Registry No. DM, 2265-22-7; Cys, 52-90-4.

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Structural Studies on the Interaction between Ferredoxin and Ferredoxin–NADP$^+$ Reductase†

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ABSTRACT: Structural studies carried out on a cross-linked complex between spinach ferredoxin–NADP$^+$ reductase (EC 1.18.1.2) and its protein substrate ferredoxin allowed the identification of peptide regions involved in the interaction between the two proteins. Carboxyl groups of ferredoxin were shown to interact with amino groups of the reductase. Two types of cross-links could be located within a few residues. The major one was found between the peptide segment 72–91 of the reductase, where Lys-85 and/or Lys-88 were identified as the amino donors in the carbodiimide-promoted linkage, and the ferredoxin region 76–97, which contains Asp-84, Glu-88, Glu-92, Glu-93, and Glu-94. Carboxypeptidase Y digestions would suggest an involvement of a glutamic residue of the acidic cluster 92–94. The minor cross-link was found between the α-NH_2 of the N-terminal residue of the reductase and the peptide region 5–37 of ferredoxin, which contains Glu-15, Asp-20, Asp-21, Asp-26, Glu-29, Glu-30, Glu-31, and Asp-34. Glu-15, Glu-31, and Asp-34 could be ruled out. Lack of chymotrypsin digestion at Tyr-23 of the cross-linked peptide would indicate Asp-20, -21, or -26 as the most likely COOH donor in the linkage. These results point out the importance of the N-terminal moiety of the reductase, where the flavin binding domain is located, for the interaction with ferredoxin.

In photosynthesis, NADP$^+$ photoreduction requires the interaction between the two terminal components of the chloroplast electron-transport chain, namely, ferredoxin and ferredoxin–NADP$^+$ reductase. The cross-linked complex between the two purified proteins, obtained through the aid of a carbodiimide (Zanetti et al., 1984), is considered to be a valid model of the in vivo noncovalent electron-transfer complex on the following bases. (a) The cross-linked complex has been

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shown to have many properties of the native one: the 1:1 stoichiometry, the spectral perturbations, the NADPH–cytochrome *c* reductase activity, the isoelectric point, and the CD and fluorescence spectra (Zanetti et al., 1984; Zanetti & Curti, 1984). (b) The covalent complex had no reductase activity toward exogenous ferredoxin (Zanetti et al., 1984), suggesting that the cross-linked ferredoxin completely masks the ferredoxin binding site on the flavoprotein. Furthermore, this site remained inaccessible even at high ionic strength, where electrostatic interactions between the two proteins are severed, as judged by ionic strength independence of protein fluorescence quenching in the covalent complex (Zanetti & Curti, 1984). Although several studies on protein–protein interactions have been performed by cross-linking using carbodiimides, to our knowledge only in one case (Waldmeyer & Bossard, 1985) have structural data been published.

In this paper we report structural studies carried out on the cross-linked complex between ferredoxin and the reductase, with the aim of identifying the peptide regions of the two proteins that are interfaced in the complex. Cross-linking occurred preferentially between the N-terminal moiety of the reductase and the C-terminal part of ferredoxin.

Materials and Methods

N-Tosylphenylalanine chloromethyl ketone treated trypsin and chymotrypsin were from Worthington; iodo[2-^{14}C]acetic acid obtained from Amersham was diluted with recrystallized iodoacetic acid to a specific radioactivity of 1500–5000 dpm·nmol^{-1}. Acetonitrile was Lichrosolv grade from Merck; all other reagents were of analytical grade. Ferredoxin–NADP$^+$ reductase and ferredoxin were purified from spinach as already described (Zanetti & Curti, 1980). The cross-linked complex between the two proteins was obtained as previously reported (Zanetti et al., 1984).

Protein Fragmentation. Apoproteins obtained by 10% trichloroacetic acid treatment (repeated twice) were dissolved in 6 M guanidine hydrochloride containing 50 mM phosphate, pH 8, and reduced by addition of a 5-fold molar excess of dithiothreitol per mole of SH, under nitrogen at 37 °C for 3 h. Then, alkylation of thiol groups was performed by incubation for 60 min with iodo[^{14}C]acetate (2 mol/mol of SH); the reaction was blocked with excess 2-mercaptoethanol. After extensive dialysis against 50 mM NH_4HCO_3, the samples were lyophilized. Tryptic digestions of carboxymethylated apoproteins were carried out in 50 mM phosphate, pH 8, containing 2 M urea, by addition of trypsin (4% w/w) for 7 h at 37 °C. Chymotryptic digestions were performed for 3 h at 37 °C in 50 mM NH_4HCO_3, pH 8, containing 0.1 mM $CaCl_2$, by addition of 3% (w/w) chymotrypsin or for 15 h at 37 °C in sodium phosphate, pH 8, containing 0.1 mM $CaCl_2$ and 2 M urea, by addition of 3% (w/w) chymotrypsin, followed by a second addition of 1% (w/w) protease and a further incubation for 4 h.

