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COMPLEX FORMATION BY FERREDOXIN-NADP$^+$ REDUCTASE WITH FERREDOXIN OR NADP$^+$

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SUMMARY

Complex formation by ferredoxin–NADP$^+$ reductase (NADPH:ferredoxin oxidoreductase, EC 1.6.99.4) with ferredoxin was measured by the independent methods based on the changes of circular dichroism, fluorescence intensity and the chromatographic behavior on a Sephadex G-75 column of the two proteins after mixing. Complex formation between the flavoprotein and NADP$^+$ was also detected from the changes of various optical properties of the protein. These experiments suggested that the optical changes accompanying the complex formation were due to a change of the chromophore group in ferredoxin–NADP$^+$ reductase, but not due to that of ferredoxin.

INTRODUCTION

It has been demonstrated in several articles[1–4] that a chloroplast flavoprotein, ferredoxin–NADP$^+$ reductase (NADPH:ferredoxin oxidoreductase, EC 1.6.99.4) participating in the photosynthetic NADP$^+$ reduction in chloroplasts, reacts with the oxidized ferredoxin to form a complex in the molar ratio of one to one. The complex formation was demonstrated by observations of the difference absorption spectrum and of the ultracentrifugal sedimentation velocity when these two proteins were mixed in solutions at low ionic strengths. The flavoprotein reacted also with its electron acceptor, NADP$^+$, in a manner suggestive of the formation of a one to one complex.

The present paper describes several independent methods providing criteria for the complex formation. These methods are based on the changes of circular dichroism, fluorescence intensity, and on the difference in the elution patterns of the proteins from a Sephadex G-75 column with and without complex formation. The circular dichroism observations have also appeared in previous reports[5,6].

MATERIALS AND METHODS

Ferredoxin–NADP$^+$ reductase and ferredoxin were purified from spinach leaves according to the methods described by Shin[5] and by Tagawa and Arnon[7], respectively. The concentrations of proteins were calculated from the millimolar extinction coefficients of 10.74 at 456 nm for the ferredoxin–NADP$^+$ reductase[8] and 9.68 at 423 nm for ferredoxin[9].

Circular dichroism measurements

The circular dichroism measurements of the complex formation were carried out by a Jouan Dichrographe II, Model CD 185, at room temperature. The difference CD spectrum of the complex between ferredoxin and ferredoxin–$NADP^+$ reductase was obtained by subtracting the additive CD spectrum of ferredoxin and ferredoxin–$NADP^+$ reductase from the CD spectrum of the mixture of the two proteins. To obtain the additive spectrum, two sample cells were placed in series on a pass of an analytical beam: one contained ferredoxin and the other ferredoxin–$NADP^+$ reductase. For the spectrum of the mixture, both of the two sample cells in series contained the mixture of almost equimolar quantities of the proteins, made by mixing an equal volume of each protein solution. The difference CD spectrum was also obtained from the difference between the two spectra of the protein mixture in 0.01 M Tris–HCl buffer, pH 7.5, alone and in the same buffer containing 0.3 M NaCl.

The difference CD spectrum of the complex between $NADP^+$ and ferredoxin–$NADP^+$ reductase was measured by subtracting the additive spectrum of $NADP^+$ and ferredoxin–$NADP^+$ reductase from the spectrum of the mixture, in the same manner as the observation of the ferredoxin complex.

Fluorimetric measurements

The fluorimetric measurements of the complex formation were carried out by using a Hitachi fluorescence spectrophotometer, Model MPF-2A, at room temperature. Prior to the measurements, the flavoprotein preparations were passed through a Sephadex G-25 column equilibrated with 0.05 M Tris–HCl buffer, pH 7.5, in order to remove the free FAD released from the flavoprotein during storage.

