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Ferredoxin-thioredoxin reductase: properties of its complex with ferredoxin *

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Ferredoxin-thioredoxin reductase formed an electrostatically stabilized 1:1 complex with ferredoxin. Complex formation could be detected, following mixing of the two proteins, either by changes in optical absorbance and circular dichroism spectra or by co-chromatography during gel filtration. Chemical modification of three or four carboxyl groups on ferredoxin, which had previously been shown to inhibit the binding of ferredoxin to several ferredoxin-dependent chloroplast enzymes, had little effect on its interaction with ferredoxin-thioredoxin reductase. The results suggest that the ferredoxin domain that binds ferredoxin-thioredoxin reductase is not completely identical to that involved in binding other ferredoxin-dependent enzymes.

Introduction

The iron-sulfur protein ferredoxin acts as the first soluble electron acceptor for Photosystem I in oxygenic photosynthesis and, when reduced, serves as the electron donor for a variety of reductant-requiring reactions [1]. Four of these ferredoxin-dependent reductions have been demonstrated to involve electrostatically stabilized complexes between ferredoxin and the chloroplast enzyme catalyzing the electron transfer reaction: (1) the reduction of $NADP^+$ [2–5] catalyzed by ferredoxin:$NADP^+$ oxidoreductase (EC 1.18.1.2, hereafter referred to as $NADP^+$ reductase); (2) the reduction of nitrite to ammonia [6–8] catalyzed by ferredoxin:nitrite oxidoreductase (EC 1.7.7.1, hereafter referred to as nitrite reductase); (3) the reductive conversion of glutamine plus 2-oxoglutarate to glutamate [8] catalyzed by glutamate synthase (EC 1.4.7.1) and (4) The reduction of sulfite to sulfide [9] catalyzed ferredoxin:sulfite oxidoreductase (EC 1.8.7.1, hereafter referred to as sulfite reductase). ^{13}C-NMR measurements [10], cross-linking studies [11,12] and chemical modification investigations [8,12–14] have implicated carboxyl groups on ferredoxin as supplying the negative charges involves in complex formation with these enzymes. These investigations have also lead to the proposal [7,8] that negatively charged ferredoxin carboxylate groups constitute a common binding domain for ferredoxin-dependent enzymes. It thus appeared or interest to examine in detail the interaction of ferredoxin with the ferredoxin-dependent enzyme, ferredoxin-thioredo-

* Third paper in a series. The first two papers are identified in Refs. 15 and 16.

Abbreviation: CD, circular dichroism.

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xin reductase, a novel enzyme that contains both an iron-sulfur cluster and a catalytically active disulfide bridge [15–17], and which has recently been shown to form a noncovalent complex with ferredoxin [15]. Ferredoxin-thioredoxin reductase is of particular interest because, unlike previously studied ferredoxin-dependent enzymes, it functions in enzyme regulation [18].

Experimental procedures

Materials. Spinach ferredoxin ($A_{422\,nm}$: $A_{277\,nm}$ = 0.43) was prepared according to the method of Tagawa and Arnon [19] and stored in 30 mM Tris-HCl buffer (pH 8.0) at liquid N_2 temperature until used. Ferredoxin was modified by treatment with glycine ethyl ester in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide according to the method of Vieira and Davis [13], and stored as described for the unmodified ferredoxin. The same batch of carboxyl-modified ferredoxin used in the experiments with ferredoxin-thioredoxin reductase described below was assayed with $NADP^+$ and nitrite reductases, and showed the same diminished effectiveness in binding to these two chloroplast enzymes as that observed previously [8]. Ferredoxin-thioredoxin reductase was purified to apparent homogeneity from spinach leaves as described previously [15].

