PHOTOREDUCTION OF MEMBRANE-BOUND PARAMAGNETIC COMPONENT X BY WATER AS ELECTRON DONOR

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1. Introduction

The use of electron paramagnetic resonance (EPR) spectrosopy for the study of photosynthetic electron transport has led in recent years to the identification in photosynthetic membranes of three bound paramagnetic components closely linked to the primary electron-transfer reactions of photosystem I. Two of the membrane-bound paramagnetic components, initially described as belonging to bound ferredoxins, are now generally known as iron–sulfur centers A and B [1–4]. The third, whose chemical identity is less certain, is known as component X [5–10] and is thought to be identical with component A_2 that was detected spectrophotometrically [11,12].

Although the midpoint redox potential of component X has not been determined directly, the potential is believed to be lower than −700 mV [13,10]; the potential of center B is about −580 mV, and that of center A is about −530 mV [4,14]. In the reduced state, center A is characterized by resonances at g = 1.86, 1.94 and 2.05 and center B, by resonances at g = 1.89, 1.92 and 2.05 [1–4]; component X has a distinctive signal at g = 1.75–1.78 and less readily distinguishable signals at g = 1.88 and 2.08 [5–10].

Component X has been assigned the role of primary electron acceptor for electrons released by the photooxidation of *P*-700, the reaction-center chlorophyll of photosystem I [5–10]. Component X has hitherto been detected only under extremely reducing conditions. Photoreduction was either reversible or irreversible at cryogenic temperatures, depending on the pretreatment of the sample prior to freezing. The experimental conditions under which reversible photoreduction took place involved illumination at low temperatures (8–13 K) after prior reduction of centers A and B by dithionite at alkaline pH at room temperature in the dark [5,6,8]. The irreversible reduction of component X was observed when the sample (with dithionite at alkaline pH) was illuminated at room temperature and then frozen in liquid nitrogen under continuous illumination [7,9].

We have described the photoreduction of centers A and B in photosynthetic membranes by a normal photosynthetic electron-transport pathway at room temperature with water (or reduced dichlorophenolindophenol) as the electron donor [15]. In this investigation, we sought similar evidence to establish that component X could also be photoreduced by water, the physiological electron donor in plant photosynthesis. We found that, under anaerobic conditions in the presence of an oxygen-trapping system, the photoreduction of component X with water as the sole electron donor could be demonstrated both in chloroplasts and in cyanobacterial membrane fragments. The EPR signal of photoreduced component X was irreversible at cryogenic temperatures. In the presence of $NADP^+$ and soluble ferredoxin, the signal was extremely small, an indication that electron transfer from component X occurs during photosynthetic electron transport from water to $NADP^+$.

2. Methods

Broken spinach chloroplasts were prepared by a procedure which, as shown [16], maintained the

integrity of the complete electron-transport chain from water to $NADP^+$ and the capacity for ferredoxin-catalyzed photophosphorylation. Cyanobacterial thylakoid membrane fragments that have a high capacity for light-induced electron transport from water to $NADP^+$ (fraction C) were prepared from cells of *Nostoc muscorum* (strain 7119) as in [17]. Chlorophyll was measured [18] and ferredoxin and ferredoxin-$NADP^+$ reductase were isolated from spinach leaves and purified by the procedures in [19,20]. Glucose oxidase (type VII) and bovine catalase were purchased from Sigma Chemical Co.

The chloroplasts and the cyanobacterial membrane fragments (in their respective reaction mixtures) were placed in quartz EPR tubes (3 mm i.d.). When the oxygen-trapping system was used, the prepared reaction mixture was immediately placed in the EPR tube and gassed with argon. The EPR tubes were illuminated at room temperature for 30 s, followed without interruption by illumination for another 30 s through a window of a silvered dewar containing liquid nitrogen in which the samples were placed to be frozen. Illumination was provided by a quartz–halogen lamp with the light passing through several heat-absorbing filters.