Protein Mapping. Peptides were separated by reverse phase HPLC.[1] An aliquot containing 1–30 nmol of digested protein was resolved with a Jasco HPLC equipped with an Aquapore RP-300 (Brownlee) column (0.7 × 25 cm). Peptides were eluted at room temperature with a linear gradient from 0.1% CF_3COOH (solvent A) to a 50% mixture with 0.075% CF_3-COOH in acetonitrile (solvent B) over 70 min at a flow rate of 2 mL/min. Further purification of fractions was achieved by rechromatography at pH 6 on an analytical Aquapore RP-300 (0.46 × 25 cm) with Waters HPLC equipment at room temperature. Elution was obtained by a linear gradient from 5 mM sodium phosphate, pH 6 (solvent A), to a 50% mixture with solvent B (acetonitrile/5 mM sodium phosphate, pH 6, 90:10 v/v) over 70 min at a flow rate of 0.7 mL/min. Absorbance at 220 nm was recorded, and radioactivity was measured by liquid scintillation counting (Packard Tri-carb 4000) on aliquots of the manually collected peaks.

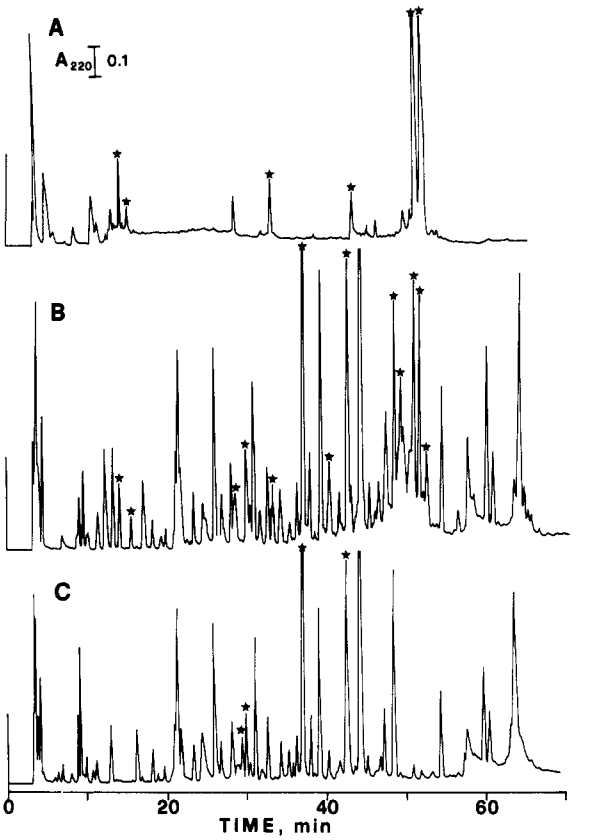

FIGURE 1: HPLC separation of tryptic peptides of ferredoxin, cross-linked complex, and reductase. An asterisk marks radioactive peptides containing (carboxymethyl)cysteine. The following amounts were loaded onto the HPLC column: (A) ferredoxin, 25 nmol; (B) cross-linked complex, 33.5 nmol; (C) FNR, 24 nmol.

Amino Acid and Sequence Analysis. Amino acid analyses were performed either on a Carlo Erba amino acid analyzer equipped for high sensitivity or on a Beckman 121 MB analyzer. Sequences were determined by the Beckman sequenator 890 M (95% repetitive yield). Release of COOH-terminal amino acid residues was performed by digestion with carboxypeptidase Y of equimolar quantities of ferredoxin and reductase or of the cross-linked complex. After treatment with 10% trifluoroacetic acid to obtain the apoproteins, protein solubilization was achieved in 50 mM *N*-ethylmorpholine acetate, pH 7. After norleucine was added as an internal standard, digestion with carboxypeptidase Y (1:10 mol/mol) was performed at 25 °C. At given times, aliquots were withdrawn, acidified to pH 1 with trifluoroacetic acid, and ultrafiltrated with an Amicon Centricon 10 to remove proteins.

Results

Peptide Maps of Fd, FNR, and Covalent Complex. In order to identify the contact sequences between ferredoxin and the reductase, structural studies have been carried out on the cross-linked complex obtained by using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) (Zanetti et al., 1984). The free and covalently bound proteins were digested

[1] Abbreviations: HPLC, high-pressure liquid chromatography; FNR, ferredoxin–NADP$^+$ reductase; Fd, ferredoxin.

