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Functional Role of Vitamin K_1 in Photosystem I of the Cyanobacterium *Synechocystis 6803*†

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Received October 6, 1987; Revised Manuscript Received November 9, 1987

ABSTRACT: The function of vitamin K_1 in the primary electron-transfer processes of photosystem I (PS I) was investigated in the cyanobacterium *Synechocystis 6803*. A preparation of purified PS I was found to contain two vitamin K_1's per reaction center. One vitamin K_1 was removed by extraction with hexane, and further extraction using hexane including 0.3% methanol resulted in a preparation devoid of vitamin K_1. The hexane-extracted PS I was functional in the photoreduction of $NADP^+$, but the PS I after extraction using hexane–methanol was totally inactive. Activity was restored by using exogenous vitamin K_1 plus the hexane extract. Vitamin K_3 would not substitute. The room temperature recombination kinetics of the PS I extracted with hexane were not significantly modified. However, following the removal of both vitamin K_1's, the 20-ms recombination between $P\text{-}700^+$ and $P\text{-}430^-$ was replaced by a dominant relaxation ($t_{1/2}$ = 30 ns) due to recombination of the primary biradical $P\text{-}700^+$ A_0^- and a slower component originating from the P-700 triplet. This kinetic behavior was consistent with an interruption of forward electron transfer to the acceptor A_1. Addition of either vitamin K_1 or vitamin K_3 to such preparations resulted in restoration of the slow kinetic phase (>2 ms), indicating significant competition by the two exogenous quinones for electron transfer from A_0^-. In the case of vitamin K_3, this change in the kinetics was completely reversed by the addition of dithionite. A similar addition of reductant did not alter the kinetics induced by vitamin K_1, suggesting successful reconstitution of the acceptor site A_1. These data support the hypothesis that acceptor A_1 is vitamin K_1 and is a component of the electron-transfer pathway for $NADP^+$ reduction.

The reaction center of photosystem I (PS I)[1] in higher plants, algae, and cyanobacteria is known to comprise the primary donor (P-700) and five acceptors. The acceptors F_X, F_B, and F_A are iron–sulfur centers thought to engage in the terminal stages of electron transfer during the reduction of $NADP^+$ (Golbeck, 1987; Rutherford & Heathcote, 1985; Sétif & Mathis, 1986). The acceptor A_0 is presumably a chlorophyll *a* monomer (Bonnerjea & Evans, 1982; Gast et al., 1983; Shuvalov et al., 1986) and forms the primary biradical with P-700, and A_1 is a transient intermediate operating between A_0 and F_X (Bonnerjea & Evans, 1982; Gast et al., 1983). It has been suggested that A_1 is a quinone on the basis of ESR experiments (Gast et al., 1983; Thurnauer & Gast, 1985), and because of the exclusive localization of vitamin K_1 in PS I, it has been suggested that the acceptor A_1 might be vitamin K_1 (Schoeder & Lockau, 1986; Takahashi et al., 1985).

Flash-induced absorption experiments have shown that the recombination of $P\text{-}700^+$ A_1^- occurs with a $t_{1/2} \simeq 120$ μs at low temperature (Mathis & Conjeaud, 1979; Sétif et al., 1984), and a spectrum of this signal corrected for the contribution in absorbance by $P\text{-}700^+$ provides strong evidence that vitamin K_1 is the acceptor A_1 (Brettel et al., 1986).

The concentration of vitamin K_1 in the PS I of higher plants and cyanobacteria was shown to be 2 mol/mol of P-700 (Malkin, 1986; Palace et al., 1987; Schoeder & Lockau, 1986; Takahashi et al., 1985). Malkin (1986) has recently shown that 1 mol of vitamin K_1 per P-700 was readily extracted from PS I by dry organic solvent. The extracted preparation containing one vitamin K_1 per P-700 was not modified in primary electron transfer as judged by low-temperature ESR. This suggests that the vitamin K_1 which was readily extracted may not function in charge separation.

This paper reports that extraction of the second vitamin K_1 per P-700 requires a slightly more hydrophilic solvent. The

† This work was supported by the Competitive Research Grants Office of the USDA (81-CRCR-1-0767), the National Science Foundation (PCM-8302983), and a Senior International Fellowship sponsored by the Fogarty International Center, NIH.
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[1] Abbreviations: PS I, photosystem I; ΔA, absorption change; DPIP, dichlorophenolindophenol; ESR, electron spin resonance; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; HPLC, high-performance liquid chromatography.

0006-2960/88/0427-1494$01.50/0

kinetic behavior of the fully extracted PS I was determined by flash absorption spectroscopy with high resolution and shown to be consistent with interruption of forward electron transfer at the acceptor site A_1. Reconstitution experiments using authentic vitamin K_1 verify that it can occupy the A_1 acceptor site at the correct redox potential and restore the electron-transfer process necessary for $NADP^+$ photoreduction.

