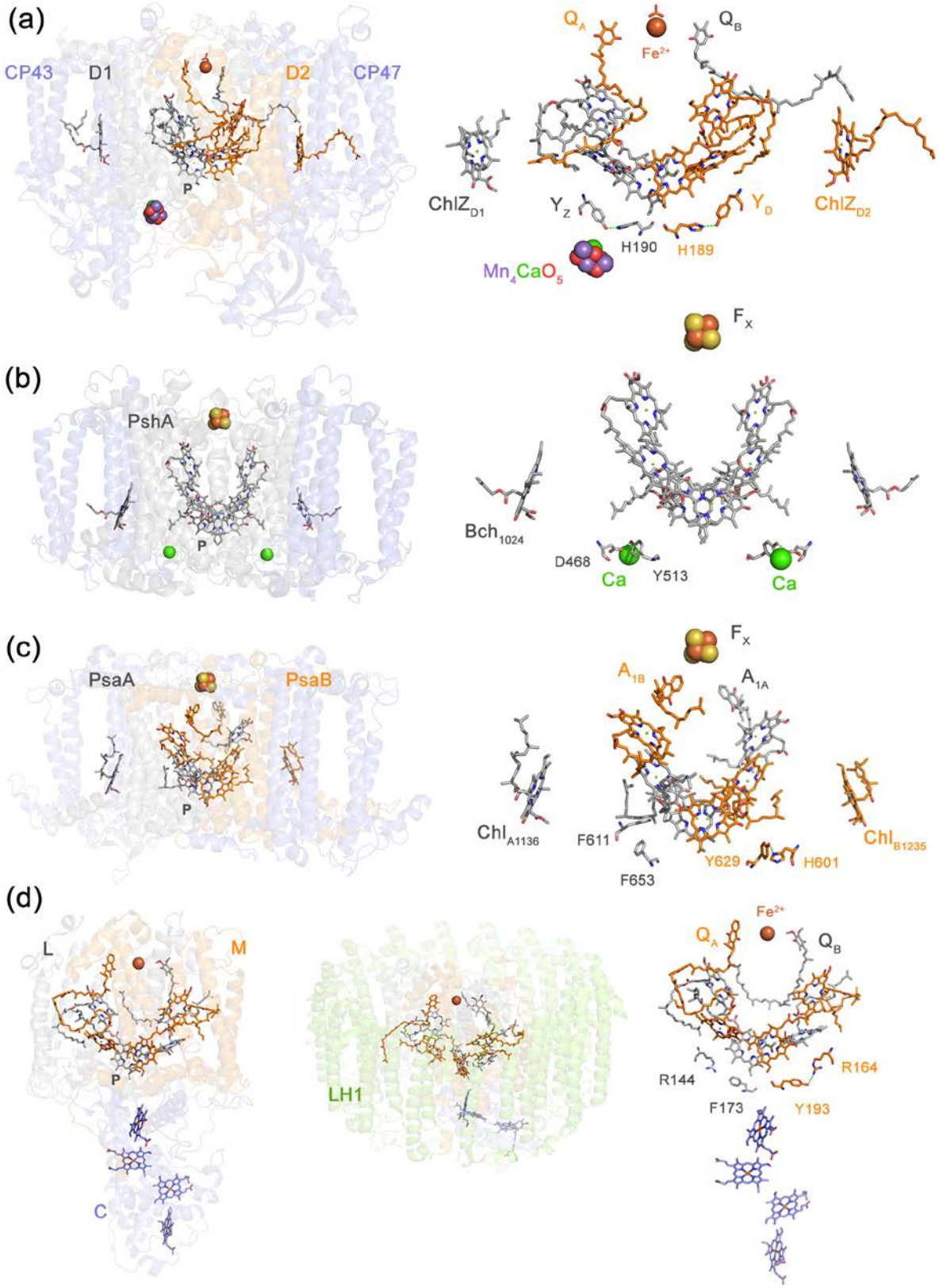

Figure 1. Photochemical reaction centres. Type II reaction centres function in quinone reduction and Type I reaction centres function in ferredoxin reduction. Polypeptides are

