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Chemical modification and cross-linking as probes of regions on ferredoxin involved in its interaction with ferredoxin : NADP reductase

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Ferredoxin which had been modified with glycine ethylester in the presence of a water-soluble carbodiimide to the extent of one carboxyl-group modified per ferredoxin was subjected to peptide mapping in an attempt to locate the site(s) of modification. The peptide mapping was done by HPLC and analysis of the resulting chromatogram allowed assignment of peaks to various segments in the amino acid sequences of the two isozymes of ferredoxin. The modified ferredoxin appeared to be a mixture of ferredoxin derivatives in which modification had occurred in three areas of the molecule. Although unable to identify the specific residues modified, it has been shown that modification is localized in the regions of residues 26–30, 65–70, and 92–94. The possibility that these regions of ferredoxin may define its binding site for ferredoxin : NADP reductase is discussed. Peptide mapping studies on a covalently linked adduct between ferredoxin and ferredoxin : NADP reductase also support these regions of ferredoxin as being important in the interaction between the two proteins.

Introduction

The nonheme iron-sulfur protein, ferredoxin, and the flavoprotein, ferredoxin : NADP reductase, participate in the terminal steps of photosynthetic electron transport mediating the reduction of NADP by electrons from the reducing side of Photosystem I. Considerable evidence has accumulated showing these two proteins to form a complex having a 1 : 1 stoichiometry [1–3]. The sensitivity of this complex to increasing ionic strength [2,3] indicates that the interaction between the two proteins is electrostatic in nature. Little is known, however, as to which portions of the two proteins are involved in their interaction.

As ferredoxin is an extremely acidic protein with about 20 of its 97 amino acids bearing carboxyl groups [4–6] it is often assumed that some of these negatively charged carboxyl groups must interact with positively charged groups on ferredoxin : NADP reductase. Data supporting this assumption have been presented in a recent paper [7] where it was shown that modification of only a few of the 20 carboxyl groups on ferredoxin dramatically decreased its ability to interact with ferredoxin : NADP reductase. Modification of 3–4 carboxyl groups per ferredoxin was sufficient to decrease the activity of ferredoxin in NADP photoreduction by chloroplast membranes by ap-

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Abbreviations: Fd, ferredoxin; Fp, ferredoxin : NADP reductase, EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Mops, 4-morpholinepropanesulfonic acid.

proximately 80%, while 50% activity was lost when one carboxyl group was modified per ferredoxin [7]. The large loss of activity accompanying the relatively small degree of modification suggest that the modified groups are likely to be involved in the interaction of ferredoxin with ferredoxin : NADP reductase. In the present paper, we report on attempts to determine the locations of these modified carboxyl groups in the ferredoxin structure.

Materials and Methods

Modification of ferredoxin. Carboxyl groups on ferredoxin were modified with ^{14}C-labelled glycine ethylester in the presence of EDC as described in Ref. 7. The extent of modification was limited to an average of one carboxyl group modified per Fd for the present studies.

Separation of ferredoxin isozymes. Separation of the two isozymes of spinach was done in a manner similar to that described by Takahashi et al. [6,8]. Purified ferredoxin was treated with 4 M urea for 10 min followed by a 30 min incubation with 20 mM dithiothreitol. After this incubation, the ferredoxin was treated with 40 mM iodoacetamide for 1 h. These treatments converted Fd into the apoprotein and modified the liberated sulfhydryl groups so that disulfide bonds could not form.

The apoprotein sample was then applied to a 1.5 × 25 cm DEAE-cellulose column (Whatman DE-52) and the two isozymes eluted with a NH_4HCO_3 gradient (0.2–0.5 M). Fractions were collected and the location of the two isozymes determined by their A_{280}.

Primary digestion and HPLC peptide mapping of ferredoxin samples. Samples of control or modified ferredoxin for peptide mapping were all treated with urea, dithiothreitol, and iodoacetamide as described above. After dialysis into 0.1 M NH_4HCO_3, the samples were treated with trypsin (0.016 mg/mg ferredoxin) for 3 h and then with chymotrypsin (0.004 mg/mg ferredoxin) for 7 h. Both treatments were done at 37°C.

Peptides obtained from this digestion procedure were resolved using a Spectra-Physics SP-8700 HPLC equipped with a Brownlee RP-300 reversed phase column. The peptides were eluted with a 0–100% methanol gradient. Trifluoroacetic acid (0.1%) was present throughout the gradient. The positions of the peptides were determined by continuous monitoring of the eluant at 210 nm using a Tracor 970A variable wavelength detector. Fractions were collected throughout the HPLC run and saved for further analysis. The digestion and HPLC mapping procedures have proved very reproducible. In over 30 runs covering a one year period, we have observed no variation in the relative positions or sizes of peaks in the resulting chromatograms.

Identification of peaks in HPLC peptide maps of ferredoxin. Each of the peaks from the HPLC run was hydrolyzed in 6 M HCl in vacuo for 24 h at 110°C prior to amino acid analysis using a microcomputer-controlled acid analyzer described by Durham and Geren [9]. When necessary, peaks from several HPLC runs were pooled to obtain sufficient material for analysis. The amino acid compositions of the various peaks were compared with the compositions of various segments of the amino acid sequences of the two Fd isozymes to identify which peaks corresponded to each segment. Compositions determined experimentally were within 15% of the predicted value for the assigned segment. No correction was made for losses of amino acids during hydrolysis.

Location of modified peptides in HPLC peptide map of modified ferredoxin. Visual comparison of peptide maps of control and modified ferredoxin showed decreases in some peaks and the appearance of some new peaks as would be expected from modification. The identity of the new peaks has been determined by amino acid analysis.

The use of ^{14}C-glycine ethylester to modify Fd also facilitated location of modified peaks. Aliquots (0.1 ml) from each fraction collected during a HPLC run were mixed with 4 ml liquid scintillation cocktail (Research Products International 3a70B) and counted using a Beckman LS100C liquid scintillation counter to determine which peaks contained radiolabel. All counts/min reported have been corrected for background.

Secondary digests of peaks from HPLC peptide map. Peaks from the HPLC run were collected and, where necessary, secondary digestions were performed using proteolytic enzymes of differing specificity. Details of these secondary digestions are provided in the appropriate figure legends.

The products of these secondary digestions were analyzed by HPLC, amino acid analysis, and liquid scintillation counting as described above.

Chemical cross-linking of ferredoxin-ferredoxin : NADP reductase complex. The complex between ferredoxin and ferredoxin : NADP reductase was cross-linked by incubation of the two proteins in the presence of EDC in a manner similar to that described by Zanetti et al. [10] with the following alterations. Ferredoxin and ferredoxin : NADP reductase were incubated with 1 mM EDC in 10 mM Mops (pH 6.5) for 2 h. In contrast to the Zanetti procedure, no NADP was present during the incubation. After the incubation, the sample was applied to a DEAE-cellulose column (Whatman DE-52) and the column washed with a 0–0.6 M NaCl gradient in 50 mM Tris-HCl (pH 8.0). The covalently linked ferredoxin-ferredoxin : NADP reductase adduct eluted between uncross-linked Fp and Fd.

