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A comparative study of the reduction of EPR signal II_{slow} by iodide and the iodo-labeling of the D2-protein in photosystem II

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The reduction kinetics of EPR signal II_{slow} in the presence of iodide were measured with EPR spectroscopy and the amount of ^{125}I incorporated into the D2-protein in the reaction center of photosystem II was quantitated in a PS II core complex preparation. The halftime of signal II_{slow} reduction in the dark was estimated at 31, 25 and 20 min in the presence of 0, 20 and 50 mM KI, respectively, reflecting the reduction of signal II_{slow} by iodide. The amount of ^{125}I that was incorporated into the D2-protein in the dark was correlated to the reduction of signal II_{slow} by iodide. This correlation indicates that the site of iodide oxidation in the dark and the component giving rise to EPR signal II_{slow} are located on the D2-protein in the PS II reaction center.

Photosynthesis; Photosystem II; Signal II_{slow}; ^{125}I labeling; D2-protein; Reaction center; EPR

1. INTRODUCTION

On the water oxidizing side of photosystem II, there are two redox components Z and D which in their oxidized forms exhibit identical radical EPR spectra. EPR signal $II_{very\,fast}$ which becomes signal II_{fast} when the oxygen evolving activity is inhibited, arises from the component Z which is the immediate electron donor to P-680, the reaction center chlorophyll [1]. The other component D gives rise to signal II_{slow}, which is quite stable in the dark and has been shown to interact in redox reactions with the S_2, S_3 and S_0 states [2–4] although it seems not to participate in the steady-state electron transfer from water to P-680. The similar structure of these EPR signals indicates that the origin and the environmental surroundings of the radicals are very similar.

Recently, the iodo-labeling technique was applied in an attempt to identify the polypeptides responsible for the binding of Z and D [5]. The preparation used in these experiments was a highly purified PS II core complex with six constituent polypeptides with apparent molecular masses of 47, 43, 32, 30, 9 and 4.5 kDa [6]. The basic idea for the experiments was that when iodide is oxidized, that is activated, the formed iodine covalently modifies a tyrosyl residue which is close to the oxidation site, thus labeling the polypeptide containing the oxidative species [7]. Upon il-

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Abbreviations: CBB, Coomassie brilliant blue; DCIP, 2,6-dichlorophenol indophenol; EPR, electron paramagnetic resonance; PS II, photosystem II

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lumination, the D1-protein (apparent molecular mass of 30 kDa) is specifically labeled with iodide which was suggested to be formed from oxidation of iodide by Z^+. In the dark, the D2-protein with an apparent molecular mass of 32 kDa was exclusively labeled by iodide. The oxidant in this reaction was an unknown component which was stable in the dark.

Here, we have determined quantitatively the radioactivity of ^{125}I incorporated into the D2-protein and the amount of signal II_{slow} reduced by iodide in the dark and we show that these reactions are correlated.

2. MATERIALS AND METHODS

The PS II core complex was prepared from digitonin extracts of spinach chloroplasts as described previously [6]. The polypeptides of the PS II core complex were iodo-labeled as described in [5] with some modifications. The reaction mixture contained the core complex (0.4 mg chl/ml), 50 mM Tris-HCl (pH 7.2), 60 mM NaCl, 20 mM KI (about 0.2 mCi $^{125}I^-$) (NEN Research Products), 50 μM DCIP and 0.1% digitonin. After iodination in the dark, the unreacted iodide was removed by chromatography on DEAE-Toyopearl 650S (Toyo Soda Co., Tokyo) [5]. After solubilization of the iodo-labeled core complex with 2.5% SDS and 2.5% 2-mercaptoethanol, the iodo-labeled polypeptides of the core complex were analyzed by SDS-polyacrylamide gel electrophoresis [5]. To estimate the radioactivity of ^{125}I incorporated in each polypeptide, the gel strips were sliced into 1 mm slices. The radioactivity in each slice was determined with an Aloka auto well gamma system JDC-751.

