Binuclear Manganese(III) Complexes as Electron Donors in D 1/D 2/Cytochrome *b* 559 Preparations Isolated from Spinach Photosystem II Membrane Fragments

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Dedicated to Professor Achim Trebst on the occasion of his 65th birthday

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The capability of different manganese complexes to act as PS II electron donors in D 1/D 2/cytochrome *b* 559 complexes has been analyzed by measuring actinic light-induced absorption changes at 680 nm (650 nm) and 340 nm, reflecting the photoaccumulation of Pheophytin$^-$ ($Pheo^-$) and the reduction of $NADP^+$, respectively. The data obtained reveal: a) the donor capacity of synthetic binuclear $Mn(III)_2$ complexes containing aromatic ligands significantly exceeds that for $MnCl_2$ in both cases, *i.e.* $Pheo^-$ photoaccumulation and $NADP^+$ reduction; b) manganese complexes can serve as suitable electron donors for light-induced $NADP^+$ reduction catalyzed by D 1/D 2/cytochrome *b* 559 complexes and ferredoxin plus ferredoxin-$NADP^+$ reductase under anaerobic conditions and c) the specific turnover rate of the system leading to $NADP^+$ reduction is extremely small.

The implications of these findings are briefly discussed.

Introduction

The essential steps of photosynthetic water cleavage take place within an integral membrane complex associated with extrinsic regulatory polypeptides. This operational unit is referred to as photosystem II (PS II) complex. The primary reactions of PS II comprise light absorption, excitation energy transfer to the photoactive pigment (a special chlorophyll *a* designated as P 680) and electron transfer from its excited singlet state to pheophytin *a* (Pheo) acting as primary acceptor and subsequent stabilization of the primary charge separation by rapid electron transfer from $Pheo^-$ to a special plastoquinone molecule Q_A (Renger, 1992). Based on similarities of the functional and structural organization of the primary processes, components P 680, Pheo and Q_A were inferred to be incorporated into a heterodimer of polypeptides D 1 and D 2 of PS II in an analogous way as the corresponding groups are incorporated into the heterodimer of the L- and M-subunit of the reaction centers of purple bacteria (Michel and Deisenhofer, 1988; Trebst, 1986). This idea is strongly supported by the isolation of PS II complexes that i) contain only D 1 and D 2 together with cytochrome *b* 559 (= Cyt *b* 559, two subunits) and at least one smaller polypeptide, and ii) retain the ability to perform the primary charge separation (Nanba and Satoh, 1987; Barber *et al.,* 1987). Although deprived of Q_A and the oxygen-evolving complex (OEC), the D 1/D 2/cytochrome *b* 559 complexes were found to catalyze the electron transport from an artificial electron donor (diphenylcarbazide) to an exogenous electron acceptor like silicomolybdate (Chapman *et al.,* 1988; Takahashi *et al.,* 1989). This observation implies that an electron transfer can take place from $Pheo^-$ to exogenous acceptors and that either this reaction or the electron donation are able competing with the rapid internal charge recombina-

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tion between $Pheo^-$ and $P680^+$. As the redox potential of Pheo/$Pheo^-$ is rather low ($E_{m,7}$ = −610 mV, see Klimov *et al.*, 1979), it appeared worthwhile to check if D1/D2/cytochrome *b*559 preparation are also able to reduce $NADP^+$ ($E_{m,7}$ = −320 mV). Recent reports claim that this can be the case (Allakhverdiev and Klimov, 1990, 1992; Arnon and Barber, 1990; Klimov *et al.*, 1986). In the present study manganese complexes were tested as electron donors for photoaccumulation of $Pheo^-$ in the absence of exogenous electron acceptors and for $NADP^+$ reduction under anaerobic conditions in the presence of ferredoxin (Fd) and Fd-$NADP^+$ reductase. The results obtained indicate that synthetic binuclear complexes are efficient electron donors in D1/D2/cytochrome *b*559 preparations.

Materials and Methods

D1/D2/cytochrome *b*559 preparations from spinach were obtained from digitonin/Triton X-100 PS II fractions (DT 20 preparations, see Allakhverdiev *et al.*, 1992; Klimov *et al.*, 1982) according to the method of Nanba and Satoh (1987) with some modifications as described in Allakhverdiev *et al.* (1992). The pigment content of the isolated D1/D2/cytochrome *b*559 was determined by HPLC. A ratio of about six chlorophylls per two pheophytins was found.

