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CHEMICAL MODIFICATION OF SPINACH FERREDOXIN WITH DIETHYLPYROCARBONATE: EVIDENCE FOR THE ESSENTIAL NATURE OF THE HISTIDYL RESIDUE

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Summary: Spinach ferredoxin contains a single ferredoxin which can be chemically modified with diethylpyrocarbonate. By varying the concentration of diethylpyrocarbonate modified ferredoxins could be prepared which had only one or both of the imidazole nitrogens of the histidine modified. A small amount of tyrosine was also modified. Ferredoxin with only one of the imidazole nitrogens modified was fully active in NADP photoreduction by chloroplast membranes. This activity was lost as the second imidazole nitrogen was modified. The results suggest an essential role for the single histidine of ferredoxin.

The iron-sulfur protein, ferredoxin, participates in the terminal steps of photosynthetic electron transport, transferring electrons from the reducing side of Photosystem I to ferredoxin:NADP reductase (1-4). This protein has a molecular weight of approximately 11,000 and contains 97-100 amino acid residues. The amino acid sequence is known for ferredoxins from a variety of plant and algal sources (5-7 and references therein) including spinach (8) and a three-dimensional structure has been determined for the ferredoxin from Spirulina platensis, a blue-green alga (9,10).

The use of chemical modification studies to assess structure:function relationships in ferredoxin have thus far been primarily directed at elucidating the roles of amino groups on the protein (11,12). In this communication we report on chemical modification of spinach ferredoxin with diethylpyrocarbonate (DEPC)[1] a reagent capable of carbethoxylation of histidyl residues (13,14). These studies were undertaken because spinach ferredoxin

1. Abbreviations used: DEPC, diethylpyrocarbonate; Tris, tris(hydroxymethyl)aminomethane; MOPS, morpholino propane sulfonic acid; Fd, ferredoxin.

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contains a single histidyl residue which is highly conserved throughout plant and algal ferredoxins. Out data indicates that modification of this histidyl residue results in loss of ability of the ferredoxin to participate in its physiological reaction, the transfer of electrons from Photosystem I to ferredoxin:NADP reductase.

MATERIALS AND METHODS

Ferredoxin was prepared in a manner similar to that described by Peterirg and Palmer (15) and was stored frozen in 50 mM Tris-Cl, pH 8.0-0.6 M NaCl. The A420/A275 ratio was greater than 0.46. Prior to modification with DEPC, the ferredoxin was dialyzed into 10 mM MOPS, pH 7.2-0.6 M NaCl.

DEPC was obtained from the Sigma Chemical Company (St. Louis, Mo., USA) and diluted to the desired concentration in acetonitrile. Fresh solutions of DEPC in acetonitrile were prepared immediately prior to the modification reaction. The modification was performed as described in the text according to the procedure of Miles (13).

Chloroplast membranes were prepared from market spinach as follows. Approximately 250 g of fresh spinach was washed and destemmed prior to homogenization using an Osterizer kitchen blender. The homogenization medium contained 0.33 M Sucrose, 4.0 mM $MgCl_2$, and 10 mM sodium pyrophosphate buffer, pH 6.5. After homogenizing for 1 min, the resulting brei was filtered through four layers of cheesecloth and centrifuged at 9000 x g for 15 min in a Beckman RC2B refrigerated centrifuge. The chloroplast membrane pellet was resuspended in 0.33 M sucrose, 1.0 mM $MgCl_2$, and 50 mM Tris-Cl, pH 8.0, centrifuged again as described above and the resulting pellet resuspended in the same buffer. After filtration through two layers of Kimwipes, the chloroplast membrane suspension was used for the measurement of NADP photoreduction. The chloroplast membranes prepared in this manner were totally stripped of ferredoxin as no NADP reduction could be detected upon illumination of the chloroplast membranes in the absence of added ferredoxin. The chloroplast suspension had a chlorophyll content of 2-3 mg/ml as determined by the method of Arnon (16).

