

On the Kinetics and Mechanism of Spontaneous Intramolecular Reduction of the Central Metal Ion in $K_2[Mn(IV)\text{-}2\text{-}\alpha\text{-hydroxyethyl isochlorin } e_4]$ Acetate in Aqueous Alkaline Solutions and its Relation to the Binding Sites of Manganese in Photosynthesis

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Manganese Binding Sites, Photosynthesis, Spontaneous Reduction of $Mn(IV)$ -chlorin, Base Catalysis

Spectrophotometric investigation of the kinetics of the spontaneous reduction of the central metal ion in $K_2[Mn(IV)\text{-}2\text{-}\alpha\text{-hydroxyethyl-isochlorine } e_4]$ acetate in aqueous alkaline solution in the absence of any reducing agent reveals that it is a pseudo-first order reaction which is specifically hydroxide ion catalyzed. The pK_a -value of the acid-base equilibrium has been estimated to be 14.4.

Electron transfer to the central metal ion is the rate limiting step. The measurements of its temperature dependence yields an activation enthalpy of $\Delta H^\ddagger = 12$ kcal/mol and an entropy of activation $\Delta S^\ddagger = -30$ e.u. thus indicating that the electron transfer step is a bimolecular reaction. The most likely reactant is water. The reduction reaction does not take place with appreciable reaction rates at physiological pH. Thus, when bound to a suitable ligand of the chlorin type, $Mn(IV)$ -compounds are sufficiently stable with respect to autoxidation to play some role in biological redox reactions as postulated recently for the photoreactivation process of the water splitting system in photosynthesis.

Introduction

In recent years evidence has been presented for the involvement of higher oxidation states of manganese in photosynthesis. It has been established by Chéniaie and Martin¹ that the metastable precursor states of oxygen evolution^{2,3} which conserve positive charges are correlated with the manganese binding sites within the water splitting system of the thylakoid membrane. Furthermore, the existence of intermediate $Mn(III)$ - and $Mn(IV)$ -compounds has been postulated in the theory of photoreactivation of the oxygen evolving centers in manganese depleted algae⁴. Thus, a direct involvement of different oxidation states of manganese in the primary processes of photosynthesis appears to be possible. It is known that manganese is bound within the protein fraction of the thylakoid membrane^{5,6}, but the binding sites of manganese have not been characterized within a molecular framework as yet. Since manganese bound to a porphyrin or a chlorin ligand is able to attain the oxidation states +2, +3, and +4, it has been suggested by several authors^{7–10} that these compounds could possibly

be regarded as model compounds for the binding site of manganese *in vivo*.

The relation of these manganese complexes to the S-states of the water splitting system^{2,3} however, is not clear. The properties of $Mn(IV)$ -porphyrin and -chlorin complexes in aqueous alkaline solutions have not been investigated extensively as yet¹¹. The oxidation state +4 of manganese in $Mn(IV)$ -hematoporphyrin IX — the only example studied to some extent thus far — turned out to be of limited stability. The complex undergoes spontaneous reduction to a $Mn(III)$ -compound without adding any reducing agent^{10,12,13}. The nature of the oxidation product is still open to question. Evidence from infrared spectra was presented which argues in favor of the oxidation of the carboxylic acid side chain of hematoporphyrin IX under alkaline conditions¹⁰. In acid media, the formation of peroxide in 30% yield among which small amounts of H_2O_2 (7%) were found has been reported by Tabushi and Sojo^{12,13}. No oxygen has been detected neither in alkaline nor in acid solution^{10,12,13}. Since oxidation of water by $Mn(IV)$ -hematoporphyrin IX has not been unequivocally demonstrated as

* Measurements were taken from M. Müller: Diplomarbeit, Frankfurt 1974.

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yet, the correlation of this compound with the S_4 -state of the water splitting system appears not to be justified at present.

