Bicarbonate-controlled reduction of oxygen by the Q$_A$ semiquinone in Photosystem II in membranes

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Edited by Eva-Mari Aro, Biochemistry, Molecular Plant Biology, Turun yliopisto, Turku, Finland; received August 31, 2021; accepted December 23, 2021

Photosystem II (PSII), the water/plastoquinone photo-oxidoreductase, plays a key energy input role in the biosphere. Q$_A^-$, the reduced semiquinone form of the nonexchangeable chlorophyll, is often considered capable of a side reaction with O$_2$, forming superoxide, but this reaction has not yet been demonstrated experimentally. Here, using chlorophyll fluorescence in plant PSII membranes, we show that O$_2$ does oxidize Q$_A^-$ at physiological O$_2$ concentrations with a $t_{1/2}$ of 10 s. Superoxide is formed stoichiometrically, and the reaction kinetics are controlled by the accessibility of O$_2$ to a binding site near Q$_A^-$, with an apparent dissociation constant of 70 ± 20 μM. Unexpectedly, Q$_A^-$ could only reduce O$_2$ when bicarbonate was absent from its binding site on the nonheme iron (Fe$^{2+}$), and it was blocked by the O$_2$-dependent decay of Q$_A^-$.

Significance

In Photosystem II (PSII), O$_2$ reduction by Q$_A^-$ is often discussed but has not been demonstrated. Here, we show in PSII membranes that Q$_A^-$ can reduce O$_2$ to superoxide, but only when bicarbonate is absent from its binding site on the nonheme Fe$^{2+}$. Bicarbonate’s role in PSII was recently shown to involve a regulatory/protective redox-tuning mechanism linking PSII function to CO$_2$ concentration. A key aspect is the presence of stable Q$_A^-$ causing release of bicarbonate from its site on Fe$^{2+}$. Here, we show that under these conditions, O$_2$ binds to the empty site on the Fe$^{2+}$ and is reduced by Q$_A^-$. This unexpected reaction may be a further indication of cross-talk between the regulation of PSII and CO$_2$ fixation.

Author contributions: A.F., V.R.I.K., and A.W.R. designed research; A.F., F.A., H.B., G.M., W.K.T., and A.P.G.-H. performed research; A.F., F.A., H.B., G.M., W.K.T., and A.P.G.-H. contributed new reagents/analytic tools; A.F., F.A., H.B., G.M., W.K.T., A.P.G.-H., V.R.I.K., and A.W.R. analyzed data; and A.F., V.R.I.K., and A.W.R. wrote the paper. The authors declare no competing interest.

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This article contains supporting information online at http://www.pnas.orglookup/suppl/doi:10.1073/pnas.2116063119/-/DCSupplemental.

Published February 3, 2022.
approaches. The results indicate the reaction constitutes an unexpected regulatory mechanism involving bicarbonate.

Results

Q_{A}^{*} Oxidation by O_{2}. Fig. 24 (orange trace) shows the stability of Q_{A}^{*} generated in low-PQ PSII membranes (SI Appendix, Fig. S1) under anaerobic conditions and with the concentration of Q_{A}^{*} monitored using chlorophyll fluorescence (17, 18). To generate this state, a dark-adapted sample was illuminated with three saturating flashes in the presence of the exogenous electron donor, NH_{3}OH, at 250 μM, a concentration that was sufficient to donate electrons to the Mn cluster but low enough to avoid overreduction and loss of the Mn cluster in the majority of centers (SI Appendix, Materials and Methods, and, for example, ref. 19). This treatment resulted in the trapping of stable Q_{A}^{*} in ~50% of the centers, based on a comparison to maximum fluorescence. The decay of Q_{A}^{*} in the other centers is mainly due to 1) forward electron transfer in the millisecond timescale (18) as a result of the incomplete removal of the PQ pool and 2) charge recombination (S_{2}Q_{A}^{*} and S_{2}Q_{B}^{*}) in the seconds timescale (20) in centers where electron donation from NH_{3}OH was insufficient to eliminate back reactions. In the absence of O_{2}, the stable fluorescence signal from Q_{A}^{*} showed a slow decay with a half-time of ~600 s. A decay rate for Q_{A}^{*} with similar kinetics has previously been correlated with the decay of Tyr_{D}, attributed and attributed to charge recombination between these two states (21), which could explain the slow decay of Q_{A}^{*} observed here.

The decay of the trapped Q_{A}^{*} state was significantly accelerated when O_{2} was added back to the medium (Fig. 24), which provided clear experimental evidence for the reduction of O_{2} by Q_{A}^{*}. Fig. 24 also shows that the rate of Q_{A}^{*} oxidation accelerated as the O_{2} concentration increased. The observed fluorescence decays were found to be biphasic, with a fast exponential decay rate that depended on the O_{2} concentration and a slow decay rate that was independent of the O_{2} concentration. The slower phase corresponded to that observed in the absence of oxygen. The kinetic data were fitted using a linear combination of the fast exponential phase and a slow, linear decay,

\[ f = e^{(k_{\text{obs}} t)} + (b \times t) + c \]  

where \( k_{\text{obs}} \) is the pseudo first-order rate constant for Q_{A}^{*} oxidation at a fixed O_{2} concentration, \( b \) is the slope, and \( c \) is the intercept of the linear decay observed in the absence of O_{2}. This biphasic behavior indicated heterogeneity in the preparation, where most of the centers were susceptible to reactions with O_{2}, but a smaller fraction remained unreactive. We demonstrated in the following that this heterogeneity appears to be related to bicarbonate binding to the nonheme iron. We investigated whether the effect of “connectivity” influenced the kinetics observed here in Fig. 2. Connectivity is a phenomenon seen in fluorescence when PSII shares an extended antenna, leading to a situation where there is a significant probability that excitation energy can visit more than one closed center before finding an open PSII. This results in a nonlinear relationship between fluorescence intensity and Q_{A}^{*} concentration (22, 23). We found that connectivity has a negligible effect on the present experiment (SI Appendix, Fig. S2).

