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A Magnesium Porphyrin Bicarbonate Complex with CO_2-Modulated Photosystem I Action**

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In memory of Richard Willstätter

Plant photosynthesis has three interrelated but distinctly separate domains, commonly known as photosystem I (PSI), the dark reaction (Calvin cycle), and photosystem II (PSII). PSI absorbs sunlight to transform light into chemical energy. This event follows the Calvin cycle[1] where the substrate CO_2, on binding to the enzyme, RuBisCo (present in the chloroplast), undergoes several biochemical steps to finally change into sugar units utilizing the chemical energy provided by PSI. The electrons released by PSI and carried by iron sulfur clusters and other electron carriers are finally used to reduce $NADP^+$ and related species, which are in turn needed to supply electrons to the trapped CO_2 in the dark reaction. The cationic form of PSI ($P700^+$) gains electrons from PSII system at the expense of water oxidation to complete the flow of electrons in photosynthesis. To initiate the activity of the enzyme, RuBisCo and one "activator CO_2 molecule" in association with a magnesium ion play a crucial role.[2] Although several plausible schemes have been proposed for the positioning of Mg^{2+} in the active site of the enzyme, including the attachment of carbon dioxide to a lysine residue in the active site forming a carbamate,[2,3] the precise mechanism of activator CO_2 is unknown in the dark reaction. Before the discovery of the dark reaction, Willstatter and Stoll[4] emphasized the binding of CO_2 to magnesium bound with chlorophyll. The suggestion was that a carbon dioxide derivative with chlorophyll would form an additive compound like the bicarbonate type. This was supposed to be the substrate binding event, which ultimately led to its assimilation producing carbohydrate. On the other front, Warburg also argued about the binding of CO_2 with chlorophyll, but his photolyte concept[5] remained evasive. The oxidation of water to molecular oxygen by photosystem II (PSII) is inhibited in bicarbonate-depleted media and the "bicarbonate effect" is an unresolved topic.[6] Our present knowledge about a complete photosynthesis system requires three well-connected basic reactions, like the light reaction, the dark reaction, and the oxygen evolution reaction. The dark reaction requires the substrate, CO_2, as well as another remotely placed activator CO_2 molecule, and the efficiency of oxygen evolving reaction (PSII) is controlled remotely by bicarbonate, a direct derivative of CO_2. But for the light-harvesting reaction of PSI, no role of CO_2 is envisaged. Herein we show in a model reaction that CO_2 in bound form to a magnesium porphyrin complex does facilitate the light-harvesting reaction. We synthesize a discrete bicarbonate-bound magnesium porphyrin complex that under sunlight or visible light-illumination releases an electron to reduce Fe^{III} to Fe^{II} concomitant with the generation of EPR-active, π cation porphyrin radical species. The electrochemical response of the bicarbonate-bound magnesium porphyrin shows more facile oxidation than that of unbound bicarbonate magnesium porphyrin. These results suggest that the coordination of bicarbonate to magnesium porphyrin facilitates easier conversion of light energy into chemical energy. Freshly isolated chlorophyll *a* under a CO_2 atmosphere shows similar facility in its electrochemical oxidation. The Hill reaction of isolated chlorophyll *a* under CO_2 compared to argon also showed a faster reaction similar to model porphyrin-based reactions. Moreover, easier generation of a chlorophyll π cation radical in a CO_2 atmosphere compared to argon is also corroborated by EPR studies. Finally, CO_2-mediated enhanced EPR intensity from PSI under ferricyanide reduction has been demonstrated. Thus carbon dioxide, in addition to its mandatory role as a carbon source,[7] plays its ubiquitous role in all the three interrelated reactions in photosynthesis.

