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Kinetics of O_2 Evolution from H_2O_2 Catalyzed by the Oxygen-Evolving Complex: Investigation of the S_1-Dependent Reaction†

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ABSTRACT: The evolution of O_2 from H_2O_2 catalyzed by the oxygen-evolving complex (OEC) in darkness was examined with photosystem II reaction center complex preparations from spinach. Flash illumination of dark-adapted reaction centers was used to make S_0-enriched or S_1-enriched complexes. The membranes catalyzed O_2 evolution from H_2O_2 when preset to either the S_0 or S_1 state. However, only the S_0-state reaction was inhibited by carbonyl cyanide *m*-chlorophenylhydrazone and dependent on chloride. These results indicate that (1) the S_0-dependent and S_1-dependent catalytic cycles can be separated by flash illumination, (2) the S_0-dependent reaction involves the formation of the S_2 state, and (3) the S_1-dependent reaction does not involve the formation of the S_2 or S_3 states. A kinetic study of the S_1-dependent reaction revealed a rapid equilibrium ordered mechanism in which (1) the binding of Ca(II) must precede the binding of H_2O_2 to the OEC and (2) the reaction of Ca(II) with the free enzyme is at thermodynamic equilibrium such that Ca(II) does not necessarily dissociate after each catalytic cycle.

The oxygen-evolving complex (OEC)[1] catalyzes the oxidation of water to molecular oxygen in order to provide the supply of electrons for photosynthetic electron transport. The OEC causes single-electron reductions of the reaction center following each charge separation. As a result, molecular oxygen is evolved only once per four photoevents (Kok et al., 1970). To accomplish these single-electron donations, the OEC cycles through five S states (S_0–S_4) and yields oxygen only upon formation of the S_4 state (Forbush et al., 1971).

The S_1 state is stable in darkness, while the S_2 and S_3 states will deactivate in the dark to S_1. The S_2 and S_3 states are in redox equilibrium with D, the precursor to signal IIs. Although the $t_{1/2}$ of deactivation of the S_2 and S_3 states is on the order of seconds, this rate can be accelerated greatly by the addition of reagents like carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) that convert D^+ to D (Renger, 1972; Yerkes & Crofts, 1983). It has been shown recently that D^+ can oxidize the S_0 state to S_1 in the dark such that 50% of the S_0 state initially formed in PSII preparations is converted to S_1 in about 1 h (Styring & Rutherford, 1987).

The S_2 state has been the most highly characterized of all the S states. Formation of the S_2 state results in the appearance of two low-temperature EPR signals known as the $g = 2$ multiline signal (Dismukes & Siderer, 1980a,b; Brudvig et al., 1983) and the $g = 4.1$ signal (Zimmermann & Rutherford, 1984; Casey & Sauer, 1984). Depletion of chloride by sulfate causes the formation of an abnormal S_2 state that lacks the multiline signal (Ono et al., 1986) and is incapable of undergoing further reactions until Cl^- has been restored (Sandusky & Yocum, 1984; Itoh et al., 1984; Theg et al., 1984). High concentrations of Tris (Frasch & Cheniae, 1980) or hydroxide (Briantais et al., 1977) will inactivate the OEC by a specific interaction with the S_2 state.

The OEC contains four manganese that are bound to intrinsic membrane proteins in the thylakoid (Cheniae & Martin, 1970), and the presence of Ca(II) is required for the enzyme to be catalytically competent (Ghanotakis & Yocum, 1986). The proteins that compose the OEC have not been positively identified to date. Mutants of *Scenedesmus*, which are in-

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[1] Abbreviations: PSII, photosystem II; OEC, oxygen-evolving complex; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; MES, 4-morpholineethanesulfonic acid; EPR, electron paramagnetic resonance; Tris, tris(hydroxymethyl)aminomethane; kDa, kilodalton; Chl, chlorophyll.