Experimental Procedures

Biological Samples. The cyanobacterium *Synechocystis 6803* was provided by Dr. F. Chauvat (Service de Biochimie, CEN-Saclay) and grown photoautotrophically on the BG-11 medium of Rippka et al. (1979) in 10-L batch culture. The cells were harvested at late logarithmic phase, washed with distilled water, and frozen. A typical preparation of PS I was conducted by using between 8 and 10 g of cells (wet weight). The cells were thawed in 0.4 M sucrose, 10 mM KCl, and 50 mM Na-Tricine, pH 7.6, and passed through a French press 3 times at 20 000 psi. The homogenate was centrifuged at 10000*g* for 10 min to remove unbroken cells and then at 200000*g* for 60 min to sediment thylakoids. The thylakoid pellet was resuspended in sucrose buffer and solubilized for 30 min using 1% octyl glucoside–0.5% sodium cholate at a chlorophyll concentration of 1.5–2 mg/mL. The suspension was then centrifuged at 10000*g* for 10 min to remove debris and then at 200000*g* for 60 min to sediment PS I. The PS I preparation was washed twice with distilled water to remove sucrose and salts and then lyophilized. The lyophilized preparation was stored in evacuated tubes at −80 °C.

Solvent Extraction. The PS I preparation was extracted with either dry hexane or hexane containing 0.3% methanol (v/v). Typically, 20 mg of lyophilized PS I was rapidly extracted 4 times at room temperature using 25 mL of solvent per extraction. Following extraction, the preparation was evacuated to remove residual solvent and rehydrated with 50 mM Na-Tricine, pH 7.6, and 0.2% Triton X-100 at a ratio of 2 mL of buffer/20 mg of PS I. After solubilization for 1 h at 5 °C, the preparation was centrifuged at 10000*g* for 10 min to remove a yellow-brown residue. The clear supernatant was retained and, if necessary, concentrated by using Centricon 30 microconcentrators (Amicon Co., Danvers, MA).

Vitamin K_1 Analysis. The PS I preparations were analyzed before and after the various solvent extraction protocols as follows: 2 volumes of chloroform–methanol (2:1) were added to the aqueous preparations and mixed thoroughly. After clarification by centrifugation, the lower chloroform layer was quantitatively recovered and taken to dryness. The residue was dissolved in a small volume of chloroform and analyzed directly by reversed-phase HPLC using a 30-cm analytical column (μBondapak C18, Waters, Millipore Corp.). Ethanol was used as eluant (1 mL/min), and retention times of about 7 min were observed for vitamin K_1 standards.

Reconstitution Protocol. Stock solutions of vitamin K_1 and vitamin K_3 were prepared in ethanol and stored at −30 °C. For reconstitution of the PS I preparations for flash absorption spectroscopy, the quinones were added directly to the extracted particles in aqueous solution (1% ethanol final concentration). To successfully reconstitute $NADP^+$ photoreduction, the hexane extract was also found to be required, and the following procedure for addition was adopted: the hexane extract was taken to dryness by evaporation and redissolved in a small volume of hexane (1 mL for 20 mg of dry PS I particles). A volume of extract, equivalent to that derived from an aliquot of extracted particles to be reconstituted, was placed in a small flat-bottomed vial (1 cm diameter × 2.5 cm) and evaporated to dryness. The extracted PS I particles (500 μL) were added to the lipid-coated vials and stirred in the dark at 4 °C. The quinone either was added directly to the aqueous phase or was included in the hexane extract prior to evaporation.

Table I: Concentration of Vitamin K_1 and Chlorophyll (Chl) Antenna Sizes in PS I Preparations of *Synechocystis 6803*[a]

preparation	Chl *a*/P-700	vitamin K_1/P-700
unextracted control	120 ± 11	2.1 (±0.2)
hexane extracted	120 ± 15	1.125 (±0.275)
hexane–methanol extracted	65 ± 6	<0.004

[a] P-700 was determined by difference spectrophotometry of samples oxidized with ferricyanide and reduced with ascorbate–indophenol (Sêtif et al., 1981a).

Enzymology. $NADP^+$ photoreduction was measured spectrophotometrically at 340 nm using an Aminco-Chance DW 2a UV–vis spectrophotometer equipped with tungsten–halogen source for actinic illumination of the sample. The actinic beam was passed through glass cutoff filters (Corning 4-96, Schott GG 435 plus Balzers Calflex IR block), and the photomultiplier was protected (Corning 7-54 plus Schott UG 11). The reaction mixture contained 50 mM Na-Tricine, pH 7.6, 10 mM $MgCl_2$, 2 mM sodium ascorbate, 30 μM DPIP, 1 mM $NADP^+$, 0.1 unit of ferredoxin–$NADP^+$ oxidoreductase (EC 1.18.1.2), and spinach ferredoxin (5 μg). The enzymes were obtained from Sigma Chemical Co.