Table I: Amino Acid Composition and Sequence Analysis of the Tryptic Peptide from the Cross-Linked Complex Found in Peak 49.4[a]

amino acid	residues/mol	amino acid	residues/mol	amino acid	residues/mol	amino acid	residues/mol
Lys	4.0 (4)	Ser	3.7 (4)	Cys[b]	1.1 (1)	Leu	3.7 (4)
His	1.9 (2)	Glx	11.3 (11)	Val	4.3 (5)	Tyr	0.5 (1)
Arg	0.0 (0)	Pro	3.2 (3)	Met	0.0 (0)	Phe	1.1 (1)
Asx	10.4 (11)	Gly	5.5 (6)	Ile	2.5 (3)	Trp[c]	+ (1)
Thr	4.8 (5)	Ala	3.0 (3)				

cycle		1	2	3	4	5	6	7	8	9	10	11	12	13
residues	FNR 72–91	Glu	Gly	Gln	Ser	Val	Gly	Val	Ile	Pro	Asp	Gly	Glu	Asp
	Fd I 53–97	Thr	Gly	Ser	Leu	Asn	Gln	Asp	Asp	Gln	Ser	Phe	Leu	Asp

[a] The amino acid analysis was not corrected for losses during hydrolysis. Numbers in parentheses are theoretical values deduced from the published sequences of the reductase (Karplus et al., 1984) and of ferredoxin (Matsubara & Sasaki, 1968). [b] Estimated by radioactivity of (carboxymethyl)-cysteine. [c] Determined qualitatively by the Ehrlich reagent.

with trypsin after carboxymethylation with iodo[^{14}C]acetate; the resulting peptides were separated by reverse phase HPLC. Parts A, B, and C of Figure 1 show the tryptic peptide mapping of ferredoxin, the cross-linked complex, and the reductase, respectively. Very good reproducibility of the covalent complex elution profile was observed in several runs. Peptide peaks of the three maps (numbered according to their retention time in minutes) were characterized for radioactivity content and amino acid composition and identified by comparison with the published sequences of the two proteins (Matsubara & Sasaki, 1968; Karplus et al., 1984). The map of ferredoxin was found more complex than expected due to a substantial amount (about 25%) of an isoform, ferredoxin II, which has a fairly different amino acid composition (25 amino acid substitutions over 97 residues; Takahashi et al., 1983). All the peptides arising from the two Fd isoforms were located in the map.

All the tryptic peptides derived from FNR have been found in both the covalent complex and free reductase maps. Comparison, on a molar basis, of the HPLC elution profiles (Figure 1) allowed us to remark the following: (a) The area of peak 9.6 in the covalent complex chromatogram (Figure 1B) was considerably decreased in comparison with the corresponding peak of the reductase (Figure 1C), which was found to contain mainly residues 86–91. (b) The region between 49 and 52 min in the elution profile of the cross-linked complex (Figure 1B) differed considerably from the ferredoxin peptide map (the flavoprotein showed no peaks in this range). The peaks found in this region, besides that containing the fragment 53–97 of Fd I, may be interpreted as arising from cross-linked peptides of the two proteins.

Locating Sites of Cross-Linking. The peaks in the region 47–53 min were thus further analyzed and, where necessary, rechromatographed on reverse-phase HPLC at pH 6. Figure 2 shows a representative chromatogram; a net separation of the two major peptides of ferredoxin I (residues 5–40 and 53–97) was achieved as well as purification of cross-linked peptides from contaminating FNR peptides. Peaks 47.3 and 48.7 (Figure 1B) contained mainly the corresponding peptides of FNR (Figure 1C), namely, residues 160–178 and 255–263, respectively.

Peak 49.4 of the covalent complex map (Figure 1B) contained both the fragments 72–91 of FNR and 53–97 of Fd I, as judged by the amino acid composition and N-termini (Table I). Furthermore, sequential analysis of this cross-linked peptide resulted in a release of two amino acids/cycle for at least 13 cycles, in full agreement with the published sequences of the corresponding peptides of the two proteins. Thus, in peak 49.4 two adjacent tryptic peptides of FNR, 72–85 and 86–91, were found, which in the free protein are eluted much earlier in the chromatogram (28.1 and 9.6 min, respectively), and a peptide of Fd (53–97) eluted at a higher retention time. This was in keeping with the large decrease

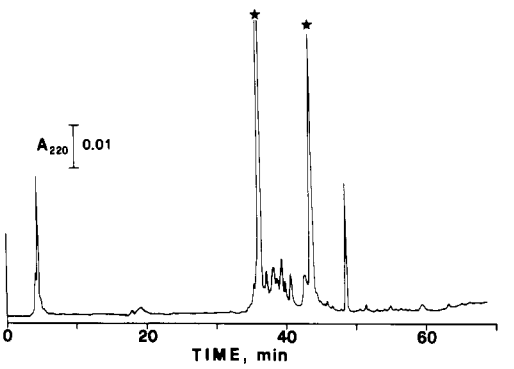

FIGURE 2: Reverse-phase chromatography at pH 6 of tryptic peptides of the cross-linked complex contained in peak 51.0. An asterisk marks radioactive peptides containing (carboxymethyl)cysteine. About 1.5 nmol of peptides (peak 51.0 of Figure 1B) was loaded onto the HPLC column.

of peak 9.6 and also of peak 28.1 in the covalent complex map (Figure 1B). It should be mentioned that similar amounts (about 25%) of the undigested FNR peptide 72–91 were recovered in the maps of both the free reductase and the covalent complex (Figure 1B,C; 27 min). Small amounts of the cross-linked peptide found in peak 49.4 were also recovered in both peaks 48.7 and 49.8.