NADP photoreduction was measured in a medium containing 0.33 M sucrose, 1.0 mM $MgCl_2$, 50 mM Tris-Cl, pH 7.2, and 0.3 mM NADP. Ferredoxin was added to a concentration of 2.0 µM and dark adapted chloroplasts to a concentration of 10 µg chl/ml. The reaction mixture was illuminated using a slide projector at a distance of 20 cm from the sample. Light from the slide projector was filtered through a 2.5 cm solution of 1% $CuSO_4$. The rate of NADP reduction was determined from the increase in absorbance at 340 nm as a function of illumination time. Linear rates were observed for illumination times up to 6 min.

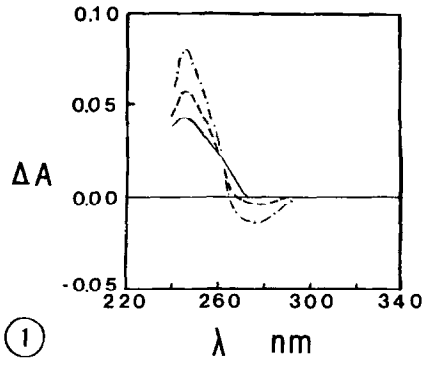
The difference spectra used to follow the modification reaction were determined using a Cary 210 spectrophotometer and NADP photoreduction was measured using an Aminco DW2a spectrophotometer.

NADP, Tris base, and MOPS were obtained from Sigma Chemical Company (St. Louis, MO., USA). All other chemicals were of reagent grade.

RESULTS

Modification of spinach ferredoxin by DEPC was followed spectrally. One ml samples of ferredoxin (60 μM) in 10 mM MOPS, pH 7.2-0.6 M NaCl were placed in both the sample and reference cuvettes and a baseline established. DEPC was added to the sample cuvette from a stock solution in acetonitrile and an equal volume of acetonitrile was added to the reference cuvette. Additions were never more than 5% of the sample volume. Difference spectra were recorded as a function of time to follow the modification reaction (Fig. 1). The difference spectra showed the appearance of a positive peak at 242 nm due to the formation of N-carbethoxyhistidine and a negative peak at 278 nm due to the formation of O-carbethoxytyrosine. Differential extinction coefficients of 3.2 $mM^{-1}cm^{-1}$ (13) and 1.3 mM^{-1} cm^{-1} (13) were used to determine the extent of histidine and tyrosine modification respectively.

Figure 2 shows the time course for the reaction of ferredoxin and DEPC. The reaction appears to go to completion in 30-40 min with no further change



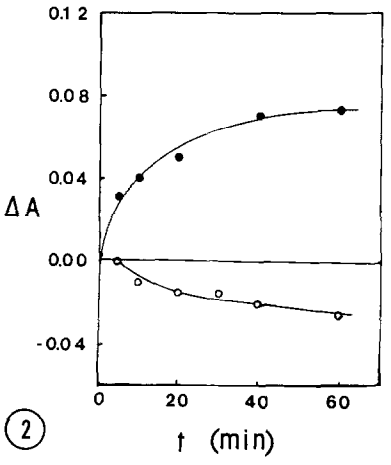


Figure 1 Difference Spectra Obtained On Treatment of Spinach Ferredoxin with DEPC The modification of ferredoxin by DEPC and the difference spectra were obtained as described in the text. For the experiment shown the DEPC concentration was 3.45 mM. Difference spectra shown are those obtained after 5 min (——), 20 min (---) and 60 min (-·-) incubation with DEPC.

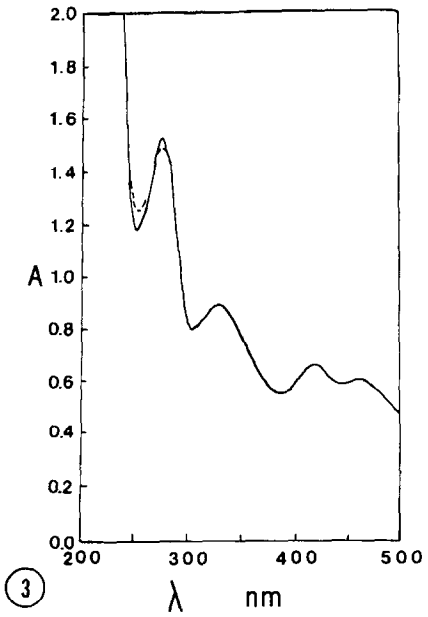
Figure 2 Time Course for Modification of Ferredoxin by DEPC ΔA_{242} and ΔA_{278} were determined from difference spectra such as those shown in Fig. 1. Conditions were as given in Fig. 1 and in the text. Closed symbols, A_{242}; open symbols, ΔA_{278}.

in absorbance taking place after that time. The onset of tyrosine modification shows a lag period of about 5 min. as substantial histidine modification occurs in the first 5 min. of reaction time while no tyrosine is modified in the same interval.