Flash Absorption Spectrophotometry. Photoinduced changes in absorbance were measured in the microsecond time range using instrumentation as described previously (Sauer et al., 1978; Van Best & Mathis, 1980) with 1-cm path lengths for both the excitation flash (10 ns, broad band ≃ 595 nm) and the measure beam. Absorption changes at 820 nm in the nanosecond time range were measured by using a modification (Brettel & Sêtif, 1987) of an apparatus developed earlier (Van Best & Mathis, 1978). The measure beam was provided by a laser diode (Telefunken, type TXSK, 820 nm) and detected by using a silicone photodiode (Lasermetrics, Model 3117). The signal was amplified (10-20-1c, Nuclêtudes, bandwidth 500 Hz–500 MHz), digitized (Tektronix, R7912), and stored in a signal averager (Didac, Intertechnique). The resolution of the instrument was between 0.5 and 1 ns. The excitation flash was provided by a frequency-doubled mode-locked neodymium YAG laser. The excitation pulses were 532 nm, 30-ps full-width, and 7 mJ before attenuation.

Results

The concentration of vitamin K_1 and the chlorophyll *a* antenna sizes of the control PS I and solvent-extracted preparations are shown in Table I. In confirmation of earlier reports for PS I from higher plants (Malkin, 1986; Palace et al., 1987; Schoeder & Lockau, 1986; Takahashi et al., 1985) and cyanobacteria (Lockau et al., 1986), the *Synechocystis* preparation also contains two vitamin K_1's per reaction center, and one is readily extracted with dry nonpolar solvent. The extraction by hexane also removed a considerable fraction of the carotenoids and uncharged lipids, but no chlorophyll *a* was removed. Consequently, the chlorophyll *a* antenna size of the hexane-extracted preparation containing one vitamin K_1 per P-700 was unchanged.

Removal of the second vitamin K_1 per P-700 required the inclusion of a small amount of a more polar solvent in the hexane. Under our conditions of extraction (rapid at room temperature, as described fully under Experimental Procedures), the inclusion of 0.3% methanol proved to be the minimum concentration of methanol required. The extraction using hexane–methanol also removed additional carotenoids and lipids and approximately 50% of the antenna chlorophyll

Table II: Photoreduction of $NADP^+$ by *Synechocystis 6803* PS I Preparations

preparation	additions	incubation time (h)	rate of $NADP^+$ reduction: μmol (mg of Chl)$^{-1}$ h^{-1}	rate of $NADP^+$ reduction: % control
unextracted control	none		53.7	100
	100 μM vit K_3, no extract	16	<0.1	0
hexane extracted	none		44.3	82.5
	100 μM vit K_1, extract	16	38.2	71.1
hexane–methanol extracted	none		<0.1	0
	10 μM vit K_1, extract	16	19.9	37.0
	20 μM vit K_1, extract	16	26.5	49.3
	100 μM vit K_1, extract	16	13.2	24.6
	200 μM vit K_1, extract	16	13.0	24.2
	200 μM vit K_1, extract	2	<0.2	0
	200 μM vit K_1, extract	4	3.7	6.9
	100 μM vit K_1, no extract	16	<0.1	0
	100 μM vit K_3, no extract	16	<0.1	0
	100 μM vit K_3, extract	16	<0.1	0
	no quinone, extract	16	<0.1	0

a. Preliminary analysis of both the hexane and hexane–methanol extracts revealed the presence of four carotenoids including β-carotene, monogalactosyl diglyceride, and two uncharged diglycerides.

$NADP^+$ Photoreduction. The activities of the control and two extracted preparations of PS I in the photoreduction of $NADP^+$ were investigated. Table II shows that following extraction by hexane (one vitamin K_1 per P-700 remaining) the activity was reduced about 17%. We suggest that this reduction in activity was due to the physical manipulation involved during solvent extraction and rehydration rather than to the loss of vitamin K_1 per se. Removal of the second vitamin K_1 per P-700 following treatment of PS I by hexane–methanol resulted in complete loss of photoreduction activity. However, in contrast to the hexane-extracted PS I, the hexane–methanol-extracted PS I responded to the addition of exogenous vitamin K_1, and partial restoration of $NADP^+$ photoreduction was observed. The conditions developed for reconstitution showed that low concentrations of vitamin K_1 were effective but only if the extracted PS I was also treated with the hexane extract with the vitamin K_1 and for relatively long incubation times. The hexane–methanol extract was not used because of the presence of extracted chlorophyll *a*. Direct addition of neither vitamin K_1 nor the extract alone was effective in reconstitution. Presumably, the concentration of vitamin K_1 in the hexane extract (50% that of control PS I) was too low.

The efficiency of the reconstitution can be estimated by reference to the photoreduction rates presented on a chlorophyll basis in Table II and after correction for the depletion of the antenna due to extraction. The reconstitution using 20 μM vitamin K_1 per 2 μM P-700 was about 25% of the control PS I. Assuming that 17% of the centers were irreversibly inactivated by the handling procedures, it appears that approximately 30% restoration was achieved. This value can be considered to be experimentally significant.

Parallel experiments were performed using vitamin K_3 added either directly or in combination with the hexane extract using an identical protocol, but no reconstitution was observed. In fact, the results show that addition of a relatively high concentration of vitamin K_3 inhibited the control PS I. As will be discussed below, this was most likely due to competition between the exogenous quinone and the primary electron-transfer process.

