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Circular dichroism, binding and immunological studies on the interaction between spinach ferredoxin and glutamate synthase

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Spinach ferredoxin and spinach ferredoxin-dependent glutamate synthase have been shown to form an electrostatically stabilized complex. Circular dichroism (CD) measurements indicate that conformational changes occur in one or both proteins during complex formation. A dissociation constant for the complex of 9 μM at low ionic strength was estimated from a titration of the CD changes. Ultrafiltration binding assays suggest that the likely ferredoxin : glutamate synthase stoichiometry of the complex is 2 : 1. Immunological experiments indicate that glutamate synthase has an antigenic determinant in common with two other spinach chloroplast ferredoxin-dependent enzymes, nitrite reductase and NADP$^+$ reductase. The immunological data have been interpreted in terms of structurally similar binding sites for ferredoxin on all three enzymes.

Introduction

Ferredoxin-dependent glutamate synthase (EC 1.4.7.1), which catalyzes the reductive conversion of glutamine plus 2-oxoglutarate to glutamate with reduced ferredoxin serving as the electron donor, plays a key role in ammonia assimilation by plants and algae [1]. Recently, glutamate synthase has been purified to homogeneity from spinach [2,3] and from the green alga *Chlamydomonas reinhardtii* [4,5]. The $M_r = 170$ kDa spinach enzyme contains a single polypeptide with FMN, FAD and at least one iron-sulfur center as prosthetic groups [2]. The spinach enzyme has been shown to form an electrostatistically stabilized complex with ferredoxin that appears to involve negatively charged groups on ferredoxin [3]. These acidic amino acids on ferredoxin appear to define a common binding domain for several ferredoxin-dependent chloroplast enzymes [3]. More recently, antigenic similarities have been demonstrated between the ferredoxin-binding sites on glutamate synthase and on ferredoxin-dependent nitrite reductase isolated from *C. reinhardtii* [6]. We present below additional evidence, obtained from spectroscopic and ultrafiltration studies, for complex formation between spinach glutamate synthase and ferredoxin and also present immunological data suggesting that the ferredoxin-binding sites on three spinach chloroplast enzymes (glutamate synthase, nitrite reductase and NADP$^+$ reductase) have common antigenic determinants.

Materials and Methods

Spinach (field grown during the 1987 growing season) was used as the starting material for the preparation of all proteins used in this study. Spinach ferredoxin ($A_{422nm}/A_{277nm} = 0.50$) was prepared as described previously [3] and stored in 30 mM Tris-HCl buffer (pH 8.0) at liquid nitrogen temperature until used. Spinach ferredoxin : nitrite oxidoreductase (EC 1.7.7.1, hereafter referred to as nitrite reductase) was prepared as described previously [3] and ferredoxin : NADP$^+$ oxidoreductase (EC 1.18.1.2, hereafter referred to as NADP$^+$ reductase) was prepared essentially as described by Shin et al. [7]. Glutamate synthase was prepared as described previously [2] except that 10 mM Tricine buffer (pH 7.7) containing 200 mM NaCl, 12.5 mM β-mercapto-

Abbreviations: CD, circular dichroism; DCPIP, 2,6-dichlorophenol indophenol; SDS, sodium dodecyl sulfate.

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ethanol and 1 mM 2-oxoglutarate was used during elution from the ferredoxin-Sepharose 4B affinity column. The specific activity of the purified enzyme was 100 units/mg protein. Mouse antiserum against spinach $NADP^+$ reductase was prepared using the procedure described previously for spinach catalase [8]. Rabbit antiserum against spinach $NADP^+$ reductase was a gift from Dr. Richard Malkin. Rabbit antiserum against the product of the spinach psaD gene was a gift from Drs. R. Malkin and R.M. Wynn (Division of Molecular Plant Biology, University of California, Berkeley). All other reagents used were of the highest purity available commercially.

