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The role of aromatic and acidic amino acids in the electron transfer reaction catalyzed by spinach ferredoxin-dependent glutamate synthase

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Abstract

Treatment of the ferredoxin-dependent, spinach glutamate synthase with *N*-bromosuccinimide (NBS) modifies 2 mol of tryptophan residues per mol of enzyme, without detectable modification of other amino acids, and inhibits enzyme activity by 85% with either reduced ferredoxin or reduced methyl viologen serving as the source of electrons. The inhibition of ferredoxin-dependent activity resulting from NBS treatment arises entirely from a decrease in the turnover number. Complex formation of glutamate synthase with ferredoxin prevented both the modification of tryptophan residues by NBS and inhibition of the enzyme. NBS treatment had no effect on the secondary structure of the enzyme, did not affect the K_ms for 2-oxoglutarate and glutamine, did not affect the midpoint potentials of the enzyme's prosthetic groups and did not decrease the ability of the enzyme to bind ferredoxin. It thus appears that the ferredoxin-binding site(s) of glutamate synthase contains at least one, and possibly two, tryptophans. Replacement of either phenylalanine at position 65, in the ferredoxin from the cyanobacterium *Anabaena* PCC 7120, with a non-aromatic amino acid, or replacement of the glutamate at ferredoxin position 94, decreased the turnover number compared to that observed with wild-type *Anabaena* ferredoxin. The effect of the change at position 65 was quite modest compared to that at position 94, suggesting that an aromatic amino acid is not absolutely essential at position 65, but that glutamate 94 is essential for optimal electron transfer. © 1998 Elsevier Science B.V.

Keywords: Aromatic amino acid; Acidic amino acid; Glutamate synthase

1. Introduction

Ferredoxin-dependent glutamate synthases (EC 1.4.7.1), found in cyanobacteria and in the stroma of chloroplasts in higher plants and algae, are soluble enzymes that catalyze the 2-electron reductive conversion of 2-oxoglutarate *plus* glutamine to two

Abbreviations: CD, circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FNR, ferredoxin:$NADP^+$ oxidoreductase; NBS, *N*-bromosuccinimide

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molecules of glutamate, using reduced ferredoxin as the electron donor [1]. The spinach enzyme has been particularly well characterized [1,2] and shown to be a monomeric protein with $M_r = 160$ kDa [2,3] that contains one [3Fe–4S] cluster [4] and one FMN [5] as the only prosthetic groups. The two prosthetic groups of spinach glutamate synthase are isopotential, with E_m values of approximately -200 mV for both the FMN and the [3Fe–4S] cluster [5,6]. The amino acid sequence, deduced from the base sequence of the corresponding cDNA and from direct amino acid sequencing of the N-terminal region of the protein [3], is known for approximately 98% of the spinach enzyme.

Spinach glutamate synthase is known to form an electrostatically stabilized complex with its physiological electron donor, ferredoxin, with ferredoxin supplying the negative charges and the enzyme the positive charges involved in this complex formation [1,7–10]. Cross-linking and ultrafiltration experiments suggested the possible presence of two ferredoxin-binding sites on the spinach enzyme [8,9]. Immunological evidence [8] suggests that the ferredoxin-binding site(s) on spinach glutamate synthase is (are) antigenically similar to the single ferredoxin-binding sites on two other chloroplast enzymes that use ferredoxin as the physiological electron donor, ferredoxin:$NADP^+$ oxidoreductase (EC 1.18.1.2, hereafter abbreviated FNR) and ferredoxin:nitrite oxidoreductase (EC 1.7.7.1, hereafter referred to as nitrite reductase). Immunological evidence also exists suggesting a possible similarity between the ferredoxin-binding sites of glutamate synthase and nitrite reductase isolated from the green alga *Chlamydomonas reinhardtii* [11,12]. Given these possible similarities, and the recent observations that treatment of spinach nitrite reductase [13] and of spinach FNR [14] with the tryptophan-modifying reagent *N*-bromosuccinimide (NBS) inhibits these two enzymes, it was of interest to examine the effect of tryptophan modification on glutamate synthase. The results of these investigations, which suggest the involvement of tryptophan at the ferredoxin-binding site(s) of glutamate synthase in the reaction catalyzed by the enzyme, and of preliminary site-directed mutagenesis experiments designed to identify amino acid residues on ferredoxin involved in electron transfer to glutamate synthase, are reported below.