However, Mn(IV)-Hm IX does not model the S_2 - and S_3 -state either. Depending on the ionic strength I of the solution, the half life time of Mn(IV)-Hm IX at pH = 7.5 was determined to be 9.5 sec ($I = 1.2$ M/L) and 12.7 sec for $I = 0.012$ M/L¹². The biological desactivation reactions of the two metastable states S_2 and S_3 of the oxygen evolving system occur *via* reduction by electron donor substances and roughly obey pseudo-first order kinetics^{3, 15, 16}. In whole algae half life times of about 17 sec and 4–7 sec, respectively, have been reported^{14–16}. In isolated chloroplasts the half life time for both states varies between 26–55 sec^{15, 16}. Somewhat higher values (about 90 sec) have been observed by Forbush *et al.*³. This shows that spontaneous reduction of Mn(IV) accompanied by autooxidation of the active manganese binding site principally might be able to seriously interfere with biological desactivation reactions. Incidentally, this rapid reversion to a Mn(III)-compound would not fit the kinetic scheme for the photoreactivation of the oxygen evolving system⁴ either.

Thus, if manganese is involved in the primary photosynthetic reactions at all, the binding hypothesis mentioned above appears questionable in the light of the results obtained with Mn(IV)-hematoporphyrin IX as model compound.

It is not clear, however, whether spontaneous reduction is also observed with a porphyrin nucleus bound to a protein molecule. This depends on the detailed reaction mechanism of the reduction reaction which has not been elucidated thus far.

In this paper we present an investigation on the mechanism of the spontaneous reduction of an analogous Mn(IV)-chlorin compound, namely that of $K_2[Mn(IV)-2-\alpha$ -hydroxyethyl-isochlorin e_4]acetate. It is shown that the reduction reaction is base catalyzed and will not occur with appreciable reaction rates at physiological pH. Thus, a chlorin ligand molecule appears to be an appropriate candidate for the binding site of manganese in photosynthesis.

Material and Methods

Preparation of substance

Isochlorin e_4 (1,3,5,8-tetramethyl-4-ethyl-2-vinyl-chlorin- γ -acetic acid-7-propionic acid) and its 2- α -hydroxyethyl derivative are the main constituents of

sodium-copper-chlorophyllin (Merck, Germany)^{17–19}. After extraction of uncomplexed pigment constituents by 20% HCl from an ethereal solution of copper chlorophyllin the metal was removed by treatment with concentrated sulfuric acid (98%). This procedure also leads to oxidation of the vinyl side chain of the chlorin with formation of the 2- α -hydroxyethyl derivative²⁰. The product was recrystallized several times from ether and further purified by column chromatography. The positions of the absorption bands in pyridine-ether were found to be (in nm): 652, 624, 599, 547, 527, 499, 397, and are identical with those of 2- α -hydroxyethyl-iso-chlorin e_4 ²¹.

This identification was further confirmed by chemical test reactions: Treatment with colourless HI in glacial acetic acid^{22, 23}, with concentrated sulfuric acid at 100 °C²³, the D.E.E.-test^{19, 24}, and the reaction with benzoylchloride in pyridine¹⁹.

Manganese was inserted into the chlorin nucleus in anhydrous glacial acetic acid at 60 °C in a manner similar to the method described by Loach and Calvin²⁵. The potassium salt of this compound was prepared according to Glikman and Zabroda⁹.

Thin layer chromatography (chloroform/methanol 1:1, Al_2O_3) of the dimethylester of Mn(III)-2- α -hydroxyethyl-isochlorin e_4 revealed only one component to be present. For this analysis, 2- α -hydroxyethyl-isochlorin e_4 was esterified (methanol containing 5 vol.% concentrated H_2SO_4). Then manganese was inserted as described above.

$K_2[Mn(IV)-2-\alpha$ -hydroxyethyl-isochlorin e_4]acetate (1) was obtained from the corresponding Mn(III)-compound (2) by oxidation with $K_3Fe(CN)_6$. The absorption spectra of these compounds are shown on Fig. 1.

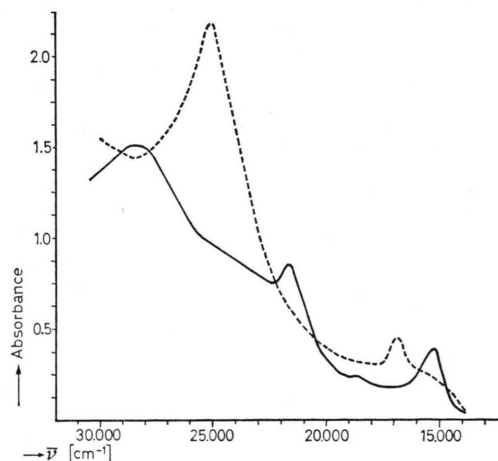


Fig. 1. Absorption spectra of $K_2[Mn(III)-2-\alpha$ -hydroxyethyl-isochlorin e_4]acetate (2) and its corresponding Mn(IV)-compound (1) in 2 N NaOH at 20 °C. — — — 1, ——— 2.

