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KINETICS OF APPEARANCE AND DISAPPEARANCE OF LIGHT-INDUCED EPR SIGNALS OF P700$^+$ AND IRON–SULFUR PROTEIN(S) AT LOW TEMPERATURE

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SUMMARY

Light-induced changes of EPR signals in Photosystem-I subchloroplast particles at temperatures between 225 and 13 °K showed that the rates of onset of photooxidation of P700 and photoreduction of iron–sulfur protein(s) are identical and instantaneous within the limits of resolution of our instruments The fraction of the P700$^+$ EPR signal that appears reversibly decreased with decreasing temperature down to 13 °K when the photoreaction was completely irreversible At temperatures below 225 °K, the reversible fraction consists of two approximately equal portions with decay halftimes of approx 3 and 75 s, respectively Light-induced absorption changes due to P700 photooxidation at low temperatures monitored at 700 nm showed a similar kinetic pattern

Since the reduced iron–sulfur protein signals can only be detected at very low temperature, their decay kinetics cannot be continuously monitored at higher temperatures Therefore, exposure at appropriate temperatures and reaction times were selected according to the decay kinetics of P700$^+$, after which decay was stopped by lowering the temperature to 13 °K and the P700$^+$ and reduced iron–sulfur protein signals were recorded and compared In the temperature range (225–13 °K) studied, the decay of P700$^+$ and reduced iron–sulfur protein signals appears identical, suggesting that the two oppositely charged species recombine in the dark These experiments support the view that iron–sulfur protein(s) is the reaction partner of P700 in the primary photochemical act of Photosystem I

INTRODUCTION

In 1971, Hiyama and Ke [1] reported kinetic–spectroscopic observations which suggested the existence of a spectral species with the behavior expected of the primary electron acceptor of Photosystem I This species was designated "P430", because the major band of the light-induced absorption changes occurred at 430 nm The possible role of P430 as primary acceptor was further supported by measurements of quantum yield and rise times [2, 3] The difference spectrum of P430 resembles closely that of soluble spinach ferredoxin, but the difference extinction based on the molarity of P700 was greater [1–4]

Malkin and Bearden [5] observed a light-induced EPR signal which resembled reduced ferredoxin when spinach chloroplasts previously freed of soluble ferredoxin were illuminated Because the reduction occurred at liquid-N_2 temperature, the authors suggested that this "bound" ferredoxin might function as the primary electron acceptor This finding was subsequently confirmed by others [6–9] Furthermore, the light-induced EPR signal attributable to an iron–sulfur protein was found to be associated with Photosystem I [8–10], and completely absent in Photosystem II [11]

In an attempt to clarify the chemical identity of P430, we compared two Photosystem-I reactions by spectrophotometric and EPR measurements These were the accumulations of $P700^+$ and $P430^-$, respectively Primary photochemical charge separation followed by a "one-way" electron discharge by $P430^-$ to an autooxidizable secondary acceptor results in a difference spectrum of exclusively $P700^+$ [12] Illumination of Photosystem-I subchloroplasts in the presence of an efficient reductant and under anaerobic conditions results in the reduction of both P700 and P430 [4] Subsequent EPR measurements of these reactions confirmed that exclusively $P700^+$ free radicals [13] or reduced iron–sulfur proteins [9] were formed in the two respective reactions

Because the EPR signals of the reduced iron–sulfur proteins involved are detected only at very low temperatures (< 25 °K), kinetic correlation requires controlled cycling of sample temperature between that desired for the reaction and that necessary for observation It would seem promising to study the decay of the charged species at low temperatures because the back reactions are expected to be much slower However, background information on the low-temperature reactions of P700 is often contradictory, and that of P430 is totally lacking Some workers found P700 photooxidation to be practically irreversible at approx 110 °K [14–17], while others reported a partial reversibility [5, 7, 18–21]

This note presents the decay kinetics of $P700^+$ monitored by EPR spectroscopy over a wider range of low temperatures The low-temperature kinetic data were then used as a guide for selecting temperatures and time intervals for examining the decay of the reduced iron–sulfur proteins According to our results, the dark decay of the signals of $P700^+$ and reduced iron–sulfur protein at low temperatures agree to an extent that the idea of P700 and iron–sulfur protein being the reaction partners in the primary photochemical act of Photosystem I receives strong support