represented as transparent ribbons and cofactors as sticks. Spheres indicate atoms of Fe (orange), S (yellow), Mn (purple), O (red), Ca (green). (**A**) Cyanobacterial PSII (pdb id: 3wu2) [4]. Only the four main core protein subunits are shown and a detailed view of the cofactors bound by the reaction centre proteins is presented on the right. (**B**) Heliobacterial Type I reaction centre (pdb id: 5v8k) [3]. Two calcium atoms are shown in green spheres. The reaction centre is a homodimer made of a single protein, PshA, with the antenna domain shown in blue. (**C**) Cyanobacterial Photosystem I (pdb id: 1jb0) [50]. The reaction centre is made of two proteins, PsaA and PsaB, with the antenna domain highlighted in blue. (**D**) Proteobacteril anoxygenic Type II reaction centre (pdb id: 3wmm) [51]. Anoxygenic Type II reaction centres use a different light harvesting system (LH1) unrelated to those in PSII and Type I reaction centres. In all Type II reaction centres, electron transfer occurs asymmetrically. After charge separation electron moves via the active branch (D1 and L), via Q_A, to finally reduce Q_B. In Type I reaction centres, electron transfer can occur via both branches. PSII is characterised by the presence of redox active tyrosine-histidine pairs. A hydrogen-bonded pair of residues at this position seems to be a feature found in all reaction centres.

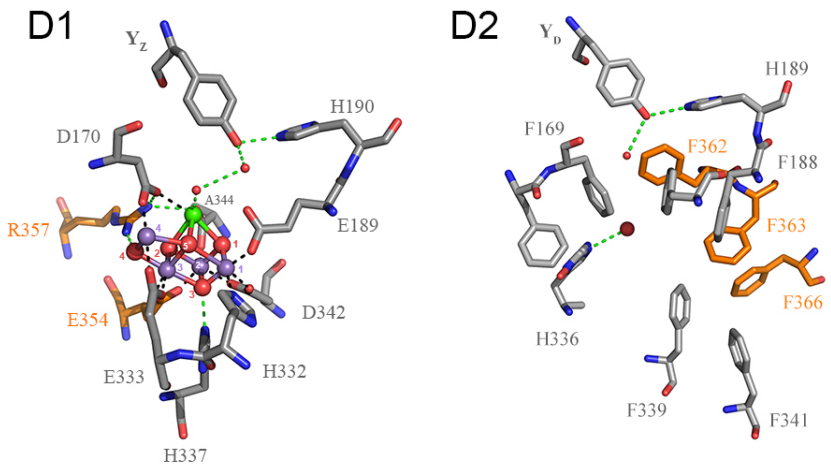

Figure 2. The Mn_4CaO_5 cluster of PSII. The cluster is coordinated by ligands from D1 (grey sticks) and CP43 (orange sticks). Ca is connected to the redox tyrosine Y_Z (Y161) via hydrogen-bonded water molecules. On the right, the homologous site in D2 is shown. Residues from D2 are shown as grey sticks and those from CP47 as orange sticks. No catalytic cluster is observed but a strictly conserved redox tyrosine is found, Y_D (Y160). D2-F169 and D2-F188 occupy the positions of D1-D170 and D1-E189 respectively; and D2-F339 and D2-F341 occupy positions similar to those of D1-D342 and D1-A344, respectively. In addition, D2-H336 provides a hydrogen bond to a water molecule found between the phenylalanine residues in a way that is quite similar to the hydrogen bond of D1-H337 to the cubane oxygen (O3). CP47-F362 occupies the position of one of the waters that provides a hydrogen-bond to the phenolic oxygen of the Y_Z and which is thought to be important for its rapid, reversible, high-potential redox chemistry [52]. It has been suggested that water oxidation started in a homodimeric photosystem, with two catalytic clusters placed symmetrically, one on each side of the reaction centre and with ligands to the ancestral antenna protein [7, 8, 27].

