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Oxygenic photosynthesis: history, status and perspective

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Review

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Abstract

Cyanobacteria and plants carry out oxygenic photosynthesis. They use water to generate the atmospheric oxygen we breathe and carbon dioxide to produce the biomass serving as food, feed, fibre and fuel. This paper scans the emergence of structural and mechanistic understanding of oxygen evolution over the past 50 years. It reviews speculative concepts and the stepped insight provided by novel experimental and theoretical techniques. Driven by sunlight photosystem II oxidizes the catalyst of water oxidation, a hetero-metallic $Mn_4CaO_5(H_2O)_4$ cluster. Mn_3Ca are arranged in cubanoid and one Mn dangles out. By accumulation of four oxidizing equivalents before initiating dioxygen formation it matches the four-electron chemistry from water to dioxygen to the one-electron chemistry of the photo-sensitizer. Potentially harmful intermediates are thereby occluded in space and time. Kinetic signatures of the catalytic cluster and its partners in the photo-reaction centre have been resolved, in the frequency domain ranging from acoustic waves via infra-red to X-ray radiation, and in the time domain from nano- to milli-seconds. X-ray structures to a resolution of 1.9 Å are available. Even time resolved X-ray structures have been obtained by clocking the reaction cycle by flashes of light and diffraction with femtosecond X-ray pulses. The terminal reaction cascade from two molecules of water to dioxygen involves the transfer of four electrons, two protons, one dioxygen and one water. A rigorous mechanistic analysis is challenging because of the kinetic enslaving at millisecond duration of six partial reactions ($4e^-$, $1H^+$, $1O_2$). For the time being a peroxide-intermediate in the reaction cascade to dioxygen has been in focus, both experimentally and by quantum chemistry. *Homo sapiens* has relied on burning the products of oxygenic photosynthesis, recent and fossil. Mankind's total energy consumption amounts to almost one-fourth of the global photosynthetic productivity. If the average power consumption equalled one of those nations with the highest consumption per capita it was four times greater and matched the total productivity. It is obvious that biomass should be harvested for food, feed, fibre and platform chemicals rather than for fuel.

Introduction

Cyanobacteria, algae and plants carry out oxygenic photosynthesis. They produce oxygen (O_2) from water and take up carbon dioxide (CO_2) to yield biomass. Cell respiration reverses this process. Oxygenic photosynthesis has formed the oxygen we breathe and most of the biomass we use as food, feed, fibre and fuel. Solar driven oxygen evolution started very early in evolution. A dramatic rise of the atmospheric oxygen level (by more than four orders of magnitude), the Great Oxygenation Event (GOE), dates 2.4 billion years back from now (Bekker *et al.*, 2004; Kump, 2008). Both geological (Planavsky *et al.*, 2014) and genomic evidence (Cardona *et al.*, 2015) have suggested that ancestors of cyanobacteria might have started oxygenic photosynthesis half a billion years before the GOE. The stabilization of land masses and the emergence of land plants about half a billion years back from now caused another rise of the atmospheric oxygen content. Photosynthetic produced biomass and oxygen have powered the vast emergence of animal life. The time window of *Homo sapiens* is just a blip on these time scales. Its impact on the consumption of photosynthesis products is tremendous, as will be discussed at the end of this paper.

Several authors have comprehensively reviewed the respective momentary status of knowledge on the mechanism of oxygenic photosynthesis. They are cited in the text. The present paper scans how our knowledge on this fundamental process has unfolded over the past 50 years. Freshmen may start by reading the excellent book on photosynthesis by Bob Blankenship (Blankenship, 2014).

Figure 1 illustrates the present concept of the key elements of the primary processes of photosynthesis in green plants (photosystem II (PSII), PSI, $cytb_6f$ and the adenosine triphosphate (ATP) synthase). It is meant as a scaffold for the reader when travelling through the originally foggy, and then increasingly detailed and complicated terrain that follows.

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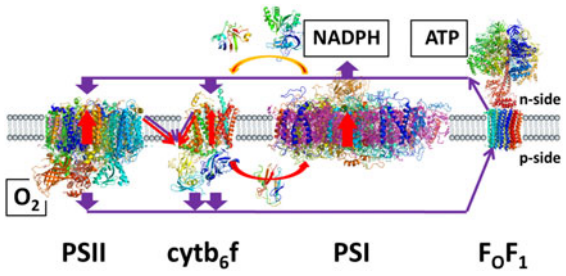


Fig. 1. Key proteins of the primary processes of photosynthesis in green plants (PSII, PSI, cytb$_6$f and the ATP synthase), electron transfer (red), proton transfer (purple) and products (O_2, NADPH and ATP) (Junge and Nelson, 2015).

Pioneering studies

Photosynthetic oxygen production from water

In 1772 Joseph Priestley reported a first systematic study on gas turnover between plants and animals (Priestley, 1772). A mouse confined in a sealed vessel suffocated if not backed up by a plant. It has meant that the mouse produces 'bad air' and the plant restores 'vital air'. In modern terms, plants take up carbon dioxide (CO_2) and produce oxygen (O_2), while animals inhale O_2 and exhale CO_2. Hundred years after Priestley Theodor Engelmann (Engelmann, 1881) introduced a less harmful bioassay for oxygen, namely the flocking behaviour of oxygen-loving, motile bacteria around illuminated chloroplasts. It yielded the first action spectrum of oxygenic photosynthesis (with typical chlorophyll peaks). Half a century later the great biochemist Otto Warburg (Nobel Prize 1931) studied by manometry the turnover of O_2 and CO_2 in respiring cells and tissues (Warburg, 1922). Robert Emerson and William Arnold used his technique to detect O_2-production by illuminated algae (Emerson and Arnold, 1932). They pioneered excitation of photosynthesis by short flashes of light (duration some 10 µs). Varying both the repetition rate of flashes and their energy they obtained two seminal results (Emerson and Arnold, 1932). (i) The rate of oxygen evolution is limited by a temperature-sensitive (dark) step of some 10 ms duration, and (ii) the maximum production per flash is 1 mol O_2 per 2500 mol chlorophyll. The relevance of these findings could only later be fully appreciated (see below).

The minimal balanced equation of oxygenic photosynthesis is $2H_2O + CO_2 = O_2 + (CH_2O)$. The substrate for oxygen production, water or carbon dioxide, was under debate. Robin Hill studied photosynthetic oxygen production in freshly isolated chloroplasts by another bio-assay, the spectral shift of added oxy-/deoxy-haemoglobin. Upon ageing of chloroplasts both, oxygen production and carbon dioxide consumption faded out. The addition of ferric ions as oxidant restored oxygen production but not carbon dioxide uptake. Hill took the decoupling of the former from the latter as evidence that plants 'do not evolve oxygen from carbon dioxide' (Hill, 1939). Using heavy oxygen as a tracer Martin Kamen and collaborators found 'the $^{18}O/^{16}O$-ratio of the evolved oxygen identical with that of the water'. The obvious conclusion has been that O_2 originates solely from water (Ruben *et al.*, 1941) (for a modern remake see Clausen *et al.* (2005*a*)). Otto Warburg had meanwhile perfected the manometric monitoring the exchange of CO_2 and O_2 in photosynthesis and respiration (Burk *et al.*, 1951). Having observed a stimulating role of bicarbonate on photosynthetic oxygen production he claimed that oxygen results from bicarbonate and not from water (Warburg *et al.*, 1965). The origin of the stimulating effect of CO_2 on PSII that drove Warburg to this erroneous view, has only been settled much later (reviewed by Govindjee and his colleagues (Shevela *et al.*, 2012)) and the regulatory function of CO_2 was detailed (Koroidov *et al.*, 2014; Brinkert *et al.*, 2016).

Electron transport, oxygen production, proton transport and ATP synthesis

Lou Duysens (Duysens *et al.*, 1956), Bessel Kok (Kok, 1956) and Horst Witt (Witt and Moraw, 1959) started to characterize a wealth of spectroscopic signatures of photosynthetic electron transfer. Excitation with short flashes allowed resolving partial reactions in time (Kok, 1956; Witt *et al.*, 1961*a*). Two PSs operate in series (Duysens *et al.*, 1961; Witt *et al.*, 1961*b*). PSII produces oxygen and delivers electrons to PSI, which in turn provides electrons for the reduction of CO_2 (see Fig. 1). The rate limiting step of the whole electron transport chain is located between the two PSs. Its duration (about 10 ms) is compatible with the one found for oxygen production by Emerson and Arnold (Emerson and Arnold, 1932). The sensitizers of both PSs are chlorophyll-a constructs, which have been coined after the wavelength of their respective red absorption peak. P700 (Kok and Gott, 1960) drives PSI and P680 (Döring *et al.*, 1967) drives PSII, respectively. These biophysical data were then not readily appreciated by some biochemists. When Warburg was confronted with Witt's detailed reaction scheme in 1962, he mused: 'Could you tell us how the chemical mechanism of photosynthesis can be described on the basis of your spectroscopic observations?' Witt countered his eminent critic, the pioneer of oxygen detection, by observing that 'it would be difficult to deduce the mechanism of a combustion engine based only on sniffing the exhaust' (see Junge and Rutherford (2007)). From their then limited perspective both were of course right (see below).