EXPERIMENTAL

A Photosystem-I subchloroplast particle fractionated from spinach by Triton treatment (TSF-I, ref 18) with a NADP-reduction activity of 1 0 mmole/mg chlorophyll per h was used for all experiments The particle had a ratio of chlorophyll/P700 of 60, and of chlorophyll/total cytochrome of 360 Samples used for EPR experiments contained TSF-I particles in 0 1 M Tricine buffer, pH 8 0, at a chlorophyll concentration of 0 55 mM, with 5 mM ascorbate, and 50 μM tetramethyl-*p*-phenylenediamine

All EPR experiments were carried out in quartz tubes with 4 mm inner diameter

White light from a G E SR8 tungsten ribbon lamp with appropriate optics was used for illumination The light beam passed through 5 cm water in order to remove heat Incident intensity at the sample tube was approximately 10^6 ergs cm^{-2} s^{-1}, and was above saturation Illumination was for 10 or 20 s

EPR spectroscopy was performed in a modified Varian spectrometer, under the same experimental conditions as described previously [9] The temperature for recording the EPR spectra of iron–sulfur protein was 13 35±0 15 °K, those for recording the P700$^+$ signals are specified in the text, power, 3 mW for the iron–sulfur protein, for P700, 1 μW at 13 °K, 10 μW at 75 °K, 30 μW between 100 and 175 °K, and 100 μW between 200 and 225 °K, modulation frequency 100 kHz, amplitude, 10 G for reduced iron–sulfur proteins and 2 G for P700$^+$, time constant, 0 25 s for kinetic experiments, 0 5 s for recording the spectra

For recording the EPR spectra and decay kinetics of P700$^+$ at 13 and 75 °K, a liquid-helium boil-off system with a heater installed in the sample dewar was used For the temperature range 100–200 °K, a separate liquid-N_2 boil-off system was used For higher temperatures, gaseous N_2 passing through a copper coil immersed in an ethanol–solid CO_2 bath was used as the coolant The EPR spectra of iron–sulfur proteins were always recorded at 13 °K

For the correlation of the decaying fractions of P700$^+$ and reduced iron–sulfur protein, the subchloroplasts were first illuminated in the cavity for 20 s at 13 °K, then the EPR spectra of P700$^+$ and reduced iron–sulfur protein were recorded The temperature of the EPR sample was then changed from 13 °K to the pre-selected temperature for a given amount of time, and then returned to 13 °K followed by re-recording the EPR spectra For 13 and 75 °K, the sample tube was retained in the EPR cavity, and the temperature was raised by producing appropriate heat input The temperature transition, either downward or upward, took less than 1 min For the measurement at 75 °K, the gold–iron/Chromel thermocouple junction was placed in the top part of the sample, and the temperature was continuously recorded After the desired temperature was reached, occasional temperature excursions lasting 0 5–1 min occurred but they were not more than +1 5 °C or −2 °C These temperature excursions did not have effects on the decay kinetics which required corrections For the temperatures 125, 150 and 175 °K, an isopentane bath cooled by liquid N_2 was used To thermostat the EPR sample, the tube was quickly transferred from the cavity to the bath and kept there for a pre-selected time period For 225 °K, an ethanol–solid CO_2 bath was used The time required for transferring the EPR tube was about 5 s, and the bath temperatures were maintained to within one degree All operations were carried out in the dark

RESULTS

Photooxidation of P700 and its dark decay at low temperatures

Light-induced changes of the EPR signal at $g = 2\,0026$ which represents P700$^+$ formation and decay at various temperatures are shown in Fig 1-A The formation of P700$^+$ free radicals was within the combined time resolution of the instruments (approx 0 25 s) Upon cessation of illumination, the decay at 225 °K appeared to follow first-order kinetics, with a half time of 7 7 s However, the signal decay was not complete, at 225 °K, about 10 % of the change was irreversible at the