Kinetic methods

The kinetics of the reduction reaction was measured by following the time course of the absorbance at 400 nm — the main absorption peak of **1** — and at 357 nm — the predominant peak of **2**. The kinetic measurements were carried out with a Cary 15 spectrophotometer. Both the initial and end product of the reduction reaction contribute appreciably to the absorbance throughout the visible part of the spectrum. This was corrected for using the expression²⁶

$$\frac{c(t)}{c_0} = \frac{E_\lambda(t) - E_{\lambda,\infty}}{E_\lambda(0) - E_{\lambda,\infty}} \quad (1)$$

c = concentration of the initial compound;
 $E_\lambda(0)$ = absorbance at wavelength λ of the initial compound;
 $E_{\lambda,\infty}$ = absorbance at wavelength λ of the end product after completion of the reaction.

Results and Discussion

If the kinetic data obtained for the spontaneous reduction of **1** are evaluated according to Eqn (1), the reaction is first order in the concentration of **1** and in the concentration of **2** (Fig. 2). The experimental rate constant k_{ex} , however, depends on OH^- -concentration in a sigmoidal manner. The investigation of the role that OH^- plays in the reaction provides an important clue for the elucidation of the reaction mechanism.

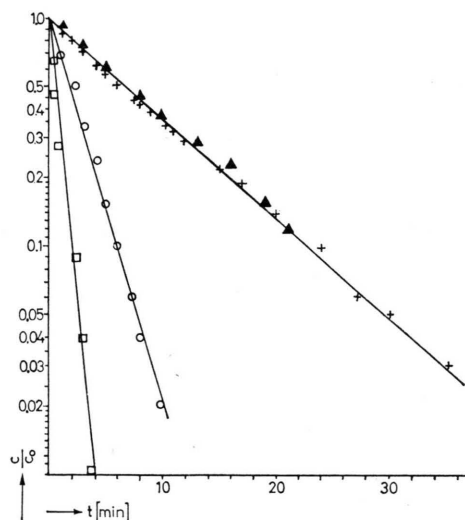
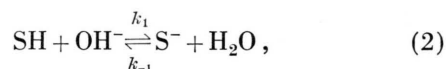


Fig. 2. Temperature dependence of the kinetics of spontaneous reduction of **1** in 2 N NaOH. Ordinate: Relative decrease of the concentration of **1** measured at 400 nm. +, 20 °C; ○, 40 °C; □, 60 °C; △, 20 °C, measured at 355 nm.

The sigmoidal OH^- -dependence of k_{ex} can be best accounted for using the reaction scheme



with the additional conditions

$$k_1 c_{\text{OH}^-} \cong k_{-1} c_{\text{H}_2\text{O}}, \quad (4)$$

$$k_2 \ll k_{-1}. \quad (5)$$

E is the end product of the reaction. The reactant A has been introduced here for reasons which will become clear later.

If A is present in excess, the kinetics of product formation is pseudo-first order

$$\begin{aligned} -\frac{d[c_{\text{S}^-} + c_{\text{SH}}]}{dt} &= -\frac{d[c_0 - c_{\text{E}}]}{dt} \\ &= \frac{k_2 f_{\text{S}^-} f_{\text{A}} c_{\text{A}} K}{f^* (1 + K)} (c_0 - c_{\text{E}}) \end{aligned} \quad (6)$$

where c_0 denotes the total concentration of the Mn(IV)-compounds, f^* the activity coefficient of the transition state, and

$$K = \frac{c_{\text{S}^-}}{c_{\text{SH}}} = \frac{K_{\text{a}} f_{\text{SH}}}{K_{\text{w}} a_{\text{H}_2\text{O}} f_{\text{S}^-}} a_{\text{OH}^-}. \quad (7)$$

K may be related to the basicity function b_- , which was introduced as an alkaline counterpart of h_0 in acid solution by Anbar *et al.*²⁷

$$b_- \equiv \frac{K_{\text{w}}}{h_-} = \frac{f_{\text{BH}}}{f_{\text{B}^-}} \frac{a_{\text{OH}^-}}{a_{\text{H}_2\text{O}}} \quad (8)$$

f_{BH} = activity coefficient of the indicator acid.