Analytical methods. Absorbance spectra were measured at 4° C using a Perkin-Elmer Lambda 5 spectrophotometer with a noise level of less than $2 \cdot 10^{-4}$ A. Difference spectra resulting from complex formation were measured using 1 cm optical path length, split cells as described previously [20]. Circular dichroism (CD) spectra were obtained using a JASCO Model J-20 spectropolarimeter. Ferredoxin-thioredoxin reductase was assayed by measuring its capacity to promote the light-dependent activation of $NADP^+$-malate dehydrogenase ($NADP^+$-MDH) in a reconstituted thylakoid system. The reconstituted system contained the following (final volume 100 µl): thylakoid membranes [15,16] equivalent to 23 µg chlorophyll; Tris-HCl buffer (pH 7.9), 100 mM; sodium ascorbate, 10 mM; 2,6-dichlorophenolindophenol, 0.1 mM; catalase, 250 units; ferredoxin-thioredoxin reductase, 2.6 µM; spinach thioredoxin *m* [21], 10 µM; corn $NADP^+$-malate dehydrogenase [21], 1.8 µM; and native or carboxyl-modified ferredoxin, as indicated. Assays were carried out under nitrogen in Eppendorf tubes. Light activation and assay of the $NADP^+$-malate dehydrogenase was carried out as described previously [15,16]. $NADP^+$ photoreduction was assayed using a reaction mixture that contained the same buffer, thylakoid membrane and electron donor concentration as those present in the ferredoxin-thioredoxin reductase assay plus 4 mM $NADP^+$. $NADP^+$ reduction was determined by the increase in absorbance at 340 nm following dilution of the sample to 1 ml with 50 mM Tris-HCl (pH 7.9), and centrifugation for 0.5 min at 13000 × *g*. Protein concentration was estimated by the method of Bradford [23] using bovine serum albumin as a standard. Gel filtration was carried out using a Sephadex G-75 column (1 × 30 cm) in 30 mM Tris-HCl buffer (pH 8.0) at 4° C. Apparent molecular masses were determined by gel filtration on calibrated columns according to the method of Andrews [24].

Results

Absorbance measurements

The formation of complexes between ferredoxin and each of several ferredoxin-dependent enzymes is known to produce significant alterations in the absorbance spectra of protein chromophore groups [2–6,8]. It therefore seemed likely that absorbance changes would also occur during complex formation between ferredoxin and ferredoxin-thioredoxin reductase, an enzyme also possessing a chromophore [15]. Such absorbance changes were detected when oxidized ferredoxin was mixed with a sample of ferredoxin-thioredoxin reductase at 15 mM ionic strength. Fig. 1 shows the difference spectrum of the complex minus the sum of the spectra of the two separate proteins. If the proteins were mixed at high ionic strength (215 mM), no absorbance changes were detected, suggesting that, in agreement with previous results [15], the interaction between the two proteins is electrostatic in nature. Fig. 1 shows that somewhat different absorbance changes were observed when native, unmodified ferredoxin was replaced by a ferredoxin containing three or four carboxyl groups that had been modified [13] by