Chromatography on a Sephadex G-75 column

Two kinds of mixture of ferredoxin and ferredoxin–$NADP^+$ reductase were prepared by mixing 30.1 nmoles of each protein in 0.2 ml of 0.01 M Tris–HCl buffer, pH 7.5, with or without 0.05 M $MgCl_2$. The mixtures were chromatographed on Sephadex G-75 columns (1 cm × 20 cm) equilibrated with the respective buffer solutions. The elution patterns of the proteins from the Sephadex columns were monitored at 460 and 540 nm. Ferredoxin was detected directly by absorbance at 540 nm. Ferredoxin–$NADP^+$ reductase was detected by absorbance at 460 nm after subtracting the absorbance of ferredoxin. The absorbance at 460 nm of ferredoxin was 1.8-fold that at 540 nm.

RESULTS AND DISCUSSION

Circular dichroism measurements

The CD spectra of ferredoxin and ferredoxin–$NADP^+$ reductase used in the experiments are shown in Fig. 1 (Curves 1 and 2). Formation of a complex was observed by the changes in a CD spectrum when the two proteins were mixed in solution. The maximum positive changes in the difference spectrum occured at 271, 395 and 456 nm and the maximum negative changes at 320, 356 and 570 nm.

The complex formation was also detectable from the measurements of the difference CD spectrum (Curve 8) obtained from the two spectra of the protein

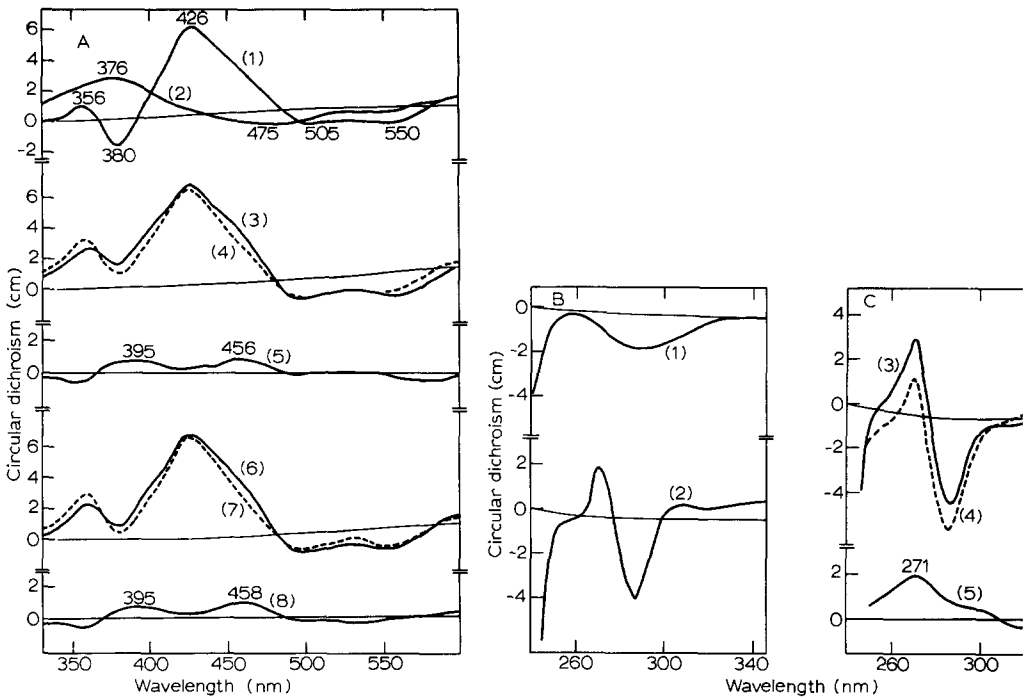

Fig. 1. Circular dichroism measurements of complex formation between ferredoxin–NADP reductase and ferredoxin. The proteins were dissolved in 0.01 M Tris–HCl buffer, pH 7.5 Measurements were made at room temperature (24 ± 1 °C) with the sensitivities, $s = 2 \cdot 10^{-5}$/mm for Curves (1), (2), (3) and (4) in A, and $s = 1 \cdot 10^{-5}$/mm for all of the others, using the sample cells with a light path of 1 cm. 1, ferredoxin, $5.17 \cdot 10^{-5}$ M in A and $1.72 \cdot 10^{-5}$ M in B and C 2, ferredoxin–NADP+ reductase, $4.66 \cdot 10^{-5}$ M in A, and $1.55 \cdot 10^{-5}$ M in B and C; 3, the mixture of 1 and 2 in two sample cells in series; 4, 1+2; 5, calculated difference spectrum between 3 and 4; 6, the mixture of ferredoxin, $2.59 \cdot 10^{-5}$ M, and ferredoxin–NADP+ reductase, $2.33 \cdot 10^{-5}$ M; 7, 6+0.3 M NaCl; 8, calculated difference spectrum between 6 and 7.