The bicarbonate adduct of MgTPP (**1**; TPP = tetraphenylporphyrin) was made by passing CO_2 through a solution of **1** in dichloromethane containing tetrabutylammonium hydroxide; on standing it crystallizes out as $[Bu_4N][MgTPP(HCO_3)]$ (**2**). Complex **2** is stable in the solid state; in solution it remains stable under CO_2, however on bubbling argon through its solution it slowly changes to **1**. FTIR spectroscopy of **2** shows the presence of $\nu(CO)$ at 1626 and 1378 cm^{-1} that are due to the coordination of bicarbonate. The ^{13}C NMR spectrum also corroborates the binding of bicarbonate with **1** (Supporting Information, Figure S3). The molecular structure of the asymmetric unit of **2** contains one bicarbonate-coordinated magnesium tetraphenylporphyrin anion (Figure 1)[8] and one tetrabutylammonium cation (not shown). The stability of the anion of **2** is due to its involvement in hydrogen bonding. In the lattice (Figure 1b), two such units of **2** are held together. The dimer is formed by hydrogen bonding using the oxygen atom of the hydroxy group of one bicarbonate and oxygen atom of the keto group of another bicarbonate, with an apparently weak interaction

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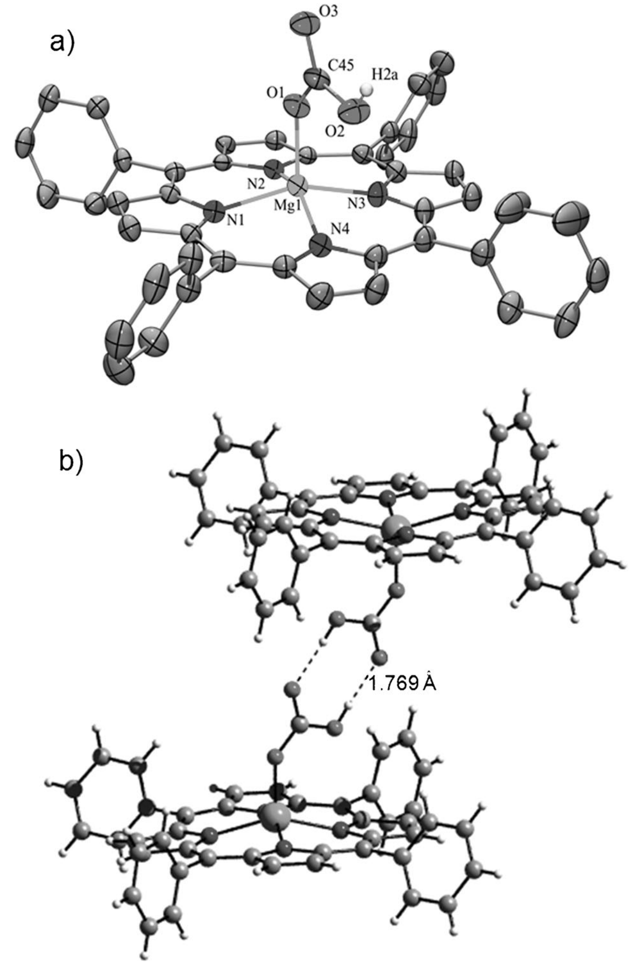

Figure 1. a) Perspective view of anion **2** with atom labeling (ellipsoids set at 50% probability, hydrogen atoms omitted except the bicarbonate hydrogen). b) Intermolecular hydrogen-bonding interaction between two units to form a dimer.