$NADP^+$ reductase [7], nitrite reductase [9] and glutamate synthase [2] were assayed as described previously. Circular dichroism (CD) spectra were measured at ambient temperature using a JASCO Model J-20 spectropolarimeter. Protein concentrations were estimated by the method of Bradford [10] using bovine serum albumin as a standard. Membrane filtration binding assays were conducted using Centricon-30 microconcentrators (Amicon Corp, 30 kDa cut-off) which were centrifuged at 4°C for 1 h at $4000 \times g$. Ouchterlony double-diffusion tests and monitoring of antiserum inhibition of enzyme activity were carried out as described previously [11]. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) and isoelectric focusing were carried out using a Pharmacia Phast Electrophoresis System according to procedures recommended by the manufacturer.

Results

Previous work in our laboratory utilized co-migration during gel-filtration chromatography and perturbations of visible absorbance spectra to provide evidence for complex formation between spinach glutamate synthase and its physiological donor, ferredoxin [2]. Another spectroscopic technique that has been used to provide evidence for protein–protein interactions between ferredoxin and other ferredoxin-dependent chloroplast enzymes involves perturbations of CD spectra resulting from the mixing of two proteins [12–15]. Before looking for changes in CD spectra arising from complex formation between glutamate synthase and ferredoxin, the CD spectrum of glutamate synthase itself was measured. Fig. 1 shows both the CD spectra of glutamate synthase as isolated and of the dithionite-reduced enzyme. The CD spectrum of the enzyme in the absence of dithionite contains positive features at 309, 339, 388 and 435 nm and negative features at 485 and 555 nm. The positive features at 388 and 435 nm are characteristic of flavin-containing enzymes [15–18], consistent with earlier analytical data showing that the enzyme contains one FMN and one FAD [2]. Mixing a saturating amount of ferredoxin with glutamate synthase at low ionic strength produces changes in the CD spectrum (Fig. 2). A broad positive feature between 420 and 480 nm and broad negative features between 330 and 400 nm and between 490 and 580 nm are seen in the CD difference spectrum ([glutamate synthase · ferre-

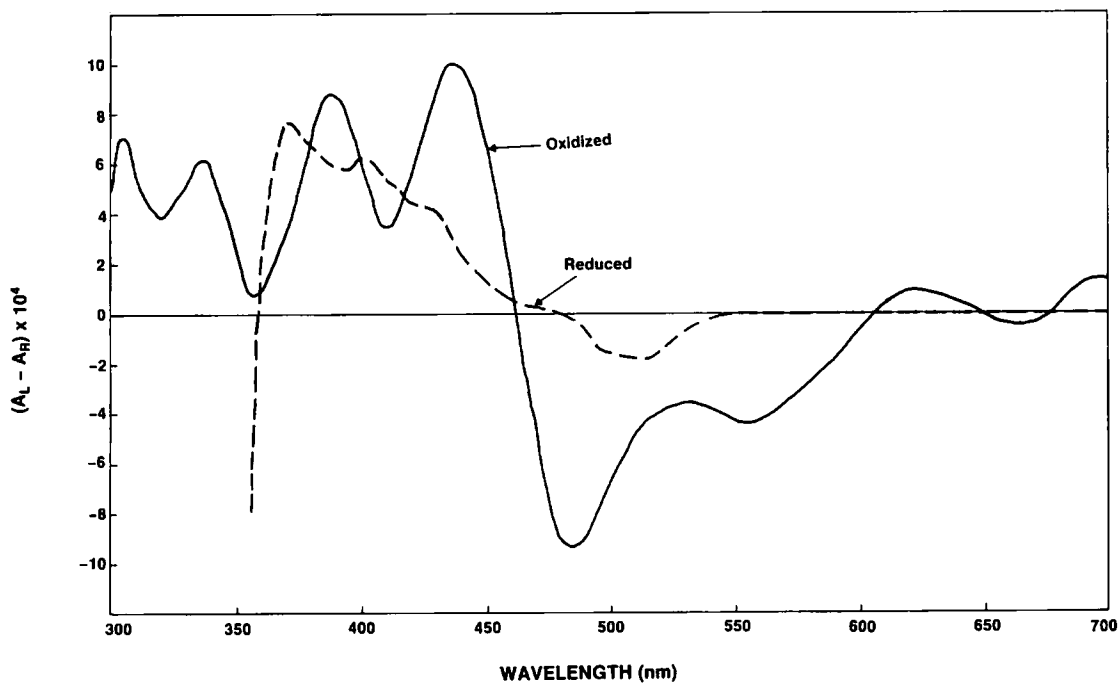

Fig. 1. CD spectra of glutamate synthase. The reaction mixture contained 62.9 μM enzyme in 10 mM Tricine·KOH buffer (pH 7.7) containing 200 mM NaCl, 1 mM 2-oxoglutarate and 0.1% (v/v) β-mercaptoethanol at ambient temperature in a 1 cm optical pathlength cell (———). The enzyme was reduced by adding a few crystals of solid sodium dithionite (– – –). Each spectrum represents the average of two runs.