A single-beam differential spectrophotometer with a phosphoroscope similar to that described previously (Klimov *et al.*, 1979, 1986; Allakhverdiev and Klimov, 1990, 1992; Allakhverdiev *et al.*, 1992) was used to monitor at 650 nm and 680 nm the light-induced photoaccumulation of pheophytin$^-$ (= $Pheo^-$). Likewise, at 340 nm the photoreduction of $NADP^+$ was measured. Optical pathlength: 1 cm; measuring light beam λ = 340 nm, 650 nm or 680 nm; I ≈ 0.15 $W \cdot m^{-2}$; actinic light: λ > 600 nm, I ≈ 100 $W \cdot m^{-2}$. All measurements were performed at 20 °C.

The binuclear complexes $[Mn(III)Mn(III)(HNQOX)_4(OAc)_2]$ and $[Mn(III)-O-Mn(III)(HNQOX)_2(OAc)_2(H_2O)_2]$ symbolized by M-2 and M-3, respectively, contained 2-hydroxy-1,4-naphthoquinone monoxime (HNQOX) as terminal and CH_3COO^- (OAc) as bridging ligands (in M-3 there exist in addition a μ-oxo-bridge). These complexes were synthesized according to previously described procedures (Charles, 1963; Wieghardt *et al.*, 1985) and characterized by spectroscopic and electrochemical methods (Khan, 1993). Ferredoxin and ferredoxin-$NADP^+$ reductase were purchased from Sigma.

The compositions of the sample suspensions used in the different experiments are given in the figure legends.

Results and Discussion

When thylakoid and PS II membrane fragments are exposed to actinic illumination in the presence of the strong reductant dithionite ($Na_2S_2O_4$), $Pheo^-$ accumulates and gives rise to a marked quenching of the chlorophyll fluorescence (Allakhverdiev and Klimov, 1990, 1992; Allakhverdiev *et al.*, 1978, 1979, 1986; Shuvalov *et al.*, 1980; Renger and Kayed, 1987). Analogously, $Pheo^-$ photoaccumulation can be achieved also in

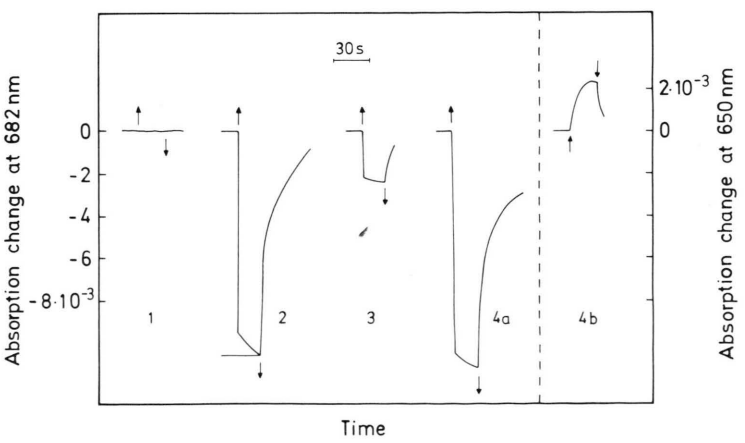

Fig. 1. Actinic light-induced absorption changes at 650 nm and 680 nm as a function of time in D1/D2/cytochrome *b*559 complexes. The sample suspension contained: D1/D2/cytochrome *b*559 complexes (5 μg chlorophyll/ml), 35 mM NaCl, 5 mM $MgCl_2$, 1 μM methylviologen, 20 mM tris(hydroxymethyl)aminoethane-HCl, pH = 8.0. Arrows indicate turning on (up) and off (down) the actinic light. Trace 1: control with no further addition; trace 2: addition of 1 mg $Na_2S_2O_4$/ml; trace 3: addition of 50 μM $MnCl_2$, traces 4a/b: addition of 5 μM manganese complex M-3.