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capable of evolving O_2 and have an altered capacity for binding manganese, contain a 36-kDa polypeptide in lieu of a 34-kDa protein found in wild-type strains (Metz et al., 1980). Approximately two manganese are bound to the extrinsic 33-kDa protein when it is purified under oxidizing conditions (Abramowicz & Dismukes, 1984). By the use of photoaffinity cross-linking reagents, proteins that retain significant amounts of bound manganese have been isolated (Bowlby & Frasch, 1986). These proteins have the approximate molecular masses of 34, 33, 29, and 22 kDa (Bowlby & Frasch, 1987).

From the observation of flash-induced yields of O_2 from thylakoids in the presence of H_2O_2, Velthuys and Kok (1978) hypothesized that hydrogen peroxide can serve as a substrate for the OEC. The conversion of H_2O_2 to O_2 without light-generated oxidizing equivalents can also be catalyzed by the OEC (Frasch & Mei, 1987). From the use of inhibitors specific to the S_2 state, we have confirmed and extended the results of Velthuys and Kok (1978) to show that the OEC is capable of catalyzing O_2 from H_2O_2 by an S_2-state-dependent reaction or by a reaction that does not involve the formation of the S_2 state. This reaction was found to require Ca(II) and, for the S_2-dependent reaction, to require Cl^- as well.

We now report the use of flash illumination of PSII reaction center complex preparations to increase the abundance of the S_0 or S_1 states prior to assay for O_2 evolution from H_2O_2. With this treatment it has been possible to separate the S_2-dependent reaction, which is shown to involve a cycle between the S_0 and S_2 states, from an S_1-dependent reaction. A kinetic study of the S_1-dependent reaction revealed that there is an ordered addition of Ca(II) followed by H_2O_2 to the oxygen-evolving complex and that the reaction of Ca(II) with the free enzyme is at thermodynamic equilibrium.

Materials and Methods

Photosystem II reaction center complexes were prepared from spinach according to the method of Ghanotakis and Yocum (1986). Photosynthetic O_2 evolution and O_2 evolution from H_2O_2 were measured by using a Clark-type electrode as described by Frasch and Mei (1987). Assays contained 100 μM KCN and, except where indicated differently, 10 mM $CaCl_2$.

Flash illumination of dark-adapted reaction center complexes (300 μg of Chl/mL) was provided by firing two xenon lamps simultaneously as described by Frasch and Chenaie (1980). The flash lamps were interfaced with a Commodore-64 computer that triggered the flashes and timed the intervals between flashes. Treatments were done at 4 °C in 40 mM MES buffer, pH 6.0, with precautions to exclude stray light from the samples. The flashes were empirically determined to saturate the reaction centers at 350 μg of Chl/mL by varying the concentration of membranes when flashed and measuring the extent of inhibition by CCCP and the dependence on chloride described in Figures 1 and 2 (data not shown). In experiments that measured the dependence of the initial rate on the concentration of Ca(II), the membranes were depleted of calcium after flash illumination by resuspension and overnight dialysis in Ca(II)-deficient 40 mM MES buffer, pH 6.0, as described (Ghanotakis et al., 1985).

Reaction center complexes enriched in the S_1 state were obtained by exposing the dark-adapted membranes to a single flash followed by incubation in darkness for a minimum of 10 min. To obtain S_0-enriched complexes, the S_1-enriched membranes were exposed to three additional flashes 1 s apart and then incubated in darkness for a minimum of 10 min. Where indicated, the S_1 state was also formed from the S_0-enriched membranes by a flash and dark adaptation such that the entire protocol consisted of 1 flash–10 min dark–3 flashes–10 min dark–1 flash–10 min dark–assay. The final dark adaptation given to all samples prior to assay for O_2 evolution allowed higher S states to deactivate to the S_1 state. An exogenous electron acceptor was not added during the flash regime in order to avoid possible complications with the subsequent H_2O_2 assay. This treatment provided samples that consisted of only the S_0 and S_1 states in various proportions. These preparations were used for the duration of the experiment. For the experiments that used S_0-enriched preparations (Figures 1 and 2), the assays were completed within 30 min after exposure to flashes. The abundance of the initial S-state populations in these membranes was estimated as described by Radmer and Cheniae (1977), assuming $\alpha = 0.1$.