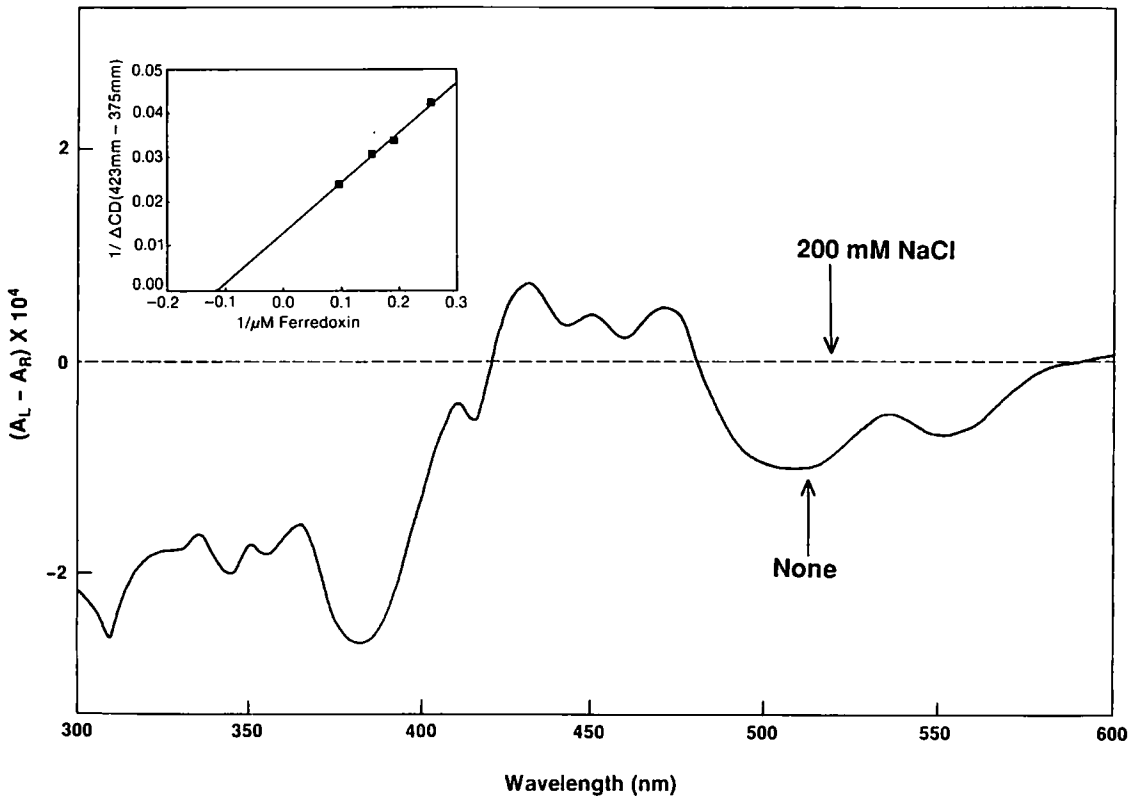

Fig. 2. CD difference spectra of the glutamate synthase·ferredoxin complex. The difference spectrum was obtained by subtracting the sum of the individual CD spectra of the two separate proteins, measured separately and then summed, from the CD spectrum of the two proteins mixed together. The reaction mixtures contained 10 mM Tricine·KOH buffer (pH 7.7), 1 mM 2-oxoglutarate and 0.1% (v/v) β-mercaptoethanol and either 200 mM NaCl (- - - - - -) or no NaCl (———). Glutamate synthase and ferredoxin concentrations were 6.5 and 28.5 μM, respectively. Other conditions were as in Fig. 1. The inset shows the effect of [ferredoxin] on the magnitude of the CD changes, plotted using reciprocals, in an experiment done in the absence of NaCl with 28.5 μM enzyme.