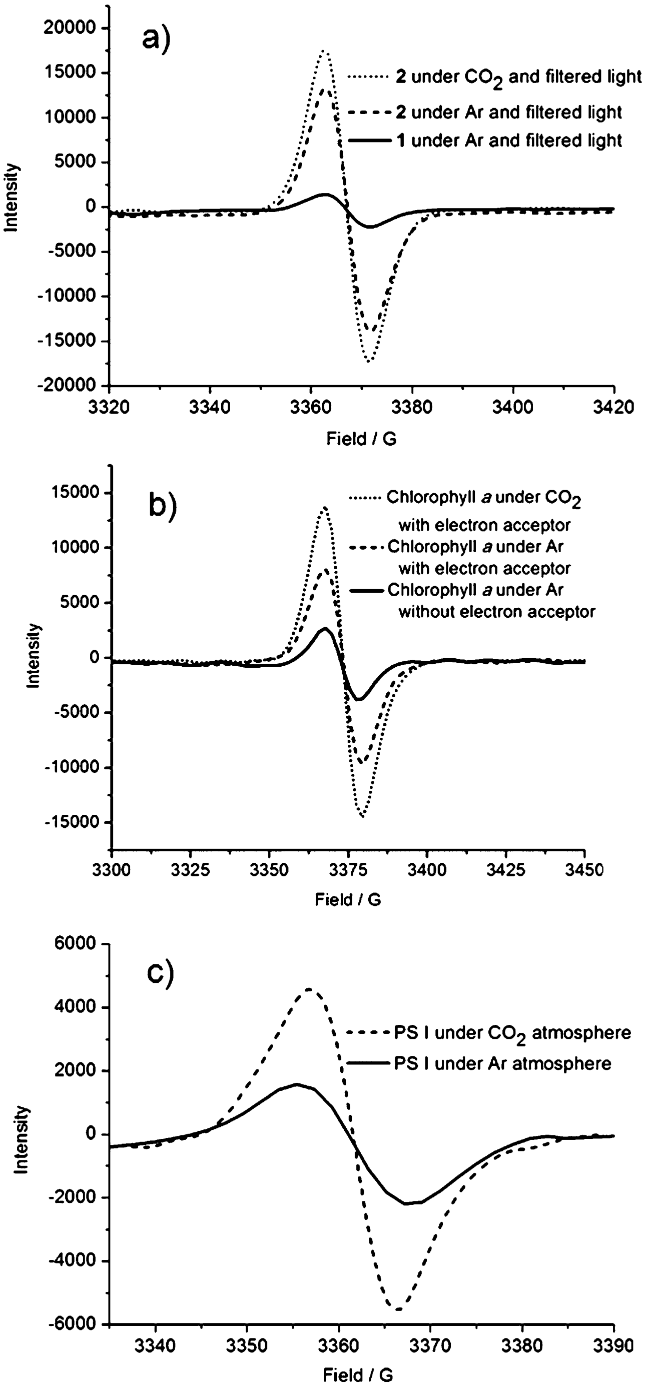

Figure 2. Low-temperature (120 K) X-band EPR spectra. a) Cation radical of complex **2** and **1** under CO_2 and an Ar atmosphere and under water-cooled tungsten light (100 Watt) illumination in CH_2Cl_2 ($g = 2.005$). b) cation radical of chlorophyll *a* under CO_2 and an Ar atmosphere ($g = 2.003$). c) P700$^+$ from PSI under CO_2 and an Ar atmosphere in MES buffer, pH 6.5 ($g = 2.003$).

with a distance of 9.653 Å. An X-ray structure of PSI has recently been reported at 3.3 Å resolution with the details of the position of the special pair.[9] The relatively weak contact between the core complex and the light-harvesting complex I (LHCI) is noteworthy.[10] The axial Mg–O distance in **2** is 1.958(2) Å, which is significantly shorter than those in [MgTPP(H_2O)] and in hydrated ethyl chlorophyllide *a* (2.072(1), 2.035(6), Å), suggesting that bicarbonate coordination to magnesium is possible by the displacement of water.[11]

The reaction in chloroplast is initiated by absorption of light by PSI, ejecting an electron and generating the P700$^+$ chlorophyll radical under sunlight illumination. To understand the influence of CO_2 on such a light reaction, we carried out a comparable EPR study of **1** and **2** under argon and also of **2** under a CO_2 atmosphere in the presence of sunlight (and also with tungsten-lamp) irradiation under identical conditions using $K_3[Fe(CN)_6]$ as the electron acceptor. To mimic the electron transfer of a lipophilic hydrophilic interface, we used 10^{-3} M of **1** and **2** in CH_2Cl_2 and 10^{-2} M of $K_3[Fe(CN)_6]$ in water. EPR studies were measured from the CH_2Cl_2 layer after photoirradiation with a 100 W tungsten lamp covered with a water jacket to prevent heating. The appearance of the relative intensity of the EPR signals (Figure 2) from these systems are easily interpreted, as **2** under a CO_2 atmosphere most readily facilitates the formation of a π cation radical. Light-induced electron transfer from zinc porphyrin to various electron acceptors is known.[12] The result of the electrochemistry of **2** concur with the EPR observation. The first oxidation potential of **2** measured is 550 mV vs Ag/AgCl, which is significantly lower than that measured with **1**[13] (620 mV vs Ag/AgCl; see the Supporting Information, Figure S7). Interestingly, upon irradiation using a 100 W tungsten lamp (with water cooling to nullify any heat produced), this oxidation step of **2** distinctly shifted to a lower potential value by 20 mV (Figure 3 a). Differential pulse polarography confirms such a shift of potential (Figure 3 b). On switching off the light, the potential is restored to its original value.