Peak 51.0 (Figure 1B), as judged by sequence analysis, contained a mixture of peptides Fd I 53–97 and Fd I 5–40. It was thus rechromatographed at pH 6; as shown in Figure 2, two major peaks were obtained. The peptide eluted at 36 min was identified with the unmodified peptide 53–97 of Fd I. The peptide eluted at 44 min yielded in the sequenator only one amino acid per cycle, confirming the presence of peptide 5–40 of Fd I. However, the amino acid analysis was not in agreement with the reported primary structure (Table II), suggesting the presence of a second peptide with a high content of proline and alanine. The only peptide rich in such amino acids is the N-terminal peptide of FNR, which eluted at a much earlier time (Figure 1C; 24.7 min). The amino acid analysis agreed very well with the theoretical sum of the two sequences. Thus, in peak 51.0 a cross-linked peptide between Fd 5–40 and the N-terminal peptide of FNR was present.

The minor peaks of the region 47–53 min (Figure 1B) were also analyzed. Peak 50.5, from sequence analysis, was found to contain at least two cross-linked peptides. Rechromatography of the material at pH 6 confirmed the presence of the two cross-linked fragments already described, namely, Fd I 53–97 cross-linked to FNR 72–91 and the Fd I 5–40 peptide cross-linked to the FNR N-terminal peptide. The unmodified peptide 5–40 of Fd I was actually recovered in peak 52.2. Peak 52.8, after rechromatography at pH 6, was found to contain both Fd I 5–40 and apparently the cross-linked peptide between Fd II 51–97 and FNR 72–91. The peptides identified

Table II: Amino Acid Composition and Sequence Analysis of the Tryptic Peptide from the Cross-Linked Complex Contained in Peak 51.0, after Rechromatography at pH 6

amino acid	expl values[a] (residues/mol)	theor values from sequence: Fd I 5–40	theor values from sequence: FNR 1–15	amino acid	expl values[a] (residues/mol)	theor values from sequence: Fd I 5–40	theor values from sequence: FNR 1–15
Lys	1.1	0	1	Ala	5.1	2	4
His	0.0	0	0	Cys[b]	1.9	2	0
Arg	1.0	1	0	Val	4.8	4	1
Asx	5.7	5	1	Met	0.0	0	0
Thr	2.9	3	0	Ile	2.7	2	1
Ser	2.3	1	1	Leu	3.2	3	0
Glx	6.5	5	2	Tyr	1.8	2	0
Pro	6.2	3	4	Phe	1.1	1	0
Gly	2.9	2	0				

cycle		1	2	3	4	5	6	7	8	9	10	11	12	13	14
residues	Fd I 5–40	Val	Thr	Leu	Val	Thr	Pro	Thr	Gly	Asn	Val	Glu	Phe	Gln	Cys

[a] The amino acid analysis was not corrected for losses during hydrolysis. [b] Estimated by radioactivity of (carboxymethyl)cysteine.

Table III: Peptides Identified in the 47–53-min Region of the Tryptic Map of the Cross-Linked Complex[a]

retention time (min)	peptides
47.3	FNR 160–178
48.7	FNR 255–263; (FNR 72–91/Fd I 53–97)
49.4	FNR 72–91/Fd I 53–97
49.8	FNR 72–91/Fd I 53–97
50.5	(FNR 72–91/Fd I 53–97); (FNR 2–15/Fd I 5–40)
51.0	Fd I 53–97; FNR 2–15/Fd I 5–40
52.2	Fd I 5–40
52.8	Fd I 5–40; (FNR 72–91/Fd II 51–97)

[a] Sequence numbers refer to the published primary structure of FNR (Karplus et al., 1984) and of ferredoxin isoforms (Takahashi et al., 1983). The peptides in parentheses were found in low amounts.

Table IV: Amino Acid Composition of a Fragment Released by Chymotryptic Digestion of the Tryptic Peptide from the Cross-Linked Complex Contained in Peak 49.4[a]

amino acid	residues/mol	amino acid	residues/mol
Lys	3.6 (4)	Ala	3.1 (3)
His	1.9 (2)	Cys[b]	1.2 (1)
Arg	0.0 (0)	Val	3.9 (4)
Asx	3.9 (4)	Met	0.0 (0)
Thr	4.0 (4)	Ile	1.8 (2)
Ser	2.1 (2)	Leu	1.4 (1)
Glx	7.3 (7)	Tyr	0.4 (1)
Pro	3.1 (3)	Phe	0.0 (0)
Gly	4.0 (4)		

residues Fd I 76–97/FNR 72–91

[a] The amino acid analysis was not corrected for losses during hydrolysis. Numbers in parentheses are theoretical values deduced from the published sequences of the reductase (Karplus et al., 1984) and of ferredoxin (Matsubara & Sasaki, 1968). [b] Estimated by radioactivity of (carboxymethyl)cysteine.

