STABILIZED FLAVIN RADICAL IN CHLOROPLAST $NADP^+$ REDUCTASE

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Received November 19, 1968

SUMMARY

Quantitative EPR data on frozen reaction mixtures of NADPH with free FAD and with chloroplast $NADP^+$ reductase respectively show that the fraction of the flavin moiety of the enzyme which exists in the radical form is at least 100 times larger than that of free FAD under identical conditions. It is concluded that the apoenzyme stabilize the radical form of the bound flavin and thereby facilitate electron transfer from chlorophyll to $NADP^+$.

The $NADP^+$ reductase isolated from spinach chloroplasts has been named ferredoxin-NADP reductase, since it catalyzes the reduction of $NADP^+$ by ferredoxin (Shin and Arnon, 1965) as well as the reverse reaction (Lazzarin and San Pietro, 1962). It has likewise been named NADPH-cytochrome f reductase, since it also catalyzes the reduction of cytochrome f as well as plastocyanine (Forti and Sturani, 1968), NAD^+ (Keister, et al., 1960) and dyes (Avron and Jagendorf, 1956) by NADPH. We have also observed this enzyme to catalyze the rapid reduction of ferricyanide, 2,6-dichlorophenyl-indophenol (DPIP), Janus Green, Tolylene Blue, Lauth's Violet and the slow reduction of Benzyl Viologen, Neutral Red, Methyl Viologen by NADPH.

Since this enzyme is highly specific only toward $NADP^+$ as an acceptor or NADPH as a donor, we shall for convenience simply call it chloroplast $NADP^+$ reductase. In the present work, a comparative study of this FAD-containing enzyme and free FAD has been made by means

of quantitative EPR measurements with NADPH as the electron-donor. The data demonstrate a remarkable stabilization of the radical state of the bound flavin moiety in this enzyme.

MATERIALS AND METHODS

The enzyme chloroplast $NADP^+$ reductase was isolated from fresh spinach leaves by the method of Forti and Sturani (1968). It exhibited well-defined absorption maxima at 275, 385 and 456 mμ as well as incompletely resolved absorption peaks at 285 and 485 mμ respectively. The enzyme concentration was calculated from the observed absorbance at 456 mμ, using the value of its molar extinction coefficient $\epsilon_{456} = 10740$ determined by Forti (1967). The ratio of absorbances at 456 and 275 mμ respectively was found to be 0.11.

Diphenylpicrylhydrazyl (DPPH) was used as the primary standard for field calibration as well as the determination of free spin concentration in the electron paramagnetic resonance measurements. A Varian E-3 EPR Spectrometer was used and all the EPR samples were sealed under high vacuum. For measurements at room temperature, a flat, fused quartz sample cell was used in order to minimize the dielectric loss.

RESULTS AND DISCUSSION

The EPR spectra at room temperature of equimolar mixtures of NADPH and FAD in aqueous phosphate buffer at pH 12.0 and 7.0 respectively are shown by (A) and (B) in Figure 1. These two EPR spectra closely resemble those obtained previously (Ehrenberg, 1962; Eriksson and Ehrenberg, 1964). By freezing the sample solution at pH 7.0 in liquid nitrogen, its EPR spectrum was observed to change to a broad resonance signal as shown by (C) in Figure 1, with $g = 2.0041$ and half-width = 20 gauss. A frozen mixture of chloroplast $NADP^+$ reduc-