2. Materials and methods

Spinach ferredoxin ($A_{422\ nm}:A_{277\ nm} = 0.46$) and glutamate synthase ($A_{438\ nm}:A_{278\ nm} = 0.112$) were purified [10,15] and stored [5,10] as described previously. Both proteins showed single Coomassie Brilliant Blue-staining bands after polyacrylamide gel electrophoresis under denaturing conditions. The specific activity of the glutamate synthase preparation used in the studies reported below was similar to that reported previously [2,15]. Glutamate synthase preparations with this specific activity and $A_{438\ nm}:A_{278\ nm}$ ratio have previously been shown by N-terminal amino acid sequencing to be at least 95% homogeneous [3]. Vegetative cell, wild type ferredoxin from *Anabaena* PCC7120, the F65A, F65I, F65W, E94Q, E94D and E95K single position mutants and the E94K/E95K double mutant of this cyanobacterial ferredoxin were expressed in *Escherichia coli* and purified to apparent homogeneity as described previously [16–18].

Ferredoxin concentrations were measured using an extinction coefficient of 9.7 cm^{-1} mM^{-1} at 422 nm for both the spinach [19] and *Anabaena* [20] ferredoxins. Glutamate synthase concentrations were measured either using an extinction coefficient of 19.5 cm^{-1} mM^{-1} at 438 nm [5] or from the protein concentration measured according to the method of Bradford [21], using bovine serum albumin as a standard and a molecular mass for glutamate synthase of 162 kDa. Glutamate synthase activity was assayed as described previously [2].

NBS, molecular mass standards for electrophoresis and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical. Isoelectric focusing standards (pI = 2.80 to 6.55), Ultrogel AcA 34 and cyanogen bromide-activated Sepharose 4B were obtained from Pharmacia LKB. Ferredoxin was coupled to cyanogen bromide-activated Sepharose 4B according to the method of Shin and Oshino [22]. Western blot kits and acrylamide (electrophoresis grade) were obtained from Bio-Rad. The 5-deazariboflavin was synthesized according to the procedure of Smit et al. [23].

Glutamate synthase tryptophan residues were modified by adding aliquots from a freshly prepared solution of NBS to a stock solution of glutamate synthase (at enzyme concentrations ranging from 0.7

to 1.2 mM) in 100 mM Tricine–KOH buffer (pH 7.5) containing 1 mM 2-oxoglutarate, 0.1% (v/v) 2-mercaptoethanol and, where indicated, 200 mM NaCl. Except where indicated in the text, all modifications were carried out using a 2-fold molar excess of NBS over glutamate synthase. The mixtures of NBS and glutamate synthase were allowed to incubate at 4°C in the dark for the times indicated. The modification reaction was stopped by diluting the sample at least 1000-fold with buffer, and the treated enzyme was then assayed for both ferredoxin-dependent and methyl viologen-dependent activities. The tryptophan contents of the native and NBS-treated enzyme were estimated from the absorbance at 280 nm using the method described by Spande and Witkop [24] and Patchornik et al. [25]. Other amino acids were analyzed at the Institutional Protein Core Facility of the University of Texas Health Science Center San Antonio after acid hydrolysis in 6 M HCl/2% phenol for 24 h at 125°C under a nitrogen atmosphere, conditions designed to minimize the loss of tyrosine and methionine (appropriate positive controls were used to correct for amino acid losses). Three replicates of each hydrolysate were treated with 6-aminoquinolyl-*N*-hydroxysuccinimyl carbamate and the modified amino acids separated on a C-18 reverse phase column, eluted with a sodium phosphate buffer/acetonitrile gradient, using a Waters Model 840 chromatography and data analysis system. The hydrolysis conditions used resulted in the complete conversion of asparagine to aspartate and glutamine to glutamate. Experimental uncertainties in the content of each amino acid are approximately $\pm 10\%$. The cysteine contents of the native and NBS-treated enzymes were also measured using the DTNB method of Janatova et al. [26].