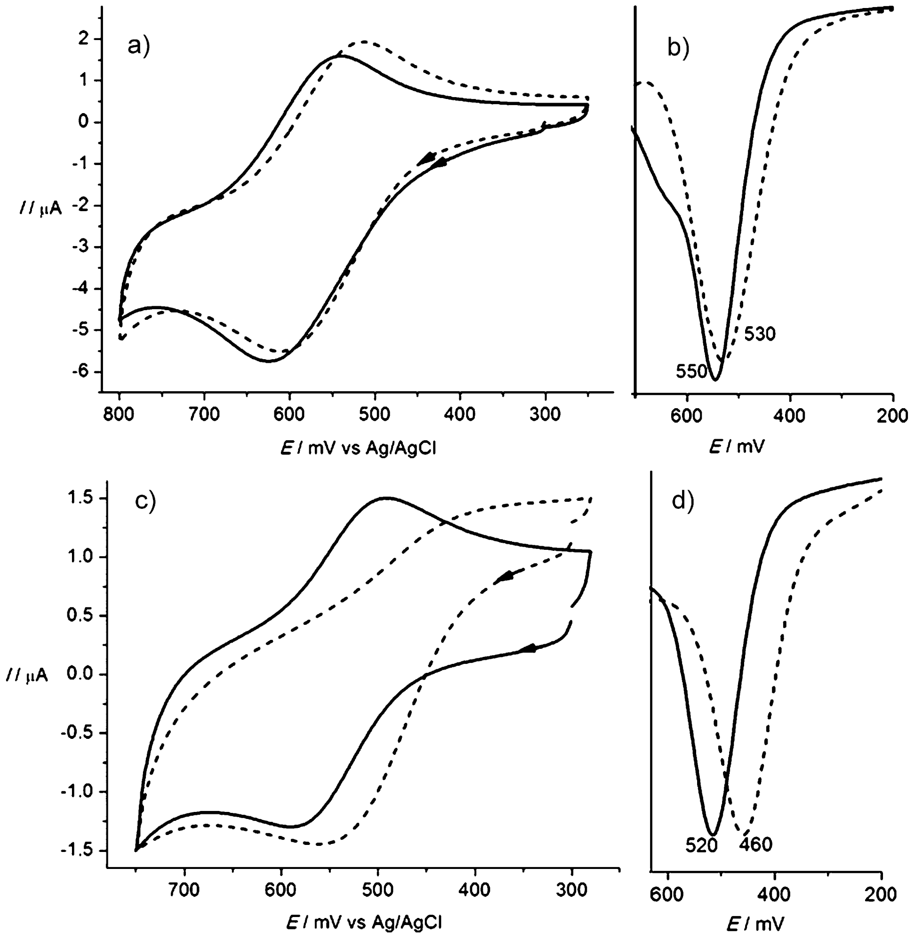

Figure 3. Electrochemistry of **2** and chlorophyll *a* in the presence of bicarbonate. a) Cyclic voltammogram of 1 mM of **2** (scan rate 100 mVs^{-1}) with 0.2 M TBAP as supporting electrolyte; b) differential pulse polarographs (scan rate 20 mV/sec, pulse width = 50 ms, pulse period = 200 ms, pulse amplitude = 50 mV) in CH_2Cl_2 (294 K) with a Pt electrode, first oxidation (+550 mV vs Ag/AgCl); —— normal conditions, ----- under light illumination. After irradiation, the first oxidation shifted to +530 mV vs Ag/AgCl. c) Cyclic voltammetry traces (——, scan rate 100 mVs^{-1}) with 0.05 M TBAP as supporting electrolyte; d) differential pulse polarographs (——, scan rate 20 mVs^{-1}, pulse width = 50 ms, pulse period = 200 ms, pulse amplitude = 50 mV) of 2.5×10^{-4} mM chlorophyll *a* in CH_2Cl_2 (294 K) with Pt electrode. ----- Using 2.5×10^{-4} mM chlorophyll *a* in CH_2Cl_2 (294 K) with a Pt electrode in the presence of bis(triphenylphosphine)iminium bicarbonate ($PNPHCO_3$).