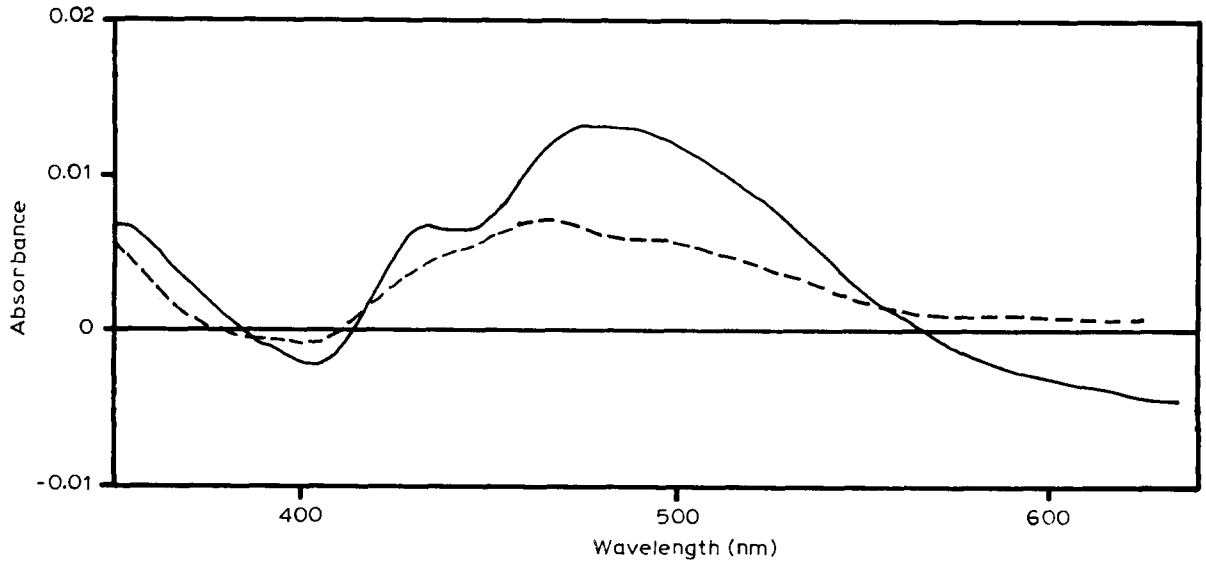

Fig. 1. Difference spectra of the complex between ferredoxin-thioredoxin reductase and either native or carboxyl-modified ferredoxin. The sample cuvette contained ferredoxin-thioredoxin reductase at a concentration of either 72 μM (———) or 36 μM (- - - - -) and equimolar native (———) or modified (- - - - -) ferredoxin in 30 mM Tris-HCl buffer (pH 8.0) in the same compartment. The reference cuvette contained the same components but with the ferredoxin and enzyme in separate compartments.

treatment with glycine ethyl ester in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The maxima in the two difference spectra are located at slightly different wavelengths and the negative feature present at 590 nm in the difference spectrum of the complex with native ferredoxin is not detectable in the complex with carboxyl-modified ferredoxin.

Fig. 2 illustrates the dependence of the magnitude of the absorbance changes at 440 minus 405 nm, indicative of complex formation, on the concentration of native or carboxyl-modified ferredoxin for a fixed amount of ferredoxin-thioredoxin reductase. Similar concentration dependencies were observed with two different wavelength pairs (370 minus 400 nm and 460 minus 405 nm). The data indicate that ferredoxin-thioredoxin reductase forms a high affinity, 1 : 1 complex with both native and carboxyl-modified ferredoxin. In control experiments (not shown), this sample of carboxyl-modified ferredoxin gave less than 15% of the complex-indicating absorbance changes produced when unmodified ferredoxin was mixed with $NADP^+$ reductase, in agreement with previous reports [13,14].

Gel-filtration chromatography experiments

Additional evidence for complex formation between ferredoxin and ferredoxin-thioredoxin reductase was obtained from gel-filtration chromatography experiments, in which the two proteins comigrated with an apparent molecular mass equal to the sum of those of the two proteins. This

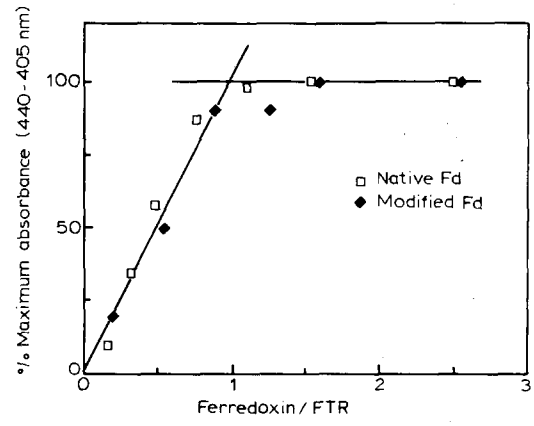

Fig. 2. Titration of ferredoxin-thioredoxin reductase with ferredoxin. Reaction mixtures were as in Fig. 1 except that the ferredoxin concentration was varied as indicated. FTR, ferredoxin-thioredoxin reductase.