Fig. 2B shows the observed rate constants (\( k_{\text{obs}} \)) for the fast, exponential phase of the Q_{A}^{*} decay, measured in pseudo first-order conditions and plotted as a function of the added concentration of O_{2}. The plot shows saturation behavior characteristic of the presence of a discrete binding site. This binding site becomes saturated above ~150 μM O_{2} (SI Appendix, Fig. S3). The data were fitted with a hyperbolic curve (Eq. 2) from which the apparent K_{d} was calculated to be 70 ± 20 μM and a rate constant, at physiological O_{2} concentrations, to be 0.07 s^{-1}, corresponding to a half-time of ~10 s (10.6 ± 0.8 s). The pseudo first-order rate constant \( k_{\text{obs}} \) for Q_{A}^{*} reoxidation at a fixed oxygen concentration was estimated as

\[ k_{\text{obs}} = \frac{\text{Fraction of Q}_{A}^{*}}{\text{Fraction of Q}_{A}^{*} - \text{Fraction of Q}_{A}^{*}} \times \frac{1}{\text{Time (s)}} \]
Influence of Herbicides on the Reactivity to Oxygen. To test if the driving force determines the rate of the electron transfer, kinetics was measured by modulating the reduction potential of Q$_{A}$/Q$_{A}^*$ by binding herbicides into the Q$_{B}$ site.

Different classes of herbicides shift the potential in different directions: by +50 mV (−144 mV to −94 mV) with 3,3′-dichlorophenyl)-1,1-dimethyurea (DCMU), and by −50 mV (−144 mV to −194 mV) with bromoxynil (24). These herbicide-induced shifts in the Q$_{A}$/Q$_{A}^*$ E$_{m}$ result in differential effects on the kinetics of Tyr D phosphate recombination (SI Appendix, Fig. S4), thereby confirming herbicide binding under our experimental conditions. Fig. 2C shows the kinetics of Q$_{A}^*$ reoxidation at 110±5 μM O$_{2}$ concentration. With DCMU, the reaction was essentially blocked, whereas with bromoxynil, the reaction was virtually unaffected. Similar behavior was observed when the experiment was performed at lower O$_{2}$ concentration (40±3 μM) (SI Appendix, Fig. S5). The effect of DCMU appears to be too drastic to be due to the small change in the Q$_{A}$/Q$_{A}^*$ reduction potential, and it is thus attributed to a structural effect of DCMU binding rather than a redox effect on the quinone (in the following, we discuss DCMU binding having a redox effect on the non-heme iron and on the binding of bicarbonate as viable options to explain its effect). The lack of a bromoxynil effect, on the other hand, suggests that the rate of O$_{2}$ reduction by Q$_{A}^*$ is not determined by the thermodynamic driving force between Q$_{A}$/Q$_{A}^*$ and O$_{2}$/Q$_{A}^*$*. As bromoxynil occupies the Q$_{B}$ site (bicarbonate binding data on flash 1 is provided in SI Appendix, Fig. S6B), this result eliminates the possibility that Q$_{A}^*$ or QaH$_{2}$ donates electrons to O$_{2}$, allowing the Q$_{A}^*$ to decay by forward electron transfer.

Influence of Bicarbonate on Q$_{A}^*$ Reoxidation Kinetics. The degassing used to make the PSII anaerobic is also expected to remove CO$_{2}$ and, at this pH (pH 6.5), bicarbonate from solution. The degassing is thus expected to result in partial loss of bicarbonate from PSII. Fig. 2D shows that addition of bicarbonate after degassing the sample, eliminated electron transfer from Q$_{A}^*$ to O$_{2}$. A control experiment using 1 mM NaCl instead of NaHCO$_{3}$ had no effect on the kinetics of Q$_{A}^*$ oxidation by O$_{2}$ (SI Appendix, Fig. S7A). Addition of bicarbonate also blocked Q$_{A}^*$ reoxidation by O$_{2}$ in the presence of the herbicides (Fig. 2B and SI Appendix, Fig. S8).

Fig. 2D thus suggests that bicarbonate binding to the non-heme Fe$^{2+}$ controls electron transfer from Q$_{A}^*$ to O$_{2}$. To test the specificity of this effect, sodium formate, which is known to bind in the bicarbonate site on the nonheme Fe$^{2+}$, was added (SI Appendix, Fig. S7B). Formate, at 100 mM, a concentration known to be competitive with bicarbonate (25, 26), behaved like bicarbonate; it prevented Q$_{A}^*$ from reducing O$_{2}$ (SI Appendix, Fig. S7B). Because the effect is not specific to bicarbonate, this suggests electron transfer from Q$_{A}^*$ to O$_{2}$ requires that nonheme Fe$^{2+}$ lacks the carboxylic acid and thus that the O$_{2}$ is reduced when bound to the Fe$^{2+}$.

Superoxide as the Product of the Reaction. The electron paramagnetic resonance (EPR) spin trap 5-(diisopropoxophosphoryl)-5-methyl-1-pyrrole-N-oxide (DIPPMPO) was used to demonstrate O$_{2}^*$ formation under continuous illumination (Fig. 3A). This spin trap forms trapped radical adducts with either OH$^*$ or the more reactive OH$^*$, but these can be distinguished by their different EPR spectra (27). The experiments in Fig. 3A were done in the presence of catalase, which removes peroxide (formed by dismutation of the O$_{2}^*$ ), which could give rise to OH$^*$ by Fenton chemistry. The presence of residual OH$^*$ forms a spectroscopically distinct adduct with DIPPMPO (28); it can be deconvoluted from the O$_{2}^*$ spin adduct and quantified as being less than 20% of the OH$^*$ adduct (SI Appendix, Figs. S9 and S10 EPR deconvolution).

Fig. 3A shows that the concentration of O$_{2}^*$ formed under these conditions was diminished by 30−50% when the experiment was done in the presence of 12 mM bicarbonate. The higher bicarbonate concentration was used to maintain the bicarbonate ratio to PSII in the EPR experiment, in which 12 times more PSII is required to obtain appropriate signal to noise.

The spin-trapping EPR experiment showing the formation of O$_{2}^*$ was insufficiently sensitive to allow its exact quantification. A more sensitive approach is to monitor the reduction of cytochrome c (cyt c) by O$_{2}^*$ by its absorption change at 550 nm (29). Fig. 3B shows the amount of reduced cyt c from the reaction of O$_{2}^*$ with O$_{2}$, in a sample containing 60 nM PSII. The sample was treated as described for the fluorescence kinetics measurements (Materials and Methods). The experiment was performed in the presence and absence of 1 mM bicarbonate, and the amount of reduced cyt c, which is equivalent to the

$$
\frac{k_{obs}}{k_{sat}} = \frac{k_{app}}{(K_{app} + [O_2])}
$$

where $k_{sat}$ is the rate constant when the binding site is fully occupied, and $K_{app}$ is the apparent dissociation constant. The second-order rate constant, calculated from the linear fit, was found to be $340 \pm 83 \text{ M}^{-1} \text{s}^{-1}$. 
The amount of \( \text{O}_2^- \) generated, was found to be 32 ± 16 nM and 68 ± 16 nM respectively. The bicarbonate-dependent decrease in the amount of reduced cyt c corresponds to ~50% of the concentration of PSII and thus approximately the same amount of \( \text{Q}^- \) present prior to \( \text{O}_2 \) addition. This confirms that \( \text{Q}^- \) decay upon the addition of \( \text{O}_2 \) is due to stoichiometric \( \text{Q}^- \) formation.