mixture, *i.e.* in the buffer alone (Curve 6) and in the buffer containing 0.3 M NaC (Curve 7).

It is difficult to conclude which protein is responsible for the changes of the CD spectrum on complex formation. However, it seems likely that the changes are mainly due to those of ferredoxin–$NADP^+$ reductase, because the difference CD spectrum is coincidental with the difference absorption spectrum previously reported[1] (see Fig. 1 in the reference), and resembles the absorption spectrum of flavins. In opposition to the present interpretation, Cammack *et al.*[6] have concluded that the changes were due to a certain change of the interaction between the iron–sulphur chromophore group and the protein moiety of ferredoxin, because the maximal negative changes of CD spectrum occurred near the regions where the major Cotton effects of the ferredoxin were observed. However, it is obvious from the present experiments that no decrease in circular dichroism was observed at 420 nm.

Measurements of circular dichroism were carried out from 210 to 230 nm

in order to find conformational changes of the proteins. No significant change was observed under the experimental conditions employed.

A complex formation between ferredoxin–$NADP^+$ reductase and $NADP^+$ was also detected by the changes of CD spectrum (Fig. 2, Curve 3). It was found that negative changes appeared at around the same region as the flavin absorption of ferredoxin–$NADP^+$ reductase, suggesting that the flavoprotein was probably responsible for the changes, and not $NADP^+$.

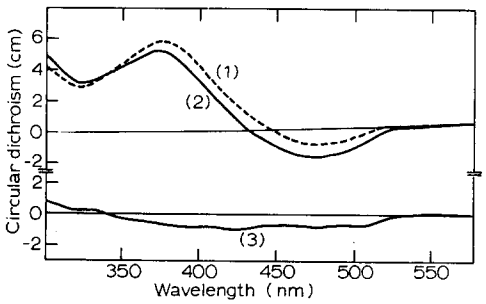

Fig. 2. Circular dichroism measurements of complex formation between ferredoxin–$NADP^+$ reductase and $NADP^+$. Measurements were made with the sensitivity, $s = 1 \cdot 10^{-5}$/mm in 0.01 M Tris–HCl buffer, pH 7.5. 1, ferredoxin–$NADP^+$ reductase, $4.66 \cdot 10^{-5}$ M and $NADP^+$, 10^{-3} M were contained separately in two sample cells in series; 2, the mixture of ferredoxin–$NADP^+$ reductase and $NADP^+$ used in 1, 3, 1–2.

Fluorimetric measurements

Through the observations of the complex formation described above, it was suggested that the optical changes accompanying the complex formation were due to the changes of the chromophore group, probably around FAD, in ferredoxin–$NADP^+$ reductase, but not due to those of ferredoxin. Therefore, an examination was carried out to determine whether the complex formation gave rise any change on fluorescence of the enzyme-bound FAD in the flavoprotein.

It was observed that the ferredoxin–$NADP^+$ reductase solution freshly prepared by passing through a Sephadex G-25 column, in order to remove free FAD in the enzyme solution, gave an extremely feeble fluorescence. The intensity of fluorescence at the maximum, 526 nm, was roughly 0.6% of that of free FAD. The fluorescence of the enzyme solution increased gradually with time as shown in Fig. 3. This indicates that fluorescence of ferredoxin–$NADP^+$ reductase is mostly due to the released or loosened FAD from the protein moiety but not due to the tightly bound FAD on the enzyme protein. In agreement with the present results, Forti[8] has reported that no fluorescence was observed in his preparation of ferredoxin–$NADP^+$ reductase in Tris–HCl buffer at pH 8.0. When ferredoxin was added to ferredoxin–$NADP^+$ reductase at high ionic strengths, whereby the complex was not formed, the fluorescence intensity at 526 nm was constantly decreased by an absorption of ferredoxin itself. At low ionic strengths the decrease of fluorescence intensity caused by ferredoxin was greater than that at high ionic strengths, and reverted after adding ferredoxin in a quantity equimolar with the flavoprotein. The fluorescence response by ferredoxin was found to be more remarkable with an old preparation of the flavoprotein than that with a fresh prep-