Absorbance spectra were measured using Shimadzu Model UV2100U and OLIS-modified Cary 15 spectrophotometers. Difference spectra were obtained using methods described previously [27,28]. Ferredoxin binding to glutamate synthase was quantitated using the spectral perturbation technique described previously [7]. Tryptophan and FMN fluorescence emission spectra were obtained using a Perkin-Elmer Model MFP-3 spectrofluorometer, with slits set at 5-nm resolution and excitation and emission wavelengths of 295 nm and 437 nm, respectively. Circular dichroism (CD) spectra were obtained using JASCO Model J-20 and Aviv Model 60 DS spectropolarimeters. The percentage-alpha helical contents of native and NBS-modified glutamate synthase were calculated using the algorithm Prosec, supplied with the Model 60 DS spectropolarimeter by Aviv Associates, using spectral data for 15 proteins (see table 3 in Ref. [29]) as a basis set. Isoelectric focusing and polyacrylamide gel electrophoresis under denaturing conditions were performed with a Pharmacia Phast system and protocols supplied by the vendor. Oxidation–reduction midpoint potentials were measured by cyclic voltammetry using self-assembled lipid bilayer membranes deposited on a gold electrode, as described previously [5,28,30,31]. Laser flash photolysis was carried out under anaerobic conditions with a sample buffer that contained 4.0 mM potassium phosphate (pH 7.5), 1 mM EDTA, 100 μM 5-deazariboflavin, using methodology and instrumentation described previously [6,32–34].

3. Results

Fig. 1 shows the effect of treating spinach glutamate synthase with a 2-fold molar excess of NBS, in the low ionic strength buffer described in Section 2. The activities of the enzyme, both with reduced ferredoxin (the physiological electron donor for glutamate synthase) serving as the source of electrons and with reduced methyl viologen (a non-physiological, alternative electron donor) serving as the source of electrons, are inhibited to equal extents. In a typical experiment, maximal inhibitions of 80% to 85% of the activity, with either electron donor, were obtained after approximately 75 min. Longer incubation times produced little or no further inhibition (Fig. 1). The effect of NBS on the ferredoxin-dependent activity of glutamate synthase was entirely an effect on the maximal turnover number of the enzyme, and NBS treatment had no detectable effect on the dependence of activity on ferredoxin concentration (data not shown). As can also be seen from Fig. 1, NBS causes a modification of approximately two tryptophan residues on the enzyme, with a time course that is very similar to that observed for the loss of activity. Essentially identical extents of inhibition and of tryptophan modification and an essentially identical time course were observed when the enzyme was