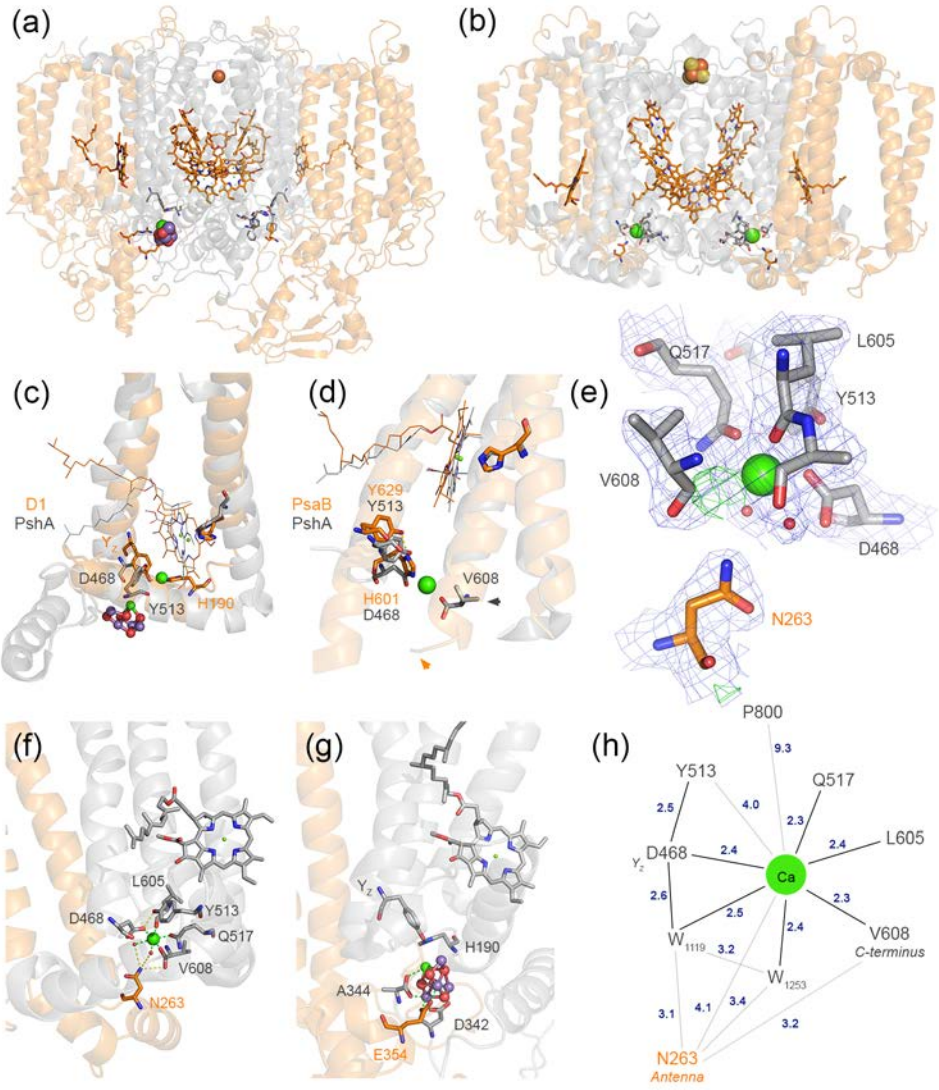

Figure 3. The Ca-binding site of the homodimeric Type I reaction centre. (**A**) Full view of PSII showing in grey transparent ribbons the core proteins and in orange the antenna proteins. (**B**) Full view of the heliobacterial Type I reaction centre showing in transparent grey the core domain and in orange the antenna domain of PshA. The green spheres are the Ca atoms located symmetrically on each side of the reaction centre. (**C**) Overlap of D1 (orange) and PshA (grey). In PshA, Ca is coordinated by D468, which occupies a structurally position similar to Y_Z in D1. Only the 9th and 10th transmembrane helices are displayed for clarity (3rd and 4th in D1). (**D**) Overlap of PsaB of cyanobacterial PSI (orange), and PshA (grey). The arrows mark the C-terminus of the core proteins. There is no Ca-binding site in PSI as the 11th transmembrane helix of the core domain is about two turns longer (orange arrow). (**E**) Electron density map of 5v8k