We extended this electrochemical study with freshly isolated chlorophyll *a* to compare the behavior of the native isolated system in the presence of bicarbonate. The result was astounding, as the appearance of the first oxidation potential of chlorophyll *a* in the presence of bicarbonate shows a decrease by 60 mV by cyclic voltammetry and by differential pulse polarography (Figure 3 c,d). The reversible nature of the cyclic voltammetric response of pure chlorophyll *a* is lost but shows more facile oxidation under bicarbonate (Figure 3 c).The drop in oxidation potential value is due to the coordination of bicarbonate. The binding of bicarbonate to the magnesium center of chlorophyll *a* is shown by ^{13}C NMR spectroscopy (Supporting Information, Figure S4). However, the bicarbonate-coordinated π cation radical of chlorophyll *a* is unstable and dissociates the bicarbonate ion, resulting in irreversibility in this process.[14] Such irreversibility may assist the drainage of the electron required in subsequent formation of reduced products to be used in the dark reaction. Under light illumination, the isolated chlorophyll *a* is notoriously sensitive to degradation,[15] which thwarted our attempts to find any meaningful drop in the oxidation potential of bicarbonate adduct of chlorophyll *a* under the exposure of light. To understand the easiness of π cation radical generation, we performed the EPR study of pure chlorophyll *a* using the electron acceptor $K_3[Fe(CN)_6]$ in an identical manner to that with compound **2**, which reveals the ability of CO_2 to facilitate the generation of a chlorophyll π cation radical (Figure 2 b).

Compound **2** reduces ferricyanide to ferrocyanide similar to the known reaction at several sites in the chloroplast electron transport chain.[16] Upon irradiation of an aqueous ferricyanide solution along with **2** dissolved in toluene, addition of Fe^{III} ion led to the appearance of blue color that finally precipitate Prussian blue. However self-photodegradation of ferricyanide is known, and control tests revealed that under light illumination, **2** loses an electron to ferricyanide, forming ferrocyanide, resulting the conversion of light into chemical energy. A better demonstration of such electron transfer has been found using the salt $[Fe_2(o\text{-phen})_4O](SO_4)_2$,[17] which changes to pink-colored ferroin on reduction from Fe^{III} to Fe^{II} by receiving an electron generated from **2** (Figure 4). The loss of an electron from **2** generates a π cation radical, which is supported in the electronic spectrum of toluene solution, which shows red-shifted and broadened Q-bands characteristic to such radical formation (Supporting Information, Figure S2). It is to be noted that the π cation radical formed in such an electron transfer reaction does not remain stable indefinitely and requires replenishment of the lost electron by an electron donor. In the native system, PSII

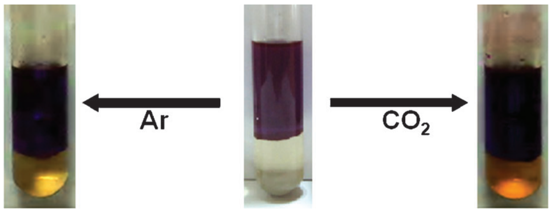

Figure 4. Reduction of ferric to ferrous. **1** (in toluene) under visible light illumination (tungsten lamp 100 W using glass chamber with cold water as filter to protect heat and UV radiation). Left side: under argon; right side: under a CO_2 atmosphere. Electron acceptor is $[Fe_2(o\text{-}phen)_4O](SO_4)_2$ in water.

remains on standby to supply the necessary electron to PSI. In the absence of an electron donor, the so-formed π cation radical undergoes degradation forming isoporphyrin-type complex.[18] Changes observed in the electronic spectrum of chlorophyll *a* upon addition of bicarbonate are very similar to those observed with the compound **1** in changing to **2**. The red-shifting of the Q-band in the electronic spectrum (Supporting Information, Figure S1) of chlorophyll *a* is thus assigned according to the coordination of bicarbonate, in support of such binding by ^{13}C NMR spectroscopy (Supporting Information, Figure S4).

The excitation of both **1** and **2** under common excitation ($\lambda_{ex} = 565$ nm) does show red-shifted fluorescence for **2** concomitant with increase of intensity (Supporting Information, Figure S6). This observation may be relevant for native chlorophyll *a* to tune further the fluorescence property of such systems under the influence of bicarbonate.