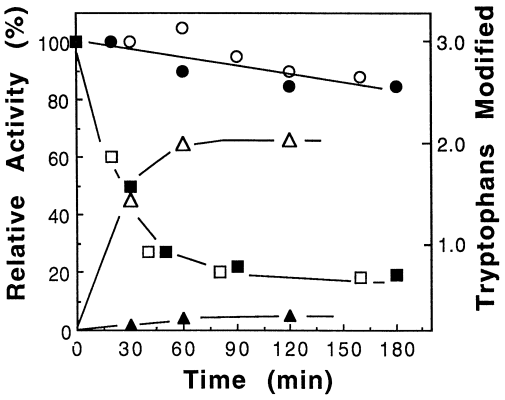

Fig. 1. The effect of NBS treatment on the activity and tryptophan content of spinach glutamate synthase. Spinach glutamate synthase, at a concentration of 900 μM, was incubated with 1.8 mM NBS in at 4°C in 10 mM Tricine–KOH buffer (pH 7.5) containing 1 mM 2-oxoglutarate and 0.1% (v/v) 2-mercaptoethanol. At the times indicated, aliquots were removed and diluted 2900-fold with the same buffer and assayed for ferredoxin-dependent (open squares) and methyl viologen-dependent (closed squares) activities. The closed and open circles represent, respectively, the ferredoxin-dependent and methyl viologen-dependent activities of enzyme that was incubated with NBS in the presence of an amount of spinach ferredoxin equal to twice that of glutamate synthase. The triangles represent the number of tryptophan residues modified in glutamate synthase that was incubated with NBS in the absence (open triangles) and presence (closed triangles) of ferredoxin. The 100% activity corresponds to 90 units per mg of protein for the ferredoxin-dependent activity and 5 units per mg of protein for the methyl viologen-dependent activity.

exposed to NBS in buffer to which 200 mM NaCl had been added to increase the ionic strength (data not shown), indicating that the kinetics of glutamate synthase modification by NBS are independent of ionic strength over the range tested. Exposure of glutamate synthase to NBS at a 8:1 molar ratio of modifier to enzyme resulted in the modification of a third tryptophan residue, but did not result in any additional inhibition of the enzyme (data not shown). Fig. 1 shows that the presence of ferredoxin, under conditions where the enzyme is known to form a complex with glutamate synthase [7], during the incubation of the enzyme with NBS results in almost complete protection of the enzyme against loss of activity, with either ferredoxin as the electron donor or methyl viologen as the electron donor. The presence of ferredoxin also prevented the modification of glutamate synthase tryptophan residues (Fig. 1). The presence of ferredoxin afforded no protection against either the loss of activity or the modification of tryptophan residues when the incubation was carried out in buffer to which 200 mM NaCl had been added to increase the ionic strength (data not shown), conditions under which the ferredoxin/enzyme complex is known to dissociate [7].

No protection against either the inhibition or the modification of tryptophan caused by NBS resulted from the presence of one of its substrates, glutamine. As highly purified spinach glutamate synthase is only fully stable in solutions that contain the other substrate, 2-oxoglutarate [2], it was not possible to conduct a rigorous test of whether this substrate is capable of protecting the enzyme against modification by NBS. However, as the inhibitory and tryptophan-modifying effects of NBS, shown in Fig. 1, occur in the presence of 1 mM 2-oxoglutarate, it appears that 2-oxoglutarate does not provide measurable protection against tryptophan modification and inhibition by NBS. The highly effective protection against inhibition and tryptophan modification by NBS provided by complex formation of glutamate synthase with ferredoxin, and the ineffectiveness of glutamine and 2-oxoglutarate as protective agents, suggest that the modified tryptophan residues are present at ferredoxin-binding site(s) of the enzyme, but are not likely to be close to the regions of the enzyme directly involved in the binding of 2-oxoglutarate or glutamine.

Although NBS preferentially modifies tryptophan residues [35], under some circumstances it can modify other amino acid residues such as histidine, cysteine, tyrosine and methionine [35]. Amino acid analysis indicated that NBS, under conditions where two glutamate synthase tryptophans were modified and enzyme turnover number was decreased by 85%, had no detectable effect on the contents of tyrosine, histidine, cysteine, methionine, aspartate *plus* asparagine, glutamate *plus* glutamine, glycine, serine, arginine, threonine, alanine, proline, valine, lysine, isoleucine, leucine and phenylalanine in spinach glutamate synthase. Thus, under the conditions used in this study, NBS appears to be quite specific for tryptophan. Titrations of both native and NBS-treated glutamate synthase with DTNB showed no differences in the number of accessible cysteine residues (2.0 ± 0.2) present, in agreement with the conclusion reached

from analysis of the amino acid composition data, i.e., that NBS does not modify cysteine residues in glutamate synthase.