Control experiments showed that the direct reduction of cyt c by PSII was negligible, consistent with previous reports (e.g., ref. 30). Interference by \( \text{H}_2\text{O}_2 \) and OH* on the cyt c reduction was prevented by the presence of catalase (Materials and Methods).

The residual cyt c reduction occurring after the addition of bicarbonate does not arise from the \( \text{Q}^- \) present prior to \( \text{O}_2 \) addition, as all of that is accounted for by the bicarbonate-sensitive \( \text{Q}^- \). Other electron transfer components have been suggested as possible reductants of \( \text{O}_2 \) forming \( \text{O}_2^- \), including cyt \( b_{599} \) (31) and \( \text{Q}_b\text{H}_2 \) (14) as well as the \( \text{PQH}_2 \) pool (15, 32), which could be present as a residual in our low PQ preparation. Furthermore, small amounts of contaminant Photosystem I could represent another possible source of \( \text{O}_2^- \). While such effects would be expected to occur to a minor extent in the dark, they could also be driven by an actinic effect of the measuring flashes. Despite their weak intensity, their frequency might result in sufficient photochemistry during the time course of the experiment to account for the observed residual \( \text{O}_2^- \)-mediated cyt c reduction (SI Appendix, Fig. S11). This would be consistent with the observation that experiments run in the presence of DCMU show a further decrease in the amount of reduced cyt c.

**O\(_2^-\) Production When Q\(^-\) Is Generated by Continuous Illumination.**

The \( \text{O}_2^- \) accumulation was also studied by cyt c reduction under constant illumination (SI Appendix, Fig. S12). After 2 min of illumination (50 \text{µmol} \text{photons} \, \text{m}^{-2} \, \text{s}^{-1} \, \text{red light}), an average of 736 ± 10 nM reduced cyt c was generated, and when 1 mM \( \text{HCO}_3^- \) was present, this was diminished by approximately half (351 ± 60 nM). This is consistent with what we observed in the single flash stoichiometric experiments. When the same experiment was done by using 20 min of illumination without \( \text{HCO}_3^- \), an average of 2,076 ± 10 nM reduced cyt c was generated, while in the presence of \( \text{HCO}_3^- \), 60 nM reduced cyt c was formed. The absence of \( \text{HCO}_3^- \) on decreasing the formation of reduced cyt c seemed to diminish at longer illumination periods, suggesting that other routes for production of \( \text{Q}^- \), such as cytochrome \( b_{599} \) and \( \text{PQH}_2 \) and perhaps involving \( \text{1} \text{O}_2 \), might play a role upon prolonged illumination. Furthermore, the prolonged illumination leads to an increased probability of \( \text{Q}^- \) being formed in turn favors bicarbonate dissociation even in the presence of 1 mM bicarbonate in solution (33, 34).

When the DCMU was present in the absence of added bicarbonate, 2 min of illumination resulted in hardly detectable amounts of reduced cyt c, while after 20 min of illumination, the amount was 626 ± 70 nM (SI Appendix, Fig. S12). These results differ from those shown in Fig. 2B, where DCMU was found to be less effective than bicarbonate for inhibiting the \( \text{Q}^- \) reduction of \( \text{O}_2 \) in the dark. The more pronounced effect of DCMU under continuous light could result from other reactions generating reactive oxygen species (ROS), such as long-lived Phco2*- and/or Chl-mediated \( \text{O}_2 \) formation.

**Mechanism of \( \text{Q}^-\) Reduction of \( \text{O}_2 \).** To probe possible molecular mechanisms of \( \text{O}_2 \) reduction, we performed quantum chemical density functional theory (DFT) calculations and hybrid quantum mechanics/molecular mechanics (QM/MM) molecular dynamics (MD) simulations to assess the electronic structure of the nonheme iron site and its reactivity with molecular oxygen (Fig. 4 and SI Appendix, Fig. S13). In our DFT models, we first optimized the structure of the nonheme iron site with either \( \text{HCO}_3^- \) or an \( \text{O}_2 \) ligand bound to the \( \text{Fe}^{2+} \) ion or in the absence of either ligand. The DFT models suggested that the \( \text{O}_2 \) can bind with a high affinity (~4 kcal mol\(^{-1} \)) to the nonheme \( \text{Fe}^{2+} \), forming a 2,4-A covalent bond between the \( \text{Fe}^{2+} \) and the \( \text{O}_2 \). We next added an electron on \( \text{Q}^- \) and reoptimized the models. The DFT calculations suggested that the electron was localized on \( \text{Q}^- \), forming \( \text{Q}^+ \) in the \( \text{HCO}_3^- \)-bound form and when no exchangeable ligand on the iron was modeled (Fig. 4A). In contrast, in the dioxygen-bound form, the electron instantly moved from \( \text{Q}^+ \) to \( \text{O}_2 \) during the calculations, leading to formation of an \( \text{Fe}^{2+} \) – \( \text{O}_2^* \) species (Fig. 4B – D). By varying the \( \text{Fe} – \text{O}_2 \) distance, we observed that the electron transfer from \( \text{Q}^+ \) was triggered at around 2.6 Å, when a chemical bond forms between the dioxygen and iron (SI Appendix, Fig. S14).

The findings thus suggest that the nonheme iron catalyzes \( \text{O}_2 \) reduction/superoxide formation (Fig. 4B and D). Our calculations also suggested that the presence of \( \text{Q}^+ \) led to weakening of the \( \text{HCO}_3^- \) affinity for the iron due to electrostatic repulsion, in accordance with experimental evidence (33). The calculations also support that the \( \text{Fe}^{2+} \) form of the nonheme iron can still bind \( \text{HCO}_3^- \), whereas both \( \text{O}_2 \) and \( \text{Q}^+ \) favor the \( \text{Fe}^{2+} \) form (SI Appendix, Fig. S15).