To verify the activating role of CO_2 in the reaction center of PSI (chlorophyll *a*) and to facilitate the electron transfer in the early photochemical stages, we followed Hill reactions, such as the bleaching of 2,6-dichlorophenolindophenol (DCPIP) in the presence and absence of CO_2 and also probed the generation of the chlorophyll *a* π cation radical using an electron acceptor, ferricyanide, in the presence and absence of CO_2 using EPR spectroscopy. The role of bicarbonate to enhance the chloroplast activity to reduce the Hill oxidants such as DCPIP or ferricyanide has been studied.[19] However, the specific species present in the chloroplasts to enhance such an activity to reduce Hill oxidants remained elusive. Several redox systems are present in the chloroplast associated with PSI and PSII systems and amongst these, chlorophyll *a* (P700) is the first species to respond to photoassociated electron transfer; however, the reduction of the Hill oxidant by isolated chlorophyll *a* has not been reported. Therefore, we demonstrate that the reduction of both DCPIP and ferricyanide by chlorophyll *a* is faster in the presence of CO_2 (Figure 5a,c). The higher intensity of EPR spectra observed in the presence of CO_2 also corroborates the easier oxidation of chlorophyll *a* in the presence of CO_2 (Figure 2b). Moreover, chlorophyll *a* can also reduce $[Fe_2(o\text{-}phen)_4O](SO_4)_2$ more quickly in a CO_2 atmosphere than in an argon atmosphere, in a similar way to that observed with **2** (Figure 5b). So, these results suggest that CO_2 may activate by binding to the magnesium of chlorophyll *a* in the PSI reaction center, resulting in more facile electron transfer in the initial stage with the photoassisted generation of P700$^+$. Our hypothesis gains credence with an in vivo study of green microalgae, which show much higher electron transport activity of PSI cells grown under high concentrations of CO_2.[20]

Encouraged by such an in vivo study,[20] the role of CO_2 on PSI has been undertaken. The stroma lamella vesicle (Y-100) fraction of a thylakoid membrane that possesses 87 % PSI[21] was investigated by ESR spectroscopy (Figure 2c). Under identical conditions, the intensity of the ESR signal under argon (Figure 2c) is appreciably weak, showing that the