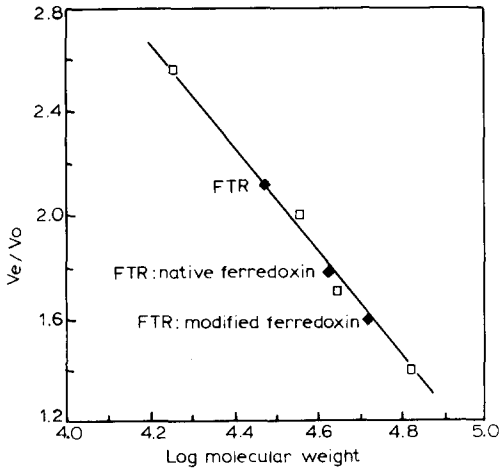

Fig. 3. Apparent molecular masses of ferredoxin-thioredoxin reductase complexes with native and carboxyl-modified ferredoxin. Gel filtration chromatography of 1:1 mixtures of ferredoxin-thioredoxin reductase (FTR) with either native or carboxyl-modified ferredoxin (■) was performed in 30 mM Tris-HCl buffer (pH 8.0) on a Sephadex G-75 column (1×30 cm). Molecular mass standards (□): β-lactoglobin; glyceraldehyde-3-phosphate dehydrogenase; ovalbumin and bovine serum albumin with molecular mass of 18.4, 36, 45 and 66 kDa, respectively.

technique has been used previously to demonstrate complex formation between ferredoxin and other ferredoxin-dependent chloroplast enzymes [8,9,25]. Fig. 3 shows that at 15 mM ionic strength, a 1:1 mixture of unmodified ferredoxin and ferredoxin-thioredoxin reductase eluted from a Sephadex G-75 gel filtration column as a single component with an apparent molecular mass of 48 ± 3 kDa. No detectable protein eluted from the column in fractions corresponding to the molecular mass of either ferredoxin-thioredoxin reductase, 30 kDa [15], or ferredoxin, 11 kDa [26], indicating that all of the ferredoxin was complexed with the enzyme. When a 2:1 mixture of ferredoxin and ferredoxin-thioredoxin reductase was subjected to gel filtration, components eluted from the column with apparent molecular masses of 48 and 11 kDa, corresponding respectively to the 1:1 ferredoxin · ferredoxin-thioredoxin complex and the excess ferredoxin (data not shown). Similar results were obtained when untreated ferredoxin was replaced by carboxyl-modified ferredoxin, except that the apparent molecular mass of the complex was 53 ± 3 kDa (Fig. 3). This same sample of carboxyl-modified ferredoxin did not comigrate with $NADP^+$ reductase during gel-filtration chromatography on Sephadex G-75, while native ferredoxin did comigrate with $NADP^+$ reductase, in agreement with previous results [8].

Evidence for the electrostatic nature of the complex between both native and carboxyl-modified ferredoxins and ferredoxin-thioredoxin reductase was obtained from gel filtration experiments in which the ionic strength was increased from 15 to 215 mM. Thus, while a 1:1 mixture of native ferredoxin and ferredoxin-thioredoxin reductase comigrated at 15 mM ionic strength, comigration was not observed at 215 mM ionic strength (Fig. 4). A similar complex between ferredoxin and ferredoxin-thioredoxin reductase, observed in