Results

Peptide map of spinach ferredoxin I and II

Before HPLC peptide mapping could be used to locate the positions of modified groups in ferredoxin, a reproducible peptide mapping procedure had to be developed. One problem encountered was the presence of two isozymes of ferredoxin (ferredoxin I = major isozyme, ferredoxin II = minor isozyme). Although amino acid sequences for these two isozyme differ in 25 positions [6], they have proved impossible to separate in an intact form. The chemical modification studies described in Ref. 7 were thus done on a mixture of the two isozymes. Takahashi et al. [6,8] have found that the two isozymes can be resolved on DEAE-Toyopearl after removal of the iron-sulfur center and blocking of the liberated sulfhydryl groups with iodoacetamide. We have found that after comparable treatment, the two ferredoxin isozymes can also be resolved on DEAE-cellulose (data not shown). The resolution and relative amounts of the two isozymes are comparable to that reported by Takahashi et al. [6,8].

Fig. 1 shows the HPLC chromatogram of native ferredoxin (a mixture of the two isozymes) and each of the isozymes run individually. All samples were digested as described in Materials and Methods after removal of the iron-sulfur center and blocking of the liberated sulfhydryl groups. It is clear that most of the peaks in the chromatogram of native ferredoxin can be correlated with peaks in the chromatogram of one or the other of the ferredoxin isozymes.

Peaks from the chromatograms shown in Fig. 1 were collected, hydrolyzed in 6 M HCl, and subjected to amino acid analysis. The composition of each peak was compared with compositions of various segments of the amino acid sequences of the two isozymes to determine which peaks corresponded to particular regions of the two sequences. The results are summarized in Fig. 2. In the case of ferredoxin I, peaks have been found which include all of the sequence excepting two small peptides (residues 38–40 and 51–52). For ferredoxin II, peaks have been found covering the entire sequence excluding residues 1–4 and 38–40. As the missing pieces are small and contain no carboxyl-containing amino acids, we have not attempted to locate them.

Peptide mapping of modified ferredoxin

Attempts to locate the positions of modified carboxyl groups on ferredoxin have utilized ferredoxin which was modified to the extent of one carboxyl group per ferredoxin. Fig. 3 shows HPLC chromatograms of digests of control and modified Fd. Several changes are apparent. Most notable are the decreases in peaks E (residues 76–97), H (residues 24–37), and K (residues 53–75) from Fd I. The integrated area under these peaks decreased by 37, 71 and 51%, respectively. (Although decreases were also seen in peaks D, F, G, and J from ferredoxin II, no attempt has been made to quantitate the decreases or further analyze these fragments due to the lower concentration of ferredoxin II in the ferredoxin preparations used in these studies.)

Two new peaks appear in the chromatogram of modified ferredoxin. These are labelled E* and K* in Fig. 3. When ferredoxin was modified in the presence of ^{14}C-glycine ethylester, both of these new peaks were found to be labelled. Analysis of their amino acid compositions identified them as modified forms of peptides E and K, respectively.

When ^{14}C-glycine ethylester was used, consid-

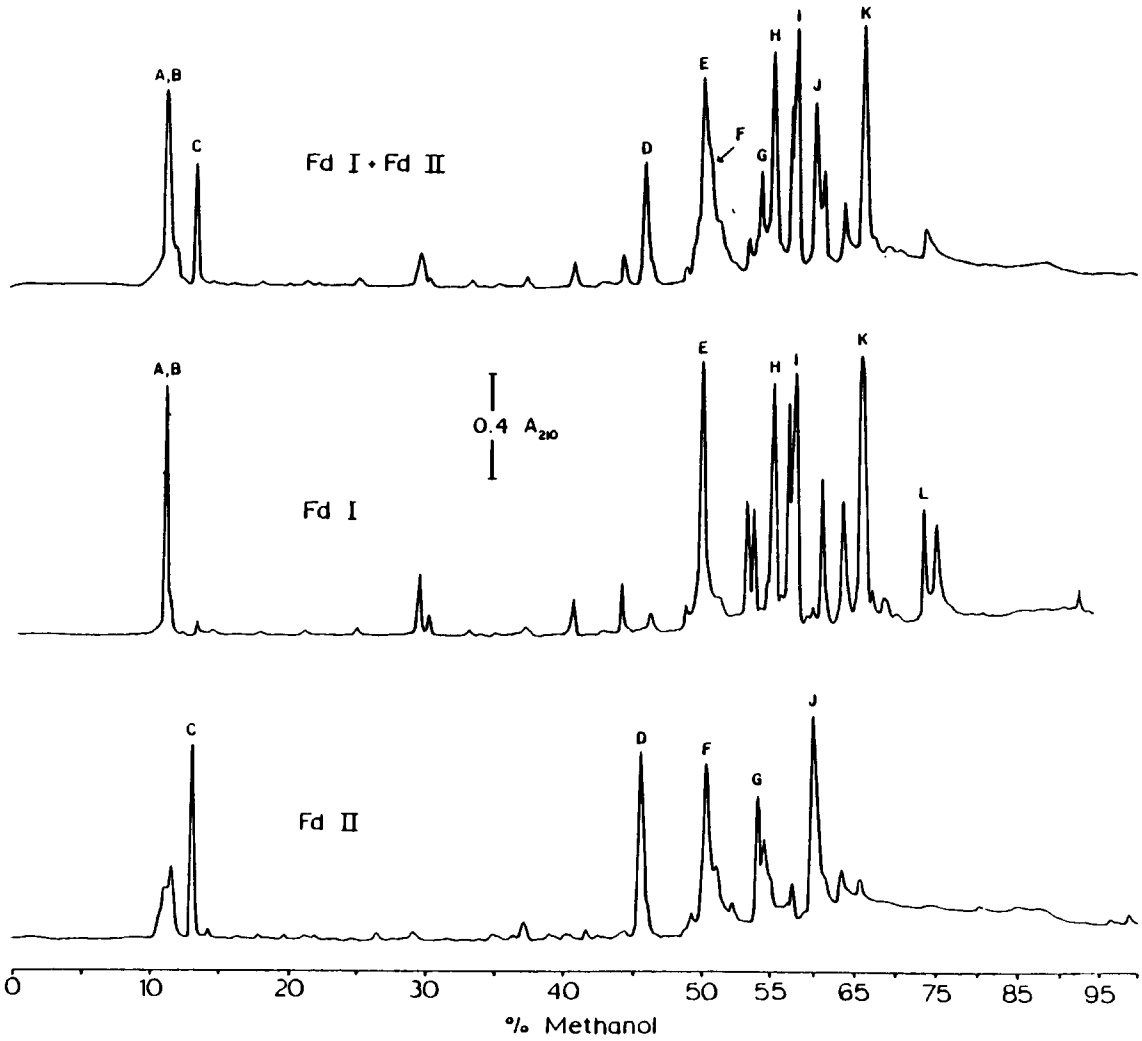


Fig. 1. HPLC peptide map of ferredoxin preparation and ferredoxin isozymes ferredoxin (a mixture of two isozymes) was converted into the apoprotein and sulfhydryl groups protected as described in Materials and Methods. The Fd or samples of the individual isozymes were subjected to proteolytic digestion as described in Materials and Methods prior to analysis of the resulting proteolysis fragments by HPLC using a methanol gradient. The gradient was increased from 0 to 55% methanol over a 60 min period followed by a increase from 55–100% methanol in 30 min.

erable labelling was also seen in the poorly resolved region of the chromatogram between peaks J and K (57–63% methanol). When this region was collected and rerun using a different methanol gradient, two prominent labelled peaks were located (Fig. 4) and identified by their amino acid compositions as being modified forms of peak H (H*) from ferredoxin I and peak J (J*) from ferredoxin II.