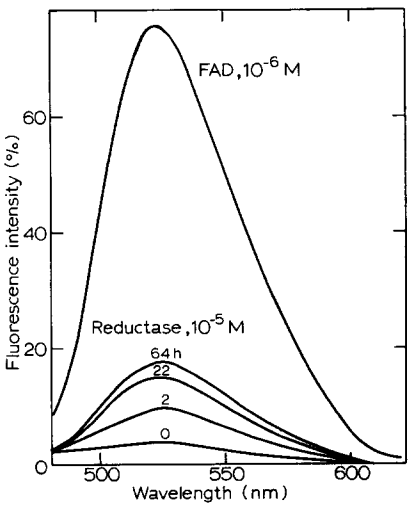

Fig. 3. Fluorescence spectra of ferredoxin–NADP+ reductase. Ferredoxin–NADP+ reductase, 10^{-5} M, and FAD, 10^{-6} M in 0.05 M Tris–HCl buffer, pH 7.5. The spectra of ferredoxin–NADP+ reductase were measured with excitation at 450 nm at the times indicated on each curve after Sephadex column treatments.

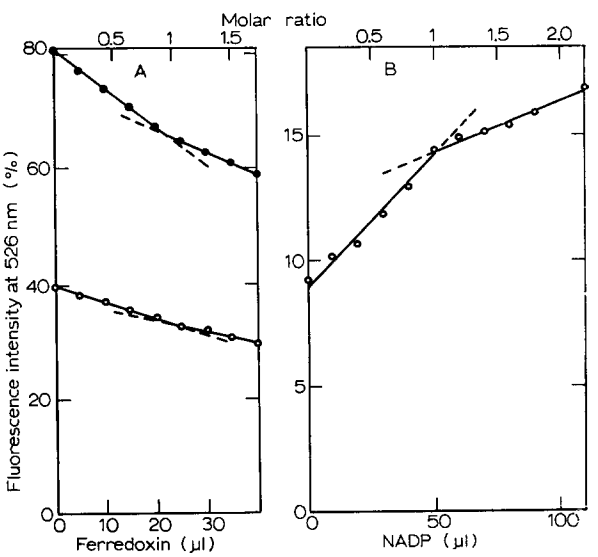

Fig. 4. Changes of fluorescence intensity during complex formation of ferredoxin–NADP+ reductase with ferredoxin or NADP+. Fluorescence intensity was measured at 526 nm with excitation at 450 nm in 0.05 M Tris–HCl buffer, pH 7.5 at room temperature. (A) Ferredoxin, $1.43 \cdot 10^{-3}$ M was added to 2.5 ml of ferredoxin–NADP+ reductase, $1.17 \cdot 10^{-5}$ M in the volume indicated on the bottom line (○—○, fresh ferredoxin–NADP+ reductase; ●—●, old ferredoxin–NADP+ reductase). (B) NADP+, $5 \cdot 10^{-4}$ M, was added to 2.5 ml of ferredoxin–NADP+ reductase, 10^{-5} M.

aration (Fig. 4A). Because the fluorescence of ferredoxin–$NADP^+$ reductase is mostly due to the released or loosened FAD, as discussed previously, these phenomena indicate that the released or loosened FAD rebinds to the apoprotein under the effects of approaching ferredoxin. Although the loosened FAD participating in the changes of the fluorescence intensity must be only a part of the FAD in the flavoprotein solution, it represents the activity of all the flavoproteins during the complex formation. The fluorimetric measurements would be, therefore, available as a tool for studies on the properties of the complex formation.