The data presented above are consistent with the hypothesis that modification of tryptophan residues at the ferredoxin-binding sites of spinach glutamate synthase directly affects catalysis by the enzyme. To provide evidence that would argue against some indirect consequence of the tryptophan modification as a possible explanation for the observed inhibition (rather than tryptophan modification per se), a detailed comparison of the properties of the native and NBS-modified enzyme was undertaken. NBS had no detectable effect on either the absorbance spectrum or on the circular dichroism (CD) spectrum of the enzyme in the visible region, nor did NBS treatment affect the emission spectrum or yield of the fluorescence originating from the FMN prosthetic group. These results indicate that no major changes in the environments of the FMN and [3Fe–4S] prosthetic group chromophores resulted from NBS treatment. Furthermore, as shown in Fig. 2, the CD spectra of native and NBS-treated glutamate synthase were virtually identical in the region from 200 to 250 nm, indicating that NBS treatment does not result in any substantial changes in the secondary structure of the enzyme. For example, calculation of the alpha helical contents of native and NBS-treated glutamate synthase gave values of 10.9% and 9.3%, respectively, values that are identical within the experimental uncertainties. Similar calculations of the beta-structure contents of native and NBS-treated glutamate synthase showed no significant differences between the two samples. The ultraviolet CD spectrum of the native glutamate synthase (Fig. 2) had not been previously reported and thus, it had not been possible until now to make preliminary estimates of the secondary structure of the enzyme. Treatment of glutamate synthase with NBS had no detectable effect on the pI of the enzyme (5.6 ± 0.1) and did not affect the ability of a rabbit polyclonal antibody [8] to recognize the enzyme, as monitored by either Western blotting or by Ouchterlouny immuno-double diffusion. These results provide additional support for the conclusion, drawn from the ultraviolet CD measurements, that NBS-treatment does not cause any major conformational changes in the enzyme.

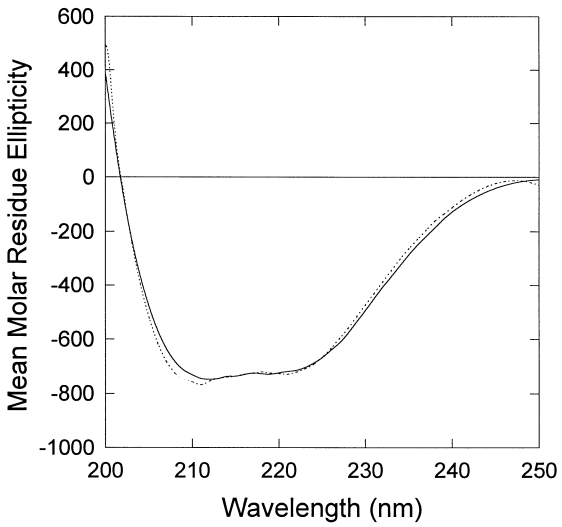

Fig. 2. The near UV circular dichroism spectra of native and NBS-treated glutamate synthase. NBS-treated GOGAT was prepared by incubating the enzyme with NBS, under the conditions described in Fig. 1, for 90 min. Both native (solid line) and NBS-treated (dotted line) glutamate synthase were present at a concentration of 0.10 μM in 0.10 mM Tricine–KOH buffer (pH 7.5).

