Recent Developments in Biological Water Oxidation

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Abstract: Rapid progress has been made in the last five years towards resolution of the structure of nature's water splitting catalyst – a $\text{Mn}_4\text{O}_5\text{Ca}$ cofactor embedded in Photosystem II - especially in the field of X-ray crystallography. In addition, recent magnetic resonance data have allowed the structure of the cofactor to be accessed in its last metastable intermediate state, prior to O-O bond formation. This activated form of the catalyst is geometrically similar to that seen by X-ray crystallography, which represents the resting state of the cofactor, but requires the coordination of an additional water molecule to the cofactor, rendering all Mn ions six coordinate. Importantly, it locates two water derived, Mn bound oxygen ligands in close proximity. It is these two oxygen ligands that likely form the product O_2 molecule, as proposed earlier by quantum chemical modeling. Current views on the molecular level events that facilitate catalyst activation, that is, catalyst/substrate deprotonation, Mn oxidation and water molecule insertion are briefly described.

The structure of Nature's water splitting cofactor in its resting state:

Crystallographic advances, including the introduction of free electron laser sources, have fundamentally altered the trajectory of research on the mechanism of biological water splitting [1]. These new methods have recently provided an atomic resolution crystal structure of the resting state of the catalyst. Specifically, the XFEL (X-ray free electron laser) structure: i) reproduces earlier EXAFS constraints (e.g. Mn-Mn distances) [2-4]; ii) clarifies the location of all oxygen bridges of the catalyst

including the O5 bridge, which in earlier X-ray models (XRD) was placed in a chemically unprecedented bonding position; and iii) constrains the net oxidation state of the cofactor in the resting state (III,IV,IV,III) by resolving the Jahn-Teller axes of two Mn^{III} ions, Mn1 and Mn4 (see **Fig. 1**). This novel structure allows the information content of complementary spectroscopies to be fully realized and together these data now provide a solid basis for the development of robust chemical models throughout is entire catalytic cycle [5].

Fig. 1 A) Comparison of the new XFEL structure with the earlier synchrotron (XRD) structure of the $\text{Mn}_4\text{O}_5\text{Ca}$ cofactor [1,6]. The cofactor resembles a distorted chair with the back of the chair shown by the light blue plane. The XFEL structure better reproduces EXAFS constraints [2,3] (panel B) and places O5 in a more chemically realistic position (panel C), more in line with earlier chemical models [5,7-9]. The cofactor contains three coordinately saturated Mn ions (Mn2, Mn3 and Mn4) with a potential substrate binding site located at Mn1, the only five coordinate Mn ion. Mn4 and the Ca^{2+} ion both carry two water derived ligands. All remaining ligands are derived from the protein backbone and oxygen bridge network. Substitution of the Ca^{2+} ion with Sr^{2+} has little effect on the structure of the catalyst [10], in line with spectroscopic results [11].

Substrate binding and delivery to the catalyst: An understanding of the mechanism of biological water splitting is greatly aided by resolving what oxygen sites of the cofactor participate in the reaction [12,13]. Recent magnetic resonance data has implicated O5 as one of the two substrates owing to its fast exchange with bulk solvent water [14-16]. This is a unique property not seen for the other four bridges, or for oxygen bridges in simpler synthetic model systems [17-19]. The identity of the 2nd substrate remains more contentious as it is unclear if it is bound in all intermediate states of the catalyst, or if bound in all states, whether its position changes during the cycle [12,20]. Water analogs such as ammonia and methanol provide a means to

address this question by probing how the substrates first access the cofactor. Substrate access forms part of a larger debate on the dynamics of the solvent/substrate interface, namely how does the catalyst selectively *activate* two water solvent molecules (when it is surrounded by solvent water) and *regulate* solvent water access to the site of the catalyst, to avoid deleterious side products such as H_2O_2 .

Fig. 2. **a)** Proposed access/egress channels to and from the $\text{Mn}_4\text{O}_5\text{Ca}$ cofactor. **b)** Binding location of NH_3 which displaces the water ligand (W1) to the outer manganese (Mn4). Its binding was used to identify the exchangeable oxygen bridge (O5) as ammonia binds near (*trans* to) the O5 bridge. **c)** Sequence of oxygen ligand movements that allow water binding to occur. Spectroscopic data support the cofactor being able to adopt open and closed cubane conformation, in support of this mechanism for substrate delivery [9,21].