The EPR measurements were carried out with a Bruker ER 200D-SRC spectrometer in a standard TE 102 cavity using a flat cell at 20°C. The reaction mixture was the same as in the iodo-labeling experiment except that $^{125}I^-$ was omitted.

3. RESULTS AND DISCUSSION

Under normal conditions, signal II_{slow} in the core complex is quite stable in the dark [8]. In the preparation used in this study, the radical spectrum was dominated by signal II_{slow} but a small radical with a lower g value was present. Fig.1 shows the effect of incubation with 50 mM KI on signal II_{slow}. 8 min after the addition of iodide, the characteristic hyperfine structure of signal II_{slow} was clearly visible (fig.1A). Longer incubation times (fig.1B and C) resulted in a decrease of the amplitude of signal II_{slow} (seen as a decrease in the shoulder peak marked with an arrow in fig.1). After 46 min incubation with iodide (fig.1C), signal II_{slow} was almost completely absent in the

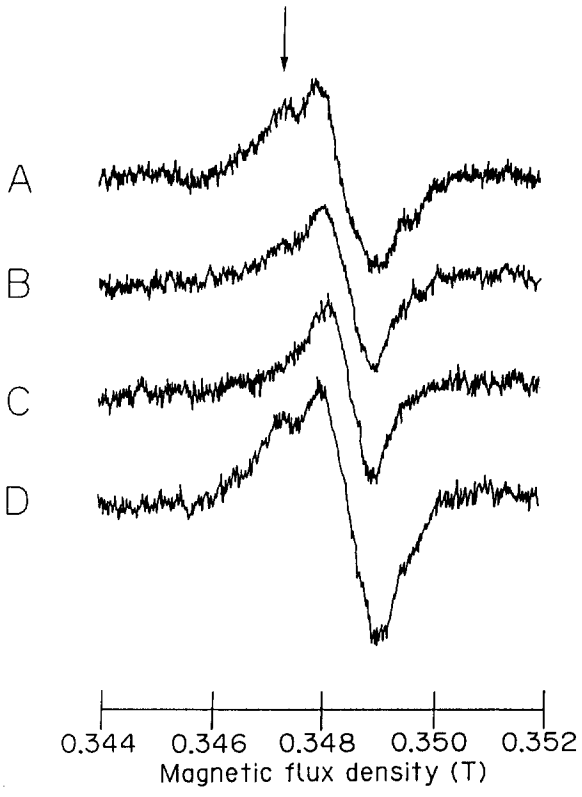

Fig.1. EPR spectra of the radical region in the PS II core complex. After the addition of 50 mM KI, the complex was kept in the dark for: 8 min in A; 27 min in B and 46 min in C. (D) After 60 min in the dark, it was illuminated for 10 s and then kept in the dark for 1 min. The reaction medium contained 50 mM Tris-HCl (pH 7.2), 50 μM DCIP, 60 mM NaCl and 50 mM KI. The chlorophyll concentration was 0.4 mg chl/ml. The amplitude of signal II_{slow} was measured at the magnetic field indicated by the arrow. The spectrometer conditions were: modulation amplitude, 2.8 G; microwave power, 20 mW; microwave frequency, 9.77 GHz. The spectrometer gain was constant.

spectrum which was dominated by another radical with $g \approx 2.0023$ and a linewidth of approx. 9 G. Signal II_{slow} could be regenerated again by a short illumination (fig.1D). The illumination also induced signal II_{fast} which rapidly (within a few seconds) disappeared when the light was switched off (not shown). Signal II_{slow}, on the other hand, disappeared slowly with similar kinetics as before illumination. Our interpretation of these results is that signal II_{slow} is slowly reduced by iodide and that the subsequent illumination regenerates D^+, the oxidized form of the radical giving rise to signal II_{slow}. The origin of the free radical that remains after the reduction of signal II_{slow} is not clear but it is possible that it originates from a chlorophyll cation which has similar EPR-parameters [9].