The relation between the indicator acidity function H_- and b_- is given by

$$H_- = -\log h_- = p K_{\text{w}} + \log b_-. \quad (9)$$

Thus, introducing b_- in Eqn (7) and (6) we obtain

$$k_{\text{ex}} = \frac{k_2 c_{\text{A}} b_-}{K_{\text{w}}/K_{\text{a}} + \frac{f^*}{f_{\text{S}^-} f_{\text{A}}} b_-} \quad (10)$$

if it is assumed, that the relation

$$\frac{f_{\text{SH}} f_{\text{A}}}{f^*} = \frac{f_{\text{BH}}}{f_{\text{B}^-}} \quad (11)$$

holds²⁹.

* It should be noted that the water concentration in this expression is normalized to unity following the convention on computation of equilibrium constants and rate constants.

H_- values for NaOH were calculated by a molar fraction scale according to Yagil and Anbar²⁸. The values of b_- can be obtained from Eqn (9). The results are presented on Table I.

Table I. Values of the indicator acidity function H_- and of the function b_- calculated by a molar fraction scale.

c_{OH^-} [M/L]	H_-	b_-	k_{ex} [min ⁻¹]
2	14.665	3.155	0.080
1	14.260	1.24	0.063
0.5	13.907	0.551	0.037
0.425	13.835	0.467	0.032
0.35	13.745	0.379	0.026
0.2	13.488	0.210	0.015

The plot $1/k_{ex}$ versus $1/b_-$ reveals a linear relationship (Fig. 3) in complete agreement with the theoretical expression (10). This result shows that the reduction reaction is specifically hydroxide ion catalyzed.

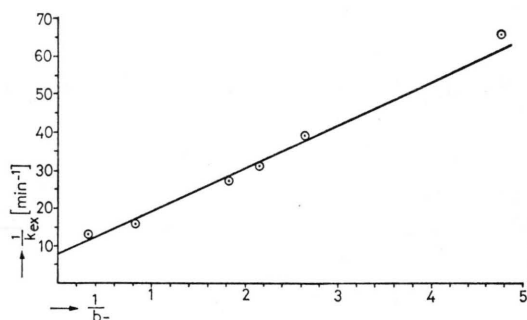


Fig. 3. Dependence of the rate constant k_{ex} on the basicity function b_- . $T = 20^\circ\text{C}$; $E_0 = 2.14$; $E_{4\infty,\infty} = 0.96$ (absorbance of the end product at 400 nm).

Spontaneous reduction proceeds faster in 2 N NaOD than in 2 N NaOH (Table II) when measured at equal complex concentration. This solvent isotope effect is in accordance with the model proposed above²⁹.

Table II. Reaction rates in 2 N NaOD and in 2 N NaOH. Concentration values are given in terms of the absorbance measured at 400 nm at the time $t=0$.

$E_{400}(0)$	base	k_{ex} [min ⁻¹]
1.69	OD ⁻	0.11
1.69	OH ⁻	0.075

From Eqn (10) the slope and the intercept with the $1/k_{ex}$ -axis of the straight line on Fig. 3 may be

calculated. For the slope we obtain

$$A = \frac{K_w}{K_a k_2 c_A} \quad (12)$$

and for the intercept with the ordinate

$$B = \frac{f^*}{f_S^- f_A k_2 c_A} \quad (13)$$

From Fig. 3 the values of the constant A and B are determined: $A = 11.5$ min; $B = 7.5$ min. Assuming that $f^*/f_S^- f_A$ is not far from unity the pK_a -value of the acid-base equilibrium can be estimated from Eqns (12) and (13) to be 14.4 at 20°C .