NBS-treatment had no detectable (i.e., to within the ± 15 mV experimental uncertainty) effect on the oxidation–reduction midpoint potentials of the enzyme prosthetic groups, as determined by cyclic voltammetry [5]. Thus, the loss of enzyme activity caused by treating glutamate synthase with NBS does not arise from a significant change in thermodynamic driving force for any electron transfer steps. Qualitative assays for complex formation between glutamate synthase and ferredoxin, such as ferredoxin–Sepharose affinity chromatography of the enzyme and co-migration of the enzyme with ferredoxin during gel filtration chromatography on Ultrogel AcA 34 at low, but not high, ionic strength [7–10], indicated that NBS-treated glutamate synthase retained the native enzyme's ability to form an electrostatically-stabilized complex with ferredoxin. Quantitative estimation of ferredoxin binding to the enzyme, using spectral perturbations to monitor complex formation [7], shown in Fig. 3, demonstrated that NBS-treatment does not decrease the affinity of ferredoxin-binding to the enzyme, but actually results in a small increase in the affinity of the enzyme for oxidized ferredoxin. Double-reciprocal plots (not shown) of the binding data displayed in Fig. 3 show, for both

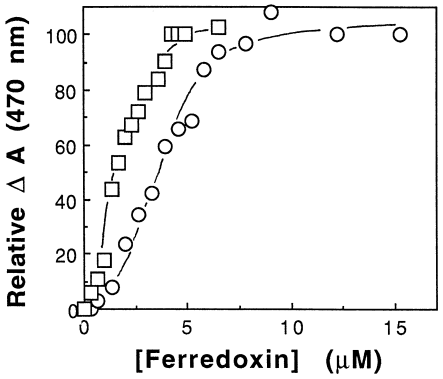

Fig. 3. The spectral perturbation titrations for the binding of spinach ferredoxin to native and NBS-treated glutamate synthase. NBS-treated GOGAT was prepared by incubating the enzyme with NBS, under the conditions described in Fig. 1, for 90 min. Native (open circles) or NBS-modified (open squares) enzyme were present in the buffer described in Fig. 1 at concentrations of 4.2 and 4.3 μM, respectively. Spinach ferredoxin was added, as a series of small aliquots from a concentrated stock solution, to give the indicated final concentrations.

native and NBS-treated glutamate synthase, show clear deviations from linearity, confirming the sigmoidal shape of the binding curves. Plots of ferredoxin-dependent activity vs. ferredoxin concentration were also sigmoidal for both native and NBS-treated glutamate synthase (data not shown). K_m values for the two substrates of the enzyme, glutamine and 2-oxoglutarate, were not affected by NBS treatment. These results strongly suggest that the inhibition of enzyme activity caused by NBS does not arise from effects on the ability of the enzyme to bind its substrates.

The shape of the tryptophan fluorescence emission spectrum of NBS-modified glutamate synthase and the position of the emission maximum are essentially identical to those observed for the native enzyme. NBS treatment decreases the magnitude of tryptophan fluorescence by only 10%, consistent with the observation that only two, out of a total of 16 tryptophan residues known to be present in the enzyme [3], are modified by NBS when the enzyme is exposed to a 2-fold excess of the modifier.

