Vol. 134, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 14, 1986 Pages 272-278

Effects of Trypsin and Calcium Chloride on Signal II_s in Oxygen-Evolving PS II Preparations

Gopinath Mavankal, Douglas C. McCain and Terry M. Bricker

The University of Southern Mississippi, Department of Chemistry, Southern Station Box 5043, Hattiesburg, MS 39406-5043

Received November 21, 1985

SUMMARY: Photosystem II oxygen-evolving preparations exhibited a reversible loss of signal II_s hyperfine structure when treated with 1.0 M $CaCl_2$. A progressive irreversible loss of hyperfine structure was observed upon trypsin treatment of these preparations. These treatments appear to alter the environment of the radical responsible for signal II_s. Gel electrophoresis of trypsin-treated photosystem II preparations indicates that three polypeptides (45, 32-34, and 26 kDa) are altered with the same kinetics as observed for the trypsin-induced loss of signal II_s. Two of these polypeptides (45 and 32-34 kDa) are core components of photosystem II.

Signal II_s is a dark-stable ESR signal which exhibits partially resolved hyperfine splitting (Commoner et al., 1956) and is thought to function on the oxidizing side of PS II (Babcock and Sauer, 1975). Recent evidence indicates that this component is highly oriented in the photosynthetic membrane (Rutherford, 1985) which implies a functional association with membrane proteins. Signal II_s is easily observed in PS II oxygen-evolving preparations (Berthold, et al., 1981) which are highly depleted in PS I (Bricker et al., 1983).

ABBREVIATIONS:

ESR, electron spin resonance; LHC-II, Light harvesting chlorophyll-protein complex II; Mes, 2-(N-morpholino)ethane sulfonic acid; PS I, photosystem I; PS II, photosystem II; PMSF, Phenylmethylsulfonylfluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPCK, L-1-Tosylamide 2-phenylethyl chloromethyl ketone; Tris, Tris(hydroxymethyl)aminomethane.

0006-291X/86 $1.50

Treatment of oxygen-evolving preparations with NaCl, alkaline Tris, and $CaCl_2$ removes various extrinsic polypeptide components of the photosystem and inhibits oxygen evolution to varying degrees (Renger and Govindjee, 1985). Trypsin treatment of oxygen-evolving preparations has also been used to probe the structural arrangement of proteins within the photosystem (Renger et al., 1984; Volker et al., 1985).

In this communication we report a dramatic alteration of signal II_s by both $CaCl_2$ and trypsin treatment. We have also identified a number of PS II polypeptides that are altered by trypsin treatment and that disappear in parallel with the loss of signal II_s.

MATERIALS AND METHODS

Chloroplasts were isolated from market spinach as previously described (Bricker et al., 1985). The oxygen-evolving preparation was prepared essentially according to the procedure of Ghanotakis and Babcock (1983). Chloroplasts were, however, incubated at 4°C for 1 hr under stacking conditions (Ford and Evans, 1983) prior to detergent treatment and were washed with resuspension buffer (300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl and 50 mM Mes-NaOH, pH 6.0) after detergent treatment. These preparations exhibited no Signal I when oxidized with ferricyanide in the dark. Extrinsic polypeptides were removed from oxygen-evolving preparations having a chlorophyll concentration of 1.0 mg/ml by treatment with 1.0 M NaCl (pH 6.0), 1.0 M $CaCl_2$ (pH 6.0) or 1.0 M Tris-HCl (pH 9.25) for 1 hr in the dark at 4° C. Polypeptide-depleted oxygen-evolving preparations were then washed with, and resuspended in, resuspension buffer (300 mM sucrose, 15 mM NaCl, 10 mM $MgCl_2$, and 50 mM Mes-NaOH, pH 6.0) prior to trypsin treatment. Oxygen-evolving preparations were treated with trypsin (TPCK-treated) at 40 ug/mg chlorophyll/ml for various lengths of time in resuspension buffer at 37°C. The reaction was stopped by the addition of 2 mM freshly prepared PMSF and 5-fold excess soybean trypsin inhibitor. The trypsin-treated oxygen-evolving preparations were either utilized directly for electrophoresis or pelleted at 14,000 x g, washed in resuspension buffer containing EDTA, and utilized for ESR spectroscopy. Electrophoresis was performed in 12.5-20% gradient SDS-PAGE overnight at 4°C. Samples were solubilized in 2% SDS, 5% 2-mercaptoethanol and 6% sucrose at 4°C for 0.5 hr. After electrophoresis the polyacrylamide gels were stained with Coomassie Blue, destained and then silver stained by the procedure of Wray et al. (1981). Q-band ESR spectra were recorded on a 35 GHz Varian E 109Q spectrometer at 90°K. Instrument conditions are as listed in figure legends. The sample size for ESR spectroscopy was 10 ul. DPPH was used as a g standard.