At that time it had been accepted that the chlorophylls and therewith the primary photosynthetic activity is embedded in the tightly folded inner membranes of chloroplasts, forming thylakoids (Menke, 1962). Peter Mitchell (Nobel Prize 1978) hypothesized a new role of this inner membrane system of chloroplasts and likewise the cristae membrane of mitochondria (Mitchell, 1961, 1966). He envisaged that the membrane is proton tight such that zigzagging electron/hydrogen transport generates a pH-difference (Mitchell, 1961), and transmembrane voltage (Mitchell, 1966), in sum coined as protonmotive force. The backflow of protons is confined to the ATP synthase where it drives ATP-synthesis. Essentials of Mitchell's hypothesis were soon experimentally established for the thylakoid membrane. André Jagendorf subjected broken chloroplasts to an acid/base-jump (pH-jump) and obtained ATP (Jagendorf and Uribe, 1966). The author with Horst Witt discovered the electrochromic origin of certain absorption transients of intrinsic chloroplast pigments (Junge and Witt, 1968). Upon excitation with flashing light they observed very rapid voltage generation by both PSs and slower proton pumping (Schliephake *et al.*, 1968). Subsequent studies of the author with Bernd Rumberg have revealed that the flash generated voltage decays in synchrony with ATP synthesis (Junge, 1970; Junge *et al.*, 1970). Titration of the voltage decay with the extremely powerful ionophore gramicidin revealed that the electrified membrane contains at least 10^5 chlorophyll molecules (Junge and Witt, 1968), i.e. more than 100 electron transport chains. This number was later raised up to more than 10^7 chlorophyll molecules (Schönknecht *et al.*, 1990). In other words, the intricately folded thylakoid membrane within a chloroplast forms one contiguous, i.e. simply connected sheet

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(Junge, 1977; Schönknecht *et al.*, 1990). It implies that almost all copies of the electron transport chain contribute driving force to all copies of ATP synthase. The lateral separation of PSII and PSI (and ATP synthase) in different areas of the folded thylakoid membrane (Boardman and Anderson, 1964) apparently does not impair their ability for concerted electron and proton transport. Losses of the chemical portion of protonmotive force between laterally separated proton pumps and ATP synthase were only later discovered. They are minor both in mitochondria (Rieger *et al.*, 2014) and in chloroplasts (Sjoholm *et al.*, 2017). Top efficiency of ATP synthesis has apparently been sacrificed for the sake of tight packing of the coupling membrane, both in chloroplasts and mitochondria.

Photosystem II with the oxygen evolving complex (BC)

Four-electron chemistry of water oxidation linked to one-electron photo-physics

In 1968 Anne and Pierre Joliot have lifted studies of photosynthetic oxygen evolution to a kinetic level by introducing a rapidly responding oxygen electrode (Joliot and Joliot, 1968). When they excited dark adapted Chlorella cells with a series of short light flashes oxygen production peaked at flash number 3 and continued with damped oscillations of period four (Joliot *et al.*, 1969). The release of oxygen occurs in 1 ms, i.e. a 10 times shorter time-interval than the overall bottleneck of the full electron transport chain (Emerson and Arnold, 1932). In 1970 Bessel Kok concluded that PSII contains an accumulator of four positive 'charges' that links the one-electron-progression of the photo-physical reaction centre to the four electron/proton-abstraction from two molecules of water to yield dioxygen (Kok *et al.*, 1970). From there on, the catalytic centre has been conceived as stepwise progressing in a cycle over five oxidation states, S_0 to S_4. With S_1 being the most stable state in dark adapted material, oxygen is only produced after three steps of the cycle during the transition $S_4 \rightarrow S_0$. Two most important features of this four-stepped mechanism were soon recognized (see Renger (1977)): (a) the levelling of the energy demand of the four successive one-electron abstractions from water to match the fixed energy input provided by red quanta of light, and (b) the control (by sequestration and/or short live-time) of potentially harmful intermediates on the way from water to dioxygen ('cryptoradicals'; Renger, 1987). The cleavage of water to produce dioxygen was expected to liberate protons into the lumen of thylakoids. The stoichiometric H^+/e^--pattern over the four-stepped progression, $S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_4 + O_2$ was 1:0:1:2 (Förster *et al.*, 1981; Förster and Junge, 1985). For some time it was controversial between various labs (reviewed in Lavergne and Junge, 1993). Different patterns were observed in partially fragmented PSII-preparations. The seeming ambiguity has later been settled in favour of a proton release pattern of 1:0:1:2. It has required the discrimination by kinetic markers between transient electrostatic proton release/uptake at the membrane periphery (membrane Bohr effect) and chemical proton production from the catalytic core of PSII (Haumann and Junge, 1994) (see review in Junge *et al.* (2002)). It has been early noted that the release of two protons during the terminal, oxygen evolving reaction is kinetically biphasic with one proton appearing at about 100 µs and the other one at 1 ms half rise (Förster *et al.*, 1981; Förster and Junge, 1985). The release of a first proton appears as a primer for the terminal reaction cascade involving four electron transfers (see Fig. 3 farther down).

George Cheniae established the necessity of manganese for oxygen evolution (Cheniae and Martin, 1967, 1970). Ken Sauer's lab in Berkeley has focused on characterizing its function. Starting with electron paramagnetic resonance (EPR) (Blankenship and Sauer, 1974), they turned with Mel Klein into X-ray spectroscopy (Kirby *et al.*, 1981*a*, 1981*b*) and, till today, into advanced X-ray diffraction (see below). When exciting dark adapted chloroplasts with repetitive flashes of light they detected by EPR that some manganese was released into solution with period of four. It suggested that manganese associated with PSII undergoes valence-changes with a period of four (Wydrzynski and Sauer, 1980). From then on it was accepted that manganese is involved in Kok's 'charge' accumulator (reviewed in Sauer (1980)). When generating Kok's state S_2 by excitation of dark adapted material with a single short flash of light Charles Dismukes and Yona Siderer observed a multiline EPR-signal which they attributed to a mixed-valence pair of manganese, Mn(III)Mn(IV), in a dimeric or tetrameric Mn-cluster (Dismukes and Siderer, 1981). For quite a while this attribution has been the stronghold for the valence of the manganese cluster during the four transitions from S_0 to S_4.

Enrichment and purification of PSII: P680, the Mn-cluster and tyrosine (Y_z)

Further progress required enrichment if not purification of PSII. Per Ake Albertson's lab pioneered the isolation of PSII-enriched membrane vesicles from spinach (Akerlund *et al.*, 1976; Andersson *et al.*, 1977). A number of consecutive studies demonstrated that the enrichment was facilitated by the intrinsic segregation between PSII in the appressed portions and PSI in the extended portions of stacked thylakoid membranes (Andersson and Anderson, 1980, 1988).

Purification of a manganese containing, oxygen evolving PSII was desirable. A first claim (Spector and Winget, 1979) was not generally reproducible and probably fraudulent. A great step forward were membrane fragments from spinach chloroplasts that revealed high rates of oxygen evolution and were virtually uncontaminated by PSI (Berthold *et al.*, 1981). They were coined BBY-particles after their parents, Berthold, Babcock and Yocum. The absolute content of PSII was quantified *via* the previously found, and chemically still unidentified EPR-signal II (Babcock and Sauer, 1973), then coined Z, supposedly an electron donor to $P680^+$ (Babcock and Sauer, 1975). In 1984 a fully competent PSII-core particle from spinach was isolated by Demetrios Ghanotakis with Gerald Babcock and Charles Yocum (Ghanotakis *et al.*, 1984*b*). It contained four Mn-ions and one Z per 250 chlorophyll molecules. In addition to the hetero-dimeric core proteins of PSII, D1 and D2, and further proteins with antennae function (CP43, CP47) the complex contained peripheral proteins of 17, 23 and 33 kDa molecular mass. The removal of the 17 and 23 kDa proteins facilitated the access of external reductants to Z. It also impaired oxygen production which was however reconstituted by added Ca (Ghanotakis *et al.*, 1984*a*, 1985*a*, 1985*b*).

At this time (i.e. before crystallization, BC) structural information on the mutual arrangement of the four Mn-ions, and a role of Ca in relation to the Mn-cluster was still lacking (for reviews see Yocum, 1991; Debus, 1992).