These data indicate that extraction procedures that remove both vitamin K_1's per P-700 result in loss of functional electron transfer for $NADP^+$ photoreduction and that vitamin K_1 can partly restore the activity.

Flash Absorption Spectroscopy. The preparations were investigated by high-resolution flash absorption spectroscopy

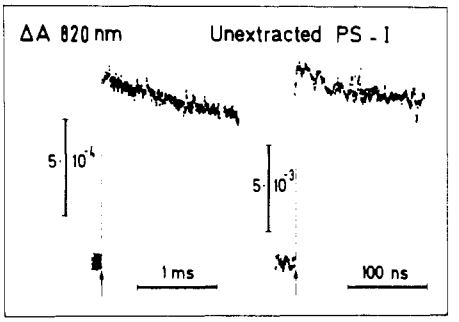


FIGURE 1: Flash-induced absorption changes in control PS I preparations from *Synechocystis 6803*. Measuring wavelength, 820 nm; preparation poised with 2 mM sodium ascorbate–30 μM DPIP in 50 mM Na-Tricine, pH 7.6, and 0.2% Triton X-100. Millisecond time range: optical path length, 10 mm; temperature, 21 °C; chlorophyll concentration, 30.2 μg/mL. Nanosecond time range: optical path length, 10 mm; temperature, 4 °C; chlorophyll concentration, 150 μg/mL.

to address whether vitamin K_1 behaves as the endogenous acceptor A_1. The kinetic behavior of the control PS I was determined, and Figure 1 shows the absorption change at 820 nm measured at room temperature. In the absence of added electron acceptor, observation of the various recombination reactions between $P\text{-}700^+$ and the reduced acceptors is generally considered to be diagnostic of the extent of forward electron transfer (Golbeck, 1987; Rutherford & Heathcote, 1985; Sétif & Mathis, 1986). Figure 1 shows that the decay of the absorption transient is slower than 2 ms, and on a nanosecond time scale on the right, only a minor rapid kinetic component ($t_{1/2} \simeq$ 20–30 ns) was detectable. This confirms that the dominant absorption change represents the back-reaction originating between the species $P\text{-}700^+$ and $P\text{-}430^-$ (Hiyama & Ke, 1971) and that the preparation was efficient in forward electron transfer to the terminal iron–sulfur centers.

The cyanobacterial PS I was then studied at low temperature to verify the existence of absorption changes previously ascribed to A_1 in the PS I of higher plants (Sétif et al., 1984). The sample was poised with ascorbate–indophenol, equilibrated, and cooled in total darkness. The absorption transient induced by the first flash, as illustrated in the inset of Figure 2 (trace a), had a decay half-time of $\simeq$160 μs. The second and third flashes induced transients with similar kinetics but of decreasing signal amplitude (not shown). After brief illumination with white light, the sample stabilized, and trace b in Figure 2 shows the final absorption change after about 50% of the centers had undergone irreversible charge separation. The absorption difference spectrum was then determined and is characteristic of $P\text{-}700^+$ minus P-700, showing a maximal

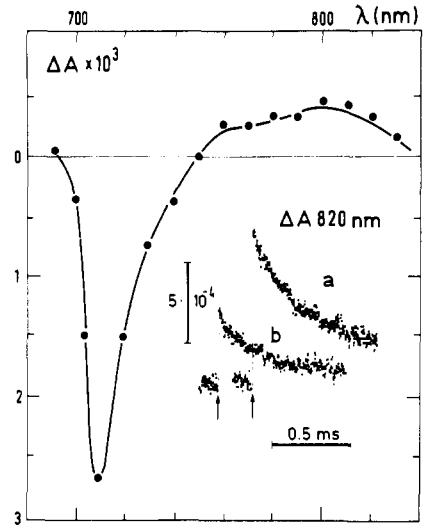


FIGURE 2: Difference spectrum for flash-induced absorption changes in PS I from *Synechocystis 6803* at 10 K. Sample conditions as in Figure 1 but containing 66% glycerol and 21.9 μg/mL chlorophyll. Optical path length, 2 mm. The inset upper trace (a) shows the absorption change at 820 nm induced by the first flash after cooling in darkness. The lower trace (b) shows the 820-nm absorption change after the sample had stabilized following illumination with white light. The spectrum was taken after the sample had stabilized.