The amplitude of signal II_{slow} could be estimated with very small interference from the low g value radical signal at the magnetic field indicated by the arrow in fig.1. The reduction kinetics of signal II_{slow} were determined by plotting the estimated signal amplitude against the incubation time in the absence and the presence of 20 or 50 mM potassium iodide (fig.2). The original levels of the signal were estimated by extrapolation assuming that the signal decreased with pseudo-first order kinetics. The halftime in the absence of iodide was 31 min (rate constant k_1 was 3.73×10^{-4} s^{-1}), which is considerably faster than that estimated in thylakoid membranes [2,4]. Compared to thylakoid membranes, the PS II core complex contains less lipids and proteins which makes it likely that signal II_{slow} is more exposed to reaction with extrinsic reductants. The reduction rate was accelerated by the addition of iodide; the halftime in the presence of 20 and 50 mM potassium iodide was 25 and 20 min, respectively. Thus the rate constant of signal II_{slow} reduction increases linearly with the concentration of iodide (fig.2, inset), which supports the view that signal II_{slow} is also reduced by iodide. The rate constant (k_2) for this reaction is 4.27×10^{-3} $M^{-1} \cdot s^{-1}$ (fig.2, inset).

Iodide, when activated by oxidation, normally attacks tyrosyl residues which are present close to the site for the oxidant in the reaction. In the previous report, it was shown that the D2-protein was specifically iodo-labeled in the dark, whereas the D1-protein was specifically iodo-labeled in the light [5]. It is possible to estimate the amount of

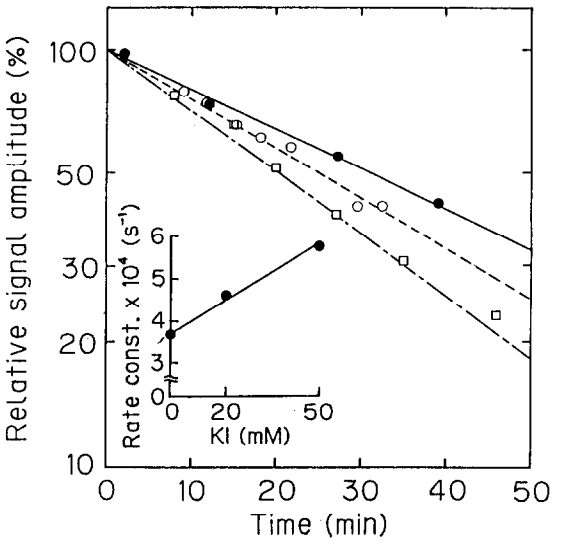

Fig.2. The reduction kinetics of EPR signal II_{slow} in the core complex. The amplitude of signal II_{slow} was measured as in fig.1. The reaction medium was the same as in fig.1, except for the concentration of KI. (●) In the absence of KI. In the presence of (○) 20 mM KI and (□) 50 mM KI.

signal II_{slow} reduced by iodide from the values of k_1 and k_2. In the presence of 20 mM KI, the fraction of signal II_{slow} reduced by iodide in the dark is calculated to be 19%. In a parallel experiment the amount of ^{125}I incorporated into the D2-protein was determined under the same conditions in order to establish the relationship between the amounts of reduced signal II_{slow} and iodo-label in the D2-protein. After iodination with 20 mM potassium iodide containing 0.2 mCi $^{125}I^-$, the core complex was solubilized and subjected to SDS-polyacrylamide gel electrophoresis. In fig.3, the D2-protein and its aggregated form of which apparent molecular masses of 32 and 60 kDa, respectively, were shown to be exclusively iodo-labeled in the dark (fig.3). After the gels were sliced into 1 mm strips, the radioactivities of the strips containing the D2-protein were counted and plotted against the iodination time in the dark (fig.4). The radioactivity incorporated into the D2-protein increased with the iodination time. The curve in fig.4 indicates the estimated amount of reduced signal II_{slow} by iodide calculated from fig.2 as stated above. It approximately follows the measured points for iodo-labeling of the

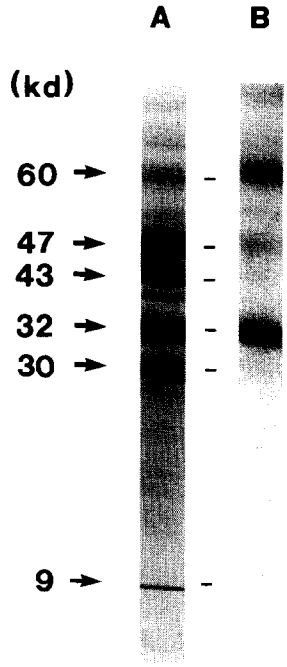

Fig.3. Labeling of polypeptides of the PS II core complex with ^{125}I in the presence of 20 mM KI for 13 min in the dark. (A) Polypeptides stained with CBB. (B) Fluorography of polypeptides labeled with ^{125}I in the dark.