doxin] minus [(glutamate synthase) + (ferredoxin)]). No changes in CD spectrum were observed when the proteins were mixed at high ionic strength (Fig. 2), consistent with our earlier conclusion that the complex was held together predominantly by electrostatic forces [3]. Plots of the reciprocals of the CD changes obtained at different ferredoxin concentrations versus $[\text{ferredoxin}]^{-1}$ yielded a straight line consistent with the presence of a single type of ferredoxin binding site on glutamate synthase with a $K_d = 9 \pm 5$ μM (see Fig. 2, inset).

The CD spectra of glutamate synthase, ferredoxin and their complex in the region from 200 to 250 nm are shown in Fig. 3. The CD spectrum of glutamate synthase contains negative features centered at 206 and 216 nm. The ultraviolet CD spectrum of glutamate synthase suggests that the enzyme has an α-helix content of approx. 60% and a β-sheet content of approx. 20% [20]. The CD spectrum of ferredoxin contains a negative feature with a minimum at 206 nm, similar to the 210 nm value reported previously [20], and a negative shoulder near 215 nm. The difference spectrum (which is presented as the sum of the two individual CD spectra minus that of the complex instead of the difference between the spectra of the complex and the individual components) is consistent with a small decrease in the α-helix content of at least one of the proteins in the complex (Fig. 3). No changes in the 200–250 nm CD spectrum were observed when the two proteins were mixed at high ionic strength (i.e., 200 mM potassium phosphate buffer (pH 7.7)).

Previous ferredoxin/glutamate synthase binding studies using gel filtration chromatography suggested a ferredoxin : glutamate synthase stoichiometry of 2 : 1 in the complex formed between the two proteins [3], a value consistent with the Hill coefficient of 1.8 observed when enzymatic activity was analyzed as a function of ferredoxin concentration [2]. In a further attempt to determine the ferredoxin : glutamate synthase stoichiometry of the complex, membrane ultrafiltration experiments were carried out. We have previously used this technique to study complex formation between *Rhodospirillum rubrum* cytochrome c_2 and the *R. rubrum* cytochrome bc_1 complex [21] and between the *Paracoccus denitrificans* proteins amicyanin and methylamine dehydrogenase [22]. Table I shows that, as expected for an 11 kDa protein [23], ferredoxin alone passed through the 30 kDa cut-off filter readily at either low or high ionic strength. At low ionic strength (10 mM potassium

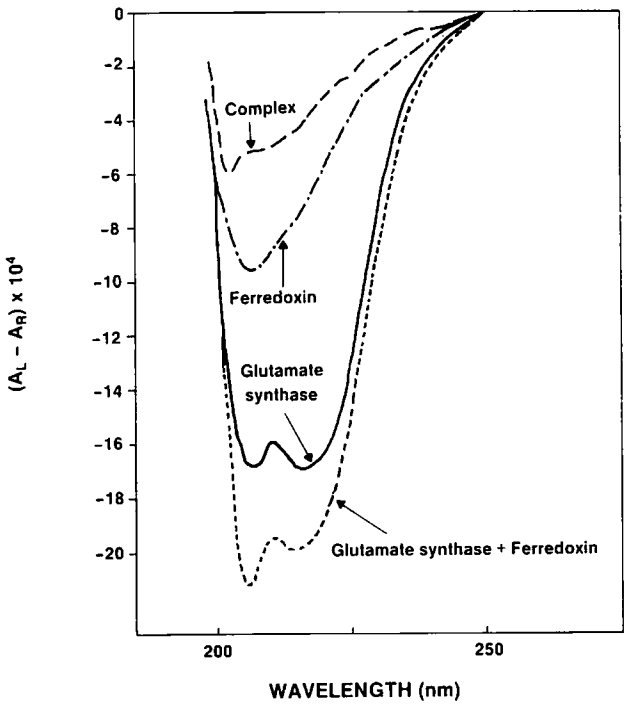

Fig. 3. CD spectra of glutamate synthase, ferredoxin and their complex in the 200–250 nm region. The difference spectrum was obtained by subtracting the CD spectrum of a glutamate synthase/ferredoxin mixture from the sum of the CD spectra of the separate proteins. Glutamate synthase (157 nM) and ferredoxin (341 nM) were dissolved in 10 mM potassium phosphate buffer (pH 7.7). Other conditions as in Fig. 1 except that each spectrum represents the average of four runs.