It was also observed that the fluorescence of ferredoxin–$NADP^+$ reductase was increased by adding $NADP^+$, in contrast with the case of adding ferredoxin. A refracting point was also observed at the molar ratio of one to one (Fig. 4B), clearly showing that a one to one complex was formed between $NADP^+$ and ferredoxin–$NADP^+$ reductase. It was previously observed[1] that an excess of $NADP^+$ (40-fold with respect to flavoprotein) was necessary for spectrophotometric measurements of the $NADP^+$ complex. The fluorescence experiments, however, predicted that the complex formation should be spectrophotometrically detectable by adding an equimolar amount of $NADP^+$. This was the case as shown in Fig. 5.

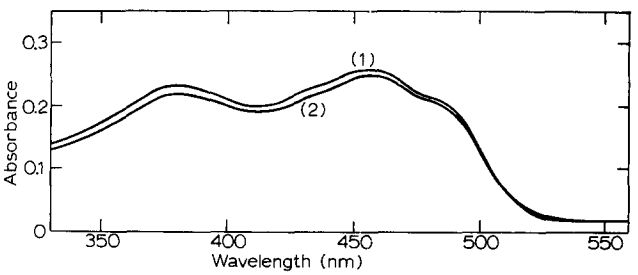

Fig. 5. Spectrophotometric measurements of complex formation between ferredoxin–NADP+ reductase and NADP+. The enzyme and NADP+ were in 0.05 M Tris–HCl buffer, pH 7.5. 1, ferredoxin–NADP+ reductase; 2, 1 + NADP+, $5 \cdot 10^{-5}$ M.

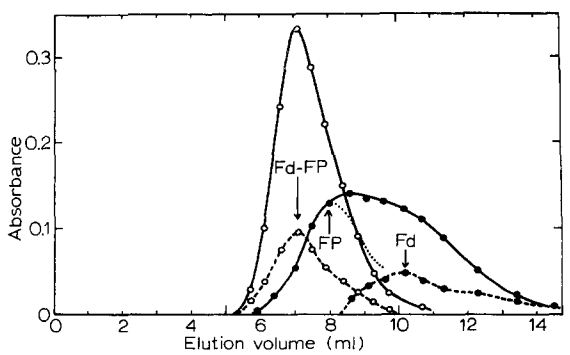

Fig. 6. Elution diagram of ferredoxin and ferredoxin–NADP+ reductase from Sepahdex G-75 column. The protein mixture added to the Sephadex G-75 column (1 cm × 20 cm) contained 30.1 nmoles of each protein in 0.2 ml 0.01 M Tris–HCl buffer, pH 7.5, with or without 0.05 M $MgCl_2$. The elution was carried out at room temperature under two different conditions: (a) 0.01 M Tris–HCl buffer, pH 7.5, alone (○); and (b) the same buffer + 0.05 M $MgCl_2$ (●). Absorbance of effluents was measured at 460 nm (—) and at 540 nm (- - - -). The dotted curve (. . . . . .) is the absorbance at 460 nm *minus* 1.8 times the absorbance at 540 nm under the conditions of (b). FP, Flavoprotein; Fd, ferredoxin.

Sephadex G-75 column chromatography

The elution patterns of ferredoxin, ferredoxin–$NADP^+$ reductase, and the complex from Sephadex G-75 columns are shown in Fig. 6. The material having an absorbance at 460 nm was eluted from the column together with that having an absorbance at 540 nm when the chromatography was carried out with 0.01 M Tris–HCl buffer, pH 7.5. This shows that ferredoxin and ferredoxin–$NADP^+$ reductase are eluted at the same position in the form of a complex. On the other hand, when 0.05 M $MgCl_2$ was added to the protein mixture and to the buffer solution in the Sephadex column, ferredoxin and ferredoxin–$NADP^+$ reductase were eluted at different position. These results indicated that ferredoxin and ferredoxin–$NADP^+$ reductase existed as a real complex in a dilute salt solution.

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