Previous flash photolysis studies carried out in our laboratories [5], in which the kinetics of reduction of the FMN and [3Fe–4S] prosthetic groups of spinach glutamate synthase by the 5-deazaflavin semiquinone radical were characterized, revealed the presence of both slow and fast phases of reduction. Second-order rate constants of 3.8×10^8 M^{-1} s^{-1} (for the fast phase) and 1.0×10^6 M^{-1} s^{-1} (for the slow phase) were measured. The fast phase was assigned to a process in which the 5-deazaflavin semiquinone, generated by a laser flash through the abstraction of a hydrogen atom from EDTA by the triplet state of 5-deazariboflavin, reduces the FMN and [3Fe–4S] centers on different glutamate synthase molecules at the same rates [5]. It was not possible to unambiguously assign the slow phase, but it was tentatively suggested that this kinetic phase arose from reduction of the FMN and [3Fe–4S] centers of the enzyme by an EDTA degradation product [5]. A kinetic pattern very similar to that described above for native glutamate synthase was observed with the NBS-treated enzyme. The rate constants obtained for the two kinetic phases with NBS-modified glutamate synthase, $3.0 \pm 0.1 \times 10^8$ M^{-1} s^{-1} and $1.3 \pm 0.1 \times 10^6$ M^{-1} s^{-1}, for the fast and slow phases, respectively, are very similar to those reported previously [5] for the native enzyme. There is, however, a significant difference between the decay kinetics of the FMN semiquinone state of glutamate synthase in the native and NBS-treated enzymes observed in these flash photolysis experiments. As shown in Fig. 4, the rapid (≤ 3 ms) initial increase in absorbance at wavelengths above 625 nm, which results from the formation of the glutamate synthase FMN semiquinone state [5], is followed by a decrease in absorbance during the subsequent 1 s, indicating the partial disappearance of the FMN semiquinone state of NBS-modified glutamate synthase during this time interval. In contrast, no appreciable disappearance of the FMN semiquinone was observed during the 200-ms period after its initial rapid formation when comparable experiments were carried out with native glutamate synthase (compare Fig. 4 from this work to fig. 5 of Ref. [5]. The longer time used in the present study was necessitated because of the use of a 2-fold smaller concentration of the NBS-treated enzyme in the current study, compared to the concentration of unmodified enzyme used in the earlier study.) Thus, although the modification of two glutamate synthase tryptophan residues by NBS does not appreciably affect either the reduction rate constants or the extent of formation of the semiquinone state of the enzyme's FMN prosthetic group (with the 5-deazariboflavin

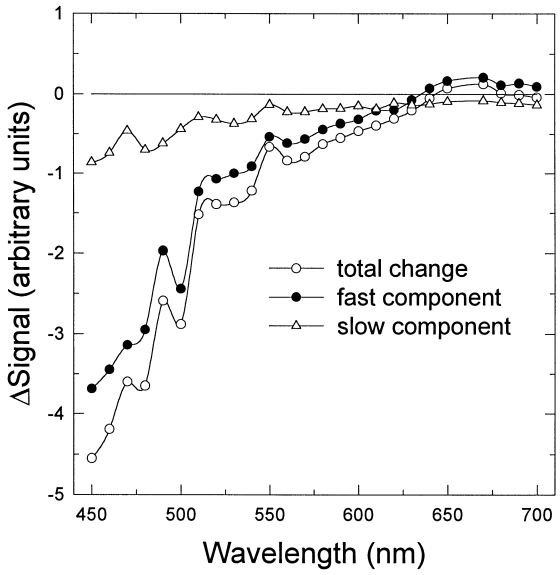

Fig. 4. The time-resolved difference spectra for the laser flash-induced reduction of NBS-modified glutamate synthase by the 5-deazariboflavin semiquinone radical. Absorbance at the indicated time minus the absorbance prior to the laser flash is plotted vs. wavelength. Differences in absorbance were recorded at 7 ms for the fast phase (closed circles) and at 1 s for the slow phase (open triangles). The total change in absorbance is indicated by open circles. The deaerated reaction mixture contained 15 μM NBS-modified glutamate synthase (prepared as described in Fig. 1), 100 μM 5-deazariboflavin and 1 mM EDTA in 4 mM potassium phosphate buffer (pH 7.7).

semiquinone serving as the electron donor), tryptophan modification does result in a destabilization of the glutamate synthase FMN semiquinone during the second kinetic phase.