First-derivative EPR spectra of the samples were recorded on a Bruker Instruments Co. EPR spectrometer (model ER200tt) equipped with 20 cm (8 inch) double-yoke magnet. The samples in the quartz tubes were cooled to the desired temperature in the cavity with liquid helium by an Oxford Instruments, Inc. temperature controller (model DTC) and cryostat (Model ESR9) (equipped with a quartz dewar cell made by J. F. Scanlon, Solvang, CA). Where indicated, g-values of signals were calculated from their relative distances from the free-radical signal ($g = 2.003$) on the spectra.

3. Results

EPR signals characteristic of photoreduced component X were produced in cyanobacterial membrane fragments and in chloroplasts by two methods. One method served as a link with earlier observations [7,21] and, as a control treatment, it involved the use of dithionite at pH 10 and illumination before and during freezing in liquid nitrogen. In the second method, the photosynthetic membranes were kept at a neutral pH, water served as the electron donor, and strict anaerobicity was maintained during illumination by the presence of an oxygen trapping system (glucose, glucose oxidase, and catalase). Here again, illumination was continued while the samples were being frozen in liquid nitrogen.

As shown in fig.1, illumination in the presence of

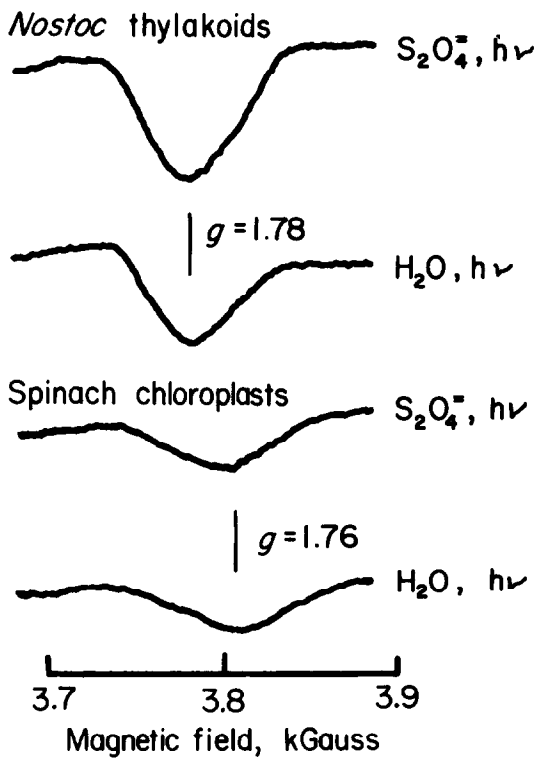

Fig.1. EPR signals of photoreduced component X. A suspension of cyanobacterial membrane fragments (*Nostoc* thylakoids) or spinach chloroplasts (1 mg chl./ml) was illuminated in a quartz EPR tube (3 mm i.d.) at room temperature with white light and then frozen quickly by immersing the tube into liquid nitrogen under continued illumination. The samples marked $S_2O_4^{2-}$ contained 10 mM Na_2SO_4 and 100 mM glycylglycine buffer (pH 10). The samples marked H_2O contained an oxygen-trapping system consisting of 10 mM glucose, 0.1 mg/ml glucose oxidase and 0.03 mg/ml catalase. The reaction mixture included, for *Nostoc* thylakoids, 50 mM Tricine buffer (pH 7.6) and 10 mM $MgCl_2$ or for spinach chloroplasts, 50 mM Tricine buffer (pH 8.2), 10 mM $MgCl_2$, 2.5 mM ADP, and 2.5 mM K_2HPO_4. The EPR spectra were recorded at 15 K; microwave frequency, 9.42 GHz; microwave power, 50 mW; magnetic field modulation, 20 G at 100 kHz.

dithionite at pH 10 produced an EPR signal at $g = 1.78$ in cyanobacterial thylakoids and at $g = 1.76$ in spinach chloroplasts. These signals, indicative of reduced component X, were recorded at the low temperature (14 K) and high microwave power (50 mW) that are required for the detection of component X (cf. [7,21]).

Figure 1 also shows that the $g = 1.78$ signal in cyanobacterial thylakoids and the $g = 1.76$ signal in chloroplasts were produced, in the absence of dithionite, by illumination at room temperature of a reaction mixture in which water was the only available reductant. The signals were induced by white or red light but not by far-red light; DCMU was inhibitory (data not shown).