Kinetics of electron transfer between $P680^+$, Y_z and the Mn-cluster

P680 is photo-oxidized in less than 300 ps (Schatz *et al.*, 1987, 1988). In PSII-preparations with small size of the antennae system

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the time of photon capture is shorter than in those with large antennae complement. In other words, PSII hosts an energetically 'shallow trap' where many transfers of excitation between the trap and its antennae complement precede the eventual charge separation (Schatz *et al.*, 1988; Barter *et al.*, 2001). $P680^+$ is then reduced in nanoseconds. For lack of time resolution in the earlier work the reduction of $P680^+$ was first detected in functionally impaired PSII where it was much delayed compared to normal (Döring *et al.*, 1967). In likewise impaired material Gerald Babcock's group found that the slow reduction of $P680^+$ kinetically matched the oxidation of the EPR-visible radical intermediate, Z (Boska *et al.*, 1983). In fully competent, oxygen evolving PSII, on the other hand, $P680^+$ is reduced in nanoseconds as later found in Horst Witt's lab. The rate of $P680^+$-reduction depends on the state of Kok's 'charge accumulator' before photon-absorption. The half-rise time ranges around 20–40 ns when starting from S_0 and S_1 and rises to about 300 ns when starting from S_2 and S_3 (Brettel *et al.*, 1984; Schlodder *et al.*, 1984; Meyer *et al.*, 1989). According to its UV-difference spectrum the electron donor to $P680^+$ was identified as a tyrosine, and the rate of its oxidation in nanoseconds matched the one for the reduction of $P680^+$ (Gerken *et al.*, 1988). By site-directed mutagenesis the electron donor was eventually identified as tyrosine-161 on the D1-polypeptide of PSII (Debus *et al.*, 1988*a*, 1988*b*). It has been coined Y_z since then. While Y_z is oxidized by $P680^+$ in nanoseconds, it is reduced by the Mn-cluster in micro- to milliseconds. The half-rise time of Y_z^+-reduction and Mn-oxidation depends on the state S_i before photon absorption, namely 30, 110, 350 and 1300 µs when starting from S_0, S_1, S_2 and S_3, respectively (Dekker *et al.*, 1984).

Around 1990 the three players of oxygen evolution, namely P680, Y_z and a $Mn_4(Ca)O_x$-entity, had been identified, and the kinetic constants of electron transfer between them quantified. The challenge was to elucidate the mutual arrangement of $P680/Y_z/Mn_4Ca$, the role of Ca, and, of course, how the dioxygen bond is formed.

Tentative structural models of the catalyst

The X-ray crystal structure of the reaction centre of purple bacteria (BRC), the first of a membrane protein ever, was published in 1985 (Deisenhofer *et al.*, 1985) (Nobel Prize to J. Deisenhofer, R. Huber and H. Michel in 1988). It was soon evident that its subunits L and M share sequence similarity with D1 and D2 of PSII (see Nitschke and Rutherford, 1991 for review). For quite a while the bacterial reaction centre served as scaffold for discussing the structure of PSII of oxygenic photosynthesis. Without any structural model of PSII proper, several labs tried to distil structural information from spectroscopic data.

Gary Brudvig's group compared the EPR-signals, attributable to state S_2 of the oxygen evolving centre, with EPR-signals of certain Fe_4S_4 proteins of known structure. Because of the similarity of their ferro- and antiferromagnetic exchange couplings they tentatively conceived a tetrameric Mn_4O_4-cluster in a cubane-like arrangement as one possibility for PSII (Depaula *et al.*, 1986). Based thereupon they hypothesized a comprehensive reaction scheme (Brudvig and Crabtree, 1986). A 'naked' Mn_4O_4-cluster (the protein was not considered!) was proposed to cycle over the five oxidation states S_0 to S_4, evolve oxygen during the transition $S_4 \rightarrow S_0$, release protons with the known stoichiometric pattern of 1:0:1:2, accommodate two water molecules to yield an Mn_4O_6-cluster in an adamantane-like conformation, and eventually to form the dioxygen bond (see Fig. 1 in Brudvig and Crabtree (1986)). Among three possible sets for the oxidation states of the four Mn ions the authors considered a very high-valence (HV) state, namely $Mn(IV)_3Mn(V)$ in S_4, as probable (Brudvig and Crabtree, 1986).

Mel Klein started investigations of the state of manganese in photosynthesis by X-ray absorption spectroscopy (XAS) (Kirby *et al.*, 1981*a*, 1981*b*), Ken Sauer's group joined this field (Goodin *et al.*, 1984), and they went on together in Berkeley. Using synchrotron radiation that was tuned to the K-edge of manganese and calcium ions they studied the X-ray absorption near edge structure (XANES), and the extended X-ray absorption fine structure (EXAFS). The former gives information on valence states, the latter yields the distance between the primarily excited ion and its neighbours. A set of Mn–Mn distances was determined (with very high (0.1 Å) precision) for the S_1- and S_2-states (Yachandra *et al.*, 1987*a*, 1987*b*). The most prominent Mn–Mn distances were two times 2.7 and one time 3.3 Å. Their K-edge showed shifts that were indicative of Mn-oxidation during $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$, and much less so during the transition $S_2 \rightarrow S_3$ (Roelofs *et al.*, 1996). Ligand centred as opposed to Mn-centred oxidation was considered as a possibility (Messinger *et al.*, 2001). The absolute valence of Mn_4 could then not be assigned unequivocally, except for S_2 with probable configuration of (III, IV, IV, IV) (Roelofs *et al.*, 1996; Iuzzolino *et al.*, 1998; Schiller *et al.*, 1998).

The results of the Berkeley group were presented in a series of reviews (Yachandra *et al.*, 1993, 1996). The Mn–Mn distance of 2.7 Å was attributed to di-µ-oxo-linked Mn–Mn and of 3.3 Å to mono-µ-oxo-linked Mn–Mn, respectively, by comparison with data on Mn-model complexes of known structure (e.g. Christou, 1989; Wieghardt, 1989). The Berkeley group proposed a Mn_4-model of two di-µ-oxo-linked Mn-dimers that are linked by one mono-µ-oxo bridge (the dimer of dimers model) (Yachandra *et al.*, 1993, 1996). However, a number of other structures for the four oxygen-linked Mn-ions were also compatible with the same set of Mn–Mn distances. In an attempt to resolve the structural ambiguity Holger Dau studied linear dichroism of X-ray absorption in oriented membrane fragments (Schiller *et al.*, 1998) (for further dichroism studies see Yano *et al.* (2006)). The additional information did not lead to an unequivocal structural model. The high precision of the Mn–Mn distances as determined by EXAFS (<0.1 Å) has, however, served to gauge the correctness of structural models by X-ray diffraction and computational chemistry.

The proximity of Ca to the Mn-ions was eventually established (Latimer *et al.*, 1995), and the distance between strontium (the only functional substitute for calcium) and Mn was determined by EXAFS (Cinco *et al.*, 1998). Sr neighbours two Mn-ions at the same distance, 3.5 Å. From there on the catalytic centre of oxygen production has been conceived as a hetero-nuclear Mn_4Ca-cluster. It has been coined 'oxygen evolving complex', in short the OEC.

Various approaches to elucidate the structure and valence of the Mn_4-cluster based on the multiline CW-EPR signal of state S_2 had produced largely different models (e.g. Ahrling and Pace, 1995; Zheng and Dismukes, 1996) because of a highly under-determined data set. Jeffrey Peloquin and David Britt increased the number of observable parameters by pulsed EPR/ENDOR (Peloquin and Britt, 2001). A trinuclear Mn_3 with one appended Mn (coined 'dangler') was better compatible with their data than the Mn_4-cubanoid that was previously suggested. They noted that 'each manganese has a unique coordination environment' and may serve 'a specific mechanistic purpose'. Based

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on their EXAFS studies on the S_0-state the Berkeley group considered a cluster with three di-μ-oxo-bridged Mn plus one outlier Mn as a possibility (Robblee *et al.*, 2002).

Tentative mechanistic models for catalysis

Several authors have speculated about the chemical mechanism of water oxidation. Most of them focused on the metal ions with one exception. Gerald Babcock emphasized an active role of Tyr^{161} of D1 ('Y_z'), namely as hydrogen acceptor for bound 'water' (Hoganson and Babcock, 1997; Tommos and Babcock, 1998). Following Lev Krishtalik (Krishtalik, 1989, 1990) they argued that a neutral tyrosyl radical abstracts hydrogen from bound water (or OH) because concerted transfer of H required less reorganization energy than the sequential transfer of an electron and proton. Babcock presented a detailed reaction scheme that was based on Brudvig's concept of a Mn_4-cubanoid. The question was whether or not tyrosine, when oxidized by $P680^+$, was a neutral radical. Michael Haumann in the author's group investigated electrostatic relaxation *versus* chemical production of protons (Haumann and Junge, 1994) plus local electrochromism (Haumann *et al.*, 1997*b*) in the reaction cascade between P680, Y_z and the Mn_4Ca-cluster. In intact PSII the oxidation of Y_z revealed strong local electrochromism and no proton release into the bulk, as if the oxidation of Y_z left a positive charge on tyrosine or closely nearby. It has been proposed that the tyrosine-proton is shifted to a neighbouring base, B, to which Y_z is hydrogen-bonded (Ahlbrink *et al.*, 1998; Junge *et al.*, 2002). B was then identified as $D1\text{-}His^{190}$ (Hays *et al.*, 1998). The sequestration of the tyrosine-proton in its vicinity holds only in intact PSII, it does not hold if smaller subunits of PSII or Ca are removed (see (Ahlbrink *et al.*, 1998; Junge *et al.*, 2002). The priming reaction for water oxidation is then conceived as follows: $P680^+Y_zHis \rightarrow P680Y_z\&HisH^+$.