Hybrid QM/MM MD simulations were also done to study the dynamics of the electron transfer process in more detail, including the effects of temperature (Fig. 4B and D, SI Appendix, Materials and Methods). This was done by initiating the simulations from a state in which \( \text{Q}^+ \) was relaxed in classical molecular dynamics (MD) simulations. The reduction of \( \text{Q}^+ \), tightened the local hydrogen bonds between the plastoquinone and His214/Phc261 relative to the neutral \( \text{Q}^- \) state, which stabilized the reduced form by further subtle conformational changes in the surrounding helices. Consistent with our results obtained from the DFT models, we found that \( \text{Q}^- \) remained reduced throughout the 2 ps QM/MM MD simulation when \( \text{HCO}_3^- \) was bound or when no exchangeable ligand on the iron was modeled. In contrast, the electron was rapidly transferred during the initial 0.2 ps to the Fe-bound \( \text{O}_2 \), forming a \( \text{Fe}^{2+} – \text{O}_2^* \) species.

To probe structural effects linked to dissociation of \( \text{HCO}_3^- \) on longer nano-to-microsecond timescales, we performed classical atomistic MD simulations of PSII embedded in a lipid membrane, with the \( \text{Q}^- \) site modeled either as oxidized or as semiquinone, \( \text{Q}^+ \). During the 200 ns MD simulations, we observed two water-filled tunnels, 1 and 2, that connect the stromal side of the membrane to the nonheme Fe center, while the \( \text{Q}^- \) site remained sequestered from water molecules (Fig. 4E–G, SI Appendix, Fig. S15). In simulations without an exchangeable ligand modeled on the nonheme iron, we observed a significant increase in the hydration state of the tunnels compared to the simulations performed when the \( \text{HCO}_3^- \) was bound (Fig. 4G). Conformational changes around Lys264 seem to regulate the accessibility of the tunnel to the nonheme iron site (Fig. 4E and F, SI Appendix, Fig. S15 A and B). We note that the water-filled tunnels are large enough for dioxygen diffusion into the site. These findings fit with the model in which occupation of the binding site on the nonheme iron prevents \( \text{O}_2 \) from binding there.

**Discussion**

**Electron Donation from Q\(^-\) to \( \text{O}_2 \).** Here, we trapped the \( \text{Q}^- \) state in PSII membranes with a depleted PQ pool by illuminating in the presence of low concentrations of the electron donor, \( \text{NH}_4\text{OH} \), under anaerobic conditions. The \( \text{Q}^- \) is stable in a significant fraction of centers because forward electron transfer is blocked due to \( \text{Qb} \) being reduced or absent in the PO-depleted PSII. In addition, \( \text{Q}^- \) cannot recombine with the stable \( \text{S}_0 \) or \( \text{S}_1 \) states on the minutes timescale of the
experiment (5). The presence of $Q_{A}^\cdot$ was monitored using chlorophyll fluorescence (17, 35). This “trapped” $Q_{A}^\cdot$ undergoes a slow decay ($t_{1/2} = 5$ min) that may correspond to the recombin-
ation of the electron on $Q_{A}^\cdot$ with a relatively stable oxidized species, such as Tyr D (21) (Fig. 2A).

We used this experimental system to test if the trapped $Q_{A}^\cdot$ would react with added $O_2$. Upon addition of $O_2$, the lifetime of the trapped $Q_{A}^\cdot$ was found to decrease, with the reaction rate increasing with increased $O_2$ concentration and saturating above ~150 μM $O_2$ (SI Appendix, Fig. S3). The kinetics of the $O_2$-induced $Q_{A}^\cdot$ decay showed a single exponential curve, consistent with a pseudo first-order reaction at a fixed $Q_{A}^\cdot$ concentration, with a transition to a zero-order reaction above 150 μM $O_2$, thus resulting in a half-time of ~10 s under physiological $O_2$ concentrations (270 μM) (Fig. 2B).

The reaction rate did not appear to be affected by the thermodynamic driving force, as judged by the lack of effect of the phenolic herbicide, bromoxynil, which is known to lower the $E_m$ of the $Q_A/Q_{A}^\cdot$ redox couple (24) (Fig. 2C and SI Appendix, Fig. S4 TyrD $Q_{A}^\cdot$ recomb). DCMU binding is expected to increase the $E_m$ of the $Q_A/Q_{A}^\cdot$ couple and thus potentially slow down the $O_2$ reduction. However, DCMU binding strongly inhibited the electron transfer from $Q_{A}^\cdot$ to $O_2$ (Fig. 2C). The DCMU inhibition was so significant that it seems unlikely to result from the predicted +50 mV change in reduction potential. Possible explanations for the DCMU-induced inhibition of $O_2$ reduction by $Q_{A}^\cdot$ include minor structural/conformational effects, redox effects on the non-heme iron, which may affect bicarbonate binding (see The Nonheme Iron as the Binding Site for $O_2$).

Fig. 4. $O_2$ reduction mechanism, bicarbonate binding, and accessibility of the nonheme Fe site of PSII. Structure and spin densities from DFT models of the (A) $HCO_3^-$ bound and (C) $O_2$-bound forms of the nonheme iron (the state without $HCO_3^-$ is shown in SI Appendix, Fig. S13G). The figure shows the spin density difference (red/blue sphere ±0.001e for alpha/beta spin) after adding an electron into the system, with only the $Q_A$ and iron/histidine residues shown for clarity (SI Appendix, Fig. S13). (B) Spin population during the QM/MM MD simulations for $HCO_3^-$ bound (in blue) and without $HCO_3^-$ bound (in orange) and the $O_2$-bound forms (in red) on the $Fe^{3+}$ (solid lines), $Q_A$ (dotted lines), and $O_2$ (dashed line). (D) The O-O bond length during electron transfer from $Q_A$. (E and F) Snapshot at 150 ns of the water-filled tunnels formed around the $Q_A$ site during MD simulations in (E) with the $HCO_3^-$ bound form and (F) without the $HCO_3^-$. (G) Histogram of water molecules in tunnels 1 and 2 connecting to the iron from the bulk solvent. The bicarbonate blocks water entry to the nonheme iron site.
Bicarbonate and Formate Inhibition of $O_2$ Reduction. Under the conditions of the fluorescence measurements, the bicarbonate was expected to be depleted from its binding site on the nonheme Fe$^{2+}$ because 1) the degassing of the sample to make it anaerobic is expected to lower the concentration of CO$_2$ and thus of the bicarbonate; and 2) the presence of Q$^*$ greatly decreases the affinity of the bicarbonate for its binding site on the nonheme iron (33). Therefore, we tested the effect of adding back bicarbonate prior to the addition of O$_2$. Bicarbonate readdition is expected to lower the $E_m$ of Q$_A$/Q$^*_A$ and thus to increase the driving force for O$_2$ reduction. Instead, we found that bicarbonate addition completely blocked electron transfer from Q$^*_A$ to O$_2$ (Fig. 2D). This finding suggests that O$_2$ reduction occurs when it is bound to the nonheme iron in the empty bicarbonate site.