The extent of histidine modification is dependent on the concentration of DEPC. At concentrations less than 7.0 mM DEPC, less than one histidine was modified per ferredoxin. At higher concentrations of DEPC, the extent of histidine modification was greater than one per molecule. This was at first surprising in that ferredoxin contains only a single histidine. It has been noted by others (13) that at high DEPC concentrations, both of the imidazole nitrogens of histidine can be carbethoxylated with DEPC and that this increased level of modification results in a further increase in the absorbance at 240 nm. Assuming that the change in extinction coefficient for the addition of a second carbethoxy group is the same as that for the addition of the first group, we have been able to prepare ferredoxins which were modified to the extent of 1.7 histidine per ferredoxin. We interpret this as indicating that one of the imidazole nitrogens is fully modified and the other is modified in about 70% of the ferredoxin molecules. The extent of tyrosine modification (0.25-0.4 tyrosine modified per ferredoxin) was not increased by higher DEPC concentrations.

The UV-visible absorption spectra of control and modified ferredoxin at the end of the 60 min incubation with DEPC are shown in Fig. 3. The increase in absorbance below 260 nm is due to the histidine modification while the slight decrease in the peak at 275 nm is due to the modification of tyrosine. The visible region of the spectrum was unchanged indicating that the ironsulfur center of the protein was intact.

Ferredoxins differing in their extent of histidine and tyrosine modification were examined for their ability to transfer electrons from Photosystem I to the flavoprotein, ferredoxin:NADP reductase, by comparing their effectiveness in catalyzing the photoreduction of NADP by chloroplast membranes. Ferredoxins with up to one histidine modified per ferredoxin were



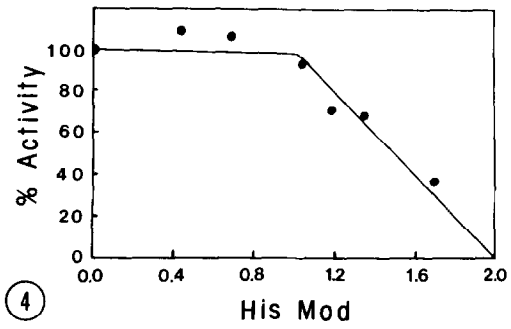


Figure 3 Spectra of Control and DEPC-Modified Ferredoxin At the end of a 60 min incubation with 3.45 mM DEPC, the UV-visible absorption spectra of control and DEPC-modified ferredoxins were determined. Control ferredoxin (———); modified ferredoxin (---).

Figure 4 Effect of Histidine Modification on Activity of Ferredoxin in NADP Reduction by Chloroplast Membranes NADP photoreduction was measured as described in Materials and Methods. Control on modified ferredoxin was present at a concentration of 0.6 μM. Data was plotted as % activity (relative to the unmodified ferredoxin) against the degree of histidine modification. The rate obtained with control ferredoxin was 16 μmoles NADP reduced/mg chl/hr. The assays were done under conditions where ferredoxin was rate-limiting.

fully active when compared to a control (unmodified) ferredoxin (Fig. 4). In contrast, ferredoxins modified to an extent of greater than one histidine per ferredoxin (i.e., ferredoxins in which some of the histidines were doubly modified) showed a decreased ability to participate in NADP photoreduction by chloroplast membranes (Fig. 4). This loss of activity correlated well with the addition of a second carbethoxy group to the single histidine of ferredoxin. No correlation was observed between loss of activity and tyrosine modification.

DISCUSSION

In this communication, we have demonstrated that the single histidine of spinach ferredoxin can be modified with DEPC, and that this modification can result in the loss of ability of the modified protein to perform

its physiological function in transferring electrons from Photosystem I to ferredoxin:NADP reductase. Our data suggest that the modification of both imidazole nitrogens of the single histidine of ferredoxin is required for this loss of activity and that the modification of only one of the imidazole nitrogens has no effect on the activity of the ferredoxin.