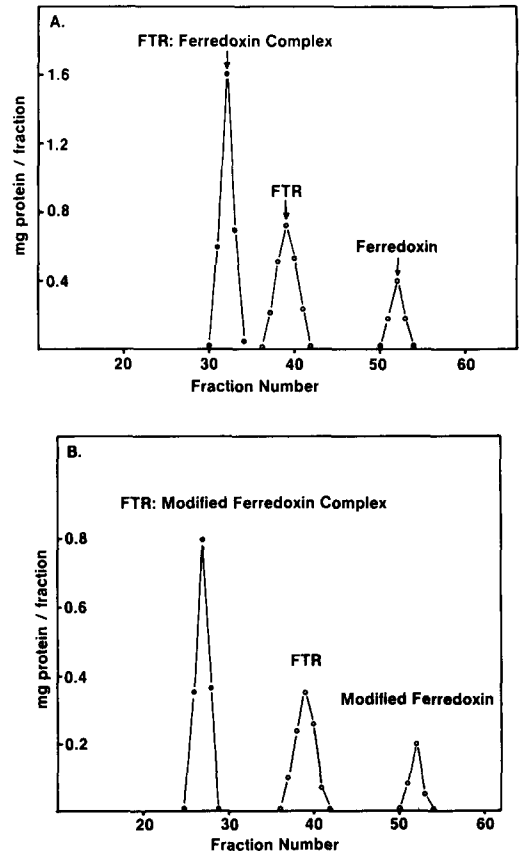

Fig. 4. Elution profile of ferredoxin-thioredoxin reductase·ferredoxin complexes. (A) Native ferredoxin. (B) Carboxyl-modified ferredoxin. Gel-filtration chromatography was carried out as in Fig. 3 (●) or in 30 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl (○).

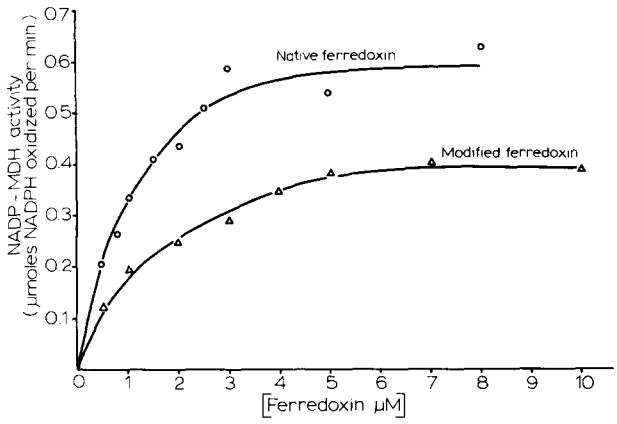

Fig. 5. Ferredoxin concentration dependence for the ferredoxin-thioredoxin reductase mediated activation of $NADP^+$-dependent malate dehydrogenase (MDH) in the presence of thioredoxin *m*. Activity was measured as described in Experimental procedures.

gel filtration experiments in earlier studies, lead to the erroneous conclusion that the complex represented a separate, ferredoxin-independent protein functional in enzyme regulation [27–29]. In the current studies, no component eluting with an apparent molecular mass of 48 kDa was detected at the higher ionic strength, but instead, two components eluted separately, with apparent molecular masses of 30 kDa (ferredoxin-thioredoxin reductase) and 11 kDa (ferredoxin). Similar results were obtained in experiments in which carboxyl-modified ferredoxin replaced native ferredoxin (Fig. 4).

Activity determinations

The observation that modification of three or four carboxyl groups on ferredoxin appeared to have very little effect on the affinity of ferredoxin binding for ferredoxin-thioredoxin reductase made it of interest to investigate the effect of carboxyl modification on the kinetic parameters of the reaction catalyzed by ferredoxin-thioredoxin reductase, i.e., the reduction of thioredoxin by reduced ferredoxin. Fig. 5 shows that carboxyl modification had virtually no effect on the K_m of the enzyme for ferredoxin. Values of 1.7 μM and 1.8 μM were measured for native and carboxyl-modified ferredoxin, respectively. However, carboxyl modification did result in a V_{max} that was only 67% of that observed with untreated ferredoxin (Fig. 5).

Circular dichroism measurements

Circular dichroism (CD) difference spectra have been used as an additional tool to study the interaction between ferredoxin and chloroplast enzymes [25,30,31]. Before examining the interaction between ferredoxin and ferredoxin-thioredoxin reductase in this manner, it was of interest to measure the CD spectrum of ferredoxin-thioredoxin reductase itself. The CD spectrum of ferredoxin-thioredoxin reductase had not yet been reported and could give information on this unique iron-sulfur protein. Fig. 6 shows the CD spectrum of ferredoxin-thioredoxin reductase in the region from 250 to 600 nm. The spectrum exhibits some

