CHEMICAL MODIFICATION OF SPINACH FERREDOXIN: EVIDENCE FOR THE INVOLVEMENT OF A COMPLEX BETWEEN FERREDOXIN AND FERREDOXIN:NADP OXIDOREDUCTASE IN NADP PHOTOREDUCTION

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Spinach ferredoxin was modified chemically with trinitrobenzene sulfonic acid (TNBS), a reagent which reacts specifically with amino groups. The trinitrophenylated ferredoxin (TNP-Fd) can accept electrons from Photosystem I as indicated by its full activity in the photoreduction of cytochrome c. The modified protein is inactive, however, in the photoreduction of NADP and cannot form a complex with the flavoprotein, ferredoxin:NADP oxidoreductase. The data presented indicate that the inactivity of the modified protein is the result of modification of a single amino group.

INTRODUCTION

The non-heme iron-sulfur protein, ferredoxin, and the flavoprotein, ferredoxin:NADP oxidoreductase (EC 1.6.99.4), participate in the terminal steps of photosynthetic electron transport (1). Ferredoxin, reduced by Photosystem I, serves as the electron donor for the flavoprotein catalyzed reduction of NADP (2-4).

Much evidence has been presented showing that ferredoxin and the flavoprotein form a stoichiometric (1:1) complex *in vitro* (5-8). The complex shows a marked sensitivity to ionic strength and undergoes dissociation as the ionic strength of the medium is increased (5-8). These observations, together with the knowledge that reactions involving both ferredoxin and flavoprotein are sensitive to ionic strength whereas those involving only ferredoxin are not (7, 8), led to the suggestion that the complex between ferredoxin and flavoprotein, observed *in vitro*, might be involved in photosynthetic electron transport. However, the direct involvement of the complex in the photoreduction of NADP (even *in vitro*) has yet to be demonstrated.

Furthermore, the realization that the flavoprotein is inactivated at high ionic strength and that the salt dependence of many ferredoxin: flavoprotein coupled reactions can be accounted for by inactivation of the flavoprotein has led others to question the involvement of the complex in photosynthetic electron transport (9).

In this communication, we report on some of the properties of a chemically modified (trinitrophenylated) ferredoxin. The data presented strongly suggest that a complex between ferredoxin and flavoprotein is required for the photoreduction of NADP.

MATERIALS AND METHODS

Ferredoxin was prepared in a manner similar to that described by Petering and Palmer (10) and ferredoxin:NADP oxidoreductase according to Forti (11). The reaction of trinitrobenzene sulfonic acid (TNBS) with ferredoxin was performed as described in the Results section.

Chloroplasts were prepared by grinding depetiolated spinach leaves in a medium consisting of 50 mM Tris-Cl, pH 7.8, 0.4 M sucrose and 10 mM NaCl. After filtration through cheesecloth, the resulting brei was centrifuged at 10,000 x g for 20 min. and the resulting pellet resuspended in 50 mM Tris-Cl, pH 8.0, containing 0.1 M sucrose and 1.0 mM $MgCl_2$. Chlorophyll was determined using the equations of Arnon (12).

The reaction mixture for the photoreduction of cytochrome c contained 33 mM Tris-Cl, pH 8.0, 67 mM sucrose, 0.67 mM $MgCl_2$, 0.067 mM cytochrome c, chloroplasts to a concentration of 0.01 mg chlorophyll/ml and 0.0017 mM of control or modified ferredoxin. The assay mixture for the photoreduction of NADP contained 33 mM Tris-Cl, pH 8.0, 67 mM sucrose, 0.67 mM $MgCl_2$, 0.33 mM sodium ascorbate, 0.0067 mM DCIP, 0.2 mM NADP, chloroplasts to a concentration of 0.01 mg chlorophyll/ml and 0.0017 mM of control or modified ferredoxin. Illumination was provided by an American Optical Corporation Lamp (power setting = 7.5) at a distance of 3 cm. The photoreduction of cytochrome c was monitored by measuring the increase in absorbance at 550 nm at 15 second intervals. The photoreduction of NADP was followed by measuring the increase in absorbance at 340 nm at 30 second intervals.