To document further that the signals at $g = 1.78$ and 1.76 were indeed due to the reduced form of component X, the effects of temperature and microwave power on signal intensity were examined in both 'water-induced' and dithionite-induced signals (fig.2,3). The temperature profiles showing a maximum signal intensity around 12 K were similar in both cases (fig.2) and were consistent with other observations [8]. Likewise, the microwave power saturation characteristics (fig.3) showed that the signals produced by physiological electron transport from water were the same as those produced by the dithionite treatment. The very high power saturation characteristics observed here were also consistent with earlier results [8,9].

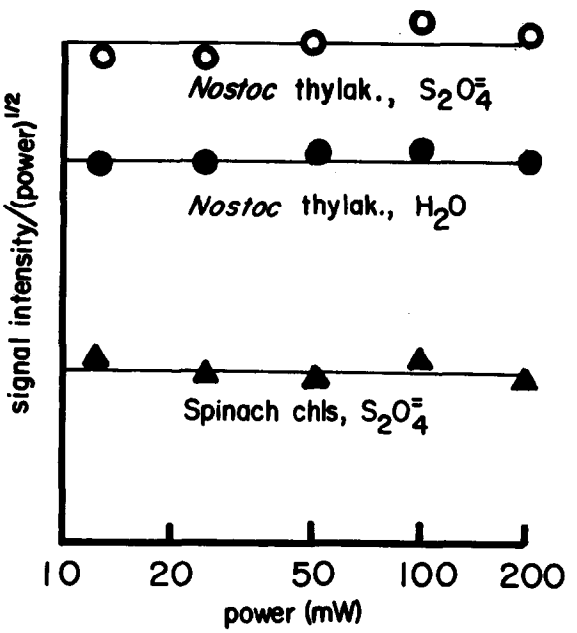

Fig.3. Microwave power saturation plots for center X signals. The ratios of signal intensity (arbitrary units) to the square root of microwave power were plotted against microwave power (cf. [9]). Except for varied microwave power, experimental conditions were as in fig.1.

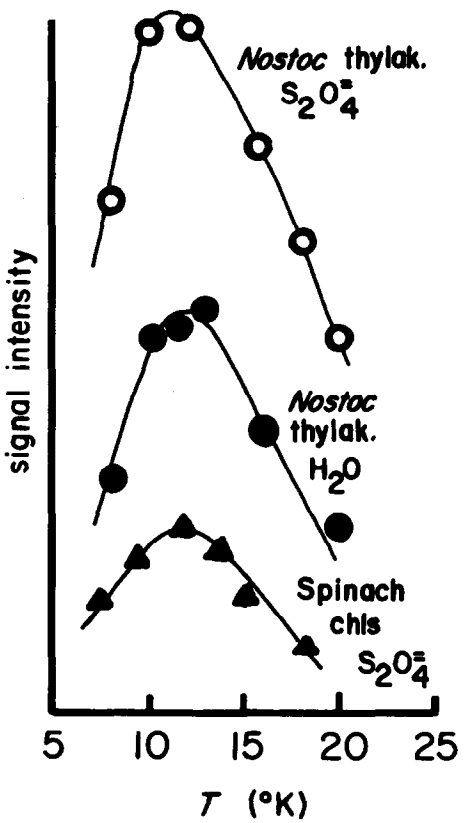

Fig.2. Effect of temperature on center X signal intensity. Except for the varied temperature, experimental conditions were as in fig.1.

With water as the reductant, the light-induced $g = 1.78$ and 1.76 signals were prominent only under strictly anaerobic conditions, in the presence of the glucose oxidase system as a trap for oxygen liberated by the photooxidation of water. The presence of air resulted in an almost complete absence of the signal (fig.4). Furthermore, even when anaerobic conditions