The single histidine of spinach ferredoxin is at position 90 in the amino acid sequence (8). This position is occupied by histidine in all plant and algal ferredoxins for which sequences are known to date (5-7 and references therein). The position may not be absolutely conserved as it has been reported that ferredoxin from Spirogyra (for which a sequence is not yet available) lacks histidine (17). Thus it may be possible that, while histidine is the preferred residue at this position, it is not absolutely required.

An examination of the three dimensional structure of ferredoxin from Spirulina platensis (which has a histidine residue in the same position) shows the molecule to be a barrel-shaped protein with a tail extending from the barrel into solution at the C-terminal end of the molecule. The histidine is located near the position where this tail breaks away from the overall barrel shape of the molecule.

In studies of the interaction of ferredoxin with ferredoxin:NADP reductase, Batie and Kamin (18) have observed a pH dependent increase in the K_d for the complex between the two proteins. They interpreted this change in K_d as being indicative of a shift in pK_a of an ionizable group on one of the proteins from 6.0 in the free protein to 8.2 in the complex between the two proteins. As the normal pK_a for the histidine imidazole group is near 6.0, we feel that the histidine of ferredoxin is a potential candidate for the ionizable group described by Batie and Kamin (18).

Further characterization of this modified ferredoxin, including its interaction with the chloroplast membrane and with purified ferredoxin:NADP reductase are in progress.

REFERENCES

1. Arnon, D.I., (1977), Encyclopedia of Plant Physiology (New Series), 5, 7-56.
2. Lazarinni, R.A. and San Pietro, A., (1963), Biochim. Biophys. Acta, 62, 417-420.
3. Davenport, H.E., (1963), Nature, 199, 149-151.
4. Shin, M., Tagawa, K., and Arnon, D.I., (1963), Biochem. Z., 338, 84-96.
5. Matsubara, H., Hase, T., Wakabayashi, S., and Wada, K., (1980), in "The Evolution of Protein Structure and Function", pp. 245-266, (Sigman, D.S., and Brazier, M.A., eds.). Academic Press, New York.
6. Wakabayashi, S., Hase, T., Wada, K., Matsubara, H., and Suzuki, K., (1980), J. Biochem., 83, 1305-1319.
7. Hase, T., Inoue, K., Matsubara, H., Williams, M.M., and Rogers, L.J., (1982), J. Biochem., 92, 1357-1362.
8. Matsubara, H. and Sadaki, R., (1968), J. Biol. Chem., 243, 1732-1757.
9. Fukuyama, K., Hase, T., Matsumoto, S., Tsukihara, T., Katsube, Y., Tanaka, N., Kakudo, M., Wada, K., and Matsubara, H., (1980). Nature, 286, 522-524.
10. Tsukihara, T., Fukuyama, K., Nakamura, M., Katsube, Y., Tanaka, N., Kakudo, M., Wada, K., Hase, T., and Matsubara, H., (1981), J. Biochem., 90, 1763-1773.
11. Davis, D.J., and San Pietro, A., (1976), Biochem. Biophys. Research Commun., 74, 33-40.
12. Masaki, R., Wada, K., and Matsubara, H., (1976), J. Biochem., 81, 1-9.
13. Miles, E.H., (1977), Methods in Enzymology, 47, 321-442, (Hirs, C.H.W. and Timasheff, S.N., eds.). Academic Press, New York.
14. Means, G.E., and Feeney, R.E., (1971), "Chemical Modification of Proteins", pp. 81-83, Holden-Day Inc., San Francisco.
15. Petering, D.H., and Palmer, G., (1970), Arch. Biochem. Biophys., 141, 456-464.
16. Arnon, D.I., (1949), Plant Physiol., 24, 1-15.
17. Matsubara, H., Wada, K., and Masaki, R., (1976), in "Iron and Copper Proteins", pp. 1-15, (Yasunebu, K.T., Mower, H.F., and Hayaishi, O., eds.), Plenum Press, New York.
18. Batie, C.J., and Kamin, H., (1981), J. Biol. Chem., 256, 7756-7763.