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Comparison of the binding sites of plant ferredoxin for two ferredoxin-dependent enzymes

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Abstract

Differential chemical modification of acidic residues was used to map the binding site of plant ferredoxin (Fd) for the chloroplast enzyme ferredoxin:thioredoxin reductase (FTR). Binding of FTR to Fd inhibits chemical modification of Fd residues D34, D65, E92, E93, E94 and C-terminal A97. The binding site demarcated by these residues differs from that for ferredoxin:$NADP^+$ reductase (FNR). The FTR site includes C-terminal residues but not helix 24–31, which is part of the FNR site. Both sites enclose the [2Fe-2S] cluster.

Key words: Carboxyl group; Chemical modification; Electrostatic interaction; Ferredoxin (spinach); Ferredoxin:thioredoxin reductase (spinach); Ferredoxin:$NADP^+$ reductase (spinach)

1. Introduction

The small, soluble [2Fe-2S] protein ferredoxin (Fd) mediates electron transfer between chloroplast photosystem I and several Fd-dependent enzymes, such as ferredoxin:$NADP^+$ reductase (FNR), ferredoxin:thioredoxin reductase (FTR), nitrite reductase, and glutamate synthase. Complexes of Fd with reaction partners are stabilized by electrostatic interactions to which Fd contributes mainly negative charges ([1] and references therein). Recently, we have assigned six carboxyl groups of Fd to the binding site for FNR, using the method of differential chemical modification of free and FNR-bound Fd [2]. The carboxyl groups protected from chemical modification in the Fd:FNR complex were located in two domains of negative surface potential on either side of the [2Fe-2S] cluster of chloroplast Fd. We noticed a complementary site of positive surface potential on FNR. This allowed us to construct a binding model of the Fd:FNR complex [2,3].

Here we ask whether Fd uses the same or a different surface area to interact with FTR. We find that the FTR site differs, though both the FTR and the FNR site enclose the [2Fe-2S] cluster.

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Abbreviations: EDC,1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Fd, ferredoxin; FNR, ferredoxin:$NADP^+$ reductase (EC 1.18.1.2); FTR, ferredoxin:thioredoxin reductase.

FTR is an iron-sulfur protein which, in the presence of ferredoxin and thioredoxins *f* and *m*, catalyzes the activation of photosynthetic enzymes such as fructose-1,6-bisphosphatase and NADP-malate dehydrogenase [4]. FNR, an FAD-containing enzyme, catalyzes the terminal step of the chloroplast thylakoid electron transport chain, namely the reduction of $NADP^+$ by two equivalents of reduced Fd [5].

2. Materials and methods

2.1. Enzymes

Fd was isolated from spinach leaves [6,7]. Isoform I was used [8]. The concentration of Fd was determined at 420 nm with $\varepsilon = 9680\ M^{-1} \cdot cm^{-1}$ [6].

FTR was isolated from 6 kg of spinach leaves by a modification of a previous procedure [9]. Steps I–V were performed as in [9]. The protein-containing fraction from step V was concentrated (Amicon YM-5), centrifuged (48,000 × *g*, 30 min) and chromatographed on Sephacryl S-100 HR (Pharmacia; 5 × 92 cm) in 30 mM buffer A (30 mM Tris-HCl, pH 7.9, 14 mM β-mercaptoethanol) containing 0.4 M NaCl (90 ml/h). Fractions with FTR activity, measured as in [10], were concentrated and chromatographed on Q-Sepharose (Pharmacia; 5 × 25 cm) equilibrated with 20 mM buffer A containing 0.1 M NaCl, and eluted with a linear gradient from 0.1 to 0.5 M NaCl in 20 mM buffer A (250 ml/h). Active fractions were combined and applied to hydroxylapatite (5 × 5 cm) equilibrated in 10 mM buffer B (10 mM $NaKP_i$, pH 6.8, 14 mM β-mercaptoethanol). Elution proceeded with 500 ml of 10 mM buffer B, followed by a linear gradient from 10 to 600 mM buffer B. Finally, FTR was affinity-purified on ferredoxin-Sepharose (2.5 × 6 cm) equilibrated with 25 mM $NaKP_i$, pH 6.8, and eluted with a linear gradient from 0 to 0.75 M NaCl in the above buffer (60 ml/h). Pure FTR (A_{408}/A_{278} ratio above 0.36) was concentrated and stored in 20 mM $NaKP_i$, pH 6.8, at liquid nitrogen temperature. FTR

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concentration was determined at 410 nm with $\varepsilon = 17{,}400\ M^{-1} \cdot cm^{-1}$ [11].