Taken together it has implied that oxidized Y_z can act as an electron but not as a hydrogen acceptor to the catalytic metal cluster.

Vincent Pecoraro and his coworkers had studied the reactivity of synthetic manganese complexes. Based thereupon they proposed a mechanistic model for photosynthetic water oxidation (Pecoraro *et al.*, 1998). 'An essential feature of the model is the nucleophilic attack by calcium-ligated hydroxide on an electrophilic oxo group ligated to high-valent manganese to achieve the critical 0–0 bond formation step.'

The involvement of Mn(V) = oxo had been previously discussed by Johannes Messinger based on studies on slow water exchange (Messinger *et al.*, 1995). The proposed pivotal role of Mn(V) = oxo for the catalytic mechanism has been taken up by others, and it is highly debated till today (see below).

By density functional theory (DFT) Per Siegbahn and Robert Crabtree scrutinized possible mechanisms for the formation of an O–O bond (Siegbahn and Crabtree, 1999) in line with Babcock's and Pecoraro's proposals. By calculations of energy surfaces they found that hydrogen abstraction from Mn–OH by a neutral tyrosine radical probably could not *directly* produce a reactive, manganese bound oxyl. As a way out they proposed an indirect pathway involving Ca that is chelated to the manganese.

Crystal structures of PSII with the Mn_4CaO_5-cluster

Starting from his PhD-work in Göttingen (right after World War II) the mechanism of water oxidation to yield dioxygen has remained Horst Witt's career-long preoccupation, his '*spröde Geliebte*' (prudish beloved, see Junge and Rutherford (2007)). But it was only after retirement that he focused on crystallization of PSII, using a PSII complex from the thermophilic cyanobacterium *Synechococcus* sp. (Rögner *et al.*, 1987). Witt joined forces with the crystallographer Wolfram Saenger, and, on their way to the structure of PSII, the Berlin group arrived first at crystals and a structural model of PSI 'of water splitting photosynthesis' (Witt *et al.*, 1988). (Three years later it was improved to a resolution of 2.5 Å (Jordan *et al.*, 2001).) Thereafter Athina Zouni characterized PSII-crystals capable of water oxidation (Zouni *et al.*, 2000). The long desired first structural model of PSII was presented (Zouni *et al.*, 2001). At a resolution of 3.8 Å it revealed a dimeric structure of PSII, the relative positions of its large subunits, the transmembrane helices, the position and orientation of cofactors (chlorophylls, Y_z, quinones, cytochromes), and the position and shape of Mn_4Ca as a whole, however without detail on the mutual arrangement of the metal ions.

Jian-Ren Shen and Nobuto Kamiya followed very closely. They characterized oxygen evolving crystals of PSII (Shen and Kamiya, 2000) and determined their structure at 3.7 Å resolution (Kamiya and Shen, 2003). The results were much alike those of the Berlin group with new features on ligands to the metal-cluster, and certain carotenoids. The inner structure of the metal-cluster was again presented as pear-shaped and without detail.

James Barber in London has not less been determined than the former two groups to solve the crystal structure of PSII (see Andersson (2005)). In 1998, in close collaboration with Werner Kühlbrandt and by electron crystallography of 2D crystals they derived a structural model of a PSII-subcomplex that was capable of photochemical electron transport but not of water oxidation. The location of four subunits and a total of 23 transmembrane helices was resolved at 8 Å (Rhee *et al.*, 1998). The structural similarity between the reaction centre of purple bacteria, PSI and PSII has been evident (see their Fig. 5). James Barber then turned to X-ray crystallography in collaboration with So Iwata, and they obtained a fully refined structural model of PSII at 3.5 Å resolution (Ferreira *et al.*, 2004). Over 5000 side chains of the heterodimeric protein complex were assigned.

Most important has been their model for the metal-cluster (see Fig. 2). In conceiving this far sighted structural model the authors relied on their own diffraction data, including the anomalous diffraction aiming at Mn (X-ray wavelength 1.89 Å) and Ca (2.25 Å), the Mn–Mn and Mn–Ca distances from EXAFS-studies of the Berkeley group (Yachandra *et al.*, 1987*b*), knowledge on μ-oxo-linked Mn-model-clusters as previously discussed (Brudvig and Crabtree, 1989), and the proposed Mn_3 + Mn(dangler)-structure (Peloquin and Britt, 2001).

The Barber-model of the metal cluster had three Mn plus one Ca forming a cubanoid and the fourth Mn dangling out. The ions were bridged by four oxygen atoms and ligated by four water molecules (though not clearly resolved). A bicarbonate was postulated to bridge the Ca-ion and the dangling Mn. Four protein residues ligated the Mn_4CaO_4-cluster, with $Y_z = D1\text{-}Tyr^{161}$ and its hydrogen-bonded partner, $D1\text{-}His^{190}$, in close vicinity to the former. In essence, this model has set the path until today. One year later the Berlin group presented a model at slightly higher resolution of 3 Å, where all carboxylate residues were now bi-dentate bridging. Even the one that was terminally bound to one manganese in Barber's model was now bridging two metals (Loll *et al.*, 2005). Again the resolution was not sufficient to resolve bridging oxygen atoms and water.

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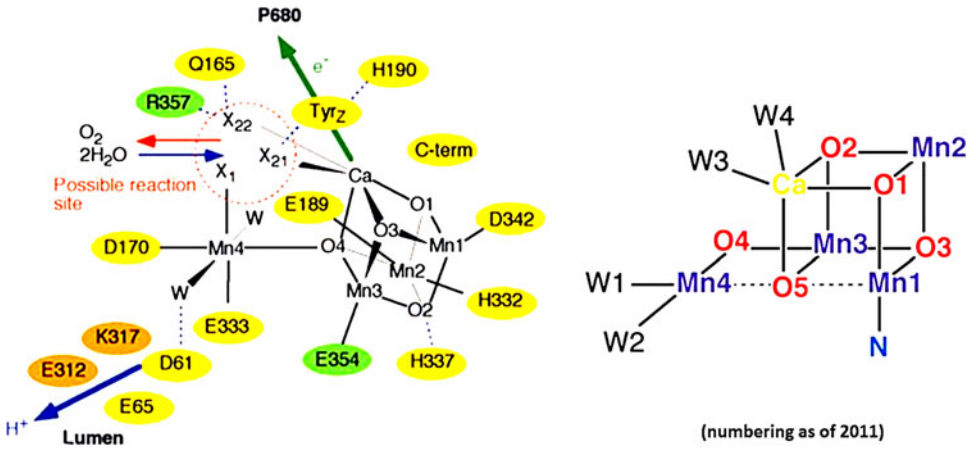


Fig. 2. Left: The first structural model of the catalytic centre (Ferreira *et al.*, 2004). Right: Schematic representation of the $Mn_4CaO_5(H_2O)_4$-complex with the numbering as used after the first model at high-resolution (Umena *et al.*, 2011).

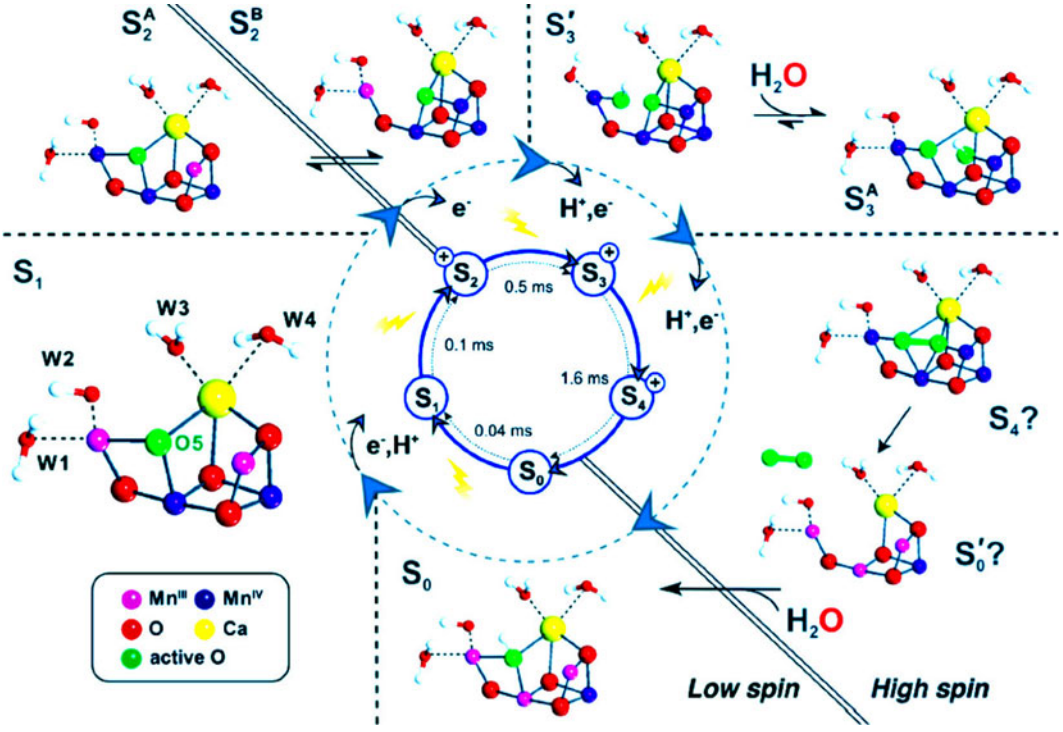


Fig. 3. Tentative scheme for the cyclic stepping of the catalytic metal cluster over Kok's states $S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_4$ and the formation of the dioxygen bond (Krewald *et al.*, 2016).