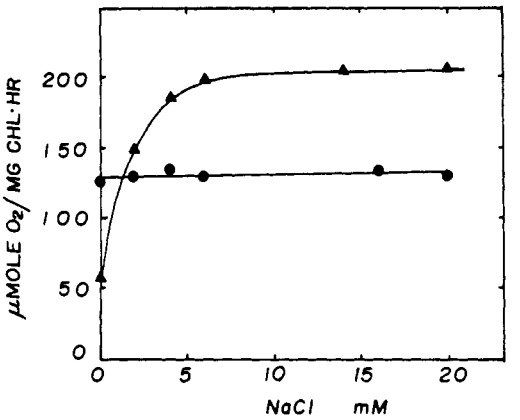


FIGURE 1: Chloride dependence of the rate of O_2 evolution from H_2O_2 catalyzed by chloride-depleted PSII reaction centers. The membranes were given a 1 flash/dark adaptation regime to increase the abundance of the S_1 state (circles) or a 1–3 flash treatment to enrich in the S_0 state (triangles) as described under Materials and Methods and then assayed for the initial rate of O_2 evolution in darkness in the presence of 129 mM H_2O_2, 50 μM $CaSO_4$, and the concentration of NaCl indicated.

The initial velocity data were expressed as double-reciprocal plots and secondary plots, used to determine the kinetic constants graphically, to check the linearity of the curves, and to determine the pattern of the plots. Analyses of the kinetic constants were then made by using the computer programs of Cleland (1967), which were translated into BASIC for use with a personal computer. Data that conformed to an asymmetrical sequential initial velocity pattern or a linear competitive inhibition were fitted to eq 1 and 2, respectively.

$$v = VAB/(K_{ia}K_b + K_bA + AB) \quad (1)$$

$$v = VA/[K_a(1 + I/K_{is}) + A] \quad (2)$$

Fitting the data to these equations gave the best estimates of the values for the kinetic constants with standard errors.

Results

Photosystem II reaction center complex preparations were given flashes of light to increase the population of centers in the S_0 or S_1 states and then used to examine the dependence of the rate of O_2 evolution from H_2O_2 in darkness on the concentration of chloride. As shown in Figure 1, chloride increased the rate catalyzed by S_0-enriched membranes by more than 3-fold. Although the rate of photosynthetic O_2 evolution in the absence of chloride was negligible, 31% of the activity remained when H_2O_2 was used as the substrate. The membranes were calculated to have an initial population of approximately 71% S_0:29% S_1 as a result of the flash regime. Thus, the fraction of activity remaining in the absence of chloride is probably due to S_1-dependent O_2 evolution from

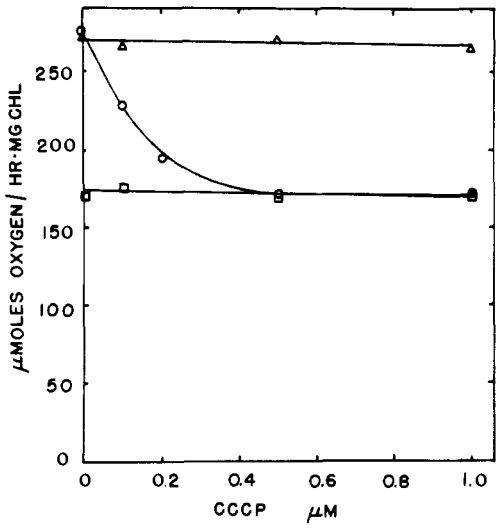


FIGURE 2: Effect of CCCP on the initial rate of O_2 evolution from H_2O_2 catalyzed by PSII reaction centers. The reaction centers were enriched in the S_1 state by 1 flash (△) or by a 1–3–1 flash treatment (□) as described under Materials and Methods. The abundance of the S_0 state was increased by a 1–3 flash treatment (○). The reaction centers were assayed in the presence of 129 mM H_2O_2 and 5 mM $CaCl_2$ in darkness with the concentration of CCCP indicated.