TABLE I

Membrane ultrafiltration of the ferredoxin · glutamate synthase complex

All experiments were carried out in potassium phosphate buffer (pH 7.7) using Amicon Centricon concentrators as described in Materials and Methods. In A and B, ferredoxin (920 μM) and, where indicated, glutamate synthase (460 μM) were present in a final volume of 0.5 ml. In C, 460 μM glutamate synthase and 1.38 mM ferredoxin were present in 0.5 ml. The ferredoxin content of the filtrate after three centrifugations was measured by monitoring the filtrate absorbance at both 420 and 280 nm and by protein determination using the method of Bradford [10]. All three methods gave essentially identical values, which are reported as a percentage of the total ferredoxin originally present in the top compartment of the microconcentrator. The results represent the average of two determinations, with the average deviation also indicated.

Component	Buffer concentration (mM)	Ferredoxin in filtrate (%)
A Ferredoxin	10	94±5
	200	94±5
B Ferredoxin plus glutamate synthase (molar ratio, 2:1)	10	<5
	200	91±5
C Ferredoxin plus glutamate synthase (molar ratio, 3:1)	10	35±4

phosphate buffer) and in the presence of glutamate synthase (ferredoxin : enzyme = 2 : 1), no ferredoxin could be detected in the filtrate. This observation, which suggests that under these conditions all of the ferredoxin is bound to glutamate synthase, is consistent with a 2 : 1 stoichiometry for the complex. Further evidence for a 2 : 1 stoichiometry was obtained from an experiment in which the ferredoxin concentration was increased (ferredoxin : glutamate synthase = 3 : 1). When this mixture, in 10 mM potassium phosphate buffer, was centrifuged through the ultrafiltration membrane, an amount of ferredoxin equal to the amount of glutamate synthase originally present was detected in the filtrate (Table I). Thus glutamate synthase can bind 2, but not 3, equivalents of ferredoxin. At high ionic strength (200 mM potassium phosphate buffer), where an electrostatically stabilized complex would be expected to dissociate, no binding of ferredoxin to glutamate synthase was observed (Table I). In control experiments it was shown that no glutamate synthase passed through the ultrafiltration membrane at either high or low ionic strength.

Chemical modification studies have suggested that a common binding site for glutamate synthase, nitrite reductase and $NADP^+$ reductase is present on spinach ferredoxin [3]. Recent immunological studies on the *C. reinhardtii* ferredoxin-dependent enzymes nitrite reductase and glutamate synthase suggested antigenic similarities between the ferredoxin binding sites on these two algal enzymes [6]. Although earlier work in our laboratory showed no effect of an antibody raised in rabbits against spinach nitrite reductase on reactions catalyzed by spinach $NADP^+$ reductase [11], the results reported for *C. reinhardtii* nitrite reductase and glutamate synthase suggested that a reinvestigation of possible antigenic similarities between spinach ferredoxin-dependent chloroplast enzymes could be of interest. Ouchterlony double immunodiffusion analysis showed that rabbit antiserum prepared against spinach $NADP^+$ reductase (provided by Professor R. Malkin) recognized both spinach nitrite reductase and glutamate synthase, as indicated by the fully fused precipitin band (Fig. 4), suggesting at least partial antigenic identity among the three enzymes. At lower enzyme and antibody concentrations, faint spurs were observed on the immunodiffusion plates (data not shown).