Johannes Messinger reviewed the status of agreements and diverging views up to 2004. He discussed two possible mechanisms for O–O bond formation, namely involving the dangler Mn and one Ca-bound water on the one hand, and involving a bridging oxygen and a dangler-bound water (Messinger, 2004).

Crystals exposed to a CW-X-ray beam from a synchrotron undergo radiation damage. By X-ray spectroscopy Holger Dau had detected dose-dependent Mn-reduction in PSII (Dau *et al.*, 1997). The larger Mn–Mn bond length in Barber's crystal structure as compared with the one inferred from EXAFS (Dau *et al.*, 2004) raised serious doubt on the valence-state of the Mn_4Ca-cluster in Barber's model. The Berlin group joined forces with the Berkeley group and they clearly demonstrated that X-ray exposure (doses by order of magnitude lower than causing loss of diffractivity) reduced Mn_4 from the expected valence of state S_1 (III,III,IV,IV) to (II,II,II,II) (Yano *et al.*, 2005). It explained why the Mn–Mn distances of the structural model were larger than very precisely determined by EXAFS. At the same time the valence-set of the four Mn-ions was determined by ^{55}Mn-pulse-NMR in Wolfgang Lubitz' lab. It was (III,III,III,IV) in S_0 and (III,IV,IV,IV) in S_2 (Kulik *et al.*, 2005).

Soon thereafter Per Siegbahn took Barber's arrangement of the metals and their ligands (with modifications by the Berlin group) as the basis for modelling Mn_4CaO_x-clusters *in silico* (Siegbahn, 2006). By DFT and energy minimization a particular configuration of Mn_4CaO_5 was selected to represent the HV state S_4

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from where the O–O bond is formed. The Mn–Mn bond lengths in the energy minimized structure were shorter than in Barber's. In this respect Siegbahn's modelling was predictive for radiation damage and not only *post mortem* descriptive as common in computational biochemistry/biophysics.

It was not astounding that, for a while, Barber's structure of the metal cluster was met with reservations, because it has been all but trivial, that the metal and ligand positions in PSII are the same if the valence of Mn_4 is (III,III,IV,IV), as supposed for S_1, or (II,II,II,II), as after radiation damage.

Subsequent work on the atomic structure has been aimed at (a) minimization of radiation damage, (b) improved spatial resolution, (c) selective characterization of the five functional states S_0 to S_4 and (d) time resolution of structural transitions between them. The latter necessitates X-ray diffraction at room temperature.

The first high-resolution structure of PSII at 1.9 Å was published by Jian-Ren Shen and Nobuo Kamiya (Umena *et al.*, 2011). Barber's cubanoid for Mn_3Ca with the dangling Mn (Ferreira *et al.*, 2004), and the ligand structure of the Berlin group (Loll *et al.*, 2005) were corroborated in essence. For the first time, the bridging oxygen atoms and four water molecules were clearly resolved, and former Mn_4CaO_4 became $Mn_4CaO_5(H_2O)_4$, as schematically illustrated in Fig. 2. Barber's numbering of the metal ions and oxygen atoms has been changed accordingly. The position of the dangler-Mn relative to the cubane was corrected by 3 Å, and Asp 170 was now a bidentate ligand (bridging Mn4 and Ca) and not monodentate, as before. This structural model was obtained by continuous synchrotron radiation (CW-XRD), and at cryogenic temperature. The Mn–Mn distances were still slightly longer than in both, EXAFS experiments and DFT calculations, and hence still indicative of some radiation damage.

A damage-free structure was desirable. X-ray diffraction with ultra-short pulses (typ. duration, <50 fs) of a free electron laser (XFEL) seemed to offer a way out. The pioneering study was published in 2012 by the joined groups of Berlin (i.a. Athina Zouni), Berkeley (i.a. Jan Kern, Junko Yano, Vittal Yachandra) and Umeå (Johannes Messinger). Very many stochastically oriented microcrystals of PSII were each exposed to a single X-ray shot to 'diffract before destruction' (Kern *et al.*, 2012). It followed a series of such XFEL studies (e.g. Kern *et al.*, 2013, 2014; Hellmich *et al.*, 2014; Kupitz *et al.*, 2014) carried out at room temperature and aiming at improved resolution.

Shen and his colleagues also conducted XFEL experiments. In contrast to the former group they used large crystals with volume-per-volume exposure at cryogenic temperature. In 2015 they presented a 'damage-free' structural model at a resolution of 1.95 Å (Suga *et al.*, 2015). The Mn–Mn distances were shorter than previously obtained by conventional CW-XRD (Ferreira *et al.*, 2004; Umena *et al.*, 2011), and they were better compatible with those from EXFAS and DFT (for the remaining slight difference, see Fig. 1 in Siegbahn (2013*b*)). The 1.95 Å-structure has become the standard for the following discussions.

A long hedged dream of inorganic chemists came true when Chunxi Zhang and his colleagues synthesized a biomimetic analogue of nature's Mn_4Ca-cluster (Zhang *et al.*, 2015).

The obvious challenge was now to determine the damage-free structure of the catalytic centre at room temperature, in its different oxidation states (S_0, …, S_4), and, if possible, time resolved.

In 2016 the Berkeley–Berlin team published a XFEL-study on structural differences between states S_1 and S_3 of the metal cluster, as function of the temperature, and with and without ammonia bound (Young *et al.*, 2016). With ammonia the authors mapped those water binding sites in the metal-cluster that are not involved in the formation of the O–O bond. While temperature affected some positions of transmembrane α-helices and redox-cofactors, the Mn_4CaO_5-cluster, proper, was practically invariant to temperature. At a resolution of 2.25 Å the authors did not detect differences between the dark state (S_1) and the 'two flashes advanced' state (mainly S_3) (Young *et al.*, 2016).

Photosystem II with the oxygen evolving complex (AC)

Mechanistic models for O–O bond formation

Adopting a chemical mechanism that had been proposed by V. Pecoraro and his colleagues (Pecoraro *et al.*, 1998) James Barber speculated that the dioxygen-bond is formed by nucleophilic attack of a water ligand to the Ca-ion on a highly electrophilic oxo supposedly bound to the dangler-Mn(V) (Ferreira *et al.*, 2004; Iwata and Barber, 2004). Barber has favoured this concept until today (Barber, 2016, 2017). His view is in line with earlier computational studies by P. Siegbahn (Siegbahn and Crabtree, 1999; Siegbahn, 2000), and it has been shared recently by others (McEvoy *et al.*, 2005; Sproviero *et al.*, 2006, 2008; Askerka *et al.*, 2017).

After the first structural model of the metal-cluster had been published Per Siegbahn investigated by DFT the energy profile of various mechanistic models for the formation of the O–O bond. Calculations of the energy demand for an O–O-bonded intermediate in the Mn_4CaO_5-structure led him to reject the nucleophilic attack mechanism. He has since then proposed a radical attack mechanism involving a new bridging oxygen (O5) within an open cubanoid (coined oxyl-oxo-mechanism) (Siegbahn, 2006) that was not included in Barber's structural model. O5 resulted from 'predictive computational chemistry', and it was only later observed in diffraction experiments at higher resolution. Siegbahn has extended this work later on (Siegbahn, 2006, 2008*a*, 2017; Siegbahn and Blomberg, 2014).

Yu Guo and his coworkers embarked on similar DFT calculations and likewise favoured an oxo-oxyl mechanism involving the same bridging oxygen (O5) in an open cubanoid (Guo *et al.*, 2017). On first inspection Guo's results are in line with Siegbahn's, however a closer look reveals that their calculated energy profile for the last transition ($S_3 \rightarrow S_0$) violates the experimentally established fact that oxygen liberation and Y_z^+-reduction are both kinetically enslaved by the same rate limiting step. In his calculated energy profile these two reactions are separated by an energy notch, 12 kcal/mol deep (see their Fig. 4; Guo *et al.*, 2017). Siegbahn's calculated energy profile on the other hand is compatible with the kinetic data.

The proponents of the leading concepts for the formation of the O–O bond, nucleophilic attack *versus* oxo-oxyl, have presented their diverging views in series of publications starting from 2004 (Ferreira *et al.*, 2004) and 2006 (Siegbahn, 2006) until today. While James Barber most recently argued in favour of nucleophilic attack by analogy with the known mechanism of carbon dioxide dehydrogenase (Barber, 2017), Per Siegbahn (Siegbahn, 2017) repeated his previous (Siegbahn, 2006) exclusion of nucleophilic attack on energetic grounds.