H_2O_2. This requirement for chloride in S_0-enriched preparations indicates that the OEC can catalyze the conversion of H_2O_2 to O_2 by a cycle that involves the S_0 and S_2 states and does not require light-induced charge separation in the reaction center.

A population of approximately 98% S_1 can be achieved by dark adaptation after a single flash. Although S_1-enriched membranes catalyzed O_2 evolution from H_2O_2, the rate was not affected by the concentration of chloride. These results indicate that the OEC can catalyze the formation of O_2 from H_2O_2 by two pathways that can be separated by presetting the S states.

The possible involvement of the S_2 and S_3 states in this catalytic process was examined by measuring the effect of CCCP on O_2 evolution from H_2O_2 catalyzed by the S_0- or S_1-enriched reaction centers. This reagent accelerates the rate of deactivation of the S_2 or S_3 states (Renger, 1972) to increase the abundance of the S_1 state (Yerkes & Crofts, 1983). As shown in Figure 2, H_2O_2-dependent O_2 evolution was observed when the reaction centers were enriched in the S_1 state by 1 flash or by the 1–3–1 flash regime (see Materials and Methods). In both cases, the rate of O_2 evolution was unaffected by CCCP, which suggests that neither the S_2 or S_3 state is involved in the S_1-dependent catalytic cycle to evolve O_2 from H_2O_2. Although the abundance of the S_1 state was about 93% in samples given the 1–3–1 flash treatment, the rate was only 61% of the S_1-enriched sample prepared by a single flash. It is evident that the flashes used to set the S states cause the loss of some activity. The flashes were found to cause a proportional loss of photosynthetic O_2-evolving activity in these preparations as well (data not shown).

The rate of O_2 evolution from H_2O_2 in the S_0-enriched sample was partially inhibited by CCCP. The rate of O_2 evolution by the S_0-enriched sample (formed by a 1–3 flash regime) decreased in the presence of CCCP to the rate of the reaction centers enriched in the S_1 state formed by the 1–3–1 flash treatment. This further supports the hypothesis that the OEC can catalyze the evolution of O_2 from H_2O_2 by a cycle between the S_0 and S_2 states.

The kinetics of the S_1-dependent process were studied further since a single preflash/dark adaptation provided a

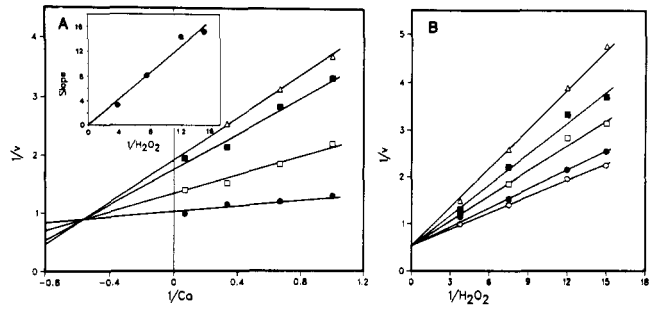


FIGURE 3: (A) Effect of Ca(II) concentration on the initial velocity of the S_1-dependent reaction at different fixed concentrations of H_2O_2. The concentrations of H_2O_2 were (▲) 66.7, (■) 83.4, (□) 133, and (●) 266 mM. Velocities are expressed as millimoles of O_2 per milligram of chlorophyll per hour. Calcium concentrations are given in (B). (Inset) Secondary replot of the slopes of the lines ($\times 10^4$) versus the reciprocal of the concentration of H_2O_2 (millimolar). (B) Effect of H_2O_2 concentration on the initial velocity of the S_1-dependent reaction at different fixed concentrations of Ca(II). The concentrations of Ca(II) added to the Ca(II)-depleted membranes were (△) 0, (■) 1, (□) 1.5, (●) 3, and (○) 15 mM. Velocities and concentrations of H_2O_2 are expressed as in (A).