*Secondary digests of peak H and H**

The labelled peaks, E*, H*, and K*, comprise over half of the sequence of Fd I and contain 17 of the 20 carboxyl groups present in ferredoxin I. Consequently, it was necessary to perform secondary digests on these peaks using proteolytic enzymes of differing specificity to identify more precisely the sites of labelling.

Peptide H (residues 24–37 of ferredoxin I) con-

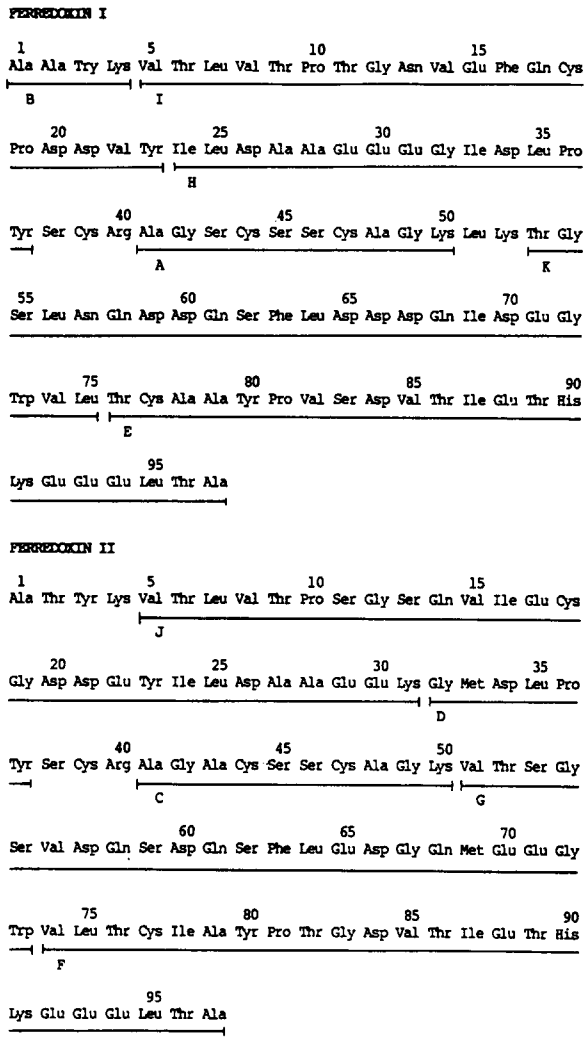


Fig. 2. Correlation between peptide map and amino acid sequences of ferredoxin I and II. The amino acid sequences for the two isozymes of spinach Fd are from Refs. 5 and 6. The underlined regions indicate portions of the sequences for which peaks in the HPLC peptide map have been found. The capital letters shown indicate that the segment indicated corresponds with the peak having that designation in the peptide map shown in Fig. 1.

tains five acidic residues. Treatment of this peptide with *Staphylococcus aureus* protease results in a cleavage after Glu-31, dividing peptide H into two smaller pieces, labelled H-1 and H-2 (Fig. 5). When ^{14}C-labelled peptide H* was treated in the same way, peak H-2 was unaltered and contained no radiolabel. Peak H-1, however, was missing and a new peak, labelled H-1*, appeared. This new peak contained nearly all of the radiolabel and was identified from its amino acid composition as being a modified form of H-1.

This data limits the possible site of modification in this region of the molecule to Asp-26, Glu-29, or Glu-30. Glu-31 cannot be the site of modification, since if it were modified no cleavage by *S. aureus* protease at that position would be observed. Asp-34 is also excluded as it is located in peptide H-2 which contains no label and chromatographs in an identical position in runs on control and modified peptide H.

*Secondary digests of peak K and K**

Peak K (residues 53–75 of Fd I) contains three regions rich in carboxyl groups: Asp 59–Asp 60, Asp 65–Asp 66–Asp 67, and Glu 70–Glu 71. Treatment of peptide K with thermolysin resulted in the production of two major peaks labelled K-1 and K-2 (Fig. 6) and several smaller uncharacterized peaks. Based on their amino acid compositions, peptides K-1 and K-2 were identified as being residues 53–63 and 64–75, respectively. Similar treatment of ^{14}C-labelled peptide K* also produced two major peaks. Peak K-1 chromatographed to the same position as did peak K-1 from control peptide K and contained no radiolabel. Peak K-2 was missing from the chromatogram from the digestion of K* and a new peak, labelled K-2*, appeared. Based on its amino acid composition, K-2* was identified as being a modified form of K-2.

Peptide K was also treated with *S. aureus* protease which cleaved the peptide into two pieces, K-3 and K-4 (Fig. 7). From their amino acid compositions, K-3 and K-4 have been identified as residues 53–71 and residues 72–75, respectively. Treatment of ^{14}C-labelled K* with *S. aureus* protease resulted in the absence of K-3 in the chromatogram and the appearance of a new doublet, labelled K-3*. The radiolabel was associated with this doublet. Amino acid compositions for each peak of the doublet suggest that the two peaks are due to singly and doubly modified peptide. Peptide K-4 from the modified Fd chromatographed in a manner identical to that of K-4 from control ferredoxin and contained no radiolabel.