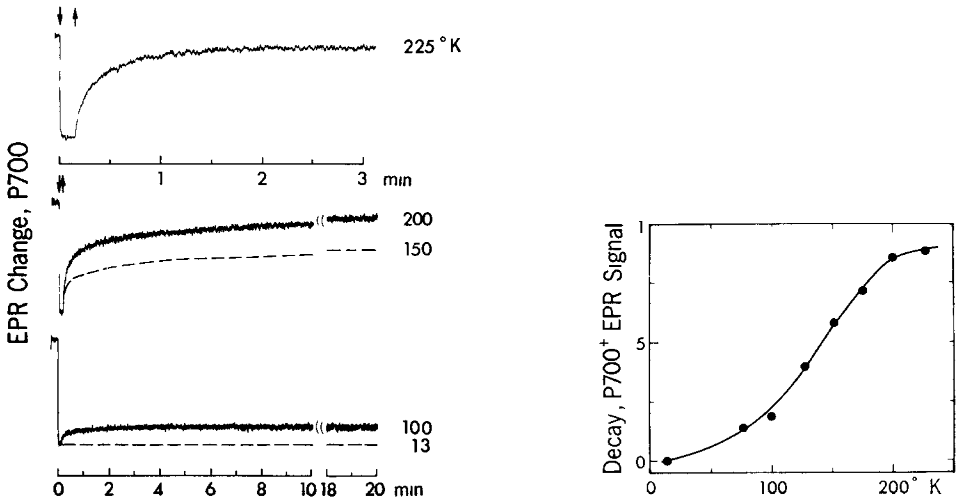


Fig 1 A Light-induced EPR signal changes due to P700 photooxidation (monitored at the high-field derivative peak of the free-radical at $g = 2\ 0026$) at various temperatures Sample composition illumination and EPR conditions are described in Experimental Illumination period, 10 s Downward arrow, light on, upward arrow, light off B Temperature dependence of the total decay of the EPR signal of P700$^+$ in the dark at the end of 20 min Data points were derived from individual experiments as those shown in A

end of 20 min It is reasonable to assume that this decay represents a recombination of the positively charged primary donor and the negatively charged primary acceptor It is of interest here that the rate of this same recombination reaction at room temperature is about 45 ms [1–4]

At 200 °K, the decay appears to be biphasic the rapid and slow phases, which are approximately equal in magnitude, had decay half times of about 3 and 75 s, respectively At 175 °K, the irreversible fraction was about 30 % At lower temperatures, the same biphasic-decay pattern remained, but the decay became less and less At 13 °K, no visible decay could be observed at the end of 20 min (cf Fig 3) The

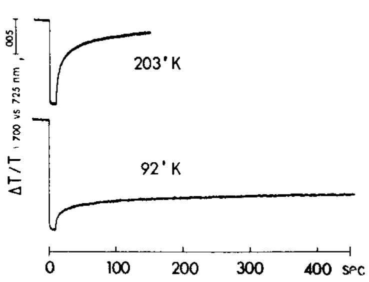


Fig 2 Light-induced absorption changes due to P700 photooxidation at low temperatures Same subchloroplast sample was suspended in a pH 8, 0 1 M Tricine buffer medium containing 2 3M sucrose, 1 mM ascorbate and 5 10^{-5} M TMPD at a chlorophyll concn of 0 05 mM Optical pathlength was 1 mm Saturating actinic light between 400 and 460 nm was isolated with interference filters, illumination period was 10 s

temperature dependence of the reversible fraction of the P700$^+$ EPR signal is plotted in Fig 1-B

Studies on low-temperature absorption changes in Photosystem-I subchloroplasts currently in progress in this laboratory yielded similar kinetics Fig 2 presents light-induced absorption changes due to P700 photooxidation in the same Photosystem-I subchloroplasts at 203 and 93 °K The general agreement between the optical and EPR signals is good Detailed kinetics of the Photosystem-I reactions at low temperatures will be reported elsewhere Although not shown in Figs 1A and 2, the EPR signal and the reversible portion of the absorption change and the EPR signal can be reversibly bleached again upon a second illumination The optical measurements support the contention that the kinetic courses obtained by EPR spectroscopy represent those of the P700$^+$ free radicals

Kinetics of P700 photooxidation and iron–sulfur protein photoreduction comparison of EPR spectra of P700$^+$ and reduced iron–sulfur protein after partial dark decay at various temperatures

If an iron–sulfur protein is indeed the reaction partner of P700 in the primary photochemical act of Photosystem I, the onset time for the formation of the two charged species and their decay kinetics should be identical As shown in Fig 3, the onset time for P700 photooxidation (top trace, also cf Fig 1-A) and that for iron–sulfur protein photoreduction monitored at $g = 1\ 86$ (bottom trace) are not resolvable and instantaneous with the instrumentation used