Table I: Kinetic Constants Associated with the S_1-Dependent Evolution of O_2 from H_2O_2

substrate	kinetic constant	value
Ca(II)	K_a	0[a]
	K_{ia}	0.990 ± 0.004[b]
H_2O_2	K_b	176.8 ± 0.7[c]
	V	1718 ± 3.3[d]

[a] Not measurable; approaches zero in a rapid equilibrium ordered reaction. [b] Apparent dissociation constant for Ca(II) (millimolar). [c] Michaelis constant for H_2O_2 (millimolar). [d] Micromoles of O_2 (mg of Chl·h)$^{-1}$.

population of complexes that were almost entirely in the S_1 state. Calcium was found to serve as an activator of this reaction (Frasch & Mei, 1987) in a manner similar to the effect of Ca(II) on photosynthetic O_2 evolution (Ghanotakis & Yocum, 1986). The effect of the concentration of calcium on the initial velocity of the reaction at various fixed concentrations of H_2O_2 is illustrated in the form of a double-reciprocal plot in Figure 3A. A family of straight lines was observed that intersected to the right of the ordinate. Although this appeared to be a normal intersecting pattern, a secondary plot of the slopes of the lines from Figure 3 versus the reciprocal of the concentration of H_2O_2 passed through the origin (Figure 3A, inset).

The results from the replot in Figure 3A predicted correctly that the dependence of the initial rate on the concentration of H_2O_2 at various fixed concentrations of Ca(II) would yield a double-reciprocal plot that consists of a family of straight lines which intersect on the ordinate at the same point (Figure 3B). A similar result has been reported for the activation by Ca(II) of the esterase activity of tetrathionate-modified transglutaminase (Chung & Folk, 1970). The data shown above gave a good fit to eq 1, and the values obtained for the kinetic constants are listed in Table I. The results of Figure 3 suggest first that O_2 evolution from H_2O_2 catalyzed by the S_1-dependent reaction has a sequential mechanism such that Ca(II) and H_2O_2 must be bound to the OEC before any product can be released. Second, it may be concluded that Ca(II) must bind to some enzyme form or forms before H_2O_2 can combine and that the reaction(s) that involve Ca(II) must be at thermodynamic equilibrium.

It is noteworthy that the reaction center complex preparations that had been depleted of Ca(II) by dialysis for more than 17 h still exhibited a low rate of O_2 evolution from H_2O_2.

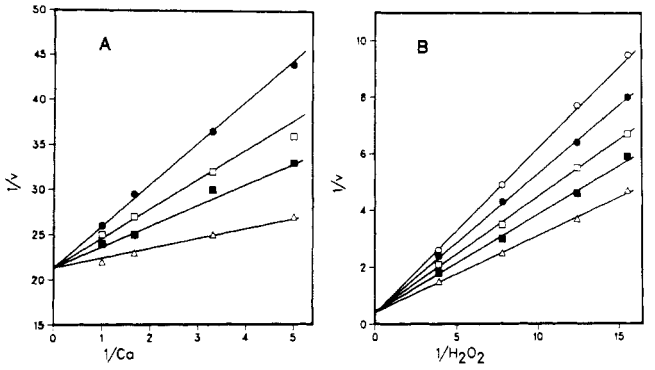


FIGURE 4: (A) Linear competitive inhibition of the S_1-dependent reaction by $LaCl_3$ when the concentration of Ca(II) added to Ca(II)-depleted membranes is varied. The concentration of H_2O_2 was held constant at 129 mM. The data are expressed as a double-reciprocal plot of the initial velocities of the reaction versus the Ca(II) concentration (millimolar). The concentrations of $LaCl_3$ were (△) 0, (■) 1, (□) 2, and (●) 3 mM. Velocities are expressed as micromoles of O_2 per milligram of chlorophyll per hour × 10^{-4}. (B) Linear competitive inhibition of the S_1-dependent reaction by $LaCl_3$ when H_2O_2 is the variable substrate. Concentrations of H_2O_2 are expressed as 10^3. The concentration of Ca(II) was held constant at 100 μM. The concentrations of $LaCl_3$ were (△) 0, (■) 1, (□) 2, (●) 3, and (○) 5 mM. Velocities are expressed as in Figure 3.

