Interaction between Ferredoxin and Ferredoxin Nicotinamide Adenine Dinucleotide Phosphate Reductase in Pyridine Nucleotide Photoreduction and Some Partial Reactions

II. COMPLEX FORMATION BETWEEN FERREDOXIN AND FERREDOXIN NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE REDUCTASE AND ITS RELEVANCE TO PYRIDINE NUCLEOTIDE PHOTOREDUCTION

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SUMMARY

It has been shown by difference spectroscopy that purified ferredoxin and ferredoxin-NADP reductase form a complex. The absorption maxima due to the interaction of the proteins are at 395, 469, and 495 nm. Bleached ferredoxin does not form such a complex. The stoichiometry of the complex is 2 molecules of ferredoxin per 1 molecule of flavoprotein. The complex is decomposed by salts but not by uncharged molecules.

Salts at concentrations which cause a decomposition of the complex inhibit the photoreduction of pyridine nucleotides, but do not inhibit electron transport with oxidants which accept electrons prior to the flavoprotein. It is suggested that changes in salt concentration can regulate the photosynthetic electron pathways.

In the preceding paper (1) we demonstrated that purified ferredoxin inhibits the activity of the chloroplast flavoprotein ferredoxin-NADP reductase. This report describes the formation of a complex between ferredoxin and the flavoprotein by protein-protein interaction and presents evidence that the complex participates in pyridine nucleotide photoreduction by isolated chloroplasts. A protein-protein interaction between two enzymes was shown recently and implicated in the regulation of glucose metabolism (2). During the course of this work an abstract was published by Foust and Massey (3) showing also the formation of a complex between ferredoxin and flavoprotein.

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of flavoprotein, the amount of complex increases and is maximal at about a ratio of 2 molecules of ferredoxin per 1 molecule of flavoprotein (Fig. 2).

The Δ O.D. of the difference spectrum of the complex at the same protein and buffer concentration (when the two proteins were present in equimolar quantities) was higher by about 50% at λ = 469 nm in the experiment of Fig. 2 as compared with that of Fig. 1. This may be a result of the fact that the experiment of Fig. 1 was performed with Swiss chard ferredoxin and lettuce flavoprotein, whereas in the experiment of Fig. 2 both proteins were obtained from Swiss chard. In subsequent experiments complex formation was measured with Swiss chard proteins only, and the maximal variability between different preparations in Δ O.D. at the peak was 15%.

Ferredoxin which was kept for 16 min at 50°C, a treatment which inactivates the carrier in photoreduction of NADP (Fig. 1; Reference 1), did not form the complex with flavoprotein. The effect of pH on complex stability could not be measured since it has been found that at pH values below 6.0 and above 9.5 ferredoxin is bleached, and, in addition, at the high pH the flavoprotein tends to aggregate.

The complex between both proteins is formed presumably by electrostatic bonds, since with increasing concentrations of NaCl of flavoprotein, the amount of complex increases and is maximal at about a ratio of 2 molecules of ferredoxin per 1 molecule of flavoprotein (Fig. 2).

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it progressively decomposes (Fig. 1). Sucrose up to a final concentration of 0.7 M did not affect the complex.

The effect of increasing concentrations of NaCl on several photo-reactions is presented in Fig. 3. High concentrations of NaCl inhibit NADP photoreduction but do not either inhibit the reduction of ferricyanide or that of cytochrome c. As can be seen in the results of Table I, NADP photoreduction was strongly inhibited by 0.25 mM monovalent salts but it was not affected by an osmotically equivalent concentration of nonionizable molecules. MgCl₂ is more effective than NaCl both in causing a decomposition of the ferredoxin-flavoprotein complex and in inhibiting the photoreduction of NADP (Table II). The photoreduction of methyl viologen, however, is not inhibited.

The effect of the polyanions phosphate, pyrophosphate, ADP, and ATP was tested on complex formation and NADP reduction. All of these substances inhibited both processes. ATP at 5 mM inhibited complex formation at λ = 465 nm by 35%; this can be accounted for by an ionic strength effect. (The solution of ATP' has an ionic strength of 0.05 and its effect is similar to NaCl at this ionic strength; compare with Fig. 1.) However, phosphate at μ = 0.1 caused an inhibition of 10% and at μ = 0.22, 63%. This is much less than expected on the basis of ionic strength (compare with Fig. 1). Thus, it seems that phosphate has some stabilizing effect on the complex.

In order to localize the inhibitory action of salts in NADP photoreduction, they were tested on a restricted part of the photosynthetic electron transport chain. As can be seen in the results of Fig. 4 the electron transport from reduced 2,6-dichloroindophenol to NADP is strongly inhibited by NaCl, but
electron transport from reduced 2,6-dichloroindophenol to methyl viologen is not. The former reaction depends on the presence of a low salt concentration, but this effect has not been studied further. The sensitivity of NAD photoreduction to monovalent salts is somewhat more pronounced when electrons are donated by ascorbate-2,6-dichloroindophenol than when donated by water (Table I).