The thermolysin digestion of K* eliminates Asp

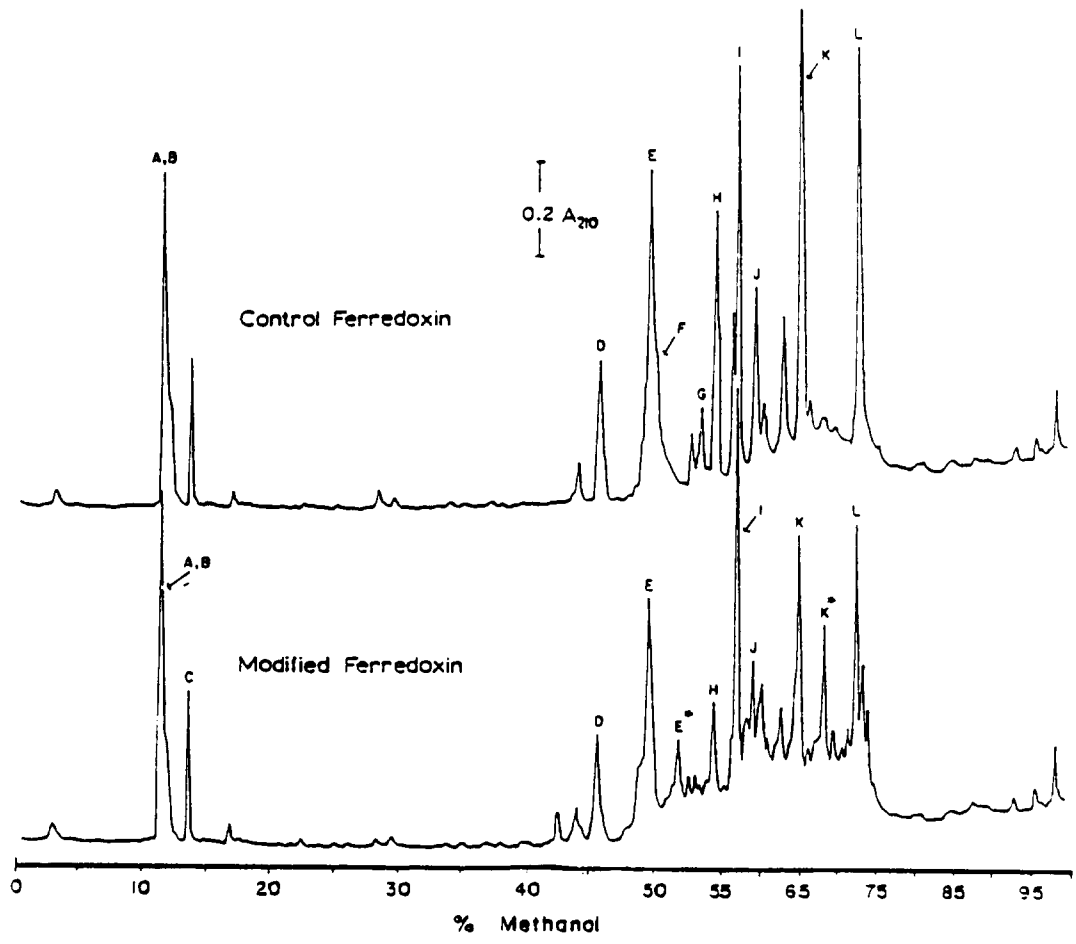


Fig. 3. HPLC peptide map of modified ferredoxin control (unmodified) ferredoxin and ferredoxin which has been modified to the extent of one carboxyl group per ferredoxin were converted into apoproteins and sulfhydryl groups blocked prior to proteolytic digestion and HPLC mapping. Procedures were as described in Materials and Methods and in the legend of Fig. 2. The upper chromatogram is that of control ferredoxin and the lower chromatogram that of modified Fd. The positions of new peaks in the chromatogram of modified ferredoxin are indicated with *.

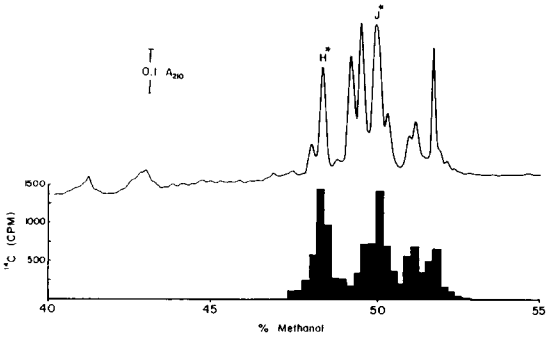


Fig. 4. Further resolution of HPLC peptide map fractions eluting between 52 and 67% methanol in the initial HPLC run on modified ferredoxin were pooled and concentrated under N_2 gas prior to reapplication to the HPLC column. The gradient was altered such that the methanol concentration was increased from 0 to 35% in 10 min followed by a further increase from 35 to 55% in 100 min. The bar graph shows the positions of fractions containing radioactivity when ^{14}C-glycine ethylester was used to modify the protein.

59 and Asp 60 as possible sites of modification. As *S. aureus* protease digestion cleaves after Glu 71 in both peptides K (from control ferredoxin) and K* (from modified ferredoxin), this residue is also excluded as a site for modification. Four carboxyl groups remain as candidates for the site(s)

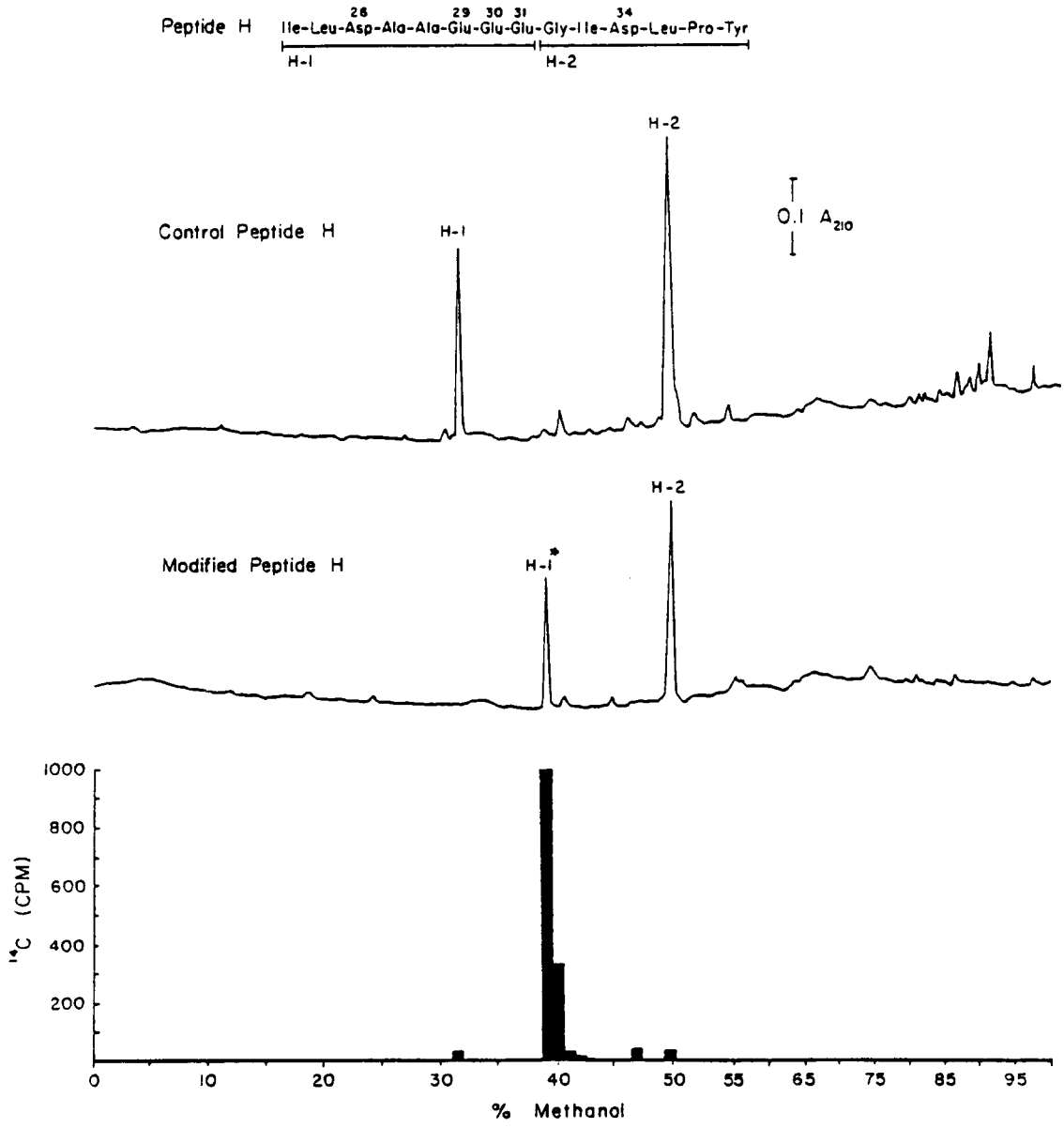


Fig. 5. Analysis of *S. aureus* protease digestion of peaks H and H* material from peak H of unmodified ferredoxin and H* of modified ferredoxin was treated with *S. aureus* protease (0.06 mg *S. aureus* protease/mg peptide) in 80 mM sodium phosphate (pH 7.8) for 12 h and the resulting fragments separated by HPLC. The bar graph shows the position of radioactive fractions when H* from ferredoxin which had been modified in the presence of ^{14}C-glycine ethylester was used. The gradient for the HPLC run was as described in Fig. 1.

of modification in this region of the molecule. These are Asp 65, Asp 66, Asp 67 and Asp 70.

*Secondary digests of peak E and E**

Peptide E contains five carboxyl groups, Asp 84, Glu 88, Glu 92, Glu 93 and Glu 94. It also contains a lysine residue at position 91. The primary digestion of control and modified Fd did not result in a cleavage after this lysine, perhaps due to its close proximity to a cluster of negatively charged groups (Glu 92–94).