The site of proton release is not known. Two different proposals can be made:

1. The pK_a -value found is close to that of water ($pK_a = 15.74$). Thus, a dissociation equilibrium of a water molecule axially bound to the Mn(IV)-compound as suggested by Loach and Calvin for Mn(IV)-hematoporphyrin IX¹⁰ can be taken into consideration. The reactive species would then be the hydroxo-complex of **1**.
2. As an alternative explanation we suggest a proton dissociation equilibrium of the alcoholic α -hydroxyethyl-group in 2-position of the chlorin ring. The pK_a -value of ethanol is close to 17, but the introduction of strong electrophilic substituents like a chlorin macrocycle is expected to increase the acidity of the proton of the OH-group considerably.

The data given on Table I show that k_{ex} decreases proportional to c_{OH^-} with decreasing OH^- -concentration, contrary to Mn(IV)-hematoporphyrin IX the rate of which is considerably enhanced at lower pH-values^{10,12}. This indicates that the reaction mechanism for spontaneous reduction is essentially different for Mn(IV)-porphyrins and Mn(IV)-chlorin complexes.

Eqn (6) shows that the reaction is first order with respect to product formation and with respect to the sum of the concentrations of the two Mn(IV)-compounds present in the solution. It has not been possible to distinguish spectrophotometrically between these compounds so that the sum of the concentrations of both is actually measured^{**}.

The correct dependence of k_{ex} from OH^- -concentration can only be obtained, if it is postulated that

^{**} The extinction coefficients of SH and S⁻ are expected to be nearly equal.

the reaction $S^- + A \rightarrow E$ is rate limiting. This is confirmed by experiment. The rate of decay of **1** turned out to be identical with the rate of formation of **2** because the rate constants are the same when measured at 400 nm and at 355 nm — the predominant absorption peak of **2** (Fig. 2). This result demonstrates that electron transfer to Mn(IV) is the rate determining step within the reaction sequence.

The rate of this reaction depends on temperature (Fig. 2). From Eqn (10) it is seen that at high OH^- -concentration K_w/K_a may be neglected in the denominator. Then, the temperature dependence of k_{ex} reflects only that of k_2 . From an Arrhenius plot of the rate constants given on Table III an

Table III. Temperature dependence of rate constants for the reduction reaction in 2 N NaOH. $E_{400}(0)$ = absorbance at 400 nm at the beginning of the reaction. $E_{400,\infty}$ = absorbance at 400 nm after completion of the reaction.

T [°C]	k_{ex} [min ⁻¹]	$E_{400}(0)$	$E_{400,\infty}$
20	0.11	2.05	0.96
40	0.38	2.10	0.99
60	1.15	2.10	0.99

activation energy of $\Delta H^\ddagger = 12$ kcal/mol is obtained which corresponds to an entropy of activation of $\Delta S^\ddagger = -30$ e.u. This shows that the transition state is of highly ordered structure. The highly negative value of ΔS^\ddagger is reminiscent to the values obtained for bimolecular ester hydrolysis reactions which range between -15 and -30 e.u.³⁰, whereas for

unimolecular reactions ΔS^\ddagger was found to be substantially more positive. (See also³¹.) This indicates that the electron transfer step (3) is a bimolecular reaction. Since pseudo-first order kinetics was observed, the reactant A must be present in excess. Assuming OH^- or an intermediate Mn-compound as reactants would lead to results which are not compatible with experiment. Thus, we suggest that the reactant A is water*.

These results convincingly show that spontaneous reduction will not occur at physiological pH-values with appreciable reaction rates. From the data given in Table I the rate constant at pH=7 can be estimated to be $10^{-10} \text{ sec}^{-1}$. Thus, when bound to a suitable chlorin ligand molecule, the oxidation state +4 of manganese is sufficiently stable with respect to spontaneous internal decay to play some role in biological redox reactions as implicated in photosynthesis.

Clearly, this result may be of importance to any electron carrier which operates with a manganese redox system. In this connection it should be noted that the existence of a second binding site of manganese on the oxidizing side of photosystem II has been established that does not seem to be involved in photosynthetic oxygen evolution³². This manganese compound probably functions as electron carrier. However, this "two pool hypothesis" as proposed by Cheniae and Martin has recently been questioned by Chen and Wang³³.

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* Unfortunately the precision of rate data does not allow for the determination of the number of water molecules participating in the reaction according to the method of Anbar *et al.*²⁷.

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