Because the EPR signals of reduced iron–sulfur proteins are only detectable at low temperatures, continuous monitoring of the course of decay as shown in Fig 1-A for P700$^+$ cannot be made Considering the fact that the EPR signals of P700$^+$ are practically completely stable at 13 °K, one could study the decay of both P700$^+$ and reduced iron–sulfur protein by exposing the EPR samples to other temperatures at which partial decay was allowed, and then rapidly refreezing the sample to 13 °K By means of procedures described in Experimental, we measured the fully developed spectra of P700$^+$ and reduced iron–sulfur protein at 13 °K after 20 s illumination, and then the spectra were measured after the sample was maintained at 75 or 125 °K

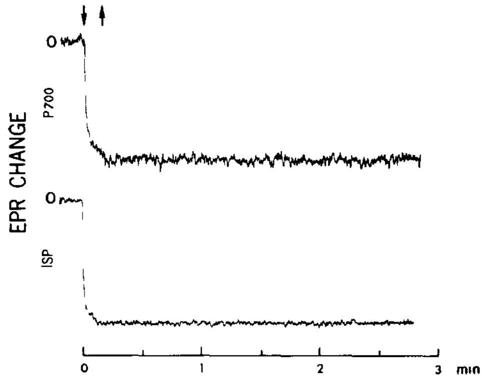
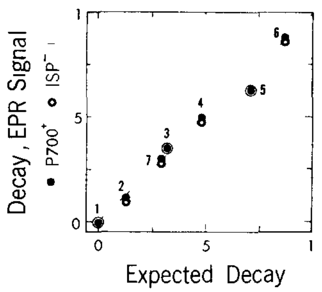


Fig 3 Light-induced EPR signal changes due to P700 photooxidation (top) and iron–sulfur protein photoreduction (bottom) at 13 °K Experimental conditions were the same as those used in Fig 1



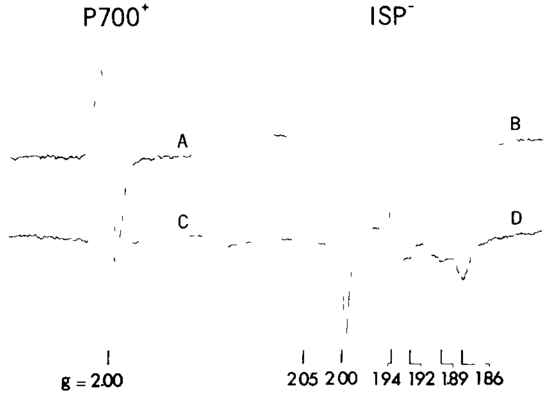


Fig 4 Plot of the actual fraction of decay of the EPR signal of P700$^+$ (solid dots) and reduced iron–sulfur protein (empty circles) vs the expected extent of decay Point sets 1, 2, and 3 are for samples maintained for 10 min at 13, 75 and 125 °K respectively, point sets 4 and 5 6 min at 150 and 175 °K, respectively, point set 6, 90 s at 225 °K, point set 7 25 s at 150 °K

Fig 5 EPR spectra of P700$^+$ (A) and reduced iron–sulfur protein (B) after the subchloroplast sample was illuminated at 13 K for 20 s (C) and (D) are the corresponding spectra (recorded at 13 °K) after the sample was maintained at 175 °K for 6 min

for 10 min, at 150 or 175 °K for 6 min, and at 225 °K for 90 s The extent of signal decay for P700$^+$ and reduced iron–sulfur protein is plotted in Fig 4 against the expected values derived from Fig 1 In addition to these values which represent final plateau levels of the decays, we also measured the EPR spectra of P700$^+$ and reduced iron–sulfur protein after the sample was maintained at 150 °K for 25 s Data in Fig 4 show that not only are the measured decays in good agreement with the expected values, but the extent of decay of P700$^+$ and reduced iron–sulfur protein also agrees very well As an illustration, the EPR spectra of a subchloroplast sample after illumination at 13 °K and after dark decay at 175 °K for 6 min are shown in Fig 5