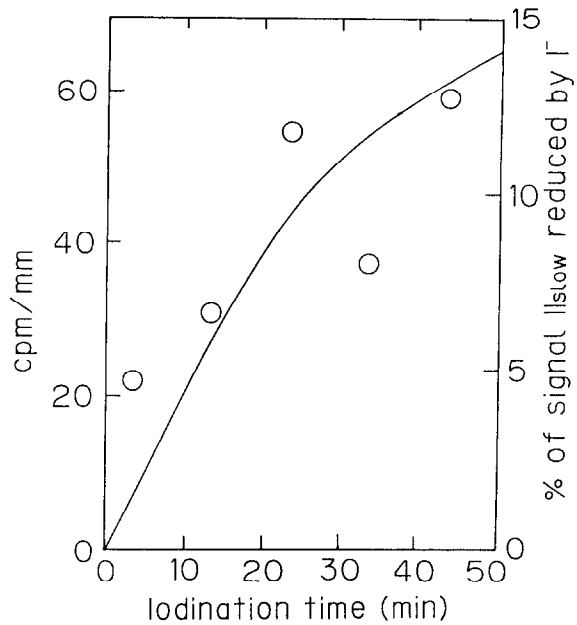

Fig.4. Iodo-labeling of the D2-protein with time in the dark in the presence of 20 mM KI. The solid curve indicates the amount of signal II_{slow} reduced by iodide in the dark in the presence of 20 mM KI as calculated from the data in fig.2.

D2-protein which suggests a correlation between the two reactions. After 43 min incubation, approximately 20% of the D2-protein was iodo-labeled. This roughly corresponds to the fraction of signal II that was reduced by iodide. Therefore, we suggest that the iodo-labeling of the D2-protein is due to oxidation of iodide by D^+ and that this component is present on the D2-protein.

In these experiments, the concentration of non-radioactive iodide was one hundred-times higher than that in the previous experiment [5]. This is the main reason for the small amount of incorporated ^{125}I as compared to earlier iodo-labeling of the D2-protein in the dark [5]. Under the present conditions, the other polypeptides were also shown to be slightly labeled (fig.3). Among them, especially any radioactivity incorporated into the D1-protein was due to the dim light used since we could not operate the iodo-labeling in complete darkness. Furthermore, none of the other polypeptides were labeled with ^{125}I in correlation to the reduction of signal II_{slow} (not shown).

Judging from the similar hyperfine structure of signal II_{slow} and signal II_{fast}, the micro environments surrounding D and Z are presumably very similar. According to the iodo-labeling experiments here and previously [5], Z^+ and D^+ can be expected to be present on the different polypeptides of D1 and D2, respectively. D^+ and Z^+ are currently considered to be semiquinone cations [10] but other candidates have not been ruled out and recently it was suggested [11] that D^+ could be a tyrosine radical. Therefore it is of interest that there is a high degree of homology between the two polypeptides D1 and D2 in the primary amino acid sequence [12–14]. Especially, a region composed of 9 amino acids from valine-157 to glutamine-165 are highly conserved. This sequence includes tyrosine-161 which is common to the two polypeptides. From the hydropathy index plot, this residue is predicted to exist in the hydrophobic part of a helix near the lumenal side of the thylakoid membrane and thus probably close to the water splitting enzyme [15]. Therefore tyrosine-161 is one of the

most promising candidates for the iodo-labeling target in both D1 and D2 and possibly also interesting candidates for the identity of Z^+ and D^+.

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