The ability of ferredoxin and chemically modified ferredoxin to complex with the flavoprotein was determined by difference spectroscopy using split cell cuvettes. Flavoprotein, 22 μM, was added to the rear compartments of both reference and sample cuvettes and control on modified ferredoxin (17 μM), to the front compartments. After a baseline was recorded, the front and rear compartments of the sample cuvette were mixed and the difference spectrum recorded.

RESULTS

Ferredoxin (0.02mM) was incubated with varying molar ratios of TNBS (TNBS/Fd) = 0-10) for two hours at room temperature in 50 mM sodium borate,

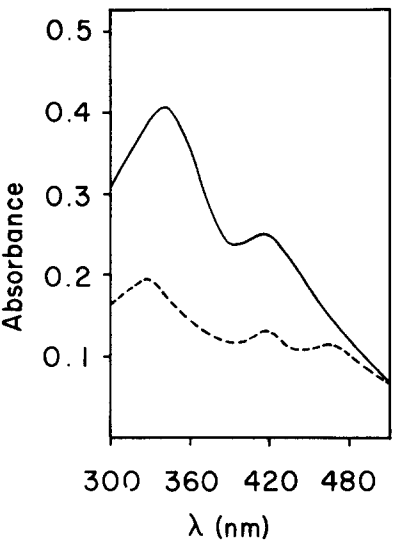

Figure 1. Integrity of Iron-Sulfur Cluster in Trinitrophenylated Ferredoxin. Top curve. Absorption spectrum of mono-trinitrophenylated ferredoxin, 16 μM. Bottom curve. Difference spectrum of mono-trinitrophenylated ferredoxin minus apomono-trinitrophenylated ferredoxin. See text for details.

pH 9.0, containing 0.4 M NaCl; thereafter, excess (and hydrolyzed) TNBS was removed by dialysis against the same buffer. The extent of modification was determined from the absorbance difference at 345 nm between modified and control ferredoxin. An extinction coefficient of 14.4 $mM^{-1}cm^{-1}$ was used to determine the extent of trinitrophenylation (13).

The spectrum of mono-trinitrophenylated ferredoxin is shown in the upper curve in Figure 1. The absorbance maxima at 345 nm and 420 nm are characteristic of a trinitrophenylated amino group. The lower curve in Figure 1 is the difference spectrum between mono-trinitrophenylated ferredoxin and mono-trinitrophenylated ferredoxin which was acidified to destroy the iron-sulfur chromophore. If the reaction of TNBS with ferredoxin does not disrupt the iron-sulfur chromophore, such a difference spectrum should be characteristic of ferredoxin. Clearly, the lower curve in Figure 1 is a typical ferredoxin spectrum and, furthermore, the absorbance at 420 nm is

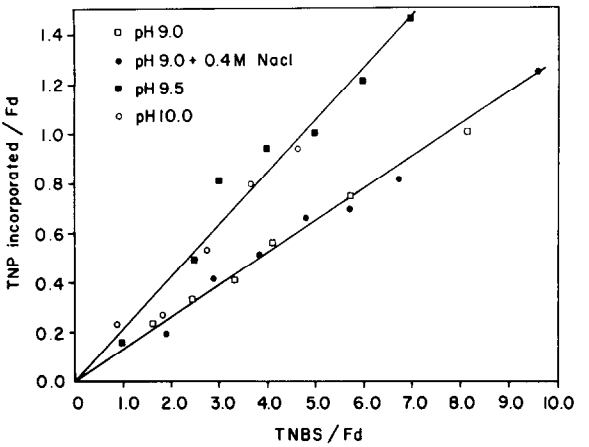

Figure 2. Effect of pH and Ionic Strength on Reaction of TNBS with Ferredoxin. Ferredoxin (17 μM) was reacted with varying molar ratios of TNBS and the extent of trinitrophenylation determined as described in the text. Conditions were as follows: (▫) 50 mM sodium borate, pH 9.0; (●) 50 mM sodium borate, pH 9.0+0.4 M NaCl; (■) 50 mM sodium borate, pH 9.5; (o) 50 mM sodium borate, pH 10.0.

about 90% of that expected based on the amount of ferredoxin present. Thus, the integrity of the iron-sulfur chromophore of ferredoxin is not disrupted by reaction with TNBS.