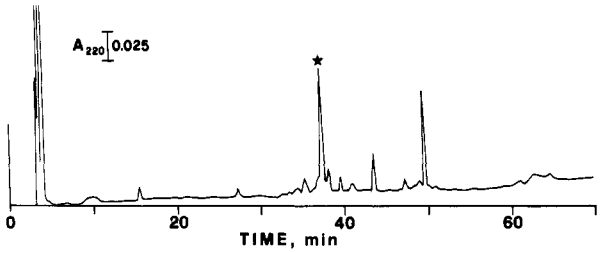

FIGURE 3: HPLC separation of chymotryptic digest of the tryptic cross-linked peptide contained in peak 49.4. An asterisk marks radioactive peptides containing (carboxymethyl)cysteine. About 4 nmol of peptide (peak 49.4 of Figure 1B) was digested with chymotrypsin for 3 h as described under Materials and Methods.

in the 47–53-min region of the cross-linked complex map (Figure 1B) are summarized in Table III.

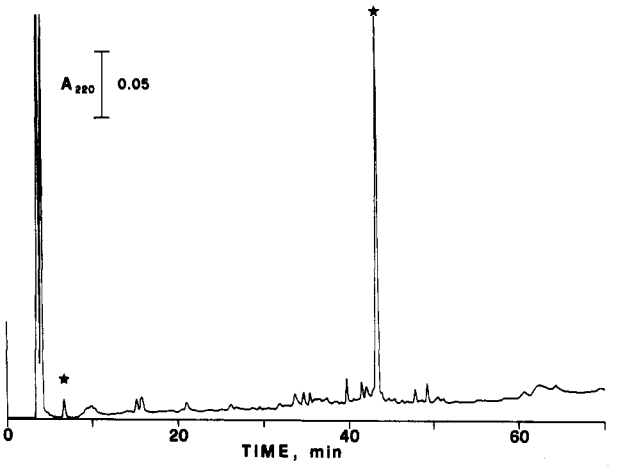

FIGURE 4: HPLC separation of chymotryptic digest of the tryptic cross-linked peptide contained in peak 51.0. An asterisk marks radioactive peptides containing (carboxymethyl)cysteine. About 6 nmol of peptide (peak 44.0 of Figure 2) was digested with chymotrypsin for 19 h as described under Materials and Methods.

Table V: Amino Acid Composition of Fragments Released by Chymotryptic Digestion of the Tryptic Peptide from the Cross-Linked Complex Contained in Peak 51.0[a]

amino acid	residues/mol: peak 6.7	residues/mol: peak 44.0	amino acid	residues/mol: peak 6.7	residues/mol: peak 44.0
Lys	0.0 (0)	1.1 (1)	Ala	0.0 (0)	5.4 (6)
His	0.0 (0)	0.0 (0)	Cys[b]	1.1 (1)	1.2 (1)
Arg	1.0 (1)	0.0 (0)	Val	0.0 (0)	5.0 (5)
Asx	0.0 (0)	5.9 (6)	Met	0.0 (0)	0.0 (0)
Thr	0.0 (0)	2.9 (3)	Ile	0.0 (0)	2.7 (3)
Ser	1.1 (1)	1.0 (1)	Leu	0.0 (0)	3.0 (3)
Glx	0.0 (0)	6.1 (6)	Tyr	0.0 (0)	1.8 (2)
Pro	0.0 (0)	6.7 (7)	Phe	0.0 (0)	1.0 (1)
Gly	0.0 (0)	2.3 (2)			

		peak 6.7	peak 44.0
residues	Fd I	38–40	5–37
	FNR		2–15

[a] The amino acid analysis was not corrected for losses during hydrolysis. Numbers in parentheses are theoretical values deduced from the published sequences of the reductase and ferredoxin. [b] Estimated by radioactivity of (carboxymethyl)cysteine.

A further characterization was pursued on the cross-linked fragments found in peaks 49.4 and 51.0. A secondary digestion with chymotrypsin, which can only cleave the Fd component of the cross-linked peptide (see FNR fragment sequences in Charts I and II), was performed. The resulting peptides were separated by reverse phase HPLC (Figures 3 and 4, respectively). The fragment eluted at 38 min (Figure 3) contained

Chart I

```
                                         *        *
       72                                85       88      91
FNR    E-G-Q-S-V-G-V-I-P-D-G-E-D-K-N-G-K-P-H-K

                            *            *           *  *  *
       76                   84           88             93        97
Fd I   T-C-A-A-Y-P-V-S-D-V-T-I-E-T-H-K-E-E-E-L-T-A
```

Chart II

```
     *
     2                         15
FNR  I-A-S-D-V-E-A-P-P-P-A-P-A-K

                         *           *  *           *           *  *  *     *
     5                   15          20             26             30       34    37
Fd   V-T-L-V-T-P-T-G-N-V-E-F-Q-C-P-D-D-V-Y-I-L-D-A-A-E-E-E-G-I-D-L-P-Y
```

Fd I residues 76–97 together with FNR residues 72–91, as judged by amino acid analysis (Table IV). From chymotrypsin digestion of the other cross-linked peptide (Figure 4) only two peptides arising from cleavage at Tyr-37 of Fd (Table V) were obtained. The unmodified peptide 5–40 of Fd I was instead completely digested at both Tyr-23 and -37. This suggests that steric hindrance by the cross-linked FNR peptide prevented the action of the protease at Tyr-23. As deduced from amino acid determinations, Ile-2 seems to be the N-terminal residue in the cross-linked FNR peptide (see Discussion).