2.2. Differential chemical modification

This was performed as described in detail before [2]. In experiment B, 40 μM Fd and 44 μM FTR were reacted with 2.78 mM EDC and 3.05 mM [^{3}H]taurine (7.84 GBq/mmol; New England Nuclear). In experiment F, FTR was omitted and 40 μM Fd alone was treated with 0.84 mM EDC and 0.92 mM [^{14}C]taurine (1.3 GBq/mol, New England Nuclear). The ratio of total protein carboxyl groups:EDC:taurine was 1:1:1.1 in both experiments. Reactions were quenched after 4 h by adding 0.2 ml of 0.5 M nonradioactive taurine in 0.2 M $NaKP_i$, pH 7.2, 3 M NaCl (to dissociate the Fd:FTR complex). Proteins from experiments B and F were combined, carrier Fd (400 nmol) was added, and Fd was separated from FTR and some covalent Fd:FTR complex on Superdex 75. The purified Fd was carboxymethylated and exhaustively modified with EDC/taurine under denaturing conditions [2].

2.3. Digestion of modified Fd, separation of peptides, sequence analysis

Exhaustively modified Fd in 0.1 M NH_4HCO_3 was digested at 37°C with 3% (w/w) trypsin (TPCK-treated, Fluka), added in 3 aliquots over a period of 24 h. Peptides were separated by reversed-phase HPLC (Nucleosil 100–5C_{18}-AB, Macherey & Nagel, Oensingen, Switzerland) with linear gradients (0.7 ml/min) of acetonitrile in 10 mM triethylammonium acetate, pH 5.7, as follows: 3–14% acetonitrile in 11 min, 14–32% acetonitrile in 36 min. Peptides eluted between 20 and 26% acetonitrile were combined and redigested with 2% (w/w) of chymotrypsin (Fluka). The C-terminal peptide (sequence 92–97) was eluted between 14% and 16% acetonitrile and was redigested with 10% (w/w) of elastase (Fluka) to obtain a mixture of peptides with sequences 92–95, 92–96, and 92–97. Re-digested peptides were separated by a 3–24% acetonitrile gradient of 70 min duration. Purified peptides were sequenced by a solid-phase method [2]. The ^{14}C/^{3}H ratio of the anilinothiazolinone derivative of each amino acid was determined.

3. Results

3.1. Differential chemical modification of carboxyl groups

In separate, parallel experiments the Fd:FTR complex (experiment B) and free Fd (experiment F) were reacted with carbodiimide and radioactive taurine (= aminoethane sulfonate) to obtain amidated protein derivatives. The Fd:FTR complex was less than 5% dissociated in experiment B, as tested by difference absorption spectroscopy (data not shown; [12]). Hence, modification of free Fd was negligible in experiment B. Labelling with radioactive taurine was kept well below 1 taurine/Fd. This ensured that modification of one carboxyl group did not influence modification of a neighbouring group. [^{3}H]Taurine was used in experiment B, [^{14}C]taurine in experiment F. Reaction products from experiment B and F were mixed after labelling. In this way it was possible to measure different degrees of carboxyl group modification from the ^{14}C/^{3}H ratio of modified Asp and Glu. The higher the ^{14}C/^{3}H ratio the less reactive was the carboxyl group in the Fd:FTR complex. Following digestion of modified Fd, peptide separation by HPLC, and sequential Edman degradation, ^{14}C/^{3}H ratios were obtained for the majority of the 21 carboxyl groups of Fd.