around the Ca-binding site (blue mesh, contour map: 1σ). V608, the C-terminus carboxylic group, was modelled in the released structure as a carbonyl, missing the oxygen that coordinates the Ca [3]. The difference between the observed and calculated maps shows the positive density between V608 and the Ca corresponding to the missing coordinating oxygen bond (green mesh, contour map: 3σ). (**F**) Close-up of the Ca-binding site showing the connection to the antenna domain via N263 and the C-terminus. (**G**) Close-up of the Mn_4CaO_5 cluster highlighting the connection to the antenna via E354. A344, the C-terminus, provides a direct ligand to Ca in PSII. (**H**) Scheme of the Ca-binding site of PshA showing the closest distances (Å) to residues in the immediate vicinity. W stands for water and the words in italics highlight structural similarities to PSII.

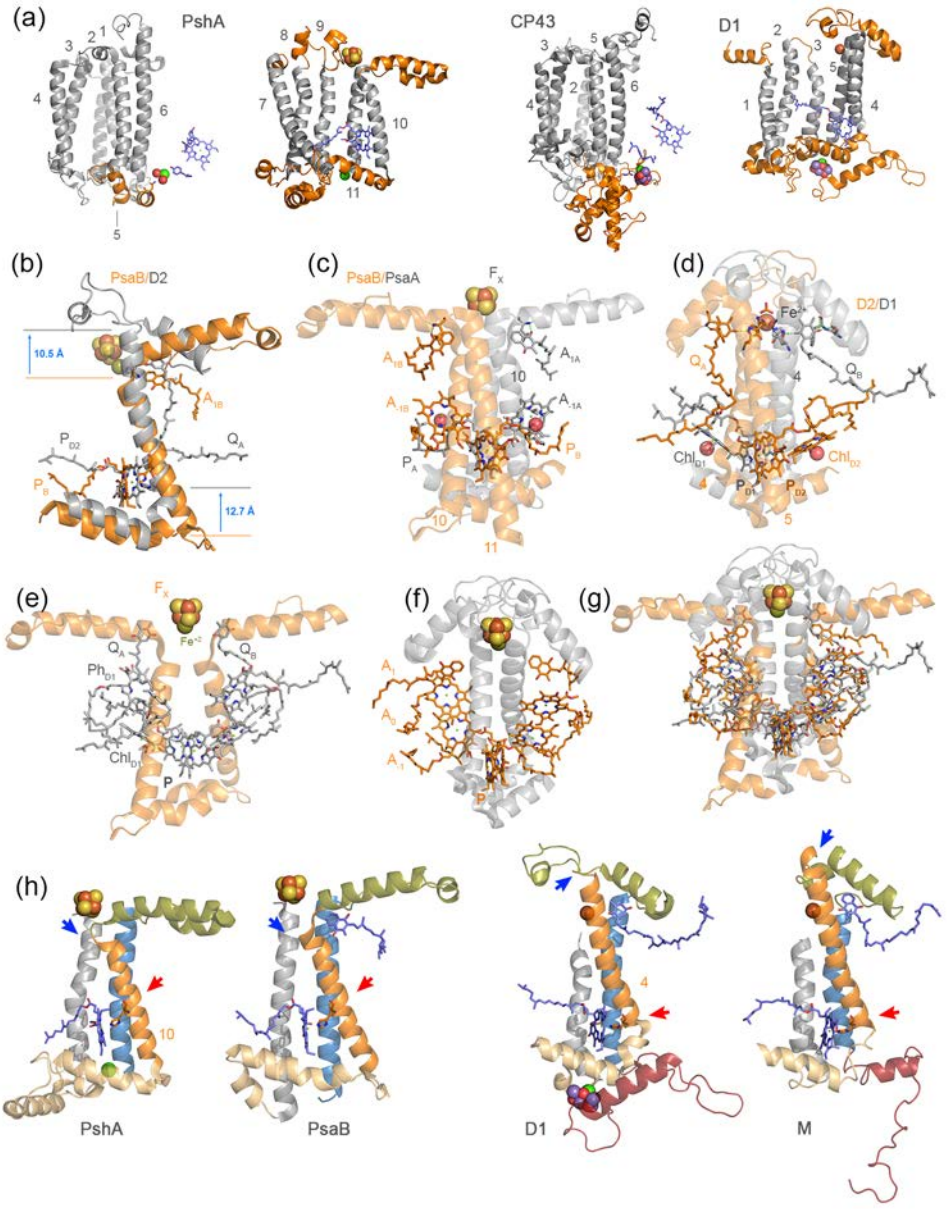

Figure 4. Structural comparisons of the reaction centre proteins. (**A**) Comparison of transmembrane helix arrangements in the heliobacterial reaction centre and PSII. The antenna and the core have been separated for clarity. (**B**) Overlap of PsaB (orange) and D2 (grey): only the helix that provides the ligand to P is shown. The start and the end of the 4th transmembrane helix in Type II reaction centres are shifted 12.7 Å and 10.5 Å respectively, relative to Type I. The position of the P chlorophylls remains unchanged. (**C**) A view of the phylloquinone binding sites in PSI relative to the position of P and A_{-1}. Only the 10th and 11th helices are shown. The red spheres represent coordinating waters. The A_0 electron acceptor chlorophylls were omitted for clarity. (**D**) A view of the plastoquinone binding sites in PSII relative to the position of P and

$Chl_{D1/D2}$. The red spheres represent coordinating waters. The pheophytin electron acceptors, the pigments at homologous positions to A_0 in Type I, were omitted for clarity. (**E**) The cofactors of PSII are shown relative to the position of the 10th helix of PsaA/PsaB. (**F**) The cofactors of PSI are shown relative to the position of the 4th helix of D1/D2. (**G**) Overlap of the 10th and 4th helices of PSI and PSII core subunits relative to their cofactors. (**H**) The last three transmembrane helices of the core reaction centre proteins are compared to highlight the structural differences and similarities between the two types. The red arrows mark the position of the histidine ligand to P. The blue arrows mark the end of the 10th helix and equivalent in Type II. All structures have been aligned to the position of the Mg atom of P and maintaining the last transmembrane helix vertical (blue helix).