Since this rate is abolished by a Tris wash, evidently this rate results from a small fraction of reaction centers that have retained the Ca(II) after dialysis.

Lanthanum was used as a dead-end inhibitor to obtain an independent determination of the order of binding of substrates. The dependence of photosynthetic O_2 evolution on Ca(II) is known to be inhibited competitively by La(III) (Ghanotakis et al., 1985). The kinetics of inhibition by La(III) versus Ca(II) catalyzed by the S_1-dependent reaction are shown in Figure 4A. Lanthanum was a linear competitive inhibitor of the reaction. Concentrations of La(III) greater than 2 mM have been observed to cause inactivation of the OEC with release of manganese (Ghanotakis et al., 1985), which would effectively decrease the concentration of the enzyme in the assay. If La(III) released significant amounts of manganese in the time scale of the assays of Figure 4, the concentration of enzyme in the assay would vary as a function of substrate and inhibitor concentration. Since this effect would be smallest when the La(III) and Ca(II) concentrations are low and high, respectively, and greatest when these concentrations are reversed, hyperbolic double-reciprocal plots would be expected. The linearity of the double-reciprocal plots, even at the highest concentrations of La(III), indicates that the extent of inactivation of the OEC by La(III) in these assays is insignificant.

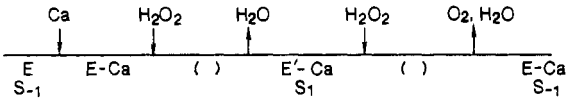
Figure 4B shows the inhibition kinetics of $LaCl_3$ versus the concentration of H_2O_2. Although La(III) is clearly an analogue of Ca(II) and not of H_2O_2, this dead-end inhibitor is competitive in a linear manner versus H_2O_2. The K_{is} values are 0.925 mM and 3.41 mM for the inhibition of O_2 evolution by La(III) versus Ca(II) and La(III) versus H_2O_2, respectively. These results support an ordered mechanism in which Ca(II) must bind to the enzyme prior to H_2O_2 for catalysis to proceed.

Discussion

The results of Figures 1 and 2 indicate that (1) the S_0-dependent and S_1-dependent catalytic cycles can be separated by flash illumination, (2) the S_0-dependent reaction involves the formation of the S_2 state, and (3) the S_1-dependent reaction does not involve the formation of the S_2 or S_3 state. Velthuys and Kok (1978) had proposed similar reactions based on the flash-induced yields of O_2 from thylakoids in the presence of H_2O_2. The results presented here and by Frasch and Mei (1987) confirm these earlier findings and extend them to show that light (charge separation in the reaction center) is not required for this reaction to occur.

Although H_2O_2 can cause a two-electron oxidation of the S_0 state to form the S_2 state, we have not observed that this reagent can oxidize the S_1 state to the S_3 state. Instead, H_2O_2 reduces the S_1 state by two electrons to form a putative S_{-1} state. The inability of the OEC to cycle between the S_1 and S_3 states in the presence of H_2O_2 may result from (1) the lack of accessibility of H_2O_2 to the S_3 state, (2) the possibility that manganese is not oxidized upon formation of the S_3 state, and/or (3) the possibility that an S_1/S_{-1} cycle operates far more efficiently than an S_1/S_3 cycle.