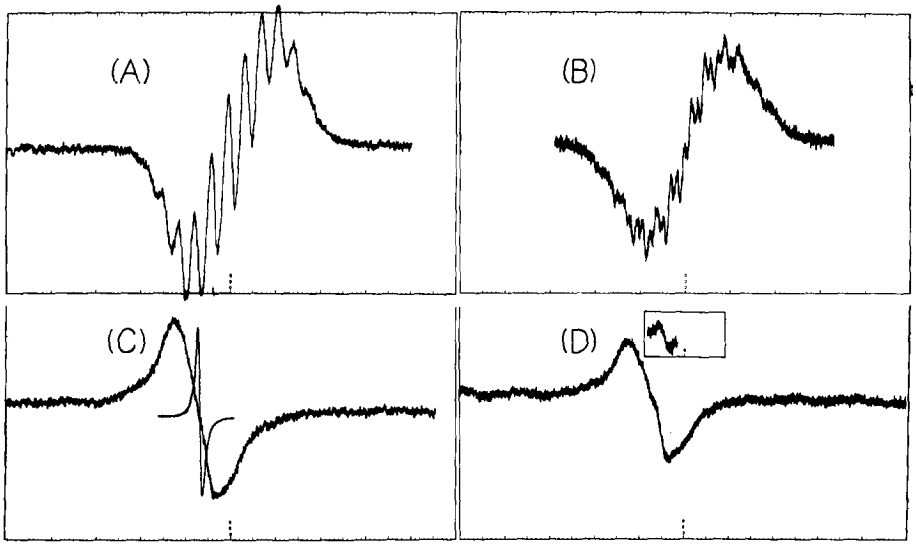

Figure 1. EPR spectra of various mixtures of NADPH and free FAD or the enzyme chloroplast $NADP^+$ reductase.

(A) Equimolar mixture of NADPH and FAD in phosphate buffer at pH 12.0 and 23°C.

(B) Equimolar mixture of NADPH and FAD in phosphate buffer at pH 7.0 and 23°C.

(C) Same mixture as in (B) but at -160°C. The sharper EPR signal at the center belongs to a DPPH calibration standard.

(D) Mixture of NADPH and the enzyme in phosphate buffer at pH 7.0 and -160°C. The solution was frozen within 20 seconds after mixing. The small asymmetry near the center is due to a trace amount of paramagnetic impurity in the sample quartz tube. The EPR spectrum of the empty tube was shown in the insert.

For spectra (A) and (B), the microwave frequency was set at 9.525×10^9 sec^{-1}, the magnetic field at the center of each diagram was set at 3378 gauss and the modulation amplitude at 1.0 gauss. For spectra (C) and (D), the frequency was set at 9.110×10^9 sec^{-1}, the field at 3250 gauss and the modulation amplitude at 10 gauss.

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tase and NADPH at pH 7.0 was also found to exhibit a broad EPR spectrum, g = 2.0042 and half-width = 20 gauss, as shown by (D) in Figure 1. Consequently we conclude that the observed EPR spectrum

TABLE I

Concentration of radicals in frozen mixtures

Composition of the frozen mixture	Enzyme (6×10^{-6} M) + NADPH	Free FAD (6.24×10^{-3} M) + NADPH
g-value	2.0042	2.0041
half-width (gauss)	20	20
X	0.19 ± 0.02 (r = 1) 1.00 ± 0.03 (r = 5)	0.002 (r = 1) 0.0037 (r = 5)

X = fraction of the flavin moiety in radical form in the frozen mixture;

r = initial ratio of the molar concentration of NADPH to that of the enzyme or free FAD.

of the frozen enzyme mixture is also due to the radical form of the flavin moiety.

The concentration of free spins in each frozen mixture was determined by double graphical integration of the EPR spectrum and comparison of the so obtained numerical result with those obtained with standard DPPH samples under identical conditions. The results are summarized in Table I.

The data in Table I show that the fraction of the flavin moiety of the enzyme which exists in the radical form is at least 100 times larger than that of free flavin under identical conditions. Consequently we conclude that the apoenzyme stabilizes the radical form of the bound flavin, either by hydrogen bonding or van der Waals interaction or both, and thereby making the flavin moiety unusually efficient in receiving electron from the photoreduced chlorophyll radical and then pass it on to $NADP^+$ during photosynthesis.

Acknowledgement

This work was supported by the National Science Foundation (GB-7459X) and the National Institutes of Health (GM-04483). We thank Miss Dolores Whelan for her assistance in preparing the enzyme.

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