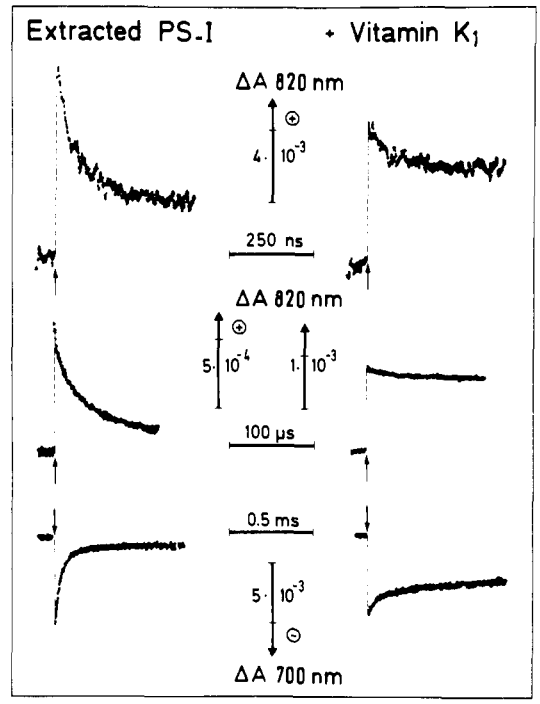


FIGURE 3: Flash-induced absorption changes in PS I from *Synechocystis 6803* after extraction of the preparation using hexane–methanol (left-hand column) and following the addition of vitamin K_1 (right-hand column). Conditions as in Figure 1. Nanosecond time range: ΔA measured at 820 nm; chlorophyll concentration, 81.75 μg/mL; temperature, 4 °C. Vitamin K_1 concentration was 25 μM. Microsecond time range: ΔA measured at 820 nm; chlorophyll concentration, 27 μg/mL; temperature, 21 °C. Vitamin K_1 concentration was 5 μM. Millisecond time range; same samples as were used in the microsecond experiment, but the ΔA was measured at 700 nm.

bleaching around 710 nm and a rather broad positive absorption at wavelengths longer than 750 nm (Figure 2). The spectrum obtained, together with the kinetic measurements, indicates that the signal was due to recombination between P-700$^+$ and A_1^- (Sétif et al., 1984).

Additional control experiments were conducted at room temperature following oxidation of the samples with 2 mM ferricyanide prior to the excitation flash. An absorption change at 820 nm was observed of approximately 10% the ΔA shown in Figure 1 and with a decay half-time of 25–30 μs, possibly due to a small contribution of P-680$^+$ (not shown). On the basis of these experiments, the PS I preparation from *Synechocystis 6803* was considered to be kinetically similar to those derived from higher plants and could be confidently compared with the PS I preparations prepared by using Triton X-100 or digitonin for thylakoid solubilization (Sétif & Mathis, 1986).

Similar optical experiments were then performed on the solvent-extracted preparations to determine their pattern of primary electron transfer and the effect of addition of exogenous vitamin K_1. The absorption transients observed from hexane-extracted PS I containing one vitamin K_1 per P-700 were very similar to the control PS I described above (Figure 1). There was a slightly greater contribution in the signal due to the 30-ns decay phase, but the addition of vitamin K_1 was without effect (not shown). In contrast, the kinetic behavior of PS I extracted with hexane–methanol containing no detectable vitamin K_1 was considerably changed. Figure 3 presents flash-induced absorption transients on three time scales for extracted PS I (left column) and following the addition of vitamin K_1 (right column). The absorption change at 820 nm, resolved in the nanosecond time range (upper trace, left-hand column), shows a dominant rapid decay phase representing 70–75% of the signal. The rapid phase had a $t_{1/2}$ = 30 ns and was most likely due to recombination between partners of the primary biradical P-700$^+$ A_0^- (Sétif et al., 1985). The slower kinetics were examined in the microsecond time range (center trace, left-hand column) and appear to be biphasic with a component of $t_{1/2}$ = 20 μs and a minor component of $t_{1/2} > 2$ ms. The faster $t_{1/2}$ = 20 μs phase was most likely due to decay of triplet P-700 originating from the P-700$^+$ A_0^- back-reaction, and this phase was observed to decrease in rate to a half-time greater than 2 ms upon the addition of dithionite. As will be discussed below, this was probably due to the removal of oxygen. The millisecond kinetic phase (lower trace, left-hand column) was most likely due to about 10% residual P-700$^+$. This overall kinetic behavior was consistent with interruption of the forward electron-transfer sequence at A_1.

The effect of addition of vitamin K_1 on the kinetic behavior of the extracted PS I is shown in the right-hand column of Figure 3. In the nanosecond range (upper trace), it can be seen that the rapid kinetic phase due to P-700$^+$ A_0^- recombination decreased concomitant with an increase in a slow phase. The slow phase comprised about 75% of the initial ΔA and corresponded to about 50% of the maximal signal amplitude from the extracted PS I shown on the left. Resolution of the slow phase on longer time scales (center and lower traces, right-hand column) shows a dominant component decaying in milliseconds and corresponding very closely with those of the unextracted control PS I (Figure 1), and a very small contribution due to residual triplet P-700. A spectrum of the slow phase was determined from absorption transients measured in the millisecond range, and the signal at 700 nm is illustrated in the lower trace, right-hand column. The spectrum (not shown) was characteristic of P-700$^+$ with a maximum bleaching around 700 nm and a broad positive absorption in the near-infrared region. We conclude from the kinetic data presented in Figure 3 that the extraction of PS I by hexane–methanol results in inhibition of forward electron transfer from the intermediate A_0 and that the addition of vitamin K_1 apparently restores functional electron transfer beyond A_1.