In conclusion, two different sites of cross-linking between Fd and the reductase were found, namely, FNR 72–91/Fd 53–97 and FNR 2–15/Fd 5–40. The recovery of the unmodified and cross-linked peptides, estimated by amino acid analysis as well as by radioactivity of [^{14}C](carboxymethyl)-cysteine content, indicated that 60–70% of the linkage was between the C-terminal moiety of Fd and residues 72–91 of FNR.

Identification of the Residues Involved in the Cross-Linking. The sequences of the major cross-linked peptide as deduced from the published primary structures of FNR (Karplus et al., 1984) and of Fd I (Matsubara & Sasaki, 1968) are presented in Chart I.

An involvement in the cross-linking of the FNR acidic residues (Glu-72, Asp-81, Glu-83, and Asp-84) could be ruled out because these residues were sequenced. Thus, FNR participated in the cross-link as the amino donor: of the three lysines present, Lys-85 seems the most likely candidate for the role, because its bond was not cleaved by trypsin as was the case for Lys-91. No data on the involvement of Lys-88 in the linkage could be obtained: the bond Lys-88–Pro-89 is trypsin resistant and the region Lys-85–Asn-86–Gly-87 is difficult to sequence, probably due to isomerization of the bond Asn-86–Gly-87 (Karplus et al., 1984; Geiger & Clarke, 1987).

In the C-terminal region of Fd there are several side-chain carboxyl groups as well as the α-COOH of C-terminal alanine. Carboxypeptidase Y released from the cross-linked complex nearly a stoichiometric amount of alanine, but at a lower rate as compared with an equimolar mixture of the free proteins. Thus, the α-COOH of Fd was not directly involved, but again there was an indication that the cross-linked peptide of FNR interfered with the peptidase action, suggesting that the site of binding could be located near the C-terminal amino acid where three glutamic residues (Glu-92–Glu-93–Glu-94) are present.

The minor site of cross-linking was formed between the peptides of FNR and Fd I as shown in Chart II.

Fd can only act as a COOH donor in the cross-link because it does not contain any lysine. Glu-15 was sequenced and was thus ruled out. Glu-31 and Asp-34 could also be excluded on the basis of the complete recovery of the unmodified peptide 32–40 of Fd II (this isoform has a Lys at position 31) and of the cross-linked peptide 5–31 (peak 40.5; Figure 1B), assuming that the interaction of the two Fd with FNR is the same (see Discussion). The lack of digestion by chymotrypsin at Tyr-23 would indicate Asp-20, -21, or -26 as the most likely COOH donor in the linkage. In the FNR peptide the only amino group available for the linkage is the α-NH_2 of Ile. The ϵ-NH_2 of Lys-15 was excluded because trypsin cleaved the peptide bond after Lys-15. This is in keeping with the fact that the FNR peptide was resistant to Edman degradation (Table II).

Discussion

The reaction of EDC with ferredoxin and the reductase appears to be highly specific, yielding a covalent complex between the two proteins stabilized by only two types of cross-links. No internal cross-links in Fd and FNR were found, as judged by comparison of the tryptic map of the covalent complex with those of the untreated proteins. Furthermore, EDC did not stabilize dimers (or oligomers) of the single proteins or of the complex. Extensive irreversible modification of other carboxyl groups (i.e., to *N*-acylurea derivatives; Hoare & Koshand, 1967) seems rather unlikely because the covalent complex maintained the p*I* of the native complex.

The free reductase in the presence of NADP$^+$ was completely active following EDC treatment, whereas it lost about 30% of the cytochrome *c* reductase activity (at saturation conditions) when treated in the presence of ferredoxin to form the covalent complex (Zanetti et al., 1984). It is tempting to relate this loss of activity to the amount of complex containing the minor cross-link, which has less chance of mimicking an ionic bond between the two proteins in vivo. The major reason resides in the fact that the spinach reductase has been reported to have a blocked N-terminal amino acid, i.e., a pyroglutamyl residue (Karplus et al., 1984). However, the presence of isoforms is a well-known phenomenon in the spinach enzyme (Gozzer et al., 1977; Karplus et al., 1984). Our FNR preparation by sequence analysis showed isoleucine, which is the second amino acid in the published sequence (Karplus et al., 1984), as the major N-terminal residue with a maximal amount of 30% of a blocked N-terminal residue calculated from the sequenator yield. A further reason is that the *Spirulina platensis* reductase lacks the 1–22 sequence (Yao et al., 1984). In this context, our determinations confirmed the published sequence of spinach FNR (Karplus et al., 1984). However, we found evidence of heterogeneity in few peptides that had amino acid substitutions in respect to the major form (Zanetti et al., unpublished results).