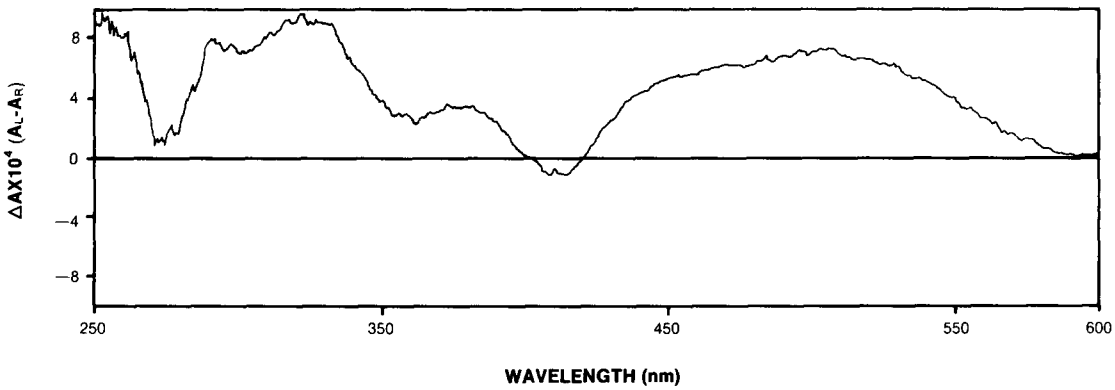

Fig. 6. Circular dichroism spectrum of ferredoxin-thioredoxin reductase. The reaction mixture contained 25 μM enzyme in 30 mM Tris-HCl buffer (pH 8.0). Optical pathlength = 1 cm.

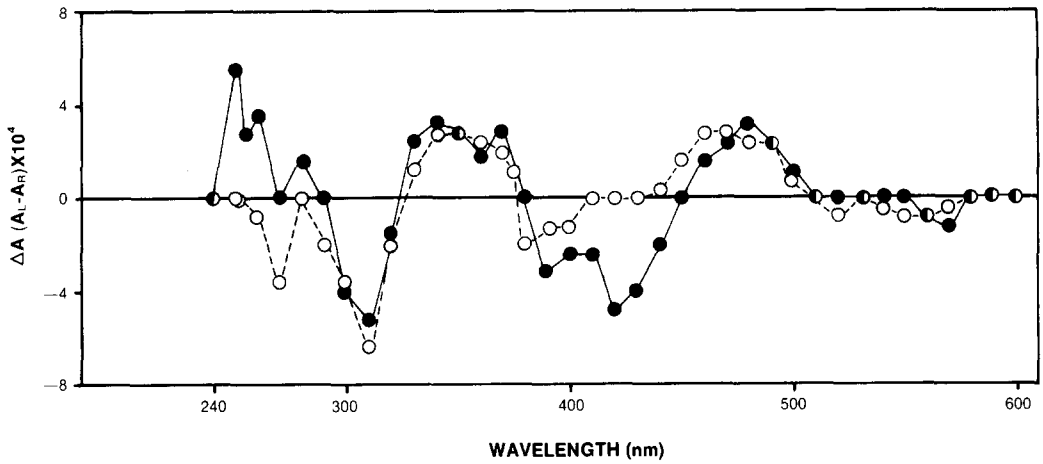

Fig. 7. Circular dichroism difference spectra resulting from complex formation between ferredoxin-thioredoxin reductase and ferredoxin. CD spectra of ferredoxin-thioredoxin reductase alone and ferredoxin alone were summed and subtracted from the CD spectrum of a solution containing both the enzyme and ferredoxin. The reaction mixture contained ferredoxin-thioredoxin reductase at a concentration of either 18 (●) or 25 μM (○) and either 18 μM native ferredoxin (●) or 25 μM carboxyl-modified ferredoxin (○) in 30 mM Tris-HCl buffer (pH 8.0). Optical pathlength = 1 cm.

similarity to that of the reduced form of the [4Fe-4S] cluster-containing high potential iron-sulfur protein from the photosynthetic purple sulfur bacterium *Thiocapsa pfennigii* [32], but bears little resemblance to that of [2Fe-2S] cluster-containing proteins, such as spinach ferredoxin [33]. Coupled with earlier evidence from visible absorbance spectra and chemical analyses [15,28,34], the CD spectrum is consistent with the hypothesis that ferredoxin-thioredoxin reductase contains a [4Fe-4S] cluster. Fig. 7 shows the CD difference spectra (enzyme-ferredoxin complex minus enzyme plus ferredoxin) resulting from complex formation between ferredoxin-thioredoxin reductase and either native or carboxyl-modified ferredoxin. While there are some minor differences, the two CD difference spectra are generally quite similar. No changes in CD spectra occurred when the enzyme was mixed with either native or carboxyl-modified ferredoxin at 215 mM ionic strength, conditions unfavorable for complex formation.