The spinach $NADP^+$ reductase sample used to prepare the rabbit antibody used for the experiment of Fig. 4 appeared to be homogeneous and its purification involved gel-filtration chromatography that should have completely separated $NADP^+$ reductase from any contaminating nitrite reductase or glutamate synthase because of the very different molecular weights of the three enzymes [3]. However, this sample of $NADP^+$ reductase was not specifically tested for nitrite reductase or glutamate synthase contaminants (Malkin, R., personal communication). To insure that the recog-

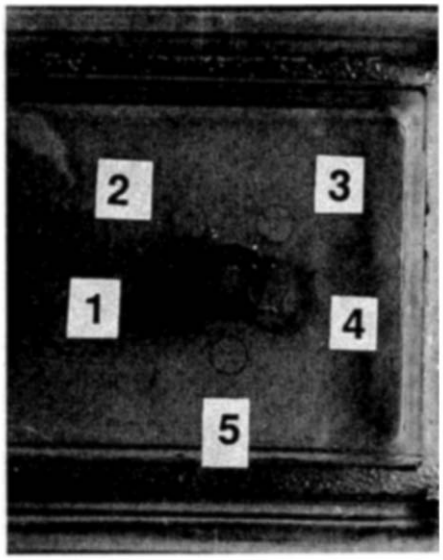

Fig. 4. Ouchterlony double-immunodiffusion of ferredoxin-dependent enzymes with $NADP^+$ reductase antiserum. The center well contained 15 μl of rabbit antiserum and the outer wells were filled with15 μl of the following samples; wells 1 and 2, nitrite reductase (3 units); wells 3 and 4, glutamate synthase (16.5 units); well 5, $NADP^+$ reductase (0.5 unit).

nition of spinach glutamate synthase and nitrite reductase by the rabbit antiserum raised against $NADP^+$ reductase described above did not arise from small amounts of contaminants in the $NADP^+$ reductase sample, we have raised antibodies against spinach $NADP^+$ reductase in mice using a sample of $NADP^+$ reductase purified in our laboratory that was homogeneous as judged by polyacrylamide gel electrophoresis in the presence of SDS and by isoelectric focusing and which showed no detectable activity when assayed for either glutamate synthase or nitrite reductase. The mouse $NADP^+$ reductase antiserum showed the same ability to recognize nitrite reductase and glutamate synthase as did the rabbit antiserum in Ouchterlony double immunodiffusion analyses (data not shown).

The rabbit antiserum raised against spinach $NADP^+$ reductase was also used in inhibition studies to demonstrate antigenic similarities between these three chloroplast ferredoxin-dependent enzymes. In control experiments, the antiserum was shown to inhibit both electron transfer from NADPH to DCPIP (dichlorophenol indophenol) and ferredoxin-dependent electron transfer from NADPH to cytochrome *c* catalyzed by $NADP^+$ reductase (Fig. 5A). The rabbit antiserum also significantly inhibited the conversion of 2-oxoglutarate plus glutamine to glutamate catalyzed by glutamate synthase if reduced ferredoxin was used as the electron donor. Considerably less inhibition was observed with identical amounts of enzyme and antibody if the non-physiological donor, reduced methyl viologen, replaced ferredoxin (15% inhibition vs. 60% inhibition, Fig. 5B). A similar pattern was observed for the nitrite reductase-catalyzed conversion of nitrite to ammonia, with complete inhibition by the rabbit antiserum observed when reduced ferredoxin was used as the electron donor, but only 15% inhibition observed with an identical antibody : enzyme ratio when ferredoxin was replaced by reduced methyl viologen (Fig. 5C). Higher amounts of $NADP^+$ reductase antiserum were required to inhibit the ferredoxin-linked activity of either glutamate syn-