In two independent experiments, the median of the ^{14}C/^{3}H ratios of individual residues was 0.39 and 0.40, respectively. These values were in good agreement with the overall ^{14}C/^{3}H ratio for the intact, carboxyl-modified Fd of 0.37 and 0.36, respectively. To normalize the values of the two experiments, each ^{14}C/^{3}H ratio was divided by the median of all ^{14}C/^{3}H ratios. Normalized ^{14}C/^{3}H ratios obtained from the two experiments and their means are shown in Table 1. Six means were above 2 (boldface in column 4 of Table 1), the remainder below 1.10. Strongest protection, i.e. highest ^{14}C/^{3}H ratios, were observed for residues next to the C-terminal end. ^{14}C/^{3}H ratios for binding of FNR obtained previously [2] are included for comparison in Table 1, column 5.

Residues D70 and E71 had ^{14}C/^{3}H ratios well below unity, indicating that these residues were more reactive in the Fd:FTR complex. We made the same observation in our previous experiments with the Fd:FNR complex (Table 1). The two residues are remote from the proposed binding sites for FTR and FNR (see below). Even a minor conformational change caused by binding to FTR and FNR may increase chemical reactivity [13].

3.2. Location of protected residues in the 3D structure of Fd

Fig. 1 shows the molecule with the [2Fe-2S] cluster facing the viewer. The structure was obtained by fitting the sequence of spinach Fd to the crystal structure of Fd

Table 1
Effect of FTR on carboxyl group modification of Fd

Residue	Exp. 1	Exp. 2	Mean Exp. 1 + 2	Mean Fd:FNR [2]
E-15	n.l.	n.l.	–	n.l.
D-20	n.d.	n.d.	–	0.60
D-21	n.d.	n.d.	–	0.58
D-26	0.81	0.75	0.78	**1.80**
E-29	0.89	0.74	0.82	**4.79**
E-30	0.73	0.87	0.80	**3.64**
E-31	0.52	0.54	0.53	0.71
D-34	2.40	1.73	**2.06**	**4.53**
D-59	0.99	n.d.	0.99	0.97
D-60	0.71	n.d.	0.71	1.15
D-65	2.24	2.07	**2.15**	10.36
D-66	1.08	1.12	1.10	**1.66**
D-67	1.00	0.88	0.94	1.01
D-70	0.30	0.38	0.34	0.21
E-71	0.08	0.09	0.09	0.41
D-84	n.l.	n.l.	–	n.l.
E-88	n.l.	n.l.	–	n.l.
E-92	12.65	9.35	**11.00**	0.82
E-93	10.41	9.14	**9.77**	0.63
E-94	14.10	6.92	**10.51**	0.57
A-97	8.22	7.36	**7.79**	n.d.

Normalized ^{14}C/^{3}H ratios of amidated Asp and Glu obtained by differential chemical modification of the Fd:FTR complex. Values for the Fd:FNR complex [2] are shown for comparison in column 5. Mean values indicating protection of a residue are in boldface. n.l. = not labelled or labelling too low to determine ^{14}C/^{3}H ratio; n.d. = not determined.