In regard to the major cross-link found in the covalent complex, it has to be noted that the FNR sequence 72–91 contains a cluster of positive charges (Lys-85, Lys-88, His-90, Lys-91) and is followed moreover by Arg-93. Most recently, such a cluster has been suggested to be involved in Fd binding on the basis of competitive inhibition experiments using the NMR probe $Cr(CN)_6^{3-}$ (Armstrong & Corbett, 1986). It is tempting to speculate that the higher loss of the diaphorase activity of FNR in the covalent complex using $Fe(CN)_6^{3-}$ as acceptor (70% at saturation conditions) with respect to that with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) (36% at saturation conditions) (Zanetti et al., 1984) is related to the relative amount of the two different cross-links formed. In this hypothesis, we assume that $Fe(CN)_6^{3-}$, being similar to $Cr(CN)_6^{3-}$, would interact with the same charge cluster that is covalently blocked by ferredoxin.

The sequence 72–91 is highly conserved in two reductases up to now sequenced; yet Lys-85 is replaced by an Asn in *S. platensis* FNR, whereas Lys-88 is conserved. Of the two possible amino donors in the cross-linkage, namely, Lys-85 and Lys-88, the former was thought of as the most likely candidate, because its peptide bond was not cleaved by trypsin. However,

FIGURE 5: Computer graphics model of spinach ferredoxin I. The van der Waals surface representation of the iron–sulfur center and the lateral chains of Glu-92, Glu-93, and Glu-94 were added to the α-carbon backbone of the protein.

because of the presence of an asparagine (Asn-86) adjacent to a glycine (Gly-87), deamidation and/or isomerization to β-aspartil as well as racemization to D-Asp is (are) taking place (Geiger & Clarke, 1987). All these reactions would likely interfere with the action of trypsin, thus preventing the cleavage at Lys-85. In support of this hypothesis, the recovery of a considerable amount (about 25%) of the uncleaved peptide 72–91 in the free reductase as well as in the covalent complex tryptic map should be recalled. On the other hand, a steric hindrance to the protease action at Lys-85 by the peptide cross-linked to Lys-88 could also be envisaged: thus, Lys-88 could well be the point of cross-linking.

The sequence 72–91 of FNR lies in the flavin binding domain, as inferred from the X-ray structure at 3.7-Å resolution (Sheriff & Herriot, 1981). Furthermore, this region is highly hydrophilic as judged by the hydropathy profile calculated according to Kyte and Doolittle (1982), and it is expected to be exposed to solvent. The conserved sequence, the location within the FAD binding domain, and the hydrophilicity of the FNR fragment 72–91 are in favor of this region being the major point of interaction of the reductase with ferredoxin.

In regard to ferredoxin, the C-terminal sequence 76–97 found cross-linked to the 72–91 region of FNR is almost completely conserved in the two isoforms of the protein. Up to now, no physiological differences between the two proteins, as far as the interaction with FNR is concerned, have been detected (Takahashi et al., 1983; Sakihama et al., 1986). Among the carboxyl groups in the fragment 76–97, only Glu-92 is totally conserved in all 27 chloroplast-type ferredoxins sequenced (Tsukiara et al., 1982). In spinach Fd, this residue is part of an acidic cluster being followed by two other acidic residues (Glu-93 and Glu-94). The same cluster has been suggested to be important for reoxidation of the iron–sulfur center of Fd by the complex $Co(NH_3)_6^{3+}$ (Adzamli et al., 1983). Furthermore, the *Halobacterium*-type ferredoxin, which lacks acidic residues in the C-terminal region, seems to interact much less effectively with spinach FNR (Werber et al., 1980). Interestingly, the Markley group (Chan et al., 1983) found by ^{13}C NMR spectroscopy that at least three glutamic residues of *Anabaena variabilis* ferredoxin are at or near the contact region with spinach FNR. Extrapolation to the acidic cluster 92–94 of spinach Fd is not straightforward, because in the *A. variabilis* Fd, Glu-94 is replaced by an aspartic residue. Most recently, Vieria et al. (1986) proposed the regions 26–30, 65–70, and 92–94 of Fd as important for binding to the reductase by chemical modifications of Fd carboxyls. Although we have found two types of cross-links involving respectively the regions encompassing the sequences 26–30 (minor site of cross-linking) and 92–94 (major site of cross-linking), we have no evidence for the involvement of the region 65–70 of Fd.