RESULTS AND DISCUSSION

Figure 1 illustrates the effects of various polypeptide-releasing washes on signal II_s. Treatment with NaCl or alkaline Tris results in little change in the structure of signal II_s. NaCl washing removes the 24 and 14 kDa polypeptides of the oxygen-evolving complex while alkaline-Tris removes the 33, 24, and 14 kDa polypeptides as well as the bound-manganese of this complex. Treatment with $CaCl_2$ (which removes the 33, 24, and 16 kDa polypeptides but not bound-manganese), however, produces a dramatic change in the ESR spectra of these preparations which is not observed with the other treatments. Signal II_s is essentially lost and appears to be replaced by a relatively narrow signal at

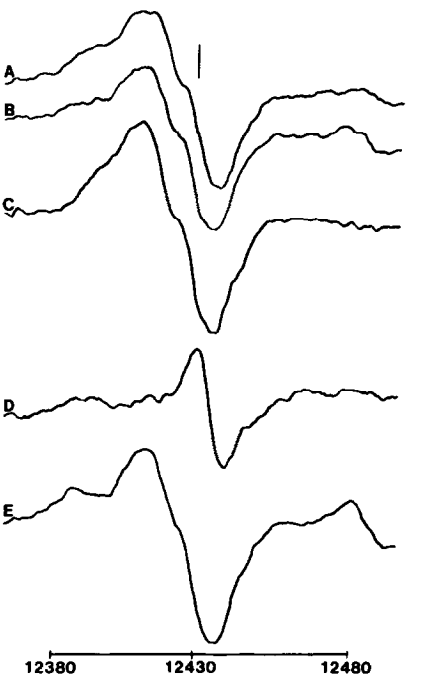

Figure 1. Q-Band ESR spectra of dark-adapted oxygen-evolving preparations recorded at 90° K. Samples were prepared as described in **MATERIALS AND METHODS.** A. Untreated; B. 1.0 M NaCl washed; C. 1.0 M Alkaline-Tris washed; D. 1.0 M $CaCl_2$ washed; E. 1.0 M $CaCl_2$ washed followed by washing with resuspension buffer. Instrument Conditions: Microwave power less than 2 x 10^{-5} W, microwave frequency 35.0 GHz, field modulation 5.0 G, modulation frequency 100 KHz, sweep rate 31.25 G/min, time constant 2 sec. Marker in field is g=2.0036 ±.0002 (DPPH).

g=2.0036 with a peak to peak width of about 9.7 gauss. This change does not appear to be caused by the removal of extrinsic polypeptides or bound-manganese since signal II_s is restored by washing with the resuspension buffer. It appears that Ca^{+2} perturbs the environment of signal II_s in a manner which leads to the reversible collapse of the semi-resolved hyperfine structure of the signal.

It is tempting to speculate as to the nature of this perturbation. The molecular species giving rise to signal II_s is probably a plastoquinone cation radical (O'Malley and Babcock, 1984) immobilized in a protein environment. Ca^{+2} may perturb the environment of the radical so that it could interact with some reducing species. Alternatively, Ca^{+2} may be capable of displacing the plastoquinone cation from its binding site. During freezing, the displaced cation may cluster with other plastoquinone molecules from the bulk lipid phase. Electron exchange reactions within these clusters could lead to loss of the hyperfine structure (Rich, 1982). A similar alteration in signal II_s has been observed in PS II preparations treated with salicyladoxime (Golbeck and Warden, 1985).

Figure 2 illustrates the effect of trypsin treatment on signal II_s. The hyperfine structure of signal II_s is progressively lost with the most dramatic changes occurring between 5 and 20 minutes of treatment. Clearly, the effect results from the action of trypsin on some protein component, since preparations treated with soybean trypsin inhibitor and PMSF prior to the addition of trypsin exhibited no such alterations in signal II_s (data not shown). Additionally, the changes observed in signal II_s are not reversible and persist after washing to remove trypsin. At limiting trypsin incubation

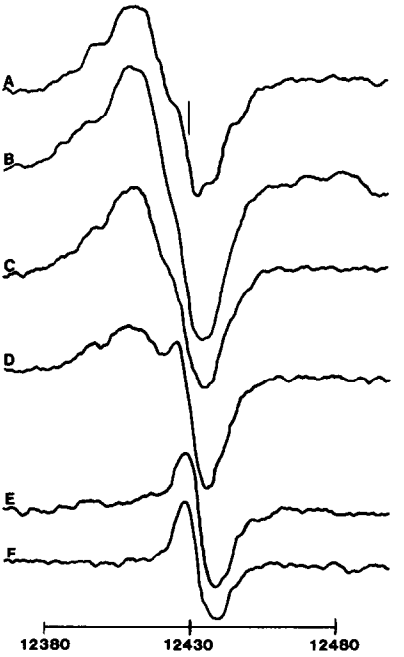

Figure 2. Effects of trypsin treatment on Q-band ESR spectra of oxygen-evolving preparations in resuspension buffer. Samples were prepared and treated with trypsin as described in **MATERIALS AND METHODS**. A. 0 min trypsin treatment, B. 2.5 min, C. 5.0 min, D. 10.0 min, E. 20.0 min, F. 40.0 min. ESR conditions are as listed in Figure 1.

times, signal II_s has disappeared and is replaced by a signal at g=2.0036 with a peak to peak width of 9.7 gauss. The signal appears identical to that observed after $CaCl_2$ treatment. These results indicate that trypsin cleavage of some protein component leads to a conformational change in the environment of the plastoquinone cation radical such that signal II_s characteristics are lost.