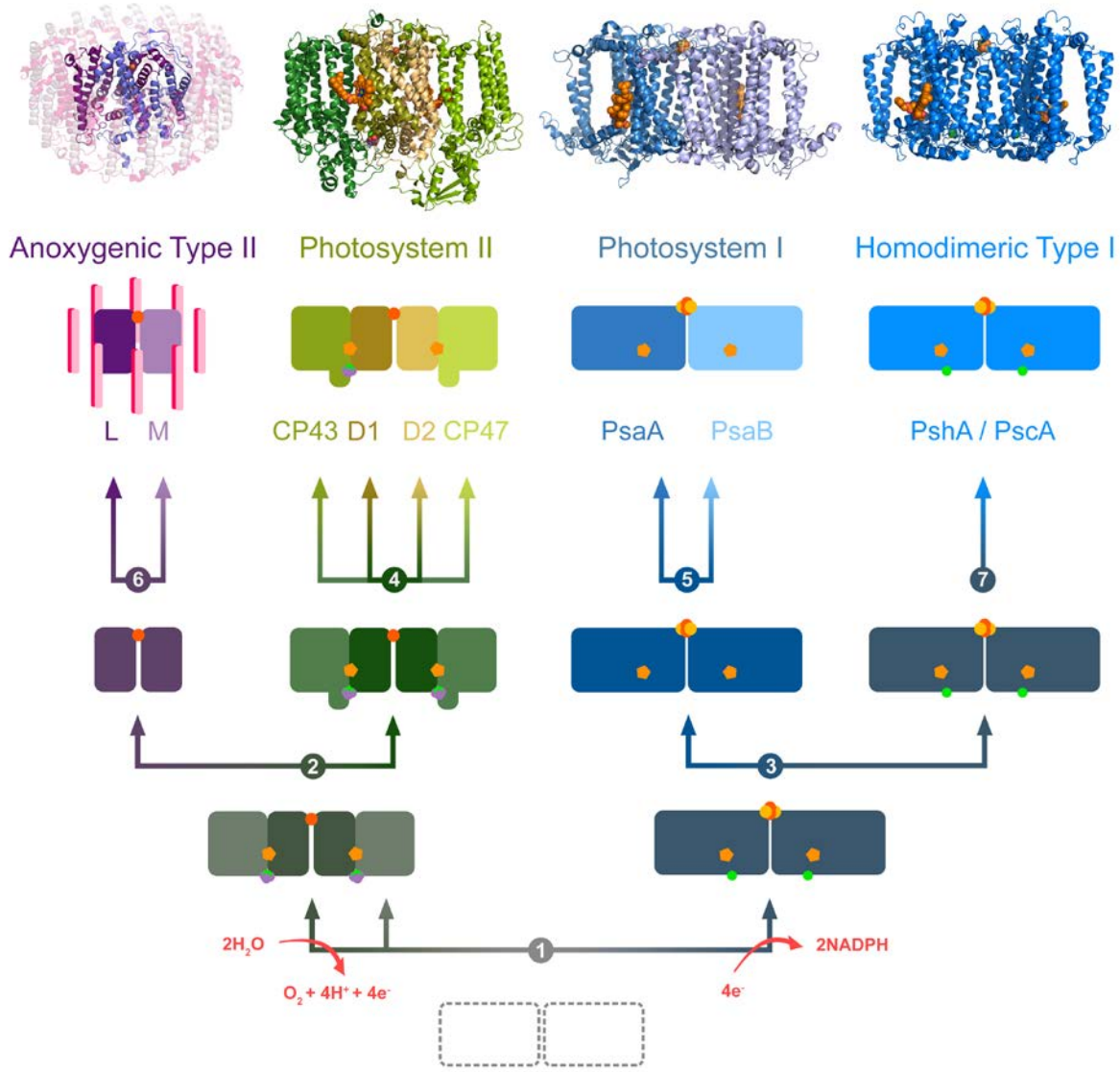

Figure I (Box 2): Evolutionary overview of structural changes leading to Type II and Type I reaction centre proteins.

Box 1. Do all homodimeric Type I reaction centres have a Ca-binding site?

Sequence alignments of PscA and PshA indicate that the Type I reaction centre of phototrophic Chlorobi and Acidobacteria could retain a Ca-binding site like that found in *Heliobacterium modesticaldum*. For example, the predicted Ca-binding site in the homodimeric Type I reaction centre of Chlorobi would be coordinated by the C-terminal A731 and by the backbone carbonyl of L729. Residue Y513 in the reaction centre of *H. modesticaldum* is conserved in all Chlorobi as Y599 (numbering from PscA of *Chlorobium limicola*). Residue D468 in *H. modesticaldum* is also

conserved as D563 in Chlorobi. Q517 is not conserved in Chlorobi, instead a glutamate residue is found in this position, which can also act as a ligand. The loop region between the 5th and 6th transmembrane helices in the PscA antenna domain is larger than that found in PshA, but smaller than in CP43 and CP47. Due to big structural differences in this extrinsic loop, a residue equivalent to N263 cannot be identified in the Chlorobi sequence, instead we suggest that in Chlorobi this region will bind the Ca via a strictly conserved glutamate at position 323 (or alternatively at position 345). Crosslinking experiments in the reaction centre of *Chlorobaculum tepidum* showed that K315 and K338, located in the extrinsic domain between the 5th and 6th transmembrane helices of the antenna domain, bound the heme-containing region of PscC, the immediate electron donor to P [53]. This is consistent with the antenna domain interacting with the electron donor site of the reaction centre as it is the case in the reaction centre of *H. modesticaldum* and PSII. It indicates that the Mn_4CaO_5 cluster evolved at the ancestral entry point of electrons into the photochemical core. Therefore, the position of PufC (the tetraheme cytochrome of anoxygenic Type II reaction centres, (see Figure 1) and its evolution as an electron donor, represent a novel adaptation rather than the primitive ancestral state of Type II reaction centres.