The spinach Fd I sequence was fitted to the X-ray structure of the *S. platensis* ferredoxin (Tsukihara et al., 1982) by means of computer graphics (Jones, 1978). In the three-dimensional model thus obtained (Figure 5), the acidic cluster 92–94 containing the residue most probably involved in the cross-link to FNR is located quite near the iron–sulfur cluster ($\sim$10 Å). The results presented emphasize the importance of the N-terminal moiety of the reductase for the interaction with ferredoxin. This region of the protein has been proposed as the flavin binding domain (Sheriff & Herriot, 1981). Thus, the two prosthetic groups (FAD and the [2Fe–2S] cluster) are brought close together in the complex for electron transfer to occur.

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SUPPLEMENTARY MATERIAL AVAILABLE

A table showing the amino acid sequences of tryptic peptides separated by HPLC in Figure 1, reported according to their retention times (3 pages). Ordering information is given on any current masthead page.

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Endogenous Glycosphingolipid Acceptor Specificity of Sialosyltransferase Systems in Intact Golgi Membranes, Synaptosomes, and Synaptic Plasma Membranes from Rat Brain†

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ABSTRACT: Preparations highly enriched in Golgi complex membranes, synaptosomes, and synaptic plasma membranes (SPM) by marker enzyme analysis and electron microscopic morphology were made from the brains of 28-day-old rats. These were incubated with cytidine 5′-monophosphate–*N*-acetyl[^{14}C]neuraminic acid (CMP–NeuAc) in a physiologic buffer, without detergents. Glycolipid sialosyltransferase activities (SATs) were measured by analyzing incorporation of radiolabeled NeuAc into endogenous membrane gangliosides. Golgi SAT was diversified in producing all the various molecular species of labeled gangliosides [2.64 pmol of NeuAc transferred (mg of protein)$^{-1}$ h^{-1}]. Synaptosomal SAT exhibited a lower activity [0.66 pmol (mg of protein)$^{-1}$ h^{-1}], but it was highly specific in its labeling pattern, with a marked preference for labeling NeuAcα2→8NeuAcα2→3Galβ1→4Glcβ1→1Cer (GD3 ganglioside). SPM prepared from the synaptosomes retained the GD3-related SAT (or SAT-2), and the total specific activity increased [1.41 pmol (mg of protein)$^{-1}$ h^{-1}], which suggests that the location of the synaptosomal activity is in the SPM. These results indicate that SAT activity in Golgi membranes differs from that in synaptosomes with regard to endogenous acceptor substrate specificity and SAT activity of synaptosomes should be located in the synaptosomal plasma membrane. This SAT could function as an ectoenzyme in concert with ecto-sialidase to modulate the GD3 and other ganglioside population in situ at the SPM of the central nervous system.

Gangliosides are sialic acid containing glycosphingolipid membrane components that exist in high amounts in brain (Brunngraber, 1979; Leskawa & Rosenberg, 1981). With their strategic location in synaptic plasma membrane (SPM), it has been popular to envision ganglioside involvement in synaptic events such as neurotransmitter release and dispersal (Rahman, 1976; Svennerholm, 1980), receptor function (Tamir et al., 1980; Fishman, 1982), Ca^{2+} flux control (Veh & Sander, 1981), and involvement in neurotrophic–neuritogenic events in neuronal development [reviewed by Ledeen (1984)]. However, it is not yet possible to clearly identify even one event in which gangliosides function unequivocally (Wiegandt, 1985).

An SPM ecto-sialidase activity has been described which splits sialic acid residues from gangliosides (Schengrund & Rosenberg, 1970; Tettamanti et al., 1972) and, more recently, an SPM ecto-sialosyltransferase (ecto-SAT) that transfers sialic acid residues to endogenous membrane gangliosides (Den et al., 1975). Through the concerted action of these two enzymes, the ganglioside characteristics of the SPM could be varied or controlled (Schengrund & Nelson, 1975).

Sialosyltransferases, along with other glycosyltransferases, traditionally have been reported as being Golgi apparatus enzymes involved in the de novo biosynthesis of gangliosides and glycoproteins. Some researchers have attributed the measured SAT activity in synaptosomes to artifact due to Golgi contamination (Ng & Dain, 1977). This is difficult to resolve because differentially subfractionated membrane preparations of brain homogenates may never be acceptably homogeneous.

In the present study, we have examined differences between the behavior of Golgi SAT and putative SPM ecto-SAT. Such differences would lend support to the existence of a separate SPM ecto-SAT activity and would lend credibility to a theoretical ganglioside desialosylation–resialosylation system in situ at the synapse.

Materials and Methods

Reagents were of the highest purity available from biochemical suppliers, and solutions were prepared with distilled–deionized water redistilled in a glass apparatus. All homogenization and centrifugation steps in the preparation of the brain subfractions were carried out at 0–4 °C.

Subfractionation of Rat Brain. After decapitation, the forebrains of 28-day-old Sprague-Dawley rat pups were removed and placed in 10 volumes of ice-cold 0.32 M sucrose [1.0 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)–0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2] and were then homogenized and fractionated by

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