The initial velocity pattern (Figure 3) and the use of La(III) as a dead-end inhibitor (Figure 4) indicate a rapid equilibrium order mechanism:



First, in such a sequential mechanism, Ca(II) must combine with some form of the enzyme and come to thermodynamic equilibrium before H_2O_2 can bind. Second, both Ca(II) and H_2O_2 must bind to the enzyme before any product can be released. Lanthanum is known to compete with Ca(II) for binding to the Ca(II)-binding site on the OEC and inhibit photosynthetic O_2 evolution (Ghanotakis et al., 1985). The competitive inhibition by La(III) versus the concentration of H_2O_2 will result only if H_2O_2 is restricted to bind exclusively to the enzyme–Ca(II) complex.

The rapid equilibrium condition results when the K_A [the Michaelis constant for Ca(II)] becomes immeasurably small compared to the K_{ia} [the dissociation constant for Ca(II) from the enzyme]. This can result if the rate constant for the release of Ca(II) from the enzyme–Ca(II) complex far exceeds V/E_t and H_2O_2 can only combine with enzyme–Ca(II). However, it is unlikely that this rate of dissociation is large because extensive dialysis is required for the effective removal of Ca(II) from the oxygen-evolving complex (Ghanotakis et al., 1985).

Alternatively, the dissociation of Ca(II) need not be rapid relative to V/E_t if Ca(II) acts as an activator of the reaction rather than as a substrate. However, to show rapid equilibrium ordered kinetics, Ca(II) must not be able to dissociate once H_2O_2 has added or during any part of the catalytic cycle until H_2O_2 is free to add again. This implies that the rate of H_2O_2 combination with the enzyme–Ca(II) complex is greater than the rate at which Ca(II) dissociates from this complex. Since the reaction of Ca(II) is at thermodynamic equilibrium and H_2O_2 combines only after the addition of Ca(II), saturation with H_2O_2 reduces to zero the steady-state concentration of the enzyme complexes that contain Ca(II) without H_2O_2. Thus, the concentration of Ca(II) need only be stoichiometric with the amount of OEC present during the assay, which suggests that Ca(II) may provide a structural role to activate the enzyme rather than act as a substrate itself. Other enzymes that bind metals in a similar manner have been found to bind the metal with high affinity after which the metal often does not dissociate for several catalytic cycles (Cleland, 1977).

The Michaelis constant for H_2O_2 (Table I) is high compared to that for many enzymes. However, it should be noted that the K_M of most enzymes is approximately the concentration of the substrates in vivo. Since the in vivo concentration of the normal substrate for the OEC (water) is present at con-

centrations of about 55 M, a low K_M for the substrate has not been a necessity.

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Steric Repulsion between Phosphatidylcholine Bilayers†

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ABSTRACT: The change in pressure needed to bring egg phosphatidylcholine bilayers into contact from their equilibrium separation in excess water has been determined as a function of both distance between the bilayers and water content. A distinct upward break in the pressure–distance relation appears at an interbilayer separation of about 5 Å, whereas no such deviation is present in the pressure–water content relation. Thus, this break is not a property of the dehydration process per se, but instead is attributed to steric repulsion between the mobile lipid head groups that extend 2–3 Å into the fluid space between bilayers. That is, electron density profiles of these bilayers indicate that the observed break in the pressure–spacing relation occurs at a bilayer separation where extended head groups from apposing bilayers come into steric hindrance. The pressure–spacing data are used to separate steric pressure from the repulsive hydration pressure, as well as to quantitate the range and magnitude of the steric interaction. An appreciable fraction of the measured steric energy can be ascribed to a decrease in configurational entropy due to restricted head-group motion as adjacent bilayers come together.

The close approach of surfaces separated by solvent is opposed by several types of repulsive pressures, including electrostatic, hydration, and steric pressures. The first two of these have been studied extensively. Electrostatic interactions between charged surfaces can be explained in terms of classical double-layer theory (Verwey & Overbeek, 1948; Israelachvili & Adams, 1978). The hydration pressure, P_h, which arises from the work of removal of polarized water molecules from between hydrophilic surfaces, has been shown empirically to

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