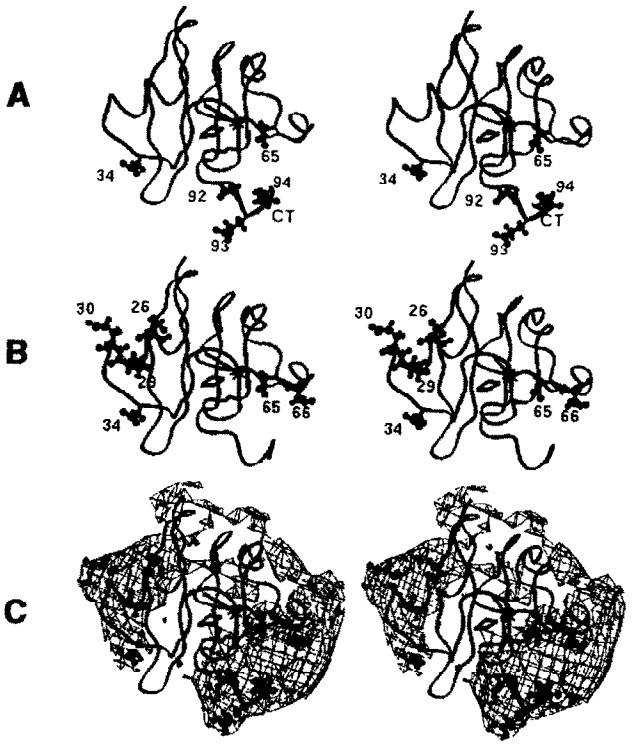

Fig. 1. Stereo ribbon model of C_α trace of ferredoxin from *Aphanothece sacrum* to which the sequence of spinach ferredoxin was fit. The [2Fe-2S] cluster is indicated by an oblong. Side chains protected by FTR and FNR, respectively, are outlined. The molecular dipole moment vector is indicated by a straight line with the negative pole marked by an asterisk. (A) Residues protected in the Fd:FTR complex (this work). CT marks the approximate location of the carboxy-terminal residue A97 of spinach Fd; this residue is missing in Fd of *A. sacrum*. (B) Residues protected in the Fd:FNR complex [2]. (C) Fd with negative electrostatic surface potential indicated at a contour level of −15 kJ/mol (calculation of surface potential as in [2]).

from *Aphanothece sacrum* [14]. The six residues protected by FTR are located on the same face of the molecule, namely D65, E92, E93, E94, and A97 to the right of the [2Fe-2S] cluster and D34 to the left (Fig. 1A). The residues form a semicircle around the [2Fe-2S] cluster. The distribution of the protected residues differs from that for the Fd:FNR complex in which four protected residues are on the left and only two on the right of the [2Fe-2S] cluster (Fig. 1B, column 5 of Table 1). The most notable difference is that FTR strongly protects the acidic sequence near the C-terminus while FNR interacts with the helix 24–31. The two binding sites overlap in the region of the [2Fe-2S] cluster.

4. Discussion

Differential chemical modification is a simple means to obtain information about the approximate location of a binding site on the surface of a protein. The method has been applied successfully to map the interface of several protein:protein complexes ([2] and references therein). We have located carboxyl groups at the interface of the complex of cytochrome *c* peroxidase with cytochrome *c* [15], and more recently of the Fd:FNR complex [2].

Fd is an acidic protein. The molecule features two distinct domains of strong negative surface potential on either side of the [2Fe-2S] cluster (Fig. 1C). The binding site for FNR (Fig. 1B and [2]) seems to coincide chiefly with the two negative surface domains. There are positive surface domains on either side of the FAD prosthetic group of FNR. The oppositely charged domains of Fd and FNR are complementary and fit together in a putative model of the Fd:FNR complex [2,3,16]. The negative end of the strong molecular dipole moment of Fd and the positive end of the corresponding moment of FNR are in the center of the proposed binding sites [2]. This led us to envisage a binding mechanism in which the two molecules are first steered toward each other through complementary orientation of the molecular dipole moments, bringing the two molecules into a roughly correct orientation. Thereafter, the complementary electrostatic surface potentials guide the molecules to optimally orient the redox prosthetic groups. Such a mechanism will increase the percentage of productive encounter complexes and speed up the reaction, in agreement with kinetic studies [17] and theoretical considerations [18].

The binding site for FTR deduced from the present experiments includes chiefly one of the two electrostatic surface potential domains of Fd (compare stereo-pairs A and C of Fig. 1). Also, the FTR site seems to be less optimally oriented than the FNR site with regard to the molecular dipole moment of Fd (compare stereo pairs A and B of Fig. 1). This could indicate that the electrostatic potential distribution on Fd is less important for interaction with FTR. Indeed, the Fd:FTR complex is less dependent on ionic strength than the Fd:FNR complex [11]. Modification of carboxyl groups of Fd interferes only slightly with the reaction of Fd with FTR in the FTR catalyzed activation of fructose-1,6-bisphosphatase [12]. In contrast, modification of 3–4 carboxyl groups of Fd leads to a large drop of the rate of FNR catalyzed reduction of $NADP^+$ [19].

It is reasonable to suppose that the recognition sites of Fd-dependent enzymes should enclose the [2Fe-2S] cluster. This can be achieved in different ways, as follows from the results presented. The situation is reminiscent of the family of cytochrome *c* dependent enzymes. Cytochrome *c* oxidase, cytochrome *c* peroxidase, sulfite oxidase, cytochrome b_5, etc., all bind in the area of the surface exposed heme edge of cytochrome *c*; the binding sites overlap but are not identical [20,21]. Finally, there is increasing evidence for some heterogeneity of the productive electron transfer complexes in a given protein pair [18,22].

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