Although ammonia and methanol do not drastically alter the binding/exchange kinetics of the two substrates, their addition is associated with a decrease of the catalyst's efficiency under steady-state conditions suggesting that they hinder or interfere with substrate access to the site(s) of catalysis. The crystal structure identifies at least three water pathways leading towards/away from the cofactor: i) a channel which includes the Ca^{2+} ion and the redox active tyrosine residue (YZ); ii) a channel which includes the outer Mn4 and the Asp61 residue [1,6]; and iii) a similar channel which also includes the Cl^- ion (**Fig. 2a**). The location of ammonia binding

was recently deduced from chemical modelling [14,15,22] and mutagenesis [23], displacing the water ligand (W1) of the outer manganese (Mn4). The binding location of methanol, however, is less well defined, with one option placing it close to the site of ammonia binding and another favouring its binding at the Ca^{2+} [24]. Competitive binding of small molecules at/near the position of W1 indicate that the water channel associated with this site (Asp61 channel) is important for water delivery [25]. As a whole, these results favor that one of the water derived ligands of the Mn4 is the second substrate. Water binding via the Asp61 channel involves a rotation of the waters on the Mn4, initiated by the facile shift of the O5 bridge [9,21]. This cascade of structural rearrangements results in a five coordinate, trigonal bipyramidal Mn ion, shown in **Fig. 2** [25]. Within this model water binding occurs on the back-face of the cofactor. As such the water molecule when first delivered is most likely not the substrate of the reaction - this is instead one of the existing water ligands (W2) - but form the substrate in the next catalytic cycle [12,20].

Fig. 3. Extended, nine intermediate S-state cycle of the $\text{Mn}_4\text{O}_5\text{Ca}$ cofactor, based on [26]. The cycle starts in the S_0^n state (n = neutral) state. Up until the S_2^+ , electron withdrawal precedes proton transfer (*ET-PT*). Subsequently, generation of the S_3^+ and S_4^+ states requires first proton transfer then oxidation (*PT-ET*). S_0^n is regenerated upon the spontaneous release of molecular oxygen and likely the binding and deprotonation of one of the substrates.

The structure of the activated catalyst - structural evolution during the S-state: It is well known that the cofactor cycles through a series of five intermediate states: S_0 ,

S_1 (dark stable), S_2 , S_3 , and S_4 where the subscript refers to the number of oxidizing equivalents stored by the cofactor. The S_4 state spontaneously decays upon release of triplet O_2 returning to the S_0 state. Oxidation of the cofactor is driven by light excitation of the Photosystem II reaction center, via a redox active tyrosine residue (Y_Z). Coupled with oxidation, protons are successively removed from the cofactor, to ensure its total charge/redox potential remains constant, termed redox tuning. As such the cycle can more accurately be described in terms of nine states which differ in terms of their net electron and proton count [26,27] (**Fig. 3**). An important observation is that at different points in the cycle the order in which electrons and protons are removed from the cofactor reverses; in the early S-state transitions (e.g. S_0 to S_1) electron transfer precedes proton transfer whereas in the late transitions (e.g. S_2 to S_3) proton transfer precedes electron transfer. As a consequence, Y_Z can be thought of having a dual role: i) it acts as an electron carrier; and ii) it promotes deprotonation of the catalyst which is presumably facilitated via the intervening H-bonding network.

The XFEL structure described above represents the resting state of the catalyst (S_1) [1], although it may contain some admixture of the S_0 state [5]. It is also thought to be a good model for the S_2 state as EXAFS results for the S_0 , S_1 and S_2 are all very similar and progression from the S_1 to the S_2 state can occur at low temperatures (< 200 K), where the protein's conformation should be fixed. The same however cannot be said of the S_3 state, the last metastable intermediate of the reaction cycle. Recent pulse EPR data has demonstrated that in this state all four Mn ions are structurally and electronically *similar*: they all exhibit a formal oxidation state 4+ and octahedral local geometry [28]. This requires the inclusion of an additional water-derived ligand at the open coordination site of Mn1 rendering all four Mn ions six-coordinate [28,29]. Importantly the proposed activated structure locates two oxygens 2.4 Å apart along the Mn1-Mn4 axis, the two likely substrates of the water splitting reaction, one of which is O5 in line with experimental results described above [7,30].

The question arises as to how this structure evolves from the resting state of the catalyst captured using X-ray crystallography. As indicated above the position of O5 is not fixed but toggles between two positions, as an oxygen bridge between Mn3 and Mn4, as seen in the X-ray structure (A form), and as a vertex of the cubane unit (B form) [9,21] (**Fig. 2c**). O5 does not display this property in all S-states, but instead evolves towards this dynamic state in the S_2 state, as it is only in this state that O5

movement can be coupled with valence isomerism within the cluster [9,21]. It is this labile nature of O5 that provides a possible mechanism via which the solvent inaccessible Mn1 is then connected to the Asp61 water channel described in the previous section (**Fig. 4**). Briefly, the shift of O5 moves the open coordination site of the cofactor to the solvent accessible Mn4 [25,31]. This site is then filled by the rotation of the water ligands of Mn4 (W2/W1, S_3'), with binding of a new water molecule via the Aps61 channel [25]. We note the Ca^{2+} channel could also provide the new water molecule. Subsequently, the protonation of the two water derived oxygens on the Mn1-Mn4 axis interchanges leading to the final activated catalyst structure (S_3^A) [32]. Importantly this pathway explains earlier spectroscopic results that suggested the net solvation increases in the S_2 to S_3 transition [12,33].