Formate had the same effect as bicarbonate. This lack of formate/bicarbonate specificity contrasts with the specific roles of bicarbonate in optimizing proton-coupled electron transfer (26, 34, 36) and in redox tuning for regulation and photoprotection (33), roles that are not shared by formate. This lack of specificity of bicarbonate-versus-formate binding is consistent with the inhibition of O$_2$ reduction being a steric effect, with either bicarbonate or formate binding occluding the O$_2$ binding site on the nonheme iron. The occlusion of the nonheme iron binding site by bicarbonate was also indicated by the MD simulations.

Formation of Superoxide. EPR spin trapping and cyc d reduction confirmed that $Q^*_A$ was the product of the reaction of Q$^*_A$ with O$_2$ (Fig. 3) and that this reaction was blocked by bicarbonate addition. The cytochrome reduction method allowed the O$_2$ to be quantified, and its concentration was found to be close to that of the Q$_A$$. A second fraction of O$_2$ was formed in the dark in a reaction that was not inhibited by bicarbonate addition and was unrelated to the oxygen-dependent decay of Q$^*_A$. We have not characterized the electron source of this fraction of O$_2$ formation, but we note that there are several possible candidates, including PO$_4$, bound to PSII or in the membrane, cyt b$_{599}$, and PSI (14, 15, 31, 32).

Physiological Significance: Rates and Conditions. (In Redox and Mechanistic Considerations, we discuss the possibility of faster rates of O$_2$ reduction by Q$^*_A$, but in this section, we discuss the slower rate measured using the experimental approach used here.) The rate of electron transfer from Q$^*_A$ to O$_2$ ($t_{1/2} \sim 10$ s) implies that under conditions where the Q$_B$ and quinone pool are oxidized, the reduction of O$_2$ is too slow to compete with the forward electron transfer rate of $t_{1/2} \sim 1$ ms (35) even when bicarbonate is absent (34). When the quinone pool is fully reduced, a large proportion of S$_2$Q$^{*+}$ and S$_3$Q$^{*+}$ will recombine with a $t_{1/2} \sim 1.5$ s (20), i.e., much faster than the rate of electron transfer of the Q$^*_A$ to O$_2$ reaction reported here. However, when forward electron transfer from Q$^*_A$ is blocked in centers where either S$_1$ or S$_0$ are present, the reaction with O$_2$ could be the dominant Q$^*_A$ reoxidation pathway, provided the bicarbonate is absent from its binding site on the nonheme iron. Such conditions are likely to occur when CO$_2$ levels are limiting, as previously discussed (33).

Physiological conditions other than those where the PQ pool is reduced are likely to exist in which electrons are trapped on Q$^*_A$ long enough to allow bicarbonate release and reduction of O$_2$ to be a relevant reaction. These circumstances could include those associated with assembly and photoactivation of PSII, photoinhibition, and repair, in which Q$^*_A$ is longer lived (9, 37–39). An intermediate state in PSII assembly is structurally modified by the binding of assembly factors (Psb27, Psb28, and Psb34) that cause the bicarbonate site on the nonheme iron to be occupied instead by a glutamate (39). This iron coordination mirrors the situation that exists in the purple bacterial reaction centers. From the present results, we expect such assembly intermediates of PSII to be unable to reduce O$_2$ from Q$^*_A$. As the Q$_B$ site is significantly modified in this assembly intermediate, it seems likely that Q$^*_A$ will recombine with $\text{TyRC}(\text{H}^+)$ via a direct tunneling step between P$^+$ and Q$^*_A$, given the high potential of the Q$_B$/Q$^*_A$ couple prior to photo-assembly of the M$_{45}$CaO$_{12}$ cluster (9, 39).

The Nonheme Iron as the Binding Site for O$_2$. The single saturable site for O$_2$ reduction and its complete inhibition by bicarbonate indicate that the nonheme Fe$^{2+}$ is the O$_2$ binding and reduction site. DFT and QM/MM calculations reported here further support this assignment. Our calculations showed that reduction of O$_2$ by Q$^*_A$ is favorable when oxygen binds to the Fe$^{2+}$ in the absence of bicarbonate binding to that site. This result contrasts with a previously proposed mechanism involving direct oxidation of Q$^*_A$ by O$_2$ (12–14), which is expected to require close contact between the oxygen and the semiquinone (40). The crystal structure (41) and the MD simulations indicate that this is unlikely because the Q$_B$ is not exposed to the solvent.

In enzymes, transition metals and Fe$^{2+}$ in particular often activate O$_2$ for reduction, overcoming the intrinsic spin-orbit coupling that makes triplet state O$_2$ anomalously stable. It seems that this is also the case in PSII. This mechanism seems reasonable given that bicarbonate dissociates from the iron when Q$^*_A$ is long-lived (33).

The DCMU inhibition of O$_2$ reduction may also be taken as an indication that the nonheme Fe$^{2+}$ is the O$_2$ binding site. DCMU binding shifts the redox potential of the Fe$^{3+}$/Fe$^{2+}$ couple 120 mV to higher values, while other herbicides/inhibitors (atrazine and o-phenanthroline) induced much smaller shifts (42–44). Whether the DCMU inhibition of O$_2$ reduction reflects a perturbation of the electronic structure of the Fe$^{2+}$ (as manifest by the redox shift), minor structural changes, or both of these is unclear. These effects could be responsible for DCMU inhibiting bicarbonate binding and dissociation (45, 46). Given the conditions of our experiment, it is possible that DCMU binding prevents bicarbonate loss, leaving the nonheme Fe$^{2+}$ site occupied. This would explain the lack of O$_2$ reduction (Fig. 2). The observation that addition of bicarbonate to a DCMU-treated sample eliminates the residual O$_2$ reduction (SI Appendix, Fig. S10) is consistent with bicarbonate depletion being a steric effect, with either bicarbonate or formate binding occluding the O$_2$ binding site on the nonheme iron. The occlusion of the nonheme iron binding site by bicarbonate was also indicated by the MD simulations.