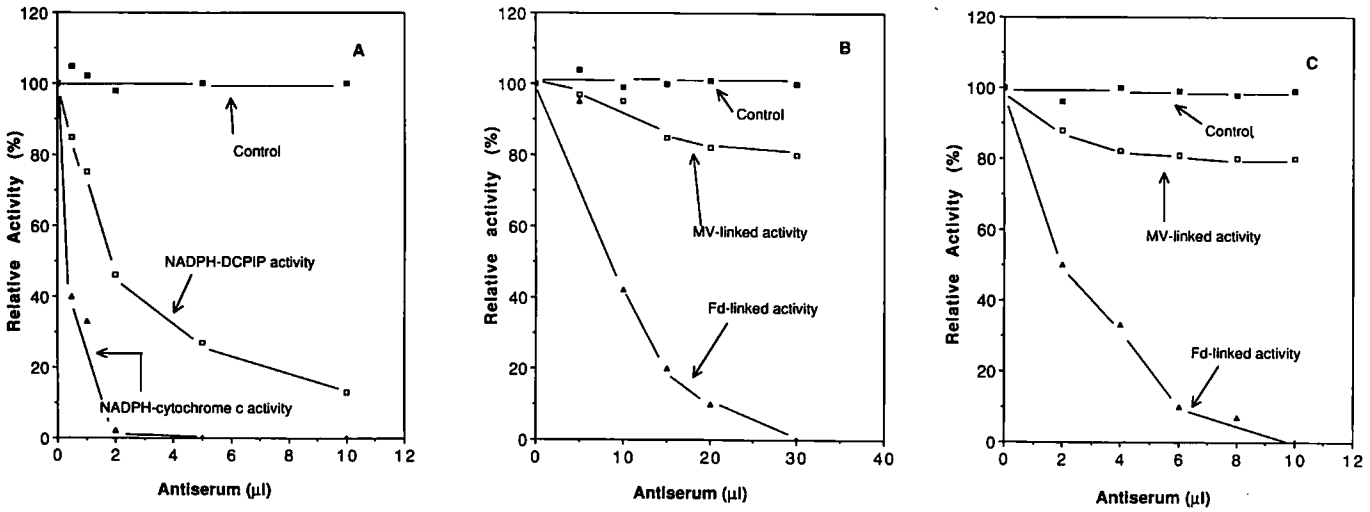

Fig. 5. Immunotitration of ferredoxin-dependent enzymes with $NADP^+$ reductase antiserum. The rabbit antiserum against $NADP^+$ reductase was incubated with constant amounts of enzyme for 2 min prior to assaying for activity as described in Materials and Methods. (A) $NADP^+$ reductase. 100% activity = 1.0 unit/ml for ferredoxin-dependent cytochrome *c* reduction (▲) and 4.0 units/ml for DCPIP reduction (□). (B) Glutamate synthase. 100% activity = 4.2 units/ml with reduced ferredoxin (▲) and 0.06 unit/ml with reduced methyl viologen (□) as the electron donors. (C) Nitrite reductase. 100% activity = 1.25 units/ml with reduced ferredoxin (▲) and 0.42 unit/ml with reduced methyl viologen (□) as electron donors. For A, B and C, ■ represents titration with pre-immune serum for both activities measured. The enzyme concentrations were 0.23 μM (cytochrome *c* reduction) and 0.086 μM (DCPIP reduction) in (A), 0.29 μM (ferredoxin-linked activity) and 0.33 μM (methyl viologen-linked activity) in (B), and 0.045 μM in (C).

thase or of nitrite reductase than of $NADP^+$ reductase itself. For all three enzymes, control experiments with pre-immune serum produced no inhibition of activity (Fig. 5). Similar inhibitions of nitrite reductase and glutamate synthase activities were observed with the mouse antiserum raised against $NADP^+$ reductase (data not shown).

Although the three enzymes used for the immunological studies described above differ greatly in molecular weight, catalytic activity and prosthetic group content [13], they share two common features: the use of reduced ferredoxin as the physiological electron donor and the ability to form relatively high-affinity, electrostatically stabilized complexes with ferredoxin [13]. This similarity, plus the finding that relatively little inhibition of nitrite reductase and glutamate synthase activity by the mouse or rabbit $NADP^+$ reductase antisera was observed when reduced methyl viologen was used as an electron donor, suggested that the common antigenic determinant between all three enzymes involves their ferredoxin binding sites. However, unlike the case for the *C. reinhardtii* nitrite reductase and glutamate synthase [6], ferredoxin did not protect the spinach enzymes against immunoprecipitation by the rabbit $NADP^+$ reductase antiserum (data not shown).

Recently, evidence has been obtained that ferredoxin binds to a specific 20–22 kDa subunit of the Photosystem I reaction center [24,25]. This protein, the product of the *psaD* gene, contains no electron-carrying prosthetic groups but appears to function by positioning ferredoxin properly for reduction by the low potential iron-sulfur clusters of the Photosystem I reaction center [26]. An antibody raised against the *psaD* gene product (provided by Drs. R. Malkin and R.M. Wynn) produced neither immunoprecipitation of $NADP^+$ reductase, nitrite reductase or glutamate synthase nor inhibition of the reactions catalyzed by any of the three enzymes (data not shown). These results are consistent with the hypothesis that the binding sites on ferredoxin for Photosystem I may be different than that for ferredoxin-dependent soluble chloroplast enzymes [27].