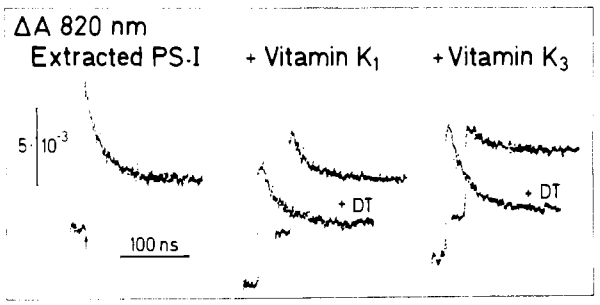


FIGURE 4: Flash-induced absorption changes at 820 nm in hexane–methanol-extracted PS I from *Synechocystis 6803*. Conditions as in Figure 1b, but the chlorophyll concentration was 98.3 μg/mL. (Left) PS I extracted with hexane–methanol. (Center) Extracted PS I following addition of 10 μM vitamin K_1. (Right) Extracted PS I following addition of 10 μM vitamin K_3. After the absorption changes were measured, 5 mM sodium dithionite was added to the samples as shown (+DT).

A wide variety of exogenous electron acceptors interact with the reducing side of PS I (Trebst, 1974), and the possibility existed that vitamin K_1, although effective in competition for electron transfer from A_0^-, may have acted as an unspecific acceptor in the above experiments rather than reconstituted the A_1 active site. To investigate this possibility, the effect of reducing the exogenous vitamin K_1 with excess dithionite was explored because it is known that the endogenous acceptor A_1 is not reducible by dithionite (Bonnerjea & Evans, 1982; Sétif et al., 1984). The results of these experiments together with a comparison of the effect of vitamin K_3 are shown in Figures 4 and 5. The absorption transients at 820 nm in Figure 4 show that the kinetic behavior of PS I in the presence of vitamin K_1 is not modified by the addition of dithionite (middle traces). Vitamin K_3 also induced similar kinetics, but its effect was completely reversed after dithionite addition (right traces). More extensive data are presented in the upper graph of Figure 5, showing that the concentration of vitamin K_1 required for saturation is 5 μM for reaction centers equivalent to 2 μM P-700. The addition of dithionite did not significantly change the kinetics at any concentration of vitamin K_1. The lower graph in Figure 5, obtained by using the same extracted PS I under identical conditions, shows that the slow kinetic phase induced by vitamin K_3 is maximal at around 10 μM, indicating effective competition with A_0^- in the $P\text{-}700^+$ A_0^- recombination. However, in contrast to the situation using vitamin K_1, the addition of dithionite resulted in complete reversal of the kinetics to those of the extracted PS I shown in Figures 3 and 4. This behavior was expected for an exogenous electron acceptor, albeit with high affinity, whereas the data for vitamin K_1 were consistent with occupancy of the A_1 acceptor site.

Experiments were performed using the protocol developed for reconstitution of $NADP^+$ photoreduction that also required inclusion of the solvent extract in addition to vitamin K_1. The absorption transients obtained were similar to those shown above for addition of vitamin K_1 alone, but the residual rapid phase ($t_{1/2}$ = 30 ns) was usually absent, suggestive of a more complete reconstitution. However, the extract did not appear to be obligatory for reconstitution of the primary processes.

Discussion

Previous investigators have shown that PS I preparations isolated following detergent treatment are functional in the photoreduction of $NADP^+$ when provided with supplemental ferredoxin, ferredoxin–$NADP^+$ oxidoreductase, and electron donor (Boardman, 1970). Although the rates reported above for the preparation from the cyanobacterium *Synechocystis*

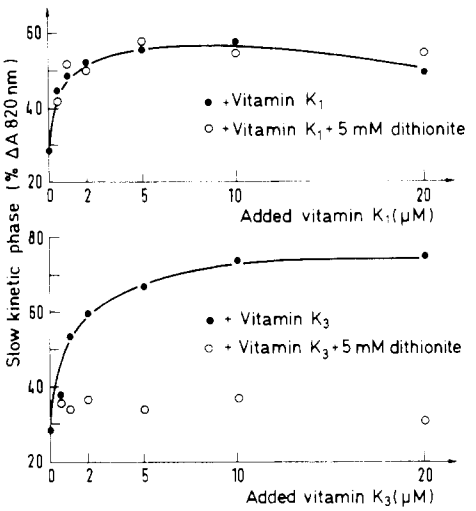


FIGURE 5: Concentration dependence for vitamin K_1 and vitamin K_3 for induction of the slow (millisecond) kinetic phase in the flash-induced absorption change in extracted PS I. The conditions were as specified in Figure 4 except for the quinone concentrations as indicated. Dithionite was 5 mM final concentration.

are low by comparison, they are significant in that vitamin K_1 is functionally implicated in the primary electron-transfer process under physiological conditions. The requirements for reconstitution of extracted PS I indicate that some component in the solvent extract is necessary in addition to the exogenous vitamin K_1. At this stage, the active component has not been identified, but the extract contains carotenoids and uncharged lipids in addition to smaller molecular weight hydrophobic compounds which may be candidates. Takahashi and Katoh (1987) have reported on the importance of carotenoids for maintaining the structural integrity of the protein fraction of PS I, and Ikegami (1983) has implicated neutral lipids in the reconstitution of antenna chlorophyll *a* to pigment-depleted PS I. Because the solvent extract does not appear to be essential for reconstitution of the primary electron-transfer processes, we suggest that the role of the solvent extract may be related to the structural requirements necessary for interaction of the ferredoxin–oxidoreductase binary complex with the PS I reaction center.