We have attempted to cleave peptide E after

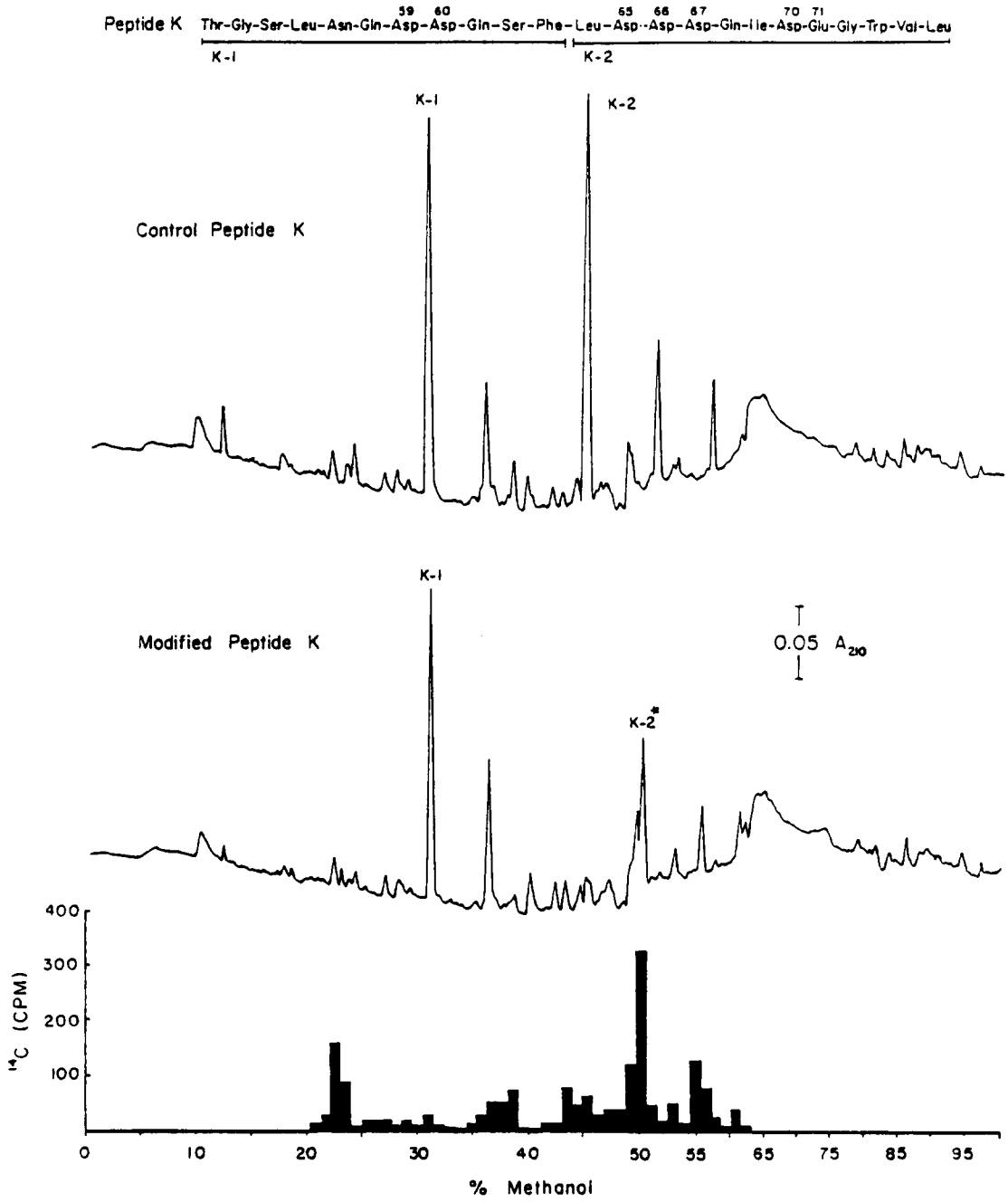


Fig. 6. Analysis of thermolysin digestion of peaks K and K*. Material from peak K of unmodified Fd and peak K* of modified Fd was treated with 0.15 mg thermolysin/mg peptide in 0.2 M ammonium acetate (pH 8.5) containing 0.5 mM $CaCl_2$ for 3.5 h. The resulting fragments were sererated by HPLC using the gradient described in Fig. 1. The bar graph shows the fractions which contained radioactivity when peak K* was obtained from Fd which had been modified in the presence of ^{14}C-glycine ethylesters.

this lysine by treatment with a higher concentration of trypsin than was used in the primary digestion. Fig. 8 shows that when incubated with trypsin at a level of 0.2 mg/mg peptide E, the peptide is split into two pieces, E-1 and E-2. Based on their amino acid compositions, E-1 is residues