We have attempted to identify the altered protein(s) responsible for the ESR changes by polyacrylamide gel electrophoresis. Figure 3 shows the results of a typical experiment. Tryptic alteration of the protein component of interest should parallel the effect of trypsin observed on signal II_s i.e. little alteration of the protein should be observed

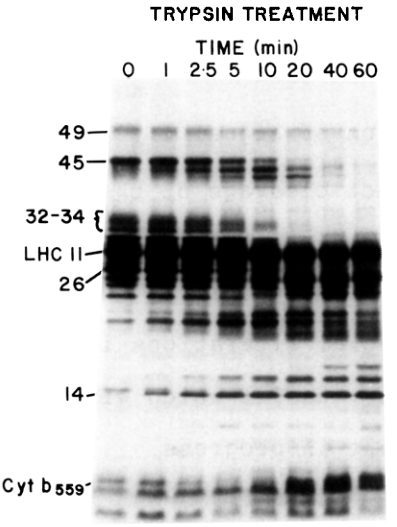

Figure 3. Effects of trypsin treatment on the constituent polypeptides of PS II oxygen-evolving preparations. Identified polypeptides are shown on the left. The length of time of trypsin treatment (40 ug/mg chlorophyll/ml) is shown above. Polypeptides at 45, 32-34, and 26 kDa are affected by trypsin in parallel with the loss of signal II_s. No PS I-associated polypeptides were observed in these oxygen-evolving preparations.

after 5 min trypsin treatment, moderate alteration of the protein should be observed at 10 min, and the protein should appear completely altered after 20 min incubation with trypsin. Three proteins appeared to be affected by trypsin in this manner. These proteins have apparent molecular masses of 45, 32-34 and 26 kDa and appear to be intrinsic components of the photosystem.. The 45 kDa polypeptide has been identified as a probable interior light-harvesting antenna for PS II (Nakatani et al., 1983). Two intrinsic proteins in the 32-34 kDa region are present in oxygen-evolving PS II preparations. D_1 is the herbicide-binding protein although it may function on both the reducing side and oxidizing side of PS II (Metz, et al., 1985), while D_2 is thought to function on the oxidizing side of the photosystem. These proteins are core components of PS II (Bricker et al., 1985). The 26 kDa polypeptide is an intrinsic PS II component of unknown function and is not present in PS II core preparations (Bricker

et al., 1985) which have been shown to possess normal signal II_s characteristics (Satoh et al., 1983).

Tryptic alteration of signal II_s characteristics could occur as the result of direct cleavage of the protein that bears the plastoquinone cation radical. It is also possible that cleavage of other proteins in close proximity to the signal II_s apoprotein could lead to substantial alterations in the environment of the plastoquinone radical. Studies which will differentiate between these possibilities are in progress.

ACKNOWLEDGEMENT

This work was partially supported by a grant from the USM Summer Research Program to T.M.B.. We also wish to thank Ms. Laurie K. Frankel for her expert technical assistance.

REFERENCES

Babcock, G.T. and Sauer, K. (1975) Biochim. Biophys. Acta 376,329-344.
Berthold, D.A., Babcock, G.T., and Yocum, C.F. (1981) FEBS Lett. 134,231-234.
Bricker, T.M., Metz, J.G., Miles, D., and Sherman, L.A. (1983) Biochim. Biophys. Acta 724,447-455.
Bricker, T.M., Pakrasi, H.B., and L.A. Sherman (1985) Arch. Biochem. Biophys. 237,170-176.
Commoner, B., Heise, J.J., and Townsend, J. (1956) Proc. Natl. Acad. Sci. (USA) 42,710-718.
Ford, R.C. and Evans, M.C.W. (1983) FEBS Lett. 160,159-164.
Ghanotakis, D.F. and Babcock, G.T. (1983) FEBS Lett. 153,231-234.
Golbeck, J.H. and Warden, J.T. (1985) Photosyn. Res. 6,371-380.
Metz, J.G., Bricker, T.M., and Seibert, M. (1985) FEBS Lett. 185,191-196.
O'Malley, P.J. and Babcock, G.T. (1984) Biochim. Biophys. Acta 765,370-379.
Renger, G., Volker, M., and Weiss, W. (1984) Biochim. Biophys. Acta 766,582-591.
Renger, G. and Govindjee (1985) Photosyn. Res. 6,33-55.
Rich, P.R. (1982) Faraday Discuss. Chem. Soc. 74,349-364.
Rutherford, A.W. (1985) Biochim. Biophys. Acta 807,189-201.
Satoh, K., Koike, H., and Inoue, Y. (1983) Photobiochem. Photobiophys. 6,267-277.
Volker, M., Ono, T., Inoue, Y., and Renger, G. (1985) Biochim. Biophys. Acta 806, 25-34.
Wray, W., Boulakis, T., Wray, V., and Hancock, R. (1981) Anal. Biochem. 118,197-203.