Several predictions may be made regarding the kinetic behavior of PS I after removal and reconstitution of the acceptor A_1. The following basic premises are pertinent:

(1) Extraction of A_1 should lead to loss of the $P\text{-}700^+$ $P\text{-}430^-$ recombination and introduction of recombination between $P\text{-}700^+$ and A_0^-. This will result in a mixture of kinetic components due to the rapid back-reaction between the partners in the primary biradical and slow (millisecond range) decay of triplet P-700 which originates as a product in this back-reaction. The triplet yield has been estimated to be between 25% and 35% using an extinction coefficient for $^3P\text{-}700 = 6000\ M^{-1}\ cm^{-1}$ (Sétif et al., 1985).

(2) Reconstitution of A_1 should restore forward electron transfer to the center F_A, and, therefore, the $P\text{-}700^+$ $P\text{-}430^-$ back-reaction (millisecond range) should appear (Hiyama & Ke, 1971) at the expense of the faster kinetics from the triplet decay and the $P\text{-}700^+$ A_0^- recombination.

(3) The amplitude of the absorption change at 820 nm following A_1 extraction should be larger than both the control PS I and the preparation reconstituted with A_1. This is because the primary acceptor A_0^- contributes to the signal in addition to the $P\text{-}700^+$. If the extinction coefficient of A_0^- is taken to be that of the chlorophyll radical anion, 5000 M^{-1} cm^{-1} (Fujita et al., 1978), and that of $P\text{-}700^+$ assumed to be

6500 M^{-1} cm^{-1} (Mathis & Sétif, 1981), then the maximal ΔA of control and A_1-reconstituted PS I preparations will be about 56% that of a preparation depleted of A_1.

(4) The acceptor A_1 must have a redox potential low enough to reduce the iron–sulfur center F_X ($E_m \simeq -0.71$ mV) (Chamarovsky & Cammack, 1982). Thus, A_1 should not be reducible by dithionite as has been shown previously in flash absorption experiments (Sétif et al., 1984).

The results presented above satisfy these four criteria and therefore provide strong support for the identity of the primary acceptor A_1 as vitamin K_1. The flash kinetic data are reinforced by the apparent specific reconstitution of $NADP^+$ reduction also by vitamin K_1. Of the two molecules of vitamin K_1 present per PS I, it is the tightly bound vitamin K_1 that is more resistant to extraction which appears to be the intermediate in electron transfer. The functional role of the second vitamin K_1, which is more readily extracted by dry solvent and is presumably located in a different environment, remains unknown.

As mentioned above under Results, the addition of dithionite decreased the rate of the decay we assumed to represent the triplet P-700, most likely due to the removal of oxygen. This is in contrast to previous observations by Sétif et al. (1981b), who showed that the triplet yield and decay kinetics were not affected by oxygen concentration. However, it should be emphasized that the preparations studied here were considerably depleted of carotenoids and oxygen may well have exerted a quenching effect. A similar effect of dithionite on the P-700 triplet lifetime has also been observed during a recent flash absorption study of the ether-extracted preparation of PS I according to Ikegami and Katoh (1975) that is totally depleted of carotenoids (Ikegami et al., 1987).

Regarding the biochemistry of the reconstitution of PS I by vitamin K_1, the concentration of vitamin K_1 necessary to restore the primary reactions was found to be within the same order as the concentration of reaction centers. A higher concentration was required for restoration of $NADP^+$ photoreduction, but the conditions were not strictly comparable, and some quinone may have bound to the lipid fraction that was also found to be required. It can also be noted (Table II) that very high concentrations were less effective possibly because the excess exogenous vitamin K_1 competed with the endogenous forward electron transfer.

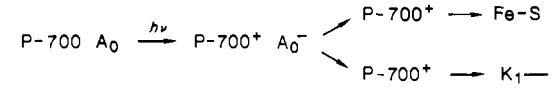
Comparison between the kinetic behavior of the extracted preparations supplied with either vitamin K_1 or vitamin K_3 indicates some constraint in the quinone structure required for successful reconstitution. Vitamin K_3 appears to act as an electron acceptor with high affinity and competes efficiently at A_0, but will not restore $NADP^+$ reduction which is an excellent indicator of complete forward electron transfer. Vitamin K_3 also does not operate as A_1 on the basis of criterion 4 that we adopted concerning the redox potential of endogenous A_1. This indicates that the 3-phytyl chain of vitamin K_1, in addition to providing an additional ca. −50 mV in potential to the substituted naphthoquinone (Rich & Bendall, 1980), may also be necessary to provide the correct interaction between vitamin K_1 and the A_1 active site. It is presumably this interaction that provides the environment necessary to confer an even lower midpoint potential on vitamin K_1 in order to function as A_1 (Brettel et al., 1986).