The effects of ionic strength and pH on the modification reaction are shown in Figure 2. At pH 9.0, the same extent of modification was obtained, for a given TNBS/Fd ratio, in the presence and absence of 0.4 M NaCl, indicating that the reaction is not affected by the ionic strength of the medium. However, the extent of modification, at a given TNBS/Fd ratio, was dependent upon the pH of the medium. At pH 9.5, the extent of trinitrophenylation was greater than that observed at pH 9.0. Little difference was noted between pH 9.5 and 10.0. The differences in extent of modification as a function of pH are most likely a manifestation of the pK of the amino group being modified.

To assess the effect of modification, the activity of modified ferredoxin in two ferredoxin dependent reactions was determined as a function of the number of trinitrophenyl groups incorporated per molecule of

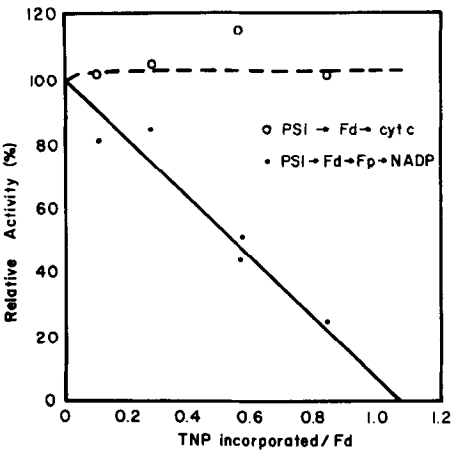

Figure 3. Effect of Trinitrophenylation on Ferredoxin Activities. The photoreduction of cytochrome c and NADP was performed as described in the Materials and Methods section. Ferredoxins with varying extents of trinitrophenylation were prepared by reacting ferredoxin with varying molar ratios of TNBS. Rates obtained with control ferredoxin were 49 μmoles Cyt c reduced/mg Chl·hr and 25 μmoles NADP reduced/mg Chl·hr.

ferredoxin. The two reactions studied were the photoreduction of cytochrome c using water as electron donor and the photoreduction of NADP using ascorbate/DCIP as electron donor. The results of these studies are shown in Figure 3.

In the range studied (0-1 trinitrophenyl groups/Fd), the modified ferredoxins were just as effective in mediating the photoreduction of cytochrome c as was the control ferredoxin. However, the ability of the modified ferredoxin to catalyze the photoreduction of NADP decreased dramatically as the incorporation of trinitrophenyl groups increased. Activity was totally abolished when one trinitrophenyl group was incorporated per molecule of ferredoxin.

The data presented in Figure 3 indicate that mono-trinitrophenylated ferredoxin can interact with, and be reduced by, Photosystem I in the same manner as does ferredoxin. Furthermore, the resulting reduced control and modified ferredoxins are equally effective in reducing (chemically) cytochrome c. In contrast, the reduced modified ferredoxin cannot serve as the electron donor for the photoreduction of NADP mediated by the flavoprotein (ferredoxin-NADP oxidoreductase).

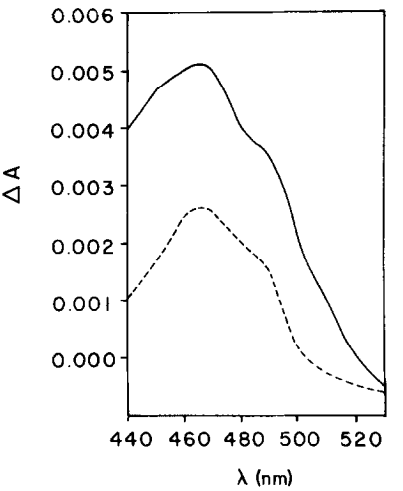

Figure 4. Comparison of Ability of Ferredoxin and Trinitrophenylated Ferredoxin to Complex with Ferredoxin:NADP Oxidoreductase. The ability of ferredoxin and a trinitrophenylated ferredoxin to complex with the flavoprotein was compared by difference spectroscopy as described on the Materials and Methods section. The trinitrophenylated ferredoxin used in these experiments contained 0.5 trinitrophenyl group per molecule of ferredoxin.