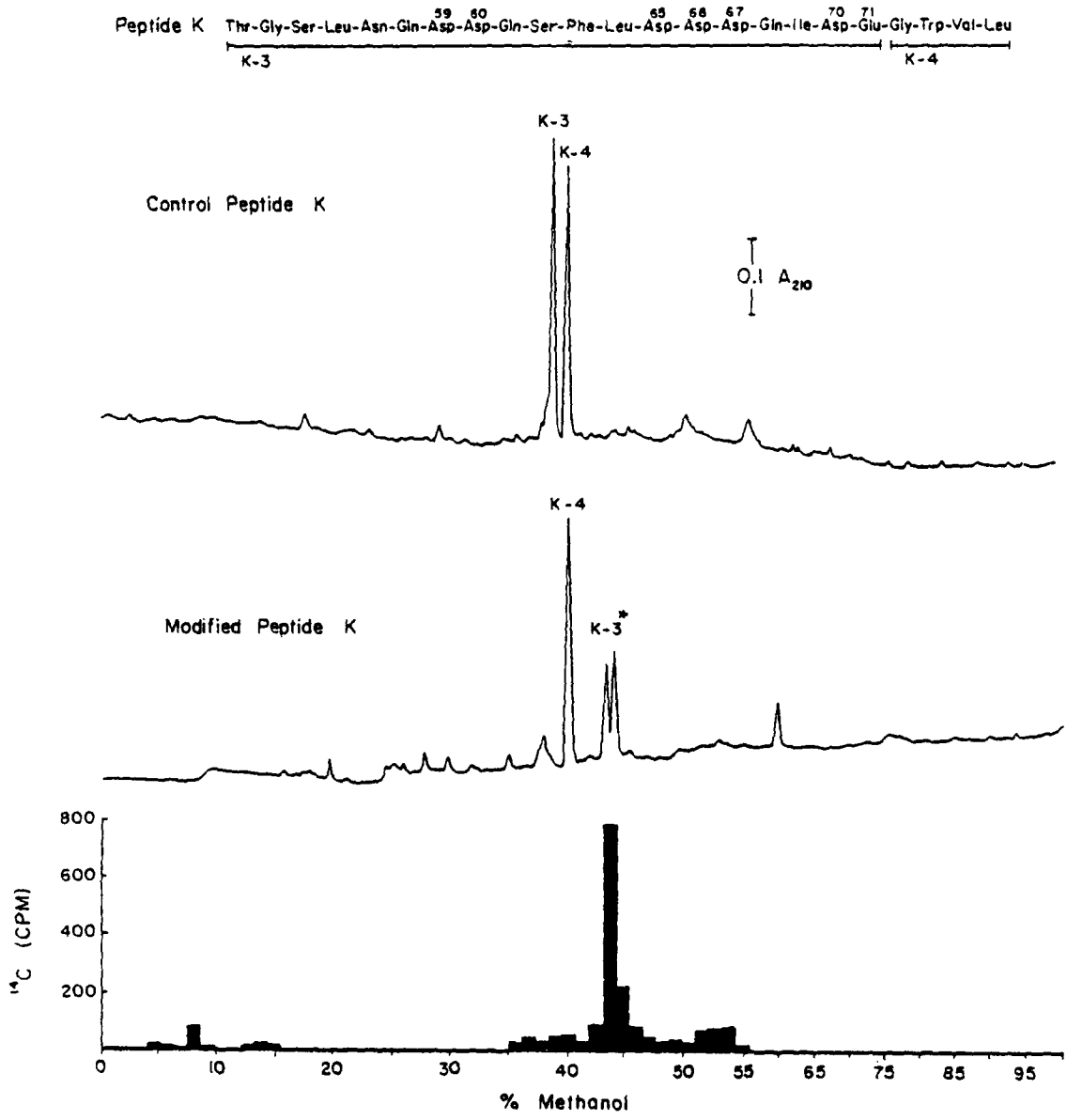


Fig. 7. Analysis of *S. aureus* protease digestion of peaks K and K*. Material from peaks K and K* was treated with *S. aureus* protease using conditions described in Fig. 5. Analysis by HPLC was done using the gradient described in Fig. 1. The bar graph shows the position of fractions containing radioactivity when peak K* from Fd modified with ^{14}C-glycine ethylester was used.

91–97 and E-2 is residues 76–90. It is noteworthy that this cleavage is after histidine 90 rather than lysine 91. Cleavage at this point is also rather inefficient as most of the peptide is not cleaved by the treatment.

Treatment of ^{14}C-labelled E* with this level of trypsin also resulted in a limited cleavage at the same position. Because of the limited nature of the cleavage, most of the label remained associated with the parent peak. A peak corresponding to E-2 is present in the chromatogram and contains no radiolabel. This observation excludes Asp 84 and Glu 88 as sites for modification. No peak characteristic of a modified form of E-1 was located in the chromatogram. A few small peaks of radioactivity were detected between the ex-

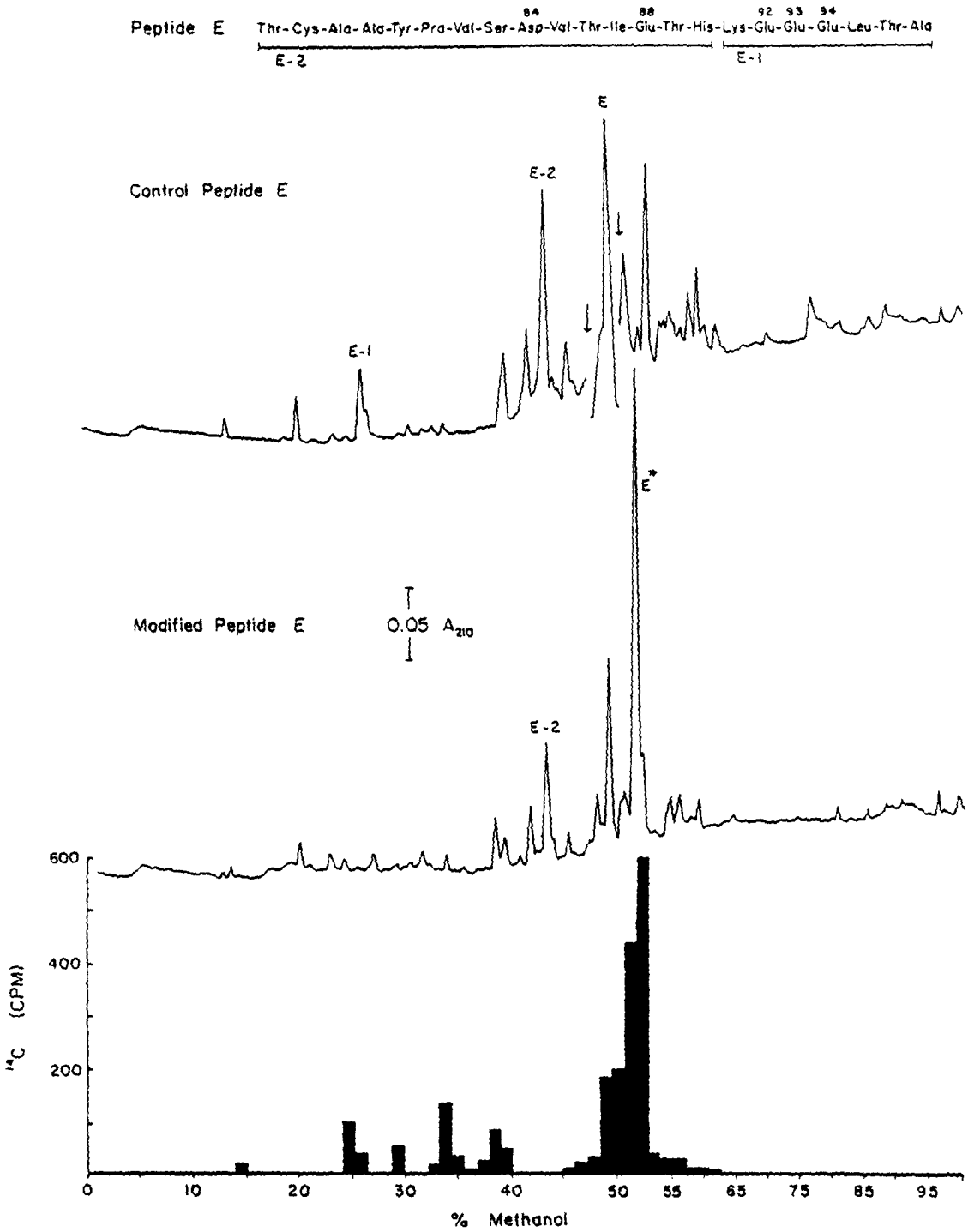


Fig. 8. Analysis of trypsin digestion of peaks E and E*. The peptides were treated with trypsin at a level of 0.2 mg trypsin/mg peptide in 0.1 M $NaHCO_3$ for 3 h. HPLC peptide mapping was done using the gradient conditions described in Fig. 1. The bar graph shows the fractions which contained radioactivity when peak E* from ferredoxin labelled with ^{14}C-glycine ethylester was used. In the chromatogram of peak E from control ferredoxin the area between the two arrows is run on an absorbance scale 4-fold greater than the rest of the chromatogram. Note that digestion of either E or E* with trypsin is rather inefficient as most of the material remains undigested.

pected location of E-1 and E-2. These peaks may represent modified forms of E-1, but are present in too small an amount to be characterized by amino acid analysis. The absence of E-1 suggests that it has been modified and limits the possible carboxyl groups modified in this region of the molecule to Glu 92, Glu 93, and Glu 94.

Peptide mapping of covalently linked ferredoxin-ferredoxin : NADP reductase adduct

A covalently linked ferredoxin-ferredoxin : NADP reductase adduct was prepared by incubation of the two proteins in the presence of EDC. Characterization of this adduct by spectral analysis and by SDS gel electrophoresis (data not shown) revealed it to have a 1 : 1 stoichiometry similar to an adduct reported by Zanetti et al. [10].