D 1/D 2/cytochrome *b* 559 preparations in the presence of methylviologen (MV) and $Na_2S_2O_4$ (Barber *et al.*, 1987; Nanba and Satoh, 1987; Allakhverdiev *et al.*, 1992; Shuvalov *et al.*, 1989) and monitored by characteristic absorption changes in the red region. Light-induced absorption changes of D 1/D 2/cytochrome *b* 559 preparations are shown in Fig. 1. In the absence of $Na_2S_2O_4$/MV virtually no absorption change can be seen at 682 nm (trace 1 of Fig. 1), a wavelength characteristic for $Pheo^-$ formation (Allakhverdiev *et al.*, 1992; Klimov *et al.*, 1979). Lack of light-induced absorption changes is understandable because the primary radical pair recombines *via* kinetics in the time domain of tens of nanoseconds (Crystall *et al.*, 1989; Danelius *et al.*, 1987; Takahashi *et al.*, 1987). On the other hand, in the presence of $Na_2S_2O_4$/MV the actinic light causes a strong bleaching at 682 nm that biphasically relaxes in the subsequent dark period (trace 2 of Fig. 1). This effect is understandable by electron donation to $P680^+$ competing with the fast recombination reaction by $Pheo^-$. For kinetic reasons the quantum efficiency of this process is rather low (for a numerical analysis in thylakoids, see ref. Renger and Kayed, 1987). $Pheo^-$ photoaccumulation therefore provides an indirect test for the efficiency of PS II donors. Accordingly, this assay was used to analyze several manganese compounds for their capacity to act as PS II donor in D 1/D 2/cytochrome *b* 559 preparations. Trace 3 of Fig. 1 reveals that a much less pronounced bleaching is observed if $Na_2S_2O_4$ is substituted by 50 μM $MnCl_2$ acting as exogenous PS II electron donor (trace 3 of Fig. 1). At this concentration, the $MnCl_2$ to P680 ratio is about 60 based on a Chl content of 6 molecules per two pheophytins in the D 1/D 2/cytochrome *b* 559 preparations used in this study. In contrast to the comparatively small $Pheo^-$ photoaccumulation achieved by $MnCl_2$ even at excess concentrations, a full restoration of this activity is achieved by addition of the binuclear manganese complex $[Mn_2O(LwOx)_2(OAc)_2(H_2O)_2]$ where LwOx symbolizes the ligand 2-hydroxy-1,4-naphthoquinone monoxime and OAc is acetate. The amount of this complex (referred to as M-3) required to elicit the same extent of 682 nm bleaching as achieved by 1 mg/ml $Na_2S_2O_4$ (compare traces 2 and 4 a in Fig. 1) is comparatively small (10–12 manganese per P680). No further increase of the signal amplitude is observed at tenfold M-3 concentrations (data not shown). This effect clearly shows that compound M-3 acts as an efficient electron donor to D 1/D 2/cytochrome *b* 559 preparations. In order to check that the photobleaching at 682 nm mediated by compound M-3 really reflects $Pheo^-$ formation, comparative measurements were performed at 650 nm (trace 4 b of Fig. 1) where the difference spectrum exhibits a positive band (Allakhverdiev *et al.*, 1992; Klimov *et al.*, 1978, 1979). The amplitude ratio $\Delta\Delta_{650}/\Delta\Delta_{682}$ of the data nicely fits with that of the difference spectrum of $Pheo^-$ *versus* Pheo.

In a recent study (Allakhverdiev *et al.*, 1994) evidence was presented that the binuclear manganese complexes M-2 and M-3 are much more efficient than $MnCl_2$ in electron donation to PS II and restoration of the water oxidase activity in PS II preparations that are almost completely deprived of their endogenous manganese by N,N,N′,N′-tetramethylendiamine (TEMED) treatment (Ananyev *et al.*, 1992). A comparison of these findings with the data presented in Fig. 1 suggests that similar structural determinants are likely to be responsible for the different efficiencies of $MnCl_2$ and M-3 (M-2) in PS II complexes and D 1/D 2/cytochrome *b* 559 preparations. Accordingly, the results of Fig. 1 (traces 2 and4 a/b) can be considered as further evidence for a special affinity of D 1/D 2/cytochrome *b* 559 complexes to manganese depending on the structure of these compounds. The possible relevance of the present data in supporting the role of amino acid residues of polypeptide D 1 in the photoassembly of the tetranuclear manganese cluster in the intact water oxidase (Nixon *et al.*, 1992) remains to be clarified in future studies. Regardless of the latter problem, the data of Fig. 1 indicate the potential donor efficiency of the binuclear manganese complex M-3 in D 1/D 2/cytochrome *b* 559 preparations.