Discussion

The data presented above provide new evidence that ferredoxin-thioredoxin reductase, like other ferredoxin-dependent enzymes (i.e., $NADP^+$ reductase, sulfite reductase, nitrite reductase and glutamate synthase), forms a complex with ferredoxin. Both the gel filtration comigration data and the titration of absorbance differences with ferredoxin suggest that the complex is one of high affinity and has a stoichiometry of 1 : 1. The shape of the titration curve of Fig. 2 suggests that the K_d for the complex is less than $1 \cdot 10^{-7}$ M at 15 mM ionic strength, a value considerably lower than those measured under similar conditions for the complexes between ferredoxin and either nitrite reductase or glutamate synthase [6–8] and comparable to that for the ferredoxin-$NADP^+$ reductase complex [3,35] (Table I). The electrostatic nature of the complex was confirmed by the finding that high ionic strength prevented complex formation, as assayed by both spectral and gel filtration measurements.

Surprisingly, modification of three or four carboxyl groups on ferredoxin caused relatively little change in the properties of its complex with ferredoxin-thioredoxin reductase, based on either absorbance or CD difference spectra or on kinetic and binding parameters. However, the difference in gel-filtration migration patterns of the ferredoxin-thioredoxin reductase complexes with native and with carboxyl-modified ferredoxin, i.e., the differences in apparent molecular weights of the complexes (cf. Fig. 3) and the differences in the

TABLE I

A COMPARISON OF FERREDOXIN-LINKED ENZYMES FROM CHLOROPLASTS

n.d., not determined due to the absence of detectable spectral changes. All values in the upper two rows are from this study, except for K_d for native ferredoxin for $NADP^+$ reductase, which was from Ref. 35. All values in the lower two rows are from Ref. 8.

Enzyme	Prosthetic group	K_d (M)		K_m^{app} (μM)		Ratio of V_{max} modified: native ferredoxin
		native ferredoxin	modified ferredoxin	native ferredoxin	modified ferredoxin	
Ferredoxin-thioredoxin reductase	Iron-sulfur cluster, disulfide	$<10^{-7}$	$<10^{-7}$	1.7	1.8	0.7
$NADP^+$ reductase	FAD	$5 \cdot 10^{-8}$	n.d.	1.5	1.6	0.3
Glutamate synthase	Iron-sulfur cluster, FAD, FMN	$1.45 \cdot 10^{-5}$	$1.0 \cdot 10^{-4}$	2.0	84	0.2
Nitrite reductase	Siroheme, iron-sulfur cluster	$6.3 \cdot 10^{-7}$	$1.0 \cdot 10^{-4}$	20	20	0.2

optical and CD difference spectra of the two complexes (cf. Figs. 1) suggest that carboxyl group modification may have some effect on the interaction of ferredoxin with the enzyme. Such carboxyl modification had earlier been shown (Table I) to inhibit complex formation between ferredoxin and $NADP^+$ reductase [8,13,14] and to decrease by 7-fold and 160-fold the respective binding affinities of glutamate synthase and nitrite reductase for ferredoxin. The results of this study on ferredoxin-thioredoxin reductase and of recent studies on the ferredoxin-dependent sulfite reductase of spinach chloroplasts [9] raise the possibility that the binding domain on ferredoxin for these two enzymes may differ in part from that of other ferredoxin-dependent enzymes studied previously (i.e., $NADP^+$ reductase, nitrite reductase and glutamate synthase). Experiments to test this possibility are currently underway in our laboratories.

Acknowledgements

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