Peptide mapping of the ferredoxin-ferredoxin : NADP reductase adduct was performed to determine which portions of ferredoxin were cross-linked to ferredoxin : NADP reductase. The proteolytic digestion and peptide mapping procedures used for analysis of chemically modified ferredoxins (see above) were applied to pure ferredoxin : NADP reductase, to a noncross-linked mixture of ferredoxin and ferredoxin : NADP reductase, and to the ferredoxin-ferredoxin:NADP reductase adduct. The peptide map of Fp alone (Fig. 9, bottom) shows the presence of about 30 major peaks and several minor peaks. When an uncross-linked mixture of ferredoxin and ferredoxin : NADP reductase was subjected to identical treatment, several additional peaks due to ferredoxin fragments could also be detected (Fig. 9, middle). These peaks are labelled with letters corresponding to those used in analysis of chemically modified ferredoxins (Fig. 1 and 2). Of particular interest are peaks E, H, and K which the modification studies suggested to contain carboxyl groups important in the interaction of ferredoxin with ferredoxin : NADP reductase. It is fortunate that all three of these peaks are distinguishable from peaks due to the digestion of Fp in this chromatogram.

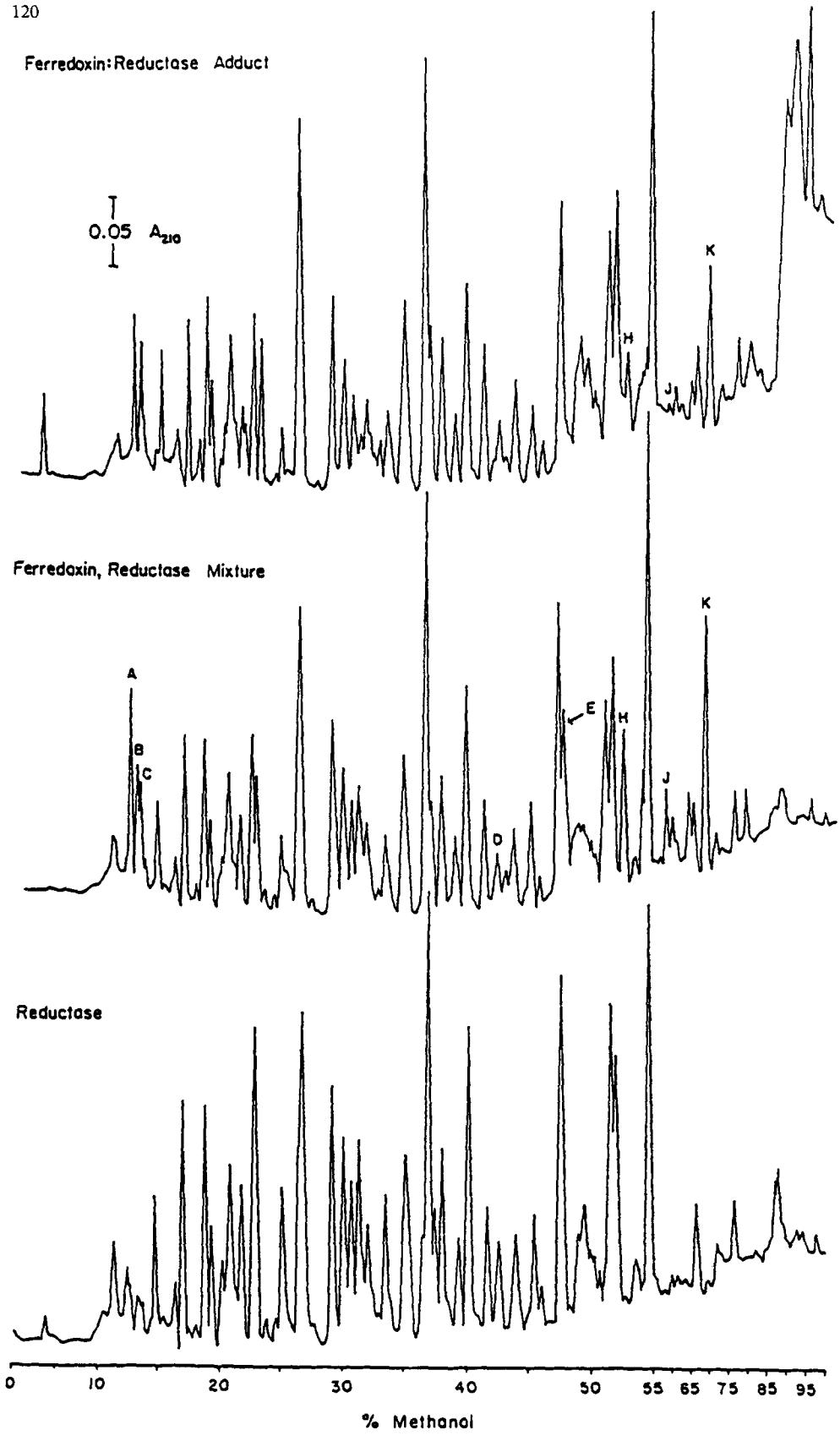
When the ferredoxin-ferredoxin : NADP reductase adduct was subjected to the same proteolytic digestion and peptide mapping procedures, changes in these three peaks occured (Fig. 9, top). Peak E disappeared entirely and the size of peak H decreased dramatically. Peak K also decreased in size, but not to the same extent as either peak E or H. As a peak from ferredoxin would only be expected to disappear or decrease in size if it were cross-linked to some undefined fragment from ferredoxin : NADP reductase, these observations support the involvement of carboxyl-groups in these three regions of the ferredoxin molecule in its interaction with ferredoxin : NADP reductase.

Discussion

Peptide mapping studies have been performed on ferredoxin modified to the extent of one carboxyl group per ferredoxin. Although we have been unable to determine single sites of modification, the data presented indicate that modification occurs primarily in three regions of the molecule: residues 26–30, 65–70 and 92–94. The apparently singly modified Fd is thus likely a mixture of monosubstituted derivatives. Attempts to further resolve this mixture by ion-exchange chromatography have not yet been successful. However, as Fd modified to this extent is only 50% active in NADP photoreduction [7], some or all of these regions may be involved in its interaction with ferredoxin : NADP reductase.

Fig. 10 shows the location of all carboxyl-containing amino acids in the 3-dimensional structure of spinach ferredoxin. (The structure shown is derived from the 3-dimensional structure of *Spirulina platensis* ferredoxin as described in Ref. 15. As the amino acid sequences for spinach and *Spirulina* ferredoxin show about 65% homology, it seems unlikely that the structures for the two proteins would be greatly different.) The positions of the possible groups modified in the present study may define the portion of the molecule interacting with ferredoxin : NADP reductase. If all three regions are involved (an assumption which may or may not be valid), it would appear that ferredoxin : NADP reductase might wrap itself around the upper left and rear portions of ferredoxin with some portion of ferredoxin : NADP reductase extending down over the right front portion of the Fd molecules.