In addition to the above two concepts for the formation of the O–O bonds several modified or even alternative mechanisms have been proposed (see e.g. Messinger, 2000; Gao *et al.*, 2009; Yamanaka *et al.*, 2011; Saito *et al.*, 2012; Cox and Messinger, 2013).

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Experimental tests of proposed mechanisms

The light driven stepping over the intermediate states of the 'charge accumulator' (Mn_4CaO_5) has been kinetically well characterized (for a comprehensive review, see Dau *et al.* (2012)). The eventually fourfold oxidized Y_zMn_4Ca-entity reacts with two molecules of water (-derivatives) which involves the transfer of four electrons. The terminal four-electron-cascade is kinetically enslaved by a bottle neck of approximately 1 ms duration. Characterizing the short-lived intermediates is difficult if not impossible. The formation of a peroxide intermediate in the penultimate state S_3 has been in focus.

The valence of the four manganese ions in states S_i (i = 0–4)

A first stronghold for the assignment of oxidation states to the four Mn-atoms has been the EPR-multiline EPR-signal of the S_2-state. It has been attributed to the interaction between Mn (III) and Mn(IV) (Dismukes and Siderer, 1981). Two valence configurations of the four Mn-ions are compatible with this notion, namely $Mn(III)_3Mn(IV)$ and $Mn(III)Mn(IV)_3$ (de Paula and Brudvig, 1985). These assignments have been extended into two schemes for the S_i-series which are usually referred to as low-valence (LV) and HV models. An enormous amount of work by different techniques had been devoted to discriminate between these models, for a while without reaching consent (reviewed in Krewald *et al.* (2015)). The comparison of NEXAFS-data with DFT-calculations supported the HV-model, although a few complications seemed to prevent an unequivocal attribution (Brena *et al.*, 2012). Related to the above controversy has been the one over ligand-centred *versus* Mn-centred oxidation in the transition $S_2 \rightarrow S_3$. The former was advocated in Yachandra *et al.* (1996) and Messinger *et al.* (2001) and the latter in Iuzzolino *et al.*, 1998; Dau *et al.*, 2003; Siegbahn, 2009; Brena *et al.*, 2012. ^{55}Mn-ENDOR studies on states S_0 and S_2 (Kulik *et al.*, 2005) and XAS-studies on S_2 and S_3 (Dau *et al.*, 2003) have established the HV concept for the four manganese ions, namely, $S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3$ = (III,III, III,IV) → (III,III,IV,IV) → (III,IV,IV,IV) → (IV,IV,IV,IV).

The oxidation of manganese during $S_2 \rightarrow S_3$ and the existence of a $Mn_4(IV)$-state has been corroborated by X-ray emission spectroscopy (Zaharieva *et al.*, 2016*a*; Schuth *et al.*, 2018).

Two lines of computational studies have agreed on that the S_2-state is interconvertible between two sub-states. In one state, coined S_2^B, the only trivalent ion is Mn4(III), the dangler outside of the cubane. In the other state, coined S_2^A, it is Mn1(III) within the cubane. Both these studies agreed in that only S_2^B can proceed to S_3 owing to the presence of a titratable ligand (Bovi *et al.*, 2013; Retegan *et al.*, 2016). While S_2^A is more stable than S_2^B the oxidation of Y_z inverts this situation such that S_2^B passes by proton-coupled electron transfer into state S_3 ($Mn(IV)_4$) (Narzi *et al.*, 2014).

Krewald *et al.* cast the available data, including their own on hyperfine coupling constants, into one consistent theoretical framework (Krewald *et al.*, 2015). They found that the most stable states, the open cubane structure of S_2 and the closed cubane of S_3, correspond to structures proposed earlier (based on DFT) by Siegbahn (Siegbahn, 2013*a*). In contrast to speculation in the literature their simulations yielded no evidence for ligand centred oxidation between S_2 and S_3. And they showed that only the HV-model complies with the data but not the LV-model (Krewald *et al.*, 2015). In light of these results, state S_3 is to be conceived as $Y_zMn_4(IV)$ or $Y_z^{+\circ}Mn_3(IV)Mn(III)$ (Cox *et al.*, 2014; Retegan *et al.*, 2014), and state S_4 as $Y_z^{+\circ}Mn_4(IV)$. The latter initiates the formation of the O–O bond.

The entry of water into the reaction cycle

The points of entry of solvent water into the reaction sequence of oxygen evolution were at first characterized by mass spectroscopy (Messinger *et al.*, 1995; Hillier *et al.*, 1998). The location of the binding sites was then unknown. The μ-oxo-bridges were initially considered as too strong for solvent exchange and catalytic relevance. Hydrogen exchange studies and ^{17}O-NMR spectroscopy has recently demonstrated that at least one μ-oxo bridge in the metal cluster, either O4 or O5, is solvent exchangeable (Rapatskiy *et al.*, 2012). The latter μ-oxo might participate in O–O bond formation as previously postulated by Siegbahn (Siegbahn, 2006).

Dimitrios Pantazis and colleagues (Retegan *et al.*, 2016) in Wolfgang Lubitz' lab in Mülheim discussed water exchange in the light of experiments on the Mn-coordination number in the S_3 state (Haumann *et al.*, 2005) and of recent work on water exchange, when progressing through the S_i states (Suzuki *et al.*, 2008). One water molecule binds *after* oxygen release. It has been implied to form the di-μ-oxo bridge in the S_0-state (Krewald *et al.*, 2015) and a fluxional bridge in the S_2-state (Bovi *et al.*, 2013). The second water binds in the $S_2 \rightarrow S_3$ transition. It is probably not the reacting water in the next turnover (Suzuki *et al.*, 2008; Cox and Messinger, 2013; Nilsson *et al.*, 2014*a*). Pantazis proposed that W2 on Mn4, it pivots close to O5, might act as reaction partner in the formation of the O–O bond (Retegan *et al.*, 2016). This view is however at odds with ammonia mapping of reactive water by the Berlin–Berkeley team which has let them to exclude W2 in this role (Young *et al.*, 2016).

Christopher Kim and Richard Debus investigated the vibrational modes of bound water by FTIR-spectroscopy (Kim and Debus, 2017). The D–O–D bending-mode of one water molecule is altered upon functional substitution of Sr for Ca. This particular hydrogen-bonded water molecule is eliminated during the transition $S_2 \rightarrow S_3$. The authors proposed that this water molecule, probably W3, is deprotonated and moves to a position close to O5 as partner for O–O bond formation in the next transition ($S_3 \rightarrow S_4$–S_0). During $S_2 \rightarrow S_3$ the vacant water position on Ca is refilled by the incoming substrate water (Kim and Debus, 2017). Their view is compatible with the reorganization of hydrogen bonds as inferred from a large H/D-kinetic isotope effect on the reduction of Y_z& as monitored by UV- (Haumann *et al.*, 1997*a*), X-ray- (Zaharieva *et al.*, 2016*b*) and FTIR-spectroscopy (Sakamoto *et al.*, 2017). Nicholas Cox and colleagues (Krewald *et al.*, 2016) cast the available spectroscopic evidence into a spin-geared reaction scheme where the O–O bond is formed within the cubane. Their scheme is illustrated in Fig. 3.

The above spectroscopic results from Wolfgang Lubitz lab and other laboratories are in line with P. Siegbahn's oxyl-oxo-mechanism (Siegbahn, 2006, 2017) and at odds with J. Barber's nucleophilic attack mechanism (Ferreira *et al.*, 2004; Barber, 2017). They qualify alternative computational models (Kusunoki, 2007, 2011), in particular those based on LV of the Mn-cluster (Sproviero *et al.*, 2008; Gatt *et al.*, 2011; Petrie *et al.*, 2012; Li *et al.*, 2013). The challenge has been back to structural biology.

Structural evidence for/against proposed mechanisms of O–O bond formation

In 2016 the Berkeley–Berlin team published a XFEL-study on structural differences between states S_1 and S_3 of the metal cluster at room temperature (Young *et al.*, 2016). At a resolution of 2.25 Å no difference in electron density was detected between

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the dark state (S_1) and the 'two flashes advanced' state (mainly S_3), as mentioned.

In 2017 Shen and coworkers in Japan reported structural differences of the metal-cluster between states S_1 and S_3 up to a resolution of 2.1 Å (Suga *et al.*, 2017). Their study was carried out at room temperature and under excitation with 10 fs pulses of a free electron laser. They found an 'apparent positive peak' in the difference Fourier map close to oxygen atom O5 which they interpreted as new oxygen, coined O6. O5 and O6 were taken as candidates for the formation of the O–O bond (Suga *et al.*, 2017) in line with Siegbahn's computational results (Siegbahn, 2006, 2008*b*, 2009). The authors discussed that Glu189, the only mono-dentate ligand to the cluster, had to move away from the cubane to accommodate O6.