A likely explanation for the lack of activity of the modified ferredoxin in NADP photoreduction is that the modified ferredoxin can not interact with the flavoprotein. To test this possibility, the abilities of control and modified ferredoxins to complex with the flavoprotein were compared. The modified ferredoxin used in this experiment contained 0.5 trinitrophenyl group per molecule of ferredoxin. It is seen in Figure 4 that the perturbations of the flavoprotein spectrum, characteristic of the ferredoxin: flavoprotein complex, produced by the modified ferredoxin are about one-half that produced by an equal concentration of unmodified (control) ferredoxin. Thus, it appears that the trinitrophenylated ferredoxin is unable to complex with the flavoprotein and, therefore, unable to participate in NADP photoreduction.

Preliminary observations indicate further that trinitrophenylated ferredoxin does not inhibit the diaphorase (NADPH to DCIP) activity of

purified flavoprotein. The inhibition of the flavoprotein diaphorase activity by ferredoxin has been attributed previously to the formation of a complex between ferredoxin and the flavoprotein (9).

CONCLUSION

The data presented herein strongly suggest that a complex between ferredoxin and the flavoprotein is required for NADP photoreduction. Chemically modified ferredoxin does not form a complex with the flavoprotein and is thereby unable to participate in NADP photoreduction even though it can mediate the photoreduction of cytochrome c.

The loss of the ability of ferredoxin to interact with the flavoprotein and to catalyze NADP photoreduction appears related to the modification of a single functional group on the protein. That is, total loss of activity is noted when one trinitrophenyl group is incorporated per molecule of ferredoxin. Since the modifying reagent (TNBS) forms a stable product only with amino groups (14, 15), it is clear that an amino group on ferredoxin is involved in complex formation with the flavoprotein. When this amino group is blocked by the reaction with TNBS, the modified ferredoxin is unable to complex with the flavoprotein.

Further characterization of modified ferredoxin and its effects on the reactions catalyzed by the purified flavoprotein are currently in progress.

ACKNOWLEDGMENTS

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REFERENCES

1. San Pietro, A., (1968), in "Biological Oxidations," (T. P. Singer, ed.), 515-532, J. R. Wiley & Sons, New York.
2. Lazarinni, R. A., and San Pietro, A., (1962), Biochim, Biophys. Acta, 62, 417-420.
3. Tagawa, K., and Arnon, D. I., (1962), Nature, 195, 537-543.
4. Davenport, H. E., (1963), Nature, 199, 149-151.
5. Shin, M., and San Pietro, A., (1968), Biochem. Biophys. Res. Commun., 33, 38-42.
6. Foust, G. P., Mayhew, S. G., and Massey, V., (1969), J. Biol. Chem., 244, 964-970.
7. Nelson, N., and Neumann, J., (1969), J. Biol. Chem., 244, 1926-1931.
8. Nelson, N., and Neumann, J., (1969), J. Biol. Chem., 244, 1932-1936.
9. Nakamura, S., and Kimura, T., (1971), J. Biol. Chem., 246, 6235-6241.
10. Petering, D. H., and Palmer, G., (1970), Arch, Biochem. Biophys., 141, 456-464.
11. Forti, G., (1971) in "Methods in Enzymology, Vol. 23, (A. San Pietro, ed.) 447-451, Academic Press, New York.
12. Arnon, D. I., (1949), Plant Physiol., 24, 1-15.
13. Goldfarb, A. R., (1966), Biochemistry, 5, 2570-2574.
14. Okuyama, T., and Satake, K., (1960), J. Biochem., (Tokyo), 47, 454-465.
15. Kotaki, A., Harada, M., and Yagi, K., (1964), J. Biochem., (Tokyo), 55, 553-561.