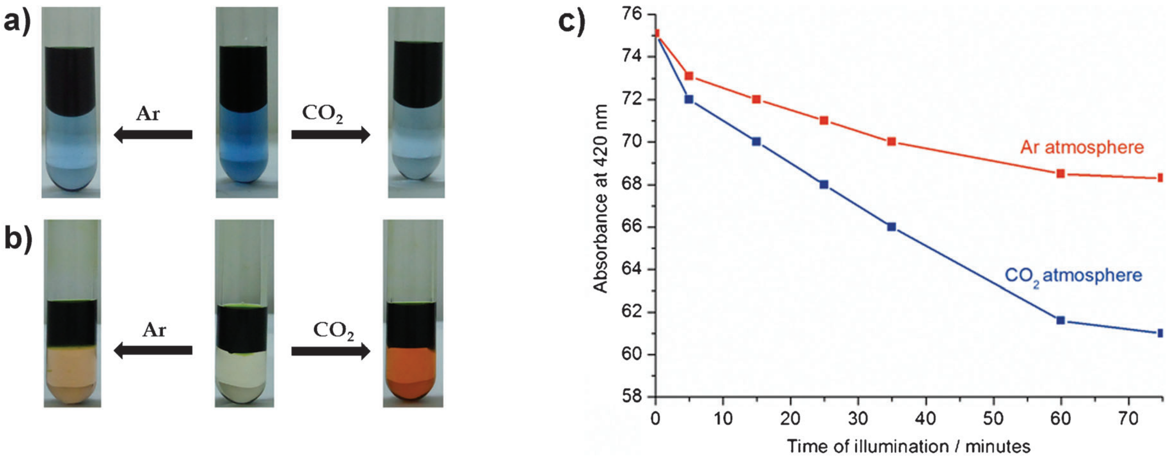

Figure 5. a) DCPIP reduction by pure chlorophyll *a* under CO_2 and Ar atmospheres. Chlorophyll *a* (10^{-3} M, 3 mL) is in petroleum ether and DCPIP in water (4 mL). After 30 min of visible light illumination (tungsten lamp, 100 W, using a glass chamber with cold water to protect from heat). b) Reduction of ferric to ferrous ions using chlorophyll *a* under visible light illumination (tungsten lamp, 100 W, using a glass chamber with cold water to protect from heat). Chlorophyll *a* is in petroleum ether(10^{-3} M, 2 mL), and the electron acceptor $[Fe_2(o\text{-}phen)_4O](SO_4)_2$ (3×10^{-4} M, 2.5 mL) is in water c) Ferricyanide reduction: absorbance of ferricyanide solution at 420 nm under CO_2 and argon atmospheres as a function of illumination time. Pure chlorophyll *a* from spinach (10^{-3} M, 3 mL in chloroform saturated with CO_2 and under argon, respectively) was added to a solution of ferricyanide (10^{-4} M, 4 mL in water). Both samples were illuminated with water-cooled 100 W tungsten light. Regarding illumination time, aliquots of ferricyanide solution were removed from the mixture and the reduction of ferricyanide was measured.

binding of CO_2 in P700 facilitates the ready ejection of electron under illumination. This additional role of CO_2 as promoter by binding onto the magnesium center of chlorophyll in PSI in the primary light absorption stage is recognized. The model study shows the binding of CO_2 in the form of bicarbonate to the magnesium center to result in such modification to facilitate the formation of a cation radical. Thus, CO_2, along with its capacity as substrate in sugar synthesis, plays a ubiquitous role in all three interrelated PSI, PSII, and dark reactions as promoters to optimize photosynthesis.

Experimental Section

The synthetic details of **1, 2**, characterization (UV/Vis and NMR spectroscopy, cyclic voltammetry, X-ray structural analysis) of **2** are provided in the Supporting Information. CCDC 778411 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Pure chlorophyll *a* was isolated from spinach leaves and purified by a standard method, and the purity was checked by electronic spectroscopy. The stroma lamella vesicle (Y-100) fraction of thylakoid membrane from spinach was isolated according to the procedure described in Ref. [21], and the separation of the fractions were verified by ESR spectroscopy.

Electrochemical measurements: Cyclic voltammetry measurements were performed with a BASi Epsilon-EC Bioanalytical systems; Inc. instrument. Cyclic voltammograms of 10^{-3} M dichloromethane solutions of **1** and **2** and 2.5×10^{-4} M for chlorophyll *a* were used with 0.2 M Bu_4NClO_4 for **1** and **2** and 0.05 M Bu_4NClO_4 for chlorophyll *a* as supporting electrolyte, Ag/AgCl as reference electrode, and a platinum auxiliary electrode and glassy carbon electrode as working electrode. All electrochemical experiments were carried out under an argon atmosphere at 298 K unless otherwise noted. Potentials are referenced against internal ferrocene (Fc) and are reported relative to the Ag/AgCl electrode ($E_{1/2}(Fc^+/Fc) = 0.459$ V vs. Ag/AgCl). For light-illuminated reactions, a tungsten bulb (100 W) was placed 40 cm away from the cyclic voltammetry cell and four mirrors were used to reflect the light to the cell; the cell was placed within a double glass jacket through which cold water was circulated to avoid any thermal heating. The CO_2 saturated water (ca. 1250 ppm), pH 3.5 was adjusted to pH around 6.5–6.8 by adding tetrabutylammonium bicarbonate and was used to carry out the reaction under CO_2 atmosphere. In two-phase reactions, the organic solvent was also saturated with CO_2 under atmospheric pressure. Distilled water (pH 7) was used when the work was carried out under an argon atmosphere.

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[8] See the Supporting Information for synthetic details. Crystallographic data for **2**: $C_{61}H_{65}MgN_5O_3$, $M_r = 940.49$, $a = 12.442(5)$, $b = 17.004(5)$, $c = 24.942(5)$ Å, $\beta = 91.194(5)°$, $V = 5276(3)$ Å^3, space group $P2_1/c$, $Z = 4$, $\lambda(Mo_{K\alpha}) = 0.71073$ Å, $T = 100(2)$ K, 33769 reflections, 12770 unique, $R_{int} = 0.1043$, $R_1 = 0.0735$, $\omega R_2 = 0.1634$.

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