When taken at face value this result could put an end to the major controversy over the mechanism of the O–O bond formation (nucleophilic attack *versus* oxyl-oxo).

At present, however, several features call for scrutiny: (a) the 'apparent positive peak' in Shen's difference map (interpreted as O6) is hardly elevated over noise and, at the given resolution, superimposed by electron density of Mn. (b) It is not yet clear to what extent it depends on particular software for the evaluation of raw data. (c) The purity of the dark state (100% S_1?) and of the '2-flash-advanced state' (45% S_3 in Shen's implies 55% S_2!) has been difficult to assess in crystals of PSII. (d) Spectroscopic evidence disfavours the presence of a peroxide intermediate in state S_3. (e) The supposedly 'moving' residue Glu189 has been point mutated into Arg, Lys and Gln without influence on the rates of various electron transfer reactions at the donor side of PSII (Clausen *et al.*, 2001).

The Berlin–Berkeley group has advanced the search technique for intermediates of the O–O bond by combining serial femtosecond X-ray crystallography with simultaneous X-ray spectroscopy under multi-flash visible laser excitation (Kern *et al.*, 2013). In principle, this technique allows time resolution of structural transitions by variation of the time delays between the X-ray and the visible flashes. The major challenge is to obtain the necessary spatial resolution (<2 Å) to reveal the expected displacement of water (oxygen) molecules in 100 µs during the transition from S_2 towards S_3 and onwards via 'S_4' to S_0 in a millisecond. Recently, this team has reported the structure of all states of Kok's cycle at 2.04–2.08 Å resolution including two transient states during $S_2 \rightarrow S_3$ at 150 and 400 µs after excitation by a flash of visible light (Kern *et al.*, 2018). Their data exclude the formation of a peroxide intermediate already in state S_3. In S_3 they found the binding of a new 'Ox' (a water derivative) in a position between Mn1 and Ca. In the next step, $S_3 \rightarrow S_4$–S_0 it could either form an O–O bond with O5 or replace O5 after formation of such a bond between O5 and another Ox. These results are in perfect agreement with the above cited results obtained by X-ray and magnetic resonance spectroscopy. There is no evidence that a peroxide intermediate is already formed in state S_3. But it is expected that one intra-cubanoid oxo-group, namely O5, is involved in the formation of the O–O bond in the next step, $S_3 \rightarrow S_4$–S_0.

Perspective for a rigorous resolution of the molecular mechanism

The rigorous resolution (in space and time) of the four one-electron steps and the eventual four-electron reaction cascade of this pivotal reaction for life on earth is complicated by two features: (a) the great stability of the (Mn_4CaO_5cage)-structure and (b) the seeming kinetic enslaving of six partial reactions by one particular step.

(a) The Mn_4CaO_5-cluster is very stable, and its expected structural changes during the terminal reaction cascade are probably subtle. Even gross valence changes of Mn like those caused by radiation damage in CW-X-ray diffraction (Dau *et al.*, 1997; Yano *et al.*, 2005) increase the bond length between metal ions only by small amount (see Siegbahn (2009)). This is why mechanistic insight can hardly be expected from tracking the motion of the metal ions proper.

As already mentioned, the effects of radiation damage have been minimized by either extremely low dose in conventional synchrotron X-ray diffraction (Tanaka *et al.*, 2017) and by femtosecond irradiation in XFEL-diffraction experiments (Kern *et al.*, 2014; Tran *et al.*, 2014; Suga *et al.*, 2015). Another type of radiation damage, namely a 'coulomb-explosion' of the metal cluster in some 10 fs has only recently been considered. MD-simulations have revealed that it is minimized if the X-ray pulse length is shorter than 10 fs (Amin *et al.*, 2017, 2016).

It came as a surprise that not only the metal-cluster is extremely stable but its protein cage as well. The Berlin group deleted the metal-cluster from PSII, and they found the protein cage very little altered, as if waiting for the insertion of di-µ-oxo-bridged pairs of manganese (Zhang *et al.*, 2017).

(b) The key to fully understand the mechanism of water oxidation are the trajectories of water, hydroxyl, oxygen, proton and electron. The motion of the former three might be tracked in the near future by time-resolved XFEL-crystallography at <2 Å resolution. Spatial tracking of protons (and electrons) is however not yet in sight. Here one is restricted to kinetic evidence based on spectroscopic data. Here, the kinetic enslaving of six partial reactions by one particular step represents a major challenge as illustrated in Fig. 4.

If the valence of Mn_4 in state S_2 is taken as a reference, namely (IV, IV, IV, III), the penultimate state before oxygen release possibly is: $S_3 = [Mn(IV)_4CaO_5(H_2O)_4Ligands]^+$. The next photo-excitation creates the sequence of events that is illustrated in Fig. 4. Oxygen is released plus two protons and one water molecule is taken up. The release of two protons is kinetically bipartite, a first step in the time range of 100 µs is followed by a slower step at 1 ms (Förster *et al.*, 1981). The fast release of one proton seems to be the priming event for the subsequent cascade of six reactions which are kinetically enslaved by one and the same bottle neck of 1 ms half-duration, namely (1) the transfer of one electron to $Y_z^{\circ}HisH^+$, (2) the reduction of three manganese ions, (3) the release of a second proton, (4) the release of di-oxygen and (5) the uptake of one molecule of water. The exact sequence of these events is subject to ongoing research. Michael Haumann and Holger Dau ventured into time-resolved calorimetry using photo-thermal beam deflection (Krivanek *et al.*, 2008; Klauss *et al.*, 2009). They obtained a wealth of signal transients that were interpreted to show the release of one proton first, oxygen release and water intake second and the release of another proton third (see Fig. 8 in Klauss *et al.* (2015)). It seems worthwhile to take up such studies with certain mutant-PSII were oxygen release is dramatically slowed down (Hundelt *et al.*, 1998; Clausen *et al.*, 2004; Nilsson *et al.*, 2014*b*).

Energetics of oxygen production by PSII

Aiming at the stabilization of transient intermediates of the terminal reaction cascade PSII was exposed to high oxygen pressure

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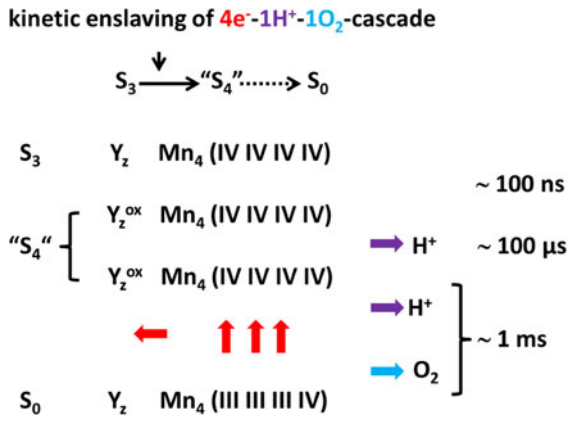


Fig. 4. The terminal reaction cascade of oxygen formation (see the text).

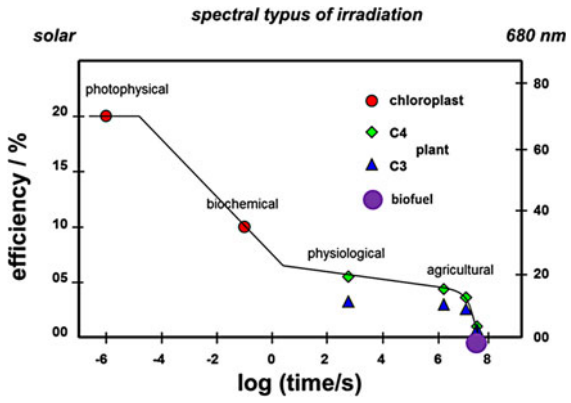


Fig. 5. The efficiency of solar energy conversion by oxygenic photosynthesis as a function of time (see the text).

(Clausen and Junge, 2004). Certain UV-absorption transients that clearly showed a phase of ~1 ms half-time during $S_3 \rightarrow S_4$–S_0 were monitored. These transients had been previously attributed to the reduction of the manganese cluster (Dekker *et al.*, 1984). If the oxygen pressure was increased 100-fold over atmospheric this phase was virtually eliminated. Half-inhibition was reached at ≈10-fold increase. It suggested a rather low driving force for photosynthetic oxygen production, implying 'little leeway for photosynthetic organisms to push the atmospheric oxygen concentration much above the present level' (Clausen and Junge, 2004) (for possible ecological and geochemical impact see Raven and Larkum (2007)). At first this observation seemed to be corroborated by another technique, delayed chlorophyll-luminescence (Clausen *et al.*, 2005*b*). Later however, no stalling effect of increased oxygen pressure was detected by (a) time-resolved X-ray absorption aiming at the K-edge of Mn (Haumann *et al.*, 2008), (b) chlorophyll-fluorescence (Kolling *et al.*, 2009) and (c) direct detection by membrane-inlet mass spectroscopy of $^{18}O_2$ produced from $H_2^{18}O$ against a background of pressurized $^{16}O_2$ (Shevela *et al.*, 2011). In the latter experiments the oxygen pressure was raised up to 20 bar without any indication for stalling of dioxygen liberation. It implies that the driving force is at least 200 meV. Recently this number was driven up by another approach. In one cyanobacterial mutant-PSII the S_0-state is stabilized for days. Johannes Messinger and his colleagues incubated this mutant for up to 3 days in $H_2^{16}O$ and $^{18}O_2$ without detecting the formation of any amount of $^{16,18}O_2$ (Nilsson *et al.*, 2016). They concluded that the driving force for dioxygen release during $S_3 \rightarrow S_4$–S_0 must be greater than 400 meV. They understood this large driving force in terms of Lev Krishtalik's pioneering discussion of the energetics of photosynthetic oxygen evolution in terms of thermodynamic principles (Krishtalik, 1989, 1990). Jérome Lavergne in Nilsson *et al.* (2016) attributed the experimentally established high driving force to the large entropic effect of dilution of bound oxygen into the solution (see free energy profile in Fig. 6 of Nilsson *et al.* (2016)). Their conclusion was that the protein has no grip on this 'extra enzyme' contribution to the driving force.