Within the context of the donor efficiency of several compounds in D 1/D 2/cytochrome *b* 559 complexes another phenomenon will be briefly analyzed in this study. Recently, evidence was presented for light-induced $NADP^+$ reduction catalyzed by D 1/D 2/cytochrome *b* 559 preparations in the presence of ferredoxin (Fd), Fd-$NADP^+$ reductase, plastocyanin and the PS II donor diphenylcarbazide (Arnon and Barber, 1990). Therefore,

it appeared worth analyzing the capacity of M-3 to support $NADP^+$ photoreduction in D1/D2/cytochrome *b*559 preparations. Experiments with different manganese compounds were performed under anaerobic conditions (glucose/glucose oxidase) in the presence of Fd and Fd-$NADP^+$ reductase. The process was monitored at 340 nm where the $NADP^+$/NADPH difference spectrum exhibits a pronounced peak. Fig. 2 shows the data obtained. In the absence of an exogenous electron donor virtually no change of the 340 nm absorption is induced by excitation of the sample with actinic light. A significant but minor increase of this absorption is observed upon illumination of D1/D2/cytochrome *b*559 preparations in the presence of 80 μM $MnCl_2$ corresponding to about 50 donor molecules per P680 (trace b of Fig. 2). The slope of the absorption increase progressively declines during the exposure to actinic illumination. After cessation of the light a decrease of the 340 nm absorption change is observed. A markedly more pronounced light-induced increase of the absorbance at 340 nm is elicited by the binuclear manganese complexes M-2 and M-3 (trace c and d, respectively, in Fig. 2). In order to confirm that these 340 nm changes are really due to $NADP^+$ reduction, the light-induced difference spectra were measured in the range from 300 to 400 nm. As expected, no light-induced change is observed in the control without any addition (trace a of Fig. 3). After addition of $NADP^+$ only a small increase of the absorbance is seen in the range from 300 to 400 nm (trace 2 of Fig. 3). In marked contrast, a pronounced difference spectrum arises in the presence of $NADP^+$ and compound M-3. The spectral feature is characteristic for $NADP^+$ reduction.

The idea of a PS II-catalyzed $NADP^+$ reduction is further supported by the finding that a special dinitrobenzene derivative recently discovered to act as electron transport inhibitor of D1/D2/cytochrome *b*559 preparations (Allakhverdiev *et al.*, 1989) completely suppressed the M-3-mediated light-induced 340 nm absorption change (data not shown).

The findings reported in this study unambiguously show that M-2 and M-3 are potent mediators of light-induced $NADP^+$ reduction in D1/D2/cytochrome *b*559 complexes. As in the case of photoactivation of the water oxidase activity in manganese-depleted DT20 PS II preparations the binuclear complex M-3 is also more efficient than M-2 in supporting $NADP^+$ reduction in D1/D2/cytochrome *b*559 samples (compare traces c and d

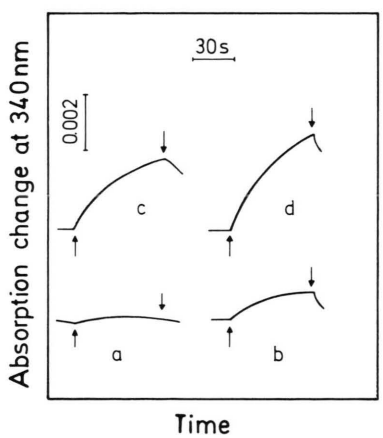

Fig. 2. Actinic light-induced absorption changes at 340 nm as a function of time in D1/D2/cytochrome *b*559 complexes. The sample suspension contained: D1/D2/cytochrome *b*559 complexes (10 μg Chl/ml), 35 mM NaCl, 3 mM $MgCl_2$, 3 mM $NADP^+$, 10 mM ferredoxin, 6 μg/ml Fd-$NADP^+$ reductase, 10 mM glucose, 50 activity units/ml glucose oxidase, 1000 activity units/ml catalase and 20 mM Tris-HCl, pH = 8.0. Arrows indicate turning on (up) and off (down) the actinic light. Trace a: no further additions; trace b: plus 80 μM $MnCl_2$; trace c: plus 10 μM binuclear manganese complex M-2; trace d: plus 10 μM binuclear manganese complex M-3.