Redox and Mechanistic Considerations. Nominally, the electron transfer from Q$^*_A$ to O$_2$ is thermodynamically unfavorable, based on the $E_m$ of Q$_A$/Q$^*_A$ = $-144$ mV, a value that is shifted to $-70$ mV in the absence of bicarbonate (33), and thus, it is more oxidizing than $E_m = -160$ mV for O$_2$/Q$^*_A$ (48). However, given the very low concentration of O$_2^*$ compared to O$_2$, its functional potential is likely to be closer to $-\sim 0$ mV (13), rendering the overall process thermodynamically favorable. In addition, it seems likely that the binding of the O$_2$ to the iron will change the redox potential of the O$_2$/Q$^*_A$ couple.

Given the thermodynamic driving force and the $\sim 8$ Å distance between Q$^*_A$ and O$_2$, rapid ($\sim$ps) reaction rates are expected, as indeed observed in the QM/MM MD simulation, when O$_2$ was already bound to the iron. The measured reaction rates are, however, much slower ($t_{1/2} \sim 10$ s), and this could be
due to the reaction rates being limited by O₂ diffusion along the constricted channels to the iron binding site (Fig. 4 E and F and SI Appendix, Fig. S14). This diffusional restriction could limit the measured rate in the experiments performed, i.e., when an anaerobic sample is mixed with oxygenated buffer. The possibility arises that under aerobic conditions, O₂ may be already within the access channel and thus have more rapid access to the nonheme iron. This raises the possibility that in vivo the reduction of O₂ by Qₐ⁻ could be faster than reported here.

The Fe³⁺/Fe²⁺ couple has an Eₘₐₖ of +430 mV at this pH (44). The Fe²⁺ is located between the quinones Qₐ and Q₉, but there is no evidence of a distinct redox role of the metal in the electron transfer process between the two quinones (49). Similarly, while the DFT and QM/MM calculations indicate that both Fe³⁺ and Fe²⁺ are stable with HCO₃⁻ bound, no Fe³⁺ is formed during the electron transfer process with O₂ bound, suggesting that the Fe²⁺ has a catalytic rather than redox role in enabling the electron transfer to O₂ (Fig. 4 and SI Appendix, Fig. S14).

**Bicarbonate Regulatory Mechanism.** Recently, it was shown that the dissociation of bicarbonate leads to an increase in the reduction potential of QA and consequently, the increase of Qₐ⁻ decreased the bicarbonate affinity for its binding site on the nonheme iron (33). The redox-tuning/bicarbonate-binding relationship suggested the following photoregulation/protection model: 1) when the intracellular CO₂ concentration is low and PSII is exposed to light, limitations in CO₂ fixation result in the overreduction of the electron transfer chain, leading to the formation of a long-lived Qₐ⁻; 2) the long-lived Qₐ⁻ triggers the dissociation of the bicarbonate by lowering its affinity for the nonheme iron; 3) the loss of the bicarbonate raises the Eₘₐₖ of QA/Qₐ⁻, increasing the energy gap between the QA/Qₐ⁻ and PheoD₁/PheoD₂ redox couples (33); 4) this increased energy gap disfavors the back-reaction, preventing the formation of P⁺/Pheo⁺, the precursor of the chlorophyll triplet state (7) that reacts with O₂ to form damaging Oₕ⁻ (9).

Our current findings that QA⁻ can reduce O₂ and that bicarbonate binding prevents this reaction suggest another layer of complexity on the regulatory and protective role of bicarbonate in PSII (Fig. 5). Under normal functional conditions (light, high intracellular CO₂ concentration), the QA⁻ lifetime is expected to be long. The bicarbonate is bound to the QA⁻ ion of the QA redox couple, preventing its release and allowing O₂ to enter the channel and to bind to the Fe³⁺⁻. (C) When the O₂ is bound to the Fe³⁺⁻, it is rapidly reduced by the electron coming from QA⁻. (D) The superoxide formed is released from the Fe²⁺ and diffuses away.

**Relevance to Previous Studies.** The phosphorylation of the D1 protein in PSII, which is associated with the migration of damaged PSII from the grana to the stroma lamellae during the repair cycle, was shown to decrease O₂⁻⁻ production (52). This was confirmed in STN8 kinase knockout mutant strains in rice that showed enhanced O₂⁻⁻ production under high light (53). In both cases, it was suggested that a conformational change induced by the phosphorylation (or the lack of it in the absence of the kinase in the knockout mutant) was responsible for the formation of O₂⁻⁻ due to the reduction of oxygen by O₅B⁻. Considering 1) that the experiments in ref. 52 were performed in high light, 2) the improved understanding of the redox properties of QA (4), and 3) the findings in the present work, it seems more likely that QA⁻ is responsible for the oxygen reduction and that the phosphorylation restricts O₂ accessibility to the nonheme iron, perhaps by favoring bicarbonate binding.

PsbS knockout mutants in rice were shown to produce more O₂⁻⁻ due to a proposed decrease in the QA/Qₐ⁻ reduction potential compared to the wild type under high light conditions (54). More recently, while investigating the role of PsbS in the efficiency of water use in field crops, a correlation between stomatal conductance and the QA redox state was reported (55). As hydrogen peroxide is a signal involved in stomatal opening, and as superoxide rapidly dismutates forming hydrogen peroxide, it is possible that the superoxide generated by QA⁻ reported here contributes to this signaling pathway.

Small carboxylic acids, such as acetate and glycolate, can replace bicarbonate at the nonheme iron binding site in vivo (56, 57) and could thereby protect against photooxidative stress under low-CO₂ conditions (33, 56, 57). The mechanism involves redox tuning of QA and the associated binding effects of bicarbonate (33). The observation here (SI Appendix, Fig. S7B) that formate can replace bicarbonate in blocking oxygen access to the iron and thus to reduction by QA⁻ extends this model suggesting a potential role for carboxylic acids in controlling superoxide-initiated signaling.
Molecular Simulations. All simulations were based on the 3.9 Å resolution crystal structure of PSII from the cyanobacterium Synechococcus vulcanus (PDB 3WU2) (41), which provided a better resolved Q_{A}/Q_{B} nonheme iron site compared to the cryoEM structure of S. oleracea (PDB ID:3JCJ) (60). The plant and cyanobacterial structures are highly similar around the region studied, and the results obtained are thus likely to apply for both systems (SI Appendix, Fig. S16). The crystal structure of PSII from T. vulcanus (PDB ID: 3WU2) (41) was embedded in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine membrane and solvated with TIP3P water molecules and 100 mM NaCl concentration. The total system comprised ca. 425,000 atoms. Parameters for all cofactors were derived from in-house DFT calculations (cf. refs. 61–63), and the remaining system was treated using the CHARMM36 force field (64). Four independent MD simulations, 200 ns each, were carried out with the Q_{A} site modeled either in the oxidized or anionic semiquinone (Q_{A}^-) state, and the nonheme iron center modeled with HCO_{3}^- bound or without the HCO_{3}^-... M. Final O_{2} concentrations, upon each addition, were monitored using the Ocean Optics Neptune Phase oxygen sensor. In the cases where oxygen was removed without bicarbonate, air was passed through Ca(OH)_{2} to remove any HCO_{3}^- dissolved in the buffer due to the equilibrium of the atmospheric CO_{2} (58). When necessary, 1 mM bicarbonate, 10 mM DCMU, or 100 μM bromoxynil was added to the PSII samples prior to the degassing step. Further experimental details are provided in SI Appendix.