The inhibition of NAD photoreduction by supraoptimal concentrations of ferredoxin described in detail in the preceding report (1) is markedly reduced when NaCl concentration is increased from 0.02 M to 0.1 M (Fig. 5).

Fig. 6 presents the effect of NaCl on the diaphorase activity of the chloroplast flavoprotein and on its inhibition by ferredoxin. It may be seen that increasing concentrations of NaCl inhibit the activity of the flavoprotein and eliminate the inhibition by ferredoxin. These effects are obtained with either purified or grana-bound flavoprotein. At an equivalent concentration, MgCl₂ was found to be more effective in the inhibition of the diaphorase than NaCl. From the results of Table III it is obvious that the salt interferes with the binding of TPNH, since by increasing the concentration of the latter the inhibition is progressively reduced.

**DISCUSSION**

From absorption measurements it is clear that a complex can be formed between ferredoxin and ferredoxin-NADP reductase when both proteins are mixed (Fig. 1). The intactness of the ferredoxin molecule is required for complex formation since slight heating (1) or treatment at low pH, which is known to remove the “labile sulfur” (4), abolished the appearance of the difference spectrum as a result of complex formation. It might be of interest that in contrast to some other flavoproteins, which contain metals, including non-heme iron, the purified ferredoxin-NADP reductase has not been shown to contain metals.

The titration data presented in Fig. 2 indicate that the complex is formed with a ratio of 2 molecules of ferredoxin per 1 molecule of NADP.
of flavoprotein. This is at variance with the reported ratio of 1:1 obtained by Foust and Massey (3) in the presence of 0.03 M phosphate. We have found that phosphate has some stabilizing effect on the complex. To assess the physiological meaning of the complex and the stoichiometry of its components, it would have been of interest to know the amount of these enzymes in the leaves. However, although the ratio of ferredoxin to chlorophyll was reported to be 1:400 (5), no comparable data are available for the flavoprotein.

As can be seen in Fig. 1, increasing concentrations of NaCl decompose the complex, but an osmotically equivalent concentration of sucrose had no effect (data not shown). MgCl₂ is more effective than NaCl (Table II). Polyansions such as pyrophosphate, ADP, and ATP were also inhibitory. With the exception of phosphate, the decomposition of the complex by salts seems to be an ionic strength effect.

The fact that the same concentrations of NaCl or MgCl₂ that cause a decomposition of the complex inhibit NADP photoreduction (Fig. 1; Table I) but do not inhibit photoreductions in which the electrons are accepted prior to flavoprotein indicates that the complex does indeed participate in the former reactions. The photoreductions which were resistant to salt included the transfer of electrons to ferricyanide, methyl viologen, and cytochrome c, which are known to accept electrons between the two photosystems, from the "system I" hypothetical reductant "X," and from ferredoxin, respectively (6, 7). If it is assumed that the high salt affects differentially the terminal segments of the photoinduced electron pathway only, it should be independent of the electron donor, and that is indeed the case (Table I, Fig. 4). The fact that the inhibition by salts is somewhat larger when reduced 2,6-dichloroindophenol is the electron donor may be the result of some additional effect of salts on the site where reduced 2,6-dichloroindophenol interacts with the electron chain.

Fewson, Black, and Gibbs (8) also found that high concentrations of NaCl or MgCl₂ that inhibit NADP photoreduction and its coupled ATP formation, but ferredoxin-supported oxygen-dependent ATP formation, a reaction in which the flavoprotein presumably does not participate, was not inhibited.

It has been shown previously that the presence of some osmotically active substance is required for ferricyanide, 2,6-dichloroindophenol, or NADP photoreduction (9). This requirement is only slightly evident in our data because of the presence of Tris buffer. Excessively high concentrations of salts (up to 1.0 M) were shown by Good (10) to stimulate ferricyanide reduction as a result of uncoupling.

Since the diaphorase activity of the flavoprotein was also inhibited by high salt it is possible that this inhibition accounts for the inhibition of NADP photoreduction. However, it has been shown that NADPH and reduced ferredoxin compete for the same site (1) on the flavoprotein; consequently, we suggest that the same site of the enzyme which complexes with ferredoxin (and dissociates by high salt) is the site which binds NADPH in the diaphorase reaction and therefore is inhibited by high salt. This, in addition, is supported by the data of Table III, which show that the inhibition of the diaphorase by MgCl₂ can be relieved by increasing concentrations of NADPH.

It is possible that local changes in vivo in the vicinity of the ferredoxin-flavoprotein complex may regulate the flow of electrons either to NADP, to oxygen, or back to the photosystem through the cyclic pathway. It should be noted that the concentrations of salt required to decompose the complex (in vitro) are within the range of the salt content of intact chloroplasts (11, 12).

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REFERENCES
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