Box 2. Evolution of reaction centre proteins

All reaction centre proteins share common ancestry. L, M, D1, and D2 make a monophyletic clade and together they are the Type II reaction centre proteins. This is also true for PsaA, PsaB, PshA and PscA, which together also make a distinct monophyletic clade, the Type I reaction centre proteins. Therefore, one of the earliest stages in the evolution of reaction centre proteins that can be inferred from sequence and structural analyses is the functional and structural changes leading to Type II and Type I reaction centre proteins, marked **1** in Figure I. However, Photosystem II share many more traits with Type I reaction centres than the anoxygenic Type II. These include: 1) the antenna CP43 and CP47; 2) the peripheral core chlorophylls $ChlZ_{D1}$ and

$ChlZ_{D2}$ (represented as orange pentagons) and sequence identity in this region; 3) the use of chlorophyll *a* as a photochemical pigment; 4) the lack of histidine ligands to the "monomeric" chlorophylls (see Fig. 4); 5) a connection between the antenna and the electron donor side conserved in PshA as a calcium-binding site (represented as green spheres). Because of these conserved traits, the transition between PSII and the anoxygenic Type II reaction centres (**2**) is better explained by a loss of these in the anoxygenic system coupled to the gain of a novel light harvesting complex (see also [1, 8]). We propose here, in line with the arguments presented in [9], that the origin of water oxidation occurred at stage **1**. Given that each reaction centre type makes a monophyletic clade, it can be deduced that stage **1** predates the diversification events leading to the phylum-specific versions of all photosynthetic reaction centres. These events needed additional evolutionary transition to occur: marked **4** to **7**. For example, the duplications leading to D1 and D2 predates the most recent common ancestor of Cyanobacteria. Or for example, the duplication leading to L and M predates the radiation events leading to the ancestor of phototrophic members of the phyla Proteobacteria and Chloroflexi, because these two inherited distinct and well differentiated L and M subunits.

Glossary

Charge separation: Light-driven charge separation is the process of an electron in chlorophyll being excited to a higher energy level by the absorption of a photon. It is then transferred from the 1st excited state orbital to a nearby electron acceptor thereby forming a radical cation and radical anion, the primary radical pair.

Mn_4CaO_5 cluster: the water-oxidising complex of Photosystem II. This is the catalytic site of Photosystem II where water oxidation occurs. It consists of 4 atoms of Mn, 1 Ca, and 5 bridging oxygens, arranged in a distorted-chair configuration.

Most recent common ancestor: also last common ancestor. The most recent common ancestor of any group of organisms is the most recent individual from which all the organisms in that group are directly descended. This concept can also be applied to gene families or proteins. The most recent common ancestor of Cyanobacteria capable of oxygenic photosynthesis is the immediate ancestor of the genus *Gloeobacter*, the earliest branch in the tree, and of all other known species. The most recent common ancestor of Photosystem II and Photosystem I is the ancestral reaction centre existing prior to the specialisation of Type I and Type II reaction centres.

Photochemical reaction centres: nature's solar cells. These are protein complexes that convert the energy of light directly into chemical energy, through the movement of electrons via a series of redox cofactors, resulting in redox reactions, chemical bond formation, electric field formation and proton movements. The reaction centres can be of two types, referred to as Type I (ferredoxin-reducing) and Type II (quinone-reducing) reaction centres. In Cyanobacteria and photosynthetic eukaryotes these are referred to as Photosystem I and Photosystem II respectively.