ROS Formation from O_{2} Reacting with a Fixed Stable Amount of Q_{A} using cyt c Reduction. Reduction of cyt c was used to quantify the concentration of Q_{A}^- generated based on known concentrations of Q_{A}^- and O_{2}. A fixed amount of Q_{A}^- was generated following the protocol previously described for the fluorescence measurements: anaerobic, 250 μM hydroxyamine, three saturating flashes, resulting in the formation of 40 to 50% Q_{A}^- being stably trapped. The experiment was initiated by the addition of oxygenated buffer corresponding to a specific final concentration of O_{2}, and after 2 min, the concentration of reduced cyt c was determined. The experiment was performed in the presence and in the absence of 1 mM bicarbonate using 60 nM PSII samples with 250 μM hydroxyamine in 40 mM MES and 15 mM MgCl_{2}, pH 6.5 with additional 20 μM cyt c and 500 μM U_{1} catalase. Catalase was added to remove any H_{2}O_{2} generated by spontaneous dismutation of O_{2}, which could oxidize cyt c by diminishing the signal. The samples containing HCO_{3}^- were treated using a Shimadzu UV-1601PC UV-Visible spectrophotometer, and the absorbance peak at 550 nm was used to determine the concentration of reduced cyt c. SI Appendix provides details on the ROS formation studied under continuous light conditions using cyt c reduction.

Q_{A}^- Formation Studied Using EPR. Q_{A}^- was trapped using DIPPMPO and spectra measured with a Magetnatic Miniscope MS5000. The 50-μl capillary tubes contained 60 μg mL^{-1} PSII, 10 mM DIPPMPO (28), 500 U mL^{-1} catalase, and 12 mM bicarbonate. Samples were illuminated for 2, 10, and 20 min using 50 μmol photons m^{-2} s^{-1} of red light (590 nm longpass filter) prior to measuring spectra. PSI in working buffer had HCO_{3}^- removed when necessary by either bubbling with dry N_{2} or with air through Ca(OH)_{2} (58). Catalase and DIPPMPO were added after HCO_{3}^- removal from the buffer. Spectra were measured at room temperature with 9.2-GHz microwave frequency, 100-kHz modulation frequency, 2-Gauss modulation amplitude, 3,363-Gauss field center, 150-Gauss sweep width, 3-mW microwave power, and 60-s sweep-time (59). Methylene blue was used to artificially generate 1 O_{2} to establish that there was no interference between 1 O_{2} generated and DIPPMPO.

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10. A. W. Rutherford, A. Osyczka, F. Rappaport, Back-reactions, short-circuits, leaks and et al.

12. R. E. Cleland, S. C. Grace, Voltammetric detection of superoxide production by photosystem II.

14. G. N. Johnson, A. Boussac, A. W. Rutherford, The origin of 40

11. R. F. C. 50

13. E. Ahlberg, I. Panas, D. J. Schiffrin, Quantum chemical modelling of the rate determining step for oxygen reduction on quinones. Phys. Chem. Chem. Phys. 8, 4189–4199 (2006).

15. Y. Umeno, K. Kavakami, J.-R. Shen, N. Yamaizumi, Crystal structure of oxygen-evolving photosystem II at a resolution of 1.8 A. Nature 473, 55-60 (2011).

16. C. A. Wright, Modulation of herbicide-binding by the redox state of Q40, an endogenous component of Photosystem II. Biochim. Biophys. Acta Bioenerg. 809, 320–330 (1985).

17. B. A. Diner, V. Petroulias, Light-induced oxidation of the acceptor-side Fe(II) of Photosystem II by exogenous quinones acting through the QB binding site. II. Blockage by inhibitors and their effects on the Fe(III) EPR spectra. Biochim. Biophys. Acta Bioenerg. 893, 138–148 (1987).

18. D. J. Blauhaug, Govindjee, Comparison of bicarbonate effects on the variable chlorophyll a fluorescence of CO2-depleted and non-CO2-depleted thylakoids in the presence of diuron. Z. Naturforsch. C 39, 378–381 (1984).

19. P. Pospisil, A. Arato, A. Krieger-Liszay, A. W. Rutherford, Hydroxyl radical generation by photosystem II. Biochemistry 43, 6783–6792 (2004).

20. P. Pospisil, Reduction potentials of one-electron couples involving free radicals in aqueous solution. J. Phys. Chem. Ref. Data 16, 1637–1755 (1989).

21. P. Chernev, I. Zaharieva, H. Dau, M. Haumann, Carboxylate shifts steer interquinone electron transfer in photosystem II. J. Biol. Chem. 286, 5368–5374 (2011).

22. J. F. H. Snel, J. S. J. van Rensen, Kinetics of the reactivation of the Hill reaction in CO2-depleted chloroplasts by addition of bicarbonate in the absence and in the presence of herbicides. Physiol. Plant. 57, 422–427 (1983).

23. D. J. Blauhaug, Govindjee, Production of reactive oxygen species by photosystem II.

24. A. W. Rutherford, A. Krieger-Liszay, A. W. Rutherford, Hydroxyl radical generation by photosystem II. Biochemistry 43, 6783–6792 (2004).

25. P. Pospisil, Reduction potentials of one-electron couples involving free radicals in aqueous solution. J. Phys. Chem. Ref. Data 16, 1637–1755 (1989).

26. R. E. Cleland, S. C. Grace, Voltammetric detection of superoxide production by photosystem II.

27. R. F. C.

28. J. Zabret, et al.

29. P. Chernev, I. Zaharieva, H. Dau, M. Haumann, Carboxylate shifts steer interquinone electron transfer in photosystem II. J. Biol. Chem. 286, 5368–5374 (2011).