The proposed type of interaction would bring the iron-sulfur center (located in the upper portion of the ferredoxin structure) in close proximity to


Ferredoxin:Reductase Adduct
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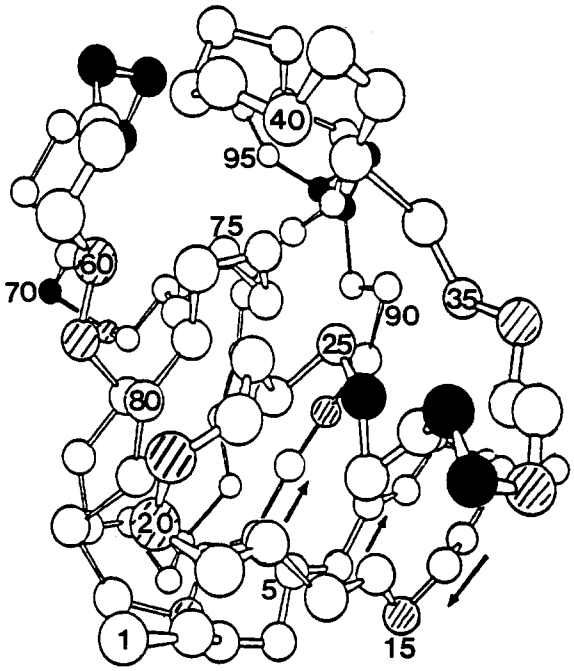

Fig. 10. Location of possible sites of modified carboxyl groups on ferredoxin. The drawing of the backbone of ferredoxin has been altered from that presented in Ref. 15 and renumbered to bring it into agreement with the numbering of the amino acid sequence of spinach ferredoxin. Only the alpha carbons are shown. The carboxyl groups which the present work indicates are candidates for modification by glycine ethylester in the presence of EDC are shown in black. All other carboxyl groups are cross-hatched. The iron-sulfur center (not shown) is attached to the protein by cysteine residues at positions 39, 44, 47, and 77.

the ferredoxin : NADP reductase such that electron transfer could occur. This view is supported by the observation that modification of histidine 90 on the rear of the ferredoxin molecule also alters its ability to interact with Fp while the modification of the single arginine at position 40 on the front side of Fd does not [7].

^{13}C-NMR studies on the interaction between ^{13}C-enriched Fd from *Anabaena variabilis* and spinach Fp [11] have indicated that the resonances of three glutamates are altered when ferredoxin binds to ferredoxin : NADP reductase. One of these three glutamates has tentatively been assigned as Glu-24 in the sequence of *Anabaena variabilis* ferredoxin. The comparable position is spinach ferredoxin I is occupied by a valine although spinach ferredoxin II does contain a Glu in this position. In all three cases, this position is preceeded by two Asp residues. The fact that we observe no modification in this region of the molecule cannot be used to exclude these (or other) carboxyl groups from being involved in the interaction with ferredoxin : NADP reductase as chemical modification experiments can only provide information on groups which have been modified.

It is also interesting to note that the regions modified in the present work are at or near clusters of carboxyl groups in the ferredoxin sequence. This is not surprising as under the mild conditions used, only the most reactive carboxyl groups would be expected to react. Work in other laboratories has shown that under similar conditions the reaction of EDC with clusters of carboxyl groups is favored [12–14]. This may explain why we were able to achieve rather specific modification of certain regions of the ferredoxin molecule rather than having carboxyl groups modified at random throughout the sequence. One potential problem with the use of EDC in protein modification is the possibility that the EDC-carboxyl group adduct initially formed may also rearrange to form stable *N*-acylurea derivatives. Under the conditions used in our work (low concentrations of EDC and high concentrations of glycine ethylester), this competing reaction should be minimal. Under conditions where this rearrangement might be favored (no glycine ethylesters present), we have observed that treatment of ferredoxin with EDC results in substantial loss of the iron-sulfur center from the protein.

Peptide mapping of a cross-linked ferredoxin-ferredoxin : NADP reductase adduct also supports the involvement of carboxyl groups in these three

Fig. 9. Peptide mapping of ferredoxin-ferredoxin : NADP reductase adduct proteolytic digestion and HPLC peptide mapping were done as described in Materials and Methods and the legend to Fig. 1. The bottom chromatogram is the peptide map of ferredoxin NADP reductase alone. The middle chromatogram is the peptide map of a 1.5 : 1 mixture of Fd an Fp and the top chromatogram is a peptide map of the covalently cross-linked Fd-Fp adduct.

regions of Fd in its interaction with ferredoxin. As the EDC is expected to link covalently the two proteins by forming amide linkages between negatively charged carboxyl groups and amino groups in close proximity, this means of cross-linking takes advantage of the electrostatic interaction between the two proteins. As peak E disappeared entirely in the peptide map of the ferredoxin-ferredoxin : NADP reductase adduct, the data would suggest that a linkage between carboxyl groups in this region of ferredoxin and amino groups on ferredoxin : NADP reductase is present in all of the ferredoxin-ferredoxin : NADP reductase adduct. The observation of decreases in peaks H and K would suggest that in some molecules of the adduct, carboxyl groups in these two regions are also cross-linked to amino groups on ferredoxin : NADP reductase with a greater degree of cross-linking involving carboxyl groups in peak H. It is not clear at the present time whether the differences in cross-linking involving these three regions of ferredoxin is a reflection of differing reactivities of the carboxyl groups or accessibility of EDC to the carboxyl groups when the two proteins are interacting. As the complete amino acid sequence of spinach Fp is now known [16], we hope to use similar approaches to determine which portions of Fp are involved in its interaction with ferredoxin.

Recent studies with the modified Fd described in this work have also indicated that its interaction with other ferredoxin-dependent enzymes such as nitrite reductase and glutamate synthase is also impaired [17]. This observation suggests that the same region of ferredoxin which is involved in its interaction with ferredoxin : NADP reductase may also be in involved in its interaction with other FD-dependent enzymes.

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References

1 Shin, M. and San Pietro, A. (1968) Biochem. Biophys. Res. Commun. 33, 33–42
2 Foust, G., Mayhew, S. and Massey, V. (1969) J. Biol. Chem. 244, 964–970
3 Batie, C. and Kamin, H. (1981) J. Biol. Chem. 256, 7756–7763
4 Tsukihara, T., Kobayashi, M., Nakamura, M., Katsube, Y., Fukayama, K., Hase, T., Wada, K. and Matsubara, H. (1982) Biosystems 15, 243–257
5 Matsubara, H., Sasaki, R.M. and Chain, R.K. (1966) Proc. Natl. Acad. Sci. USA 57, 439–445
6 Takahashi, Y., Hase, T. Wada, K. and Matsubara, H. (1983) Plant Cell Physiol. 24, 189–198
7 Vieira, B.J. and Davis, D.L. (1986) Arch. Biochem. Biophys. 247, 140–146
8 Takahashi, Y., Hase, T. and Matsubara, H. (1981) J. Biochem. 90, 1825–1828
9 Durham, B. and Geren, C.R. (1981) Anal. Biochem. 116, 331–334
10 Zanetti, G., Aliverti, A. and Curti, B. (1984) J. Biol. Chem. 259, 6153–6157
11 Chan, T.-M., Ulrich, E.L. and Markley, J.L. (1983) Biochemistry 22, 6002–6007
12 Dailey, H. and Strittmatter, P. (1979) J. Biol. Chem. 254, 5388–5396
13 Millett, F., De Jong, C., Paulson, L. and Capaldi, R.A. (1983) Biochemistry 22, 546–552
14 Geren, L.M., O'Brien, P., Stonehuerner, J. and Millett, F. (1984) J. Biol. Chem. 259, 2155–2160
15 Fukuyama, K., Hase, T., Matsumoto, S., Tsukihara, T., Katsube, Y., Tanaka, N., Kakudo, M., Wada, K. and Matsubara, H. (1980) Nature 286, 522–524
16 Karplus, P.A., Walsh, K.A. and Herriott, J.R. (1984) Biochemistry 23, 6576–6583
17 Hirasawa, M., Boyer, J.M., Gray, K.A., Davis, D.J. and Knaff, D.B., (1986) Biochim. Biophys. Acta 850, 23–28