In oxygenic photosynthesis the entropy of dilution favours the release of freshly formed and still bound dioxygen. What about cell respiration where cytochrome oxidase has to overcome this huge entropic barrier?

The 'equilibrium constant' for oxygen in cytochrome oxidase was studied by Marten Wikström's group (Krab *et al.*, 2011). The authors determined the apparent $K_m(O_2)$ *for steady turnover* of the enzyme, at neutral pH and in the absence of protonmotive force, $K_m(O_2) = 25$ nM (see their Fig. 4A). The free energy of oxygen binding from O_2-saturated water is in the order of −450 mV, very strong indeed, when considering the more than the +300 mV of Krishtalik's entropic contribution to oxygen dilution (see discussion in Nilsson *et al.* (2014*b*)).

Oxygenic photosynthesis and a sustainable future of mankind

Oxygenic photosynthesis has produced the oxygen we breathe and the biomass serving as food, feed, fibre and fuel, as mentioned. Its driving force is sunlight. The mean power of sunlight at the surface of earth is huge, 124×10^{15} W = 124 PW. Only a very small fraction thereof is captured by photosynthetic organisms, 89×10^{12} W = 89 TW, about half and half on land and in the sea (Field *et al.*, 1998; Falkowski *et al.*, 2000). The low proportion of solar energy capture is partially owed to unfavourable climate, substrate, seasons and the day–night cycle. Another loss of energy capture is intrinsic to the physico-chemical mechanism of photosynthesis, as described above. The solar energy conversion efficiency of photosynthesis is limited even under the most favourable conditions of agriculture, and even more so, if one aims at processed products like biofuels (see Fig. 5).

In the range of microseconds after absorption of a quantum of light the efficiency reaches up to 20% if related to the solar spectrum at the sea level (right scale in Fig. 5). It is about 80% under excitation with red light (see right scale in Fig. 5). These figures refer to a thin canopy (for a thick canopy, see Dau and Zaharieva (2009)). Therewith the efficiency matches the one of photovoltaic cells (Blankenship *et al.*, 2011). The initial drop (to 20%) is mainly caused by (a) limited use of the full solar spectrum (~50%) and (b) internal energy conversion ('blue → red') within chlorophyll. In the test tube and within the time range of 100 ms to yield NADPH the efficiency drops by another factor of two. William Rutherford has coined this drop as 'sacrificing energy efficiency for directionality' (Rutherford *et al.*, 2012). In other words, wasteful back-reactions are overcome by high speed of the forward reaction which requires an energy drop. On the physiological time scale (h) the efficiency drops further from the best vegetative month of the year to the average over a full year. The maximum yearly averaged efficiency at the given level of atmospheric CO_2 (380 ppm in 2010) has been estimated

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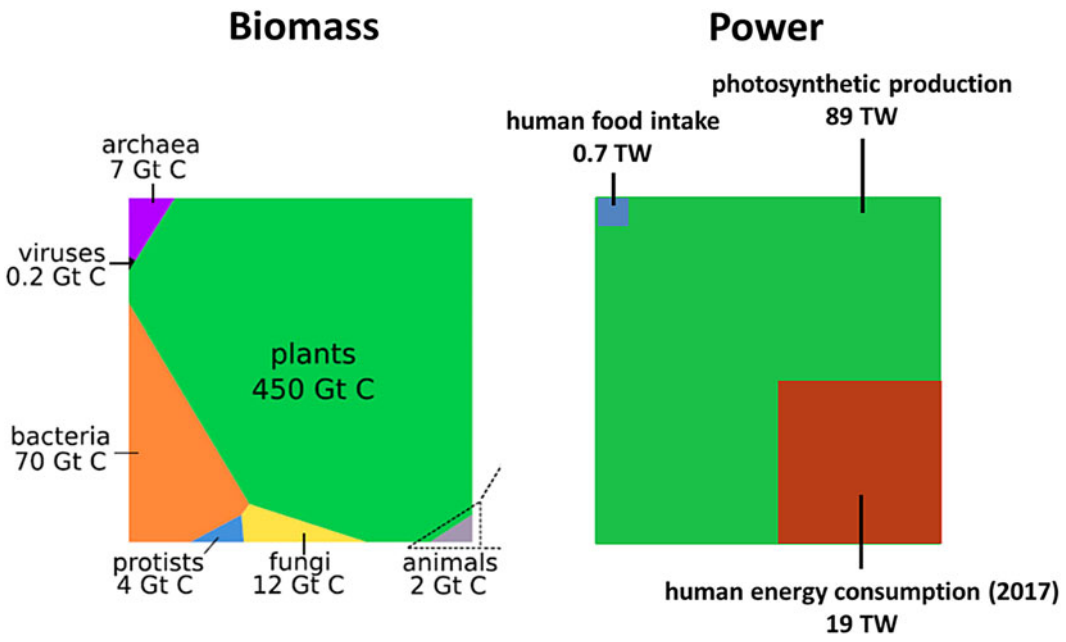


Fig. 6. Left: Distribution over taxa of the biomass on earth in terms of Gt of fixed carbon (Bar-On *et al.*, 2018). Right: Solar energy fixation by global photosynthesis in TW (green) and energy consumption of mankind, both metabolically (blue) and technically (brown).

by Don Ort's group as 4.6% for C3- and 6% for C4-plants (Zhu *et al.*, 2008). The efficiency of crops in the field is mostly lower. Take Brazil's large scale energy farming as an example. In 2010 the area specific yield of sugarcane was 80 t_{dwt} ha^{-1} yr^{-1}. It implies a solar energy conversion efficiency of about 2%. The consecutive conversion of sugarcane into bio-ethanol yielded 6300 l ha^{-1} yr^{-1} EthOH, equivalent of an overall energy efficiency of about 0.2%. Slops and slurry were utilized in the process, and the fossil energy input was not counted in this estimate. Various well-to-wheels analyses for biofuel production in the USA and in Europe (e.g. by the JRC in 2012: ISBN 978-9279-21395-3, ISSN 1831-9424, EUR 24952 EN, doi:10.2788/79018 and Torchio and Santarelli, 2010; Wang *et al.*, 2012; Orsi *et al.*, 2016) came to the same conclusion. The overall energy yield of biofuel production is at best 10% of the energy content of the biomass if not being negative (i.e. requiring higher fossil fuel input than biofuel output). Energy farming for biofuels is a questionable option, at least for densely populated countries that cannot waste arable and/or ecologically valuable land.

In a recent meta-study Ron Milo and his colleagues reviewed the distribution over taxa of the biomass on earth (Bar-On *et al.*, 2018). Figure 6 (left) shows their result. In terms of fixed carbon, plants (83%) dominate over bacteria (13%) and animals (0.4%). *Homo sapiens* account for less than 0.01% of total. (By the way, the total amount of fixed carbon in *sapiens* is by one order of magnitude greater than that of all wild mammals taken together.) Confrontation of the biomass distribution over taxa (Fig. 6, left) with the energy provision by photosynthesis (Fig. 6, right, green) and the global primary energy consumption of mankind (Fig. 6, right, brown), shows that mankind, summed over all nations, uses 22% of what is provided by photosynthesis. Mankind's metabolic energy intake (Fig. 6, right, blue) is much less than the global energy consumption by technical civilization (Fig. 6, right, brown). It is noteworthy that the energy consumption *per capita* in industrialized countries like Japan and Germany is twice the global average, and in the USA it is more than four times greater. In other words, if the present global population cared to live in the style of USA citizens, they had to use the whole biomass production at almost 100% energy conversion efficiency, an absurd scenario.

Accordingly the engineering of plants and algae for higher efficiency (see e.g. Ort *et al.* (2015); South *et al.* (2018)) should be directed towards improved supply of food, feed, fibre and platform chemicals rather than of fuel. Having reached the Anthropocene, mankind cannot rely on oxygenic photosynthesis to satisfy its energy needs.

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