30. F. J. Schmitt, et al.

31. N. Bondarava, et al.

32. M. M. Gómez, R. C. Rio, J. R. Dacosta, D. J. Schiffrin, Quantum chemical modelling of the rate determining step for oxygen reduction on quinones. Phys. Chem. Chem. Phys. 8, 4189–4199 (2006).

33. Y. Umeno, K. Kavakami, J.-R. Shen, N. Yamaizumi, Crystal structure of oxygen-evolving photosystem II at a resolution of 1.8 Å. Nature 473, 55-60 (2011).

34. D. Shevela, J. J. Eaton-Rye, J.-R. Shen, Govindjee, Photosystem II and the unique role of cytochrome b559. Proc. Natl. Acad. Sci. U.S.A. 103, 2751–2756 (2006).

35. R. de Wijn, H. J. van Gorkom, Kinetics of electron transfer from Q(a) to Q(b) in photosystem II. Biochim. Biophys. Acta Bioenerg. 893, 138–148 (1987).

36. B. A. Diner, V. Petroulias, Q40, the non-heme iron of the photosystem II iron-iron quinone complex. A spectroscopic probe of quinone and inhibitor binding to the reaction center. Biochim. Biophys. Acta Bioenerg. 895, 107–125 (1987).

37. J. F. H. Snel, J. S. J. van Rensen, Kinetics of the reactivation of the Hill reaction in CO2-depleted chloroplasts by addition of bicarbonate in the absence and in the presence of herbicides. Physiol. Plant. 57, 422–427 (1983).

38. D. J. Blauhaug, Govindjee, Production of reactive oxygen species by photosystem II.

39. J. Zabret, et al.

40. J. R. T. J. Wass, E. Ahlberg, I. Panas, D. J. Schiffrin, Quantum chemical modelling of the rate determining step for oxygen reduction on quinones. Phys. Chem. Chem. Phys. 8, 4189–4199 (2006).

41. F. J. Schmitt, et al.

42. J. Zabret, et al.

43. J. Zabret, et al.

44. J. Zabret, et al.

45. J. Zabret, et al.

46. J. Zabret, et al.

47. P. Pospisil, A. Arato, A. Krieger-Liszay, A. W. Rutherford, Hydroxyl radical generation by photosystem II. Biochemistry 43, 6783–6792 (2004).

48. P. Pospisil, Reduction potentials of one-electron couples involving free radicals in aqueous solution. J. Phys. Chem. Ref. Data 16, 1637–1755 (1989).

49. P. Chernev, I. Zaharieva, H. Dau, M. Haumann, Carboxylate shifts steer interquinone electron transfer in photosystem II. J. Biol. Chem. 286, 5368–5374 (2011).

50. F. J. Schmitt, et al.

51. J. Zabret, et al.

52. J. Zabret, et al.

53. R. S. Poudyal, K. Nath, I. S. Zulfugarov, C.-H. Lee, Production of superoxide from photosystem II-light harvesting complex II supercomplex in STN8 kinase knock-out rice plants. J. Biol. Chem. 285, 240–247 (2016).

54. I. Ugur, A. W. Rutherford, V. R. I. Kaila, Redox-coupled substrate water reorganization in the active site of Photosystem II in a rice (Oryza sativa L.) mutant lacking Phb. BMC Plant Biol. 14, 242 (2014).

55. K. Glowacka, et al.

56. J. Zabret, et al.

57. J. Zabret, et al.

58. J. Zabret, et al.

59. C. Fufezan, A. W. Rutherford, A. Krieger-Liszkay, Singlet oxygen production in barley. Photosynthesis Research 129, 342–356 (2018).

60. W. Humphrey, A. Dalke, K. Schulten, VMD: Visual molecular dynamics. J. Comput. Chem. 14, 1395–1402 (1993).

61. I. Ugur, A. W. Rutherford, V. R. I. Kaila, Redox-coupled substrate water reorganization in the active site of Photosystem II—the role of calcium in substrate water delivery. Biochemistry. Biochim. Biophys. Acta 1857, 740–746 (2016).

62. A. Bousaccou, et al.

63. D. J. Blauhaug, A. W. Rutherford, V. R. I. Kaila, Redox-coupled substrate water reorganization in the active site of Photosystem II—the role of calcium in substrate water delivery. Biochemistry Biochim. Biophys. Acta 1857, 740–746 (2016).

64. A. Bousaccou, et al.

65. J. Zabret, et al.

66. W. Humphrey, A. Dalke, K. Schulten, VMD: Visual molecular dynamics. J. Mol. Graph. 14, 33–38, 27–28 (1996).

67. E. Chavouacova, et al.

68. J. Zabret, et al.

69. J. Zabret, et al.

70. A. Schäfer, H. Horn, R. Ahlrichs, Fully optimized contracted Gaussian basis sets for atoms Li to Kr. J. Chem. Phys. 97, 2571–2577 (1992).

Funtuzi et al.

Bicarbonate-controlled reduction of oxygen by the Qm semiquinone in Photosystem II membranes.
71. A. Klamt, G. Schüürmann, COSMO: A new approach to dielectric screening in solvents with explicit expressions for the screening energy and its gradient. J. Chem. Soc., Perkin Trans. 2, 799–805 (1993).
72. M. Reiher, O. Salomon, B. Artur Hess, Reparameterization of hybrid functionals based on energy differences of states of different multiplicity. Theor. Chem. Acc. 107, 48–55 (2001).
73. T. Yanai, D. P. Tew, N. C. Handy, A new hybrid exchange-correlation functional using the Coulomb-attenuating method (CAM-B3LYP). Chem. Phys. Lett. 393, 51–57 (2004).
74. Y. Shao, Y. Mei, D. Sundholm, V. R. I. Kaila, Benchmarking the performance of time-dependent density functional theory methods on biochromophores. J. Chem. Theory Comput. 16, 587–600 (2020).
75. R. Ahlrichs, M. Bar, M. Häser, H. Horn, C. Kölmel, Electronic structure calculations on workstation computers: The program system turbomole. Chem. Phys. Lett. 162, 165–169 (1989).
76. S. Riahi, C. N. Rowley, The CHARMM-TURBOMOLE interface for efficient and accurate QM/MM molecular dynamics, free energies, and excited state properties. J. Comput. Chem. 35, 2076–2086 (2014).