DISCUSSION

The close match of the onset kinetics of the photo-production of P700$^+$ and reduced iron–sulfur proteins as well as their dark decays strongly support the concept that an iron–sulfur protein is the reaction partner of P700 in the primary photochemical act of Photosystem I At the same time these findings add evidence for our previous conclusion that P430 may be an iron–sulfur protein as suggested from the difference spectrum [1–4]

The biphasic decay kinetics of the reversible back reaction and the temperature dependence of the extent of the reversible fraction indicate an apparent heterogeneity in the reaction-center complex The two decay times represent presumably electron-tunneling phenomena in two reaction-center matrices where the distances between the oppositely charged species are slightly different [22] The temperature dependence of the dark decay shown in Fig 1-A is reminiscent of a similar phenomenon observed earlier in the photosynthetic bacterial reaction centers with electrostatically bound mammalian cytochrome *c* [23] In this case, the fraction of dark re-reduction of the photooxidized reaction-center bacteriochlorophyll was proportional to the fraction of bound ferrocytochrome *c*, but the kinetics always remained the same Analogously, one could suggest that the decreasing amount of decay observed in this work might have been caused by some as yet unknown change in the reaction-center species, as a result of decreasing temperatures

In their recent studies of the low-temperature photoreactions, Lozier and Butler [21] observed approximately 50 % reversibility of P700 photooxidation at 77 °K, the irreversible portion was considered to be the result of electron trapping by a secondary acceptor in close proximity of the reduced primary acceptor The authors concluded that the heterogeneity in the Photosystem-I complex involves two types of reaction centers They further proposed that the reaction center consists of a dimer of two chlorophylls, with one chlorophyll serving as the primary donor and the other as the primary acceptor At low temperatures, there is presumably an equal distribution of orientations of the polarized chlorophyll dimers relative to the secondary acceptor which would account for the 50 % reversibility observed at 77 °K However, our present experimental results do not support the "secondary acceptor" role for iron–sulfur proteins

Redox titrations [9] of the Photosystem-I subchloroplasts showed that possibly three bound iron–sulfur proteins are present in the reaction center of Photosystem I, whose midpoint potentials are −530 and $\leqq -580$ mV, respectively It was also found that photochemical reduction of the different iron–sulfur proteins is temperature-sensitive Only illumination at room temperature followed by freezing the sample under continued illumination results in reduction of the iron–sulfur proteins corresponding to the chemical reduction to the lower potential stage, while illumination at low temperature of samples previously frozen in the dark leads to reduction corresponding only to the chemical reduction to the higher potential stage This latter reaction has also been reconfirmed in the present work (see Fig 5)

From a purely energetic viewpoint, it might be proposed that the more negative (or more reducing) iron–sulfur protein could be the true primary acceptor, while the more positive (or less reducing) iron–sulfur protein could function as a "secondary" acceptor, presumably lying intermediate between the primary acceptor and soluble ferredoxin However, available evidence appears not to support this contention Kinetic spectroscopic studies with soluble ferredoxin as the secondary acceptor showed that ferredoxin receives electrons directly from P430 [23] Low-temperature EPR studies showed that dithionite alone under anaerobic conditions can chemically reduce most of the more positive iron–sulfur protein in the subchloroplasts and at the same time maintains P700 in the reduced state [9] If the proposal that the more negative iron–sulfur protein is the primary acceptor is correct, then subsequent illumination of the dithionite-reduced subchloroplasts should cause a photochemical reduction of the more negative iron–sulfur protein in a photochemical charge-separation reaction Earlier studies showed that such an illumination only brought out some additional EPR signal of the higher-potential iron–sulfur protein plus a small signal of the lower-potential iron–sulfur protein [9] Recording of the EPR spectra of this same sample under continuous illumination yielded the same signal (not shown here) Another argument against the suggestion that the more negative iron–sulfur protein functions as the primary acceptor is provided by data shown in this study namely, that EPR spectra recorded under continuous illumination at all low temperatures yielded only the more positive iron–sulfur proteins, and that the reduced iron–sulfur protein recovered in parallel with P700$^+$ Such a recombination is unlikely to occur at such low temperatures if the more positive iron–sulfur protein were a secondary acceptor

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ADDENDUM (Received August 19th, 1974)

After conclusion of our work, a report by Bearden and Malkin [25] appeared, which described a parallel decay of the EPR signals of P700 and bound ferredoxin at 150 °K

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