Fig. 4 Self consistent structures of the $\text{Mn}_4\text{O}_5\text{Ca}$ cofactor during its catalytic cycle developed on the Mülheim campus [5,25,34]. Chemical models shown rationalize many experimental constraints and reproduce all structural and spectroscopic observables. Substrate oxygens are shown in green: O5 and the rotated W2 Mn4 ligand [12,25,34]. 2D electronic coupling maps of the cofactor are shown below each structure, showing how the spin state of the cofactor evolves from low spin configuration in the resting states (dominantly antiferromagnetic coupling between adjacent Mn sites, orange pathways) to high spin configuration in the active states (dominantly ferromagnetic coupling between adjacent Mn sites, grey pathways).

It is likely that the sequential uptake of the substrate waters, with the delayed repositioning of the 2nd substrate, is critical for efficient catalytic function yielding a

single product. While the sequence described above provides a plausible access pathway for substrate binding/inclusion, the critical question is how this process is regulated. Experimental and theoretical results point to a gating mechanism involving proton shuffling from W1 to the Asp61 [31,35-37], potentially by a rearrangement of the net electric dipole of the cofactor induced by Y_Z oxidation [38]. This redox tuning event then allows cofactor oxidation and subsequently substrate binding to the Mn4, which in the 4+ oxidation state is a better Lewis acid [25,34]. In this way connections between the conversion of the catalyst from its resting to its active form with the two phases of the catalytic cycle (**Figs. 3 and 4**) can begin to be made. In the resting-like states (**Fig. 4**, low panel: S_0^A , S_1^A , S_2^A) the two putative substrates are kept away from each other and the cofactor is uncoupled from the protein surrounding i.e. *ET-PT*. In contrast, in the active-like states (**Fig. 4**, upper panel: S_2^B , S_3' , S_3^B) the two putative substrates are proximal to each other and the cofactor is coupled to the protein surrounding i.e. *PT-ET*. It is also noted that the two basic forms of the catalyst have a different electronic structure as evidenced by their ground spin state [34]. The resting state forms (S_0^A , S_1^A , S_2^A) are all low spin whereas the active forms (S_2^B , S_3' , S_3^B) of the catalyst are high spin. The significance of this change in the electronic structure, triggered by the S_2 to S_3 water binding step, is not yet understood but it has been suggested as critical for stabilization of the O-O bond forming transition state [28,34].

The O-O bond formation step: While recent results described above do not yet constrain the mechanism of water oxidation, they do provide a solid foundation to address this question. Importantly they point towards a mechanism involving the two proximal Mn-bound oxygens, seen in the activated catalyst structure, which are located along the Mn1 and Mn4 axis. There are two popular proposals in the literature explaining how the O-O bond formation could proceed, either by: i) a radical coupling of the two oxygens as proposed by Siegbahn [30] or instead ii) an acid base type reaction involving nucleophilic attack [39]. Current experimental data favor a coupling-type reaction, involving oxidation of one of the substrate oxygens, in part because no Mn^V intermediate has yet been observed [40]. The challenge though is to devise new experiments to better tease apart the catalytic phase of the cycle (**Fig. 3**, green area) to conclusively answer this question; namely to address what is the nature of the deprotonation step to form the $S_3^n(Y_Z^{\bullet})$ state and what is the subsequent fate of this state. This however remains difficult. Obvious routes to capture these

intermediates, such as using high external O₂ pressures do not affect photosynthetic oxygen production [41-43], requiring the equilibrium constant for the S₄ → S₀ transition to be large ($>1.0 \times 10^7$) [44]. A more promising approach is to use structurally perturbed systems in which the catalyst activity is diminished [45]. These include site directed mutants (e.g. Asp61, Val185) or replacement of the redox-inactive ions (the exchange of the Ca²⁺ ion for Sr²⁺, the Cl⁻ ion(s) for Br⁻ etc) which have been shown to slow the kinetics of O-O bond formation by a factor of up to 100 fold [46,47]. This latter approach has allowed water exchange to be studied during the catalytic phase (**Fig. 3**). Interestingly substrate exchange is arrested in S₃ⁿ(Y_Z[•]), favoring a O-O bond mechanism involving substrates that are tightly bound immediately prior to O-O bond formation [47]. This would be consistent with the proposed substrate identities described above i.e. O5 and rotated W2 [12,25,34].

A molecular description of why these structural modifications slow the kinetics of O₂ production is not immediately obvious. In these systems the intrinsic O-O bond formation step could be slower, or these changes could instead act to retard the advancement of the cofactor through its cycle (**Fig. 4**), by decoupling cofactor tautomerism from proton release and 2nd substrate inclusion. Clearly further spectroscopic characterizations of these perturbed systems are needed. Of these, new X-ray methods using free electron laser sources have the potential to provide key information, supplementing data already available on the structural and electronic evolution of the native cofactor during the catalysis phase of the cycle [48]. The goal for the future is to determine complete structures of short lived intermediates of the whole catalytic cycle, as free electron laser sources allow diffraction patterns to be collected within femtoseconds of X-ray exposure, providing snapshots of the O-O bond formation step even at ambient temperature [48,49].

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