Discussion

Glutamate synthase has been shown to contain 4 mol of non-heme iron and 4 mol of acid-labile sulfide [2]. However, the nature of the iron-sulfur cluster(s) present in the enzyme has not yet been established. The CD spectra of Fig. 1 provide some evidence that the enzyme may contain two [2Fe-2S] clusters. The positive feature at 339 nm in the CD spectrum of the enzyme and its CD spectrum at wavelengths longer than 400 nm show some similarities to those of the oxidized forms of the [2Fe-2S] center-containing proteins ferredoxin [28] and adrenodoxin [20]. Neither flavin-containing proteins [15–18], nor [4Fe-4S] cluster-containing proteins [28,29], nor [3Fe-xS] cluster-containing proteins [30] exhibit positive CD features near 339 nm. The CD spectrum of the reduced enzyme exhibits little resemblance to those of [4Fe-4S] or [3Fe-xS]-containing proteins, but does show some resemblance to those of [2Fe-2S]-containing proteins [28]. The CD spectra of glutamate synthase presented above, combined with the earlier analytical data [2], appear more consistent with the hypothesis that the enzyme contains two [2Fe-2S] clusters rather than a single [4Fe-4S] cluster. The CD spectra provide no evidence for the presence of a [3Fe-xS] cluster, although iron content data reported for the *C. reinhardtii* enzyme are consistent with the possible presence of a [3Fe-xS] cluster [5]. A definitive identification of the chemical nature of the iron-sulfur cluster(s) must await the results of detailed electron paramagnetic resonance studies.

Results obtained using CD difference spectra and membrane ultrafiltration binding assays support our earlier conclusion [3] that spinach glutamate synthase forms an electrostatically stabilized complex with its physiological electron donor, the iron-sulfur protein ferredoxin. The dissociation constant calculated for the complex from plots of changes in CD vs. ferredoxin concentration, 9 μM, is in reasonable agreement with the value of 14.5 μM calculated earlier [3] from absorbance changes measured at the same ionic strength, considering that the small magnitude of the CD changes limited the accuracy of the measurement. The results of these studies suggest that one or both of the proteins undergoes significant conformational changes during complex formation. The binding studies described above suggest that the most likely stoichiometry of ferredoxin : glutamate synthase in the complex is 2 : 1. The latter conclusion is supported by the observation (Table I) that at low ionic strength, one molecule of glutamate synthase can bind two molecules of ferredoxin in an ultrafiltration assay but cannot bind three molecules of ferredoxin. Preliminary cross-linking experiments carried out in our laboratory (Hirasawa, M., Chang, K.-T. and Knaff, D.B., unpublished observations) also support a 2 : 1 stoichiometry. A glutamate synthase : ferredoxin covalent adduct formed by treatment with the cross-linking reagent 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC) had an apparent molecular mass 22 kDa greater than that of glutamate synthase alone. Since ferredoxin has a molecular mass of 11 kDa, the simplest explanation for the cross-linking data is that two ferredoxins are bound to each molecule of enzyme.

The most striking result obtained in this study was that mouse and rabbit antisera raised against highly purified, apparently homogeneous $NADP^+$ reductase recognized nitrite reductase and glutamate synthase, implying that these three enzymes have at least one structurally similar region. The observation that the antisera inhibit reactions with all these enzymes to a

much greater extent when ferredoxin is used a substrate than when a non-physiological reaction partner (e.g., methyl viologen) is supplied suggests that the ferredoxin-binding region of the three enzymes may be the structurally similar domain. We had earlier proposed that these three spinach enzymes have similar ferredoxin binding sites based on the effect of chemical modification of ferredoxin carboxyl groups on ferredoxin binding by the enzymes and on kinetic parameters for the enzymes [3]. Romero et al. have presented evidence for related ferredoxin-binding sites on the nitrite reductase and glutamate synthase isolated from *C. reinhardtii* [6], suggesting that such structural relationships between different ferredoxin-dependent chloroplast enzymes may occur both in higher plants and green algae.

Acknowledgements

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