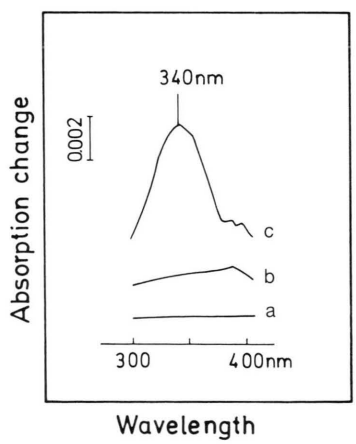

Fig. 3. Extent of actinic light-induced absorption changes as a function of wavelength in D1/D2/cytochrome *b*559 complexes. Experimental conditions as in Fig. 2 but without $NADP^+$. Trace a: no addition; trace b: plus 3 mM $NADP^+$; trace c: plus 3 mM $NADP^+$ and 10 μM complex M-3.

of Fig. 2). Two conclusions can be drawn from the result of this study: a) the donor capacity of manganese compounds depends on their chemical nature, *i.e.* binuclear manganese complexes are much more efficient than $MnCl_2$, and b) D1/D2/cytochrome *b*559 complexes can catalyze $NADP^+$ reduction with PS II donors under anaerobic conditions.

At first glance, the latter effect seems to be spectacular. However, it has to be emphasized that the absolute rate of $NADP^+$ reduction is rather slow. An evaluation of the data presented in Fig. 2 reveals that at most a rate of 2 μmol NADPH/mg Chl·h can be achieved under our experimental conditions. This value has to be compared with the more than 100-fold rates obtained with silicomolybdate as electron acceptor and either 1 mM DPC (about 900 μeq/mg Chl) or 1 mM $MnCl_2$ (400–500 μeq/mg Chl·h) as PS II donors (Chapman *et al.*, 1988). Therefore, the electron transfer from the acceptor side of D1/D2/cytochrome *b*559 preparations to the enzymatic system consisting of ferredoxin and Fd-$NADP^+$ reductase appears to be extremely slow. Based on a $NADP^+$ reduction rate of 2–4 μeq/mg Chl·h and a chlorophyll content of 6 per D1/D2/cytochrome *b*559 complex a rough estimation leads to values for the electron transfer time of the rate-limiting step that are of the order of 2–3 min. This phenomenon could be simply explained within the framework of the classical Marcus theory of thermally activated non-adiabatic electron transfer (Marcus and Sutin, 1985) by assuming a comparatively large distance between $Pheo^-$ and the iron sulfur cluster of Fd. Using the empirical relation recently derived for the distance dependence of the matrix element for electron coupling between redox groups in proteins (Moser *et al.*, 1992) distances are obtained in the range of 25–30 Å. These values are reasonable if one takes into account that the enzymes and the D1/D2/cytochrome *b*559 complexes form aggregates that are not favourable for electron transfer.

Therefore, it is concluded, that in principle $Pheo^-$ of PS II can provide the reducing power for $NADP^+$ reduction. However, for kinetic reasons owing to structural constraints only marginally small rates can be achieved. It has to be emphasized that this extremely low activity was observed under optimal conditions where $Pheo^-$ is photoaccumulated in its reduced state (see Fig. 1). In a PS II complex with open reaction center the $Pheo^-$ becomes reoxidized by Q_A with a reaction time of about 300 ps (Bernarding *et al.*, 1994; Eckert *et al.*, 1988; Nuijs *et al.*, 1986). Therefore, the probability of an electron transfer to $NADP^+$ is virtually zero, *i.e.* this reaction does practically not occur under normal conditions. For theoretical reasons it might be interesting to clarify whether or not the conditions for an electron transfer from $Pheo^-$ to $NADP^+$ can be drastically improved (*e.g.* by using lipophilic redox mediators).

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