Recently, Palace et al. (1987) reported that vitamin K_1 is not an obligatory component in PS I electron transfer based upon similar experiments but using the photoinactivation of vitamin K_1 in situ rather than solvent extraction procedures to remove vitamin K_1. They showed that after the total destruction of vitamin K_1, as monitored by reversed-phase HPLC, the PS I was still functional in the room temperature recombination between $P\text{-}700^+$ and $P\text{-}430^-$. Their conclusions are in direct contradiction to the results presented here, and no obvious explanation can be forwarded at this time.

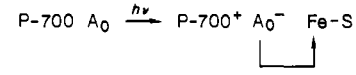
An additional anomaly is that the extracted cyanobacterial PS I described here and a spinach PS I preparation extracted with ether according to the procedure of Ikegami and Katoh (1975) were studied by ESR at 10 K, and both preparations showed photoreduction of the iron–sulfur centers, F_X, F_A, and F_B (Sétif et al., 1987). The ether-extracted spinach PS I also showed the $P\text{-}700^+$ A_0^- back-reaction at room temperature (Ikegami et al., 1987). The fact that both vitamin K_1 depleted preparations appear to be blocked at A_0, as revealed by room temperature flash absorption spectroscopy, but are apparently functional in electron transfer to iron–sulfur centers as detected by ESR at 10 K was addressed in detail by Sétif et al. (1987). They suggested alternate models of PS I electron transfer that included the following:

(1) Vitamin K_1 is not involved in PS I electron transfer.

(2) Vitamin K_1 is not on the main pathway to iron–sulfur centers but may be on a second pathway, e.g.:



(3) Modification of the electron-transfer pathway results from structural changes caused by freezing the preparations for the 10 K ESR measurements, e.g.:



The reconstitution experiments reported here clearly rule out the first possibility, particularly with the demonstration of the recovery of $NADP^+$ photoreduction upon addition of vitamin K_1. The second possibility should be seriously considered and cannot be ruled out until an absorbance difference spectrum of $P\text{-}430^-$/P-430 is determined at room temperature on the PS I preparations reconstituted with vitamin K_1. This experiment is in progress. The third model bears generally on the validity of data acquired at 10 K to those obtained at physiological temperature. In this instance, we emphasize that extraction of the PS I using organic solvent removed a considerable amount of lipid and carotenoid. Such lipid-depleted preparations may be more susceptible to structural modification during the freezing process required for the ESR measurements at 10 K.

ACKNOWLEDGMENTS

We thank S. Andrianambinintsoa for growing the cyanobacteria and M. G. Berger for assistance with the HPLC.

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Identification of the Altered Pyrrole in the Isomeric Sulfmyoglobins: Hyperfine Shift Patterns as Indicators of Ring Saturation in Ferric Chlorins†

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ABSTRACT: Analysis of the ^{1}H NMR hyperfine shift patterns of isomeric sulfmyoglobins is carried out in the met-aquo and met-cyano states to determine the site of saturation in each protein. The utility of the patterns for structure elucidation is established by specific deuterium labeling of the heme methyls of the terminal base product. On the basis of the known saturation of ring B in this isomer [Chatfield, M. J., La Mar, G. N., Lecomte, J. T. J., Balch, A. L., Smith, K. M., & Langry, K. C. (1986) *J. Am. Chem. Soc. 108*, 7108-7110], the methyl resonance of the saturated ring is found to have strongly attenuated contact shift. Thus, the heme methyl contact shift pattern is diagnostic for the saturated pyrrole in the high-spin state. This rationale is then applied to analyze the assigned NMR spectra of the initial and terminal acid sulfmyoglobin products, revealing that the same ring B is saturated in each isomer. In contrast, the heme methyl contact shift pattern in low-spin ferric complexes reveals that the methyls both on the affected pyrrole and on the trans pyrrole are influenced similarly on sulfmyoglobin formation, precluding the use of this methyl shift pattern as a unique indicator of the site of saturation. Identification of exchangeable proximal histidine resonances for met-aquo sulfmyoglobin complexes with shifts similar to that in native myoglobin dictates inconsequential axial alterations in the sulfmyoglobins, while location of downfield meso proton resonances analogous to those of the native protein demonstrates the retention of the coordinate water in the active site of met-sulfmyoglobin.

Sulfmyoglobin (SMb)[1] is a green heme derivative of myoglobin (Mb) in which the native heme, **1**, has reacted with a sulfur atom in a manner that leads to saturation of the aromatic skeleton (Berzofsky et al., 1971). The chemical nature

† This work was supported by grants from the National Institutes of Health (GM 26226 and HL 22252).
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[1] Abbreviations: SMb, sulfmyoglobin; S_AMb, S_BMb, and S_CMb, isomeric forms of sulfmyoglobin; Mb, myoglobin; metMb, ferric myoglobin; NMR, nuclear magnetic resonance; ppm, parts per million; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

0006-2960/88/0427-1500$01.50/0