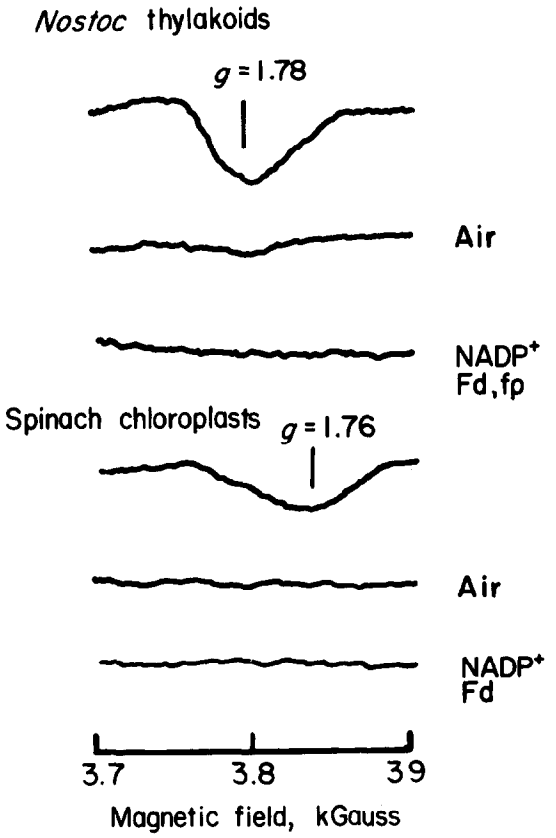

Fig.4. Effect of air and $NADP^+$ on EPR signals of center X. Traces marked 'Air' were obtained with reaction mixtures that were aerated briefly before illumination and did not contain the glucose oxidase oxygen-trapping system. Traces marked '$NADP^+$,Fd' were obtained with reaction mixtures that included 0.01 mM ferredoxin, a saturating amount of spinach ferredoxin-NADP reductase, and 2 mM NADP. Other conditions were as in fig.1.

were maintained, the $g = 1.78$ and 1.76 signals were almost completely abolished in the presence of ferredoxin and $NADP^+$ (fig.4). The signal of photoreduced component X was also observed under anaerobic conditions with reduced dichlorophenolindophenol as the electron donor (in the presence of DCMU). Here again, the signal disappeared upon the addition of ferredoxin-$NADP^+$ (data not shown).

4. Discussion

Earlier investigations have shown that the detection of component X was possible only when both iron-sulfur centers A and B were fully reduced [5–7]. This was also the case in the present experiments when water served as the electron donor for component X: parallel EPR measurements of centers A and B confirmed that both centers were in the fully reduced form.

The EPR signals of reduced component X that were induced at room temperature by photosynthetic electron transport from water were subsequently immobilized by freezing the reaction mixtures in liquid nitrogen. Once frozen, the signal of reduced component X remained stable for a long time: it showed no appreciable change when the samples were stored for more than 1 week under liquid nitrogen. The irreversible photoreduction of component X and its stability were observed earlier in samples prepared under strongly reducing conditions with dithionite [7,21]. In our preparations, this stability of reduced component X is even more remarkable in view of the fact that no strong external reductant was used: the photochemically-generated reducing power depended solely on water as the electron donor.

The difference in g-values between the signals of cyanobacterial and chloroplast membrane preparations should be noted. The g-value of the high-field signal (in its first derivative form) of component X in spinach chloroplast preparations has been reported to be 1.75 [5,6], 1.76 [7,9] and 1.78 [8], and to be 1.78 in cyanobacterial membrane fragments [21]. We have consistently found this signal to be at $g = 1.76$ in spinach chloroplasts and at $g = 1.78$ in *Nostoc* membrane fragments. Other properties of our signals, including temperature and microwave power saturation characteristics, are very similar to those previously reported.

The photoreduction of component X by water and the apparent oxidation of reduced component X by the ferredoxin-$NADP^+$ system are consistent with the idea that component X is an electron carrier in noncyclic electron flow from water to $NADP^+$. However, the possibility cannot be excluded that component X may also play a role in a cyclic electron flow linked with photosystem I. Other work [16,22–24] with similar chloroplast preparations has shown that a cyclic electron flow (and photophosphorylation) operates concurrently with a noncyclic electron flow from water to $NADP^+$ and is regulated by NADPH.

The possible role in cyclic electron flow of component X and its relation to *P*-430 [25–27] and to iron–sulfur centers A and B are under investigation.

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