The possible involvement of tryptophan, an aromatic amino acid, at the ferredoxin-binding site(s) of glutamate synthase in electron transfer from ferredoxin to the enzyme raised the question of whether aromatic amino acids on ferredoxin might also be involved in the electron transfer process. This possibility seemed particularly worth exploring because of the observation that replacement of the phenylalanine at position 65 in the ferredoxin isolated from the cyanobacterium *Anabaena* PCC7120 (a [2Fe–2S] ferredoxin with high sequence homology to spinach ferredoxin), results in a dramatic decrease in the rate of electron transfer from the cyanobacterial ferredoxin to three *Anabaena* ferredoxin-dependent enzymes, FNR [16–18,36], nitrite reductase [36] and nitrate reductase [36]. As can be seen in Fig. 5, wild-type *Anabaena* ferredoxin can serve as an electron donor for the reaction catalyzed by spinach glutamate synthase with a maximum velocity that is essentially identical to that observed with spinach ferredoxin and with a similar concentration dependence to that observed for spinach ferredoxin. The binding of wild-type *Anabaena* ferredoxin to spinach glutamate synthase, measured by determining the dependence of the magnitude of spectral perturbations on ferredoxin concentration (data not shown), is characterized by a sigmoidal absorbance difference vs. ferredoxin concentration plot that is quite similar to the one shown for spinach ferredoxin in Fig. 3. Fig. 5 shows that v_{max} obtained for the reaction catalyzed by spinach glutamate synthase with the F65A mutant of *Anabaena* ferredoxin serving as an electron donor is only 60% of that observed with wild-type *Anabaena* ferredoxin. As can also be seen from Fig. 5, the effect of this F65A substitution is not confined to a 40% decrease in v_{max}. In fact, there was a substantially larger effect on the ferredoxin concentration required to achieve half-maximal velocity, which is approximately 10-fold smaller when the F65A mutant is used than is the case when wild-type *Anabaena* ferredoxin is used. Direct binding measurements, using spectral perturbation titrations (not shown), indicated that the concentration of the F65A ferredoxin

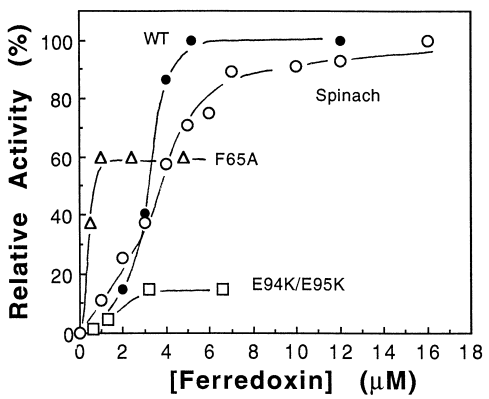

Fig. 5. The initial velocity of the reaction catalyzed by spinach glutamate synthase as a function of ferredoxin concentration for different ferredoxins. The initial velocities of the glutamate synthase catalyzed reaction, using ferredoxin as the electron donor, were measured as a function of ferredoxin concentration for spinach ferredoxin (open circles), wild-type (WT) *Anabaena* ferredoxin (closed circles) and the F65A (open triangles) and E94K/E95K (open squares) mutants of *Anabaena* ferredoxin.

Table 1
The effect of ferredoxin mutations on glutamate synthase activity

Anabaena ferredoxin	Concentration (μM)	Relative activity (%)
Wild type	10.0	100.0
	5.0	100.0
E94D	10.0	33.0
	5.0	33.0
E94Q	14.9	12.0
	7.4	6.9
E94K	10.0	15.0
	5.0	9.8
E95K	11.4	100.0
	5.7	96.9
E94K/E95K	6.6	27.5
F65W	11.0	107.2
	5.5	89.3
F65I	11.4	88.9
	5.7	69.5
F65A	4.8	66.3

mutant required for half-maximal binding to spinach glutamate synthase is approximately 7-fold lower than that observed with wild-type *Anabaena* ferredoxin. As can be seen in Table 1, substitution of a different non-aromatic amino acid, isoleucine, for the aromatic phenylalanine residue present in the wild-type ferredoxin, has a less dramatic effect on the maximal velocity, but still results in a decrease of 11% in v_{max} for this F65I mutant. A similar modest decrease in the rate of the reaction catalyzed by the ferredoxin-dependent glutamate synthase from the cynaobacterium *Synechocystis* PCC 6803 when the F65I mutant of *Anabaena* ferredoxin replaced the wild-type *Anabaena* ferredoxin has been reported [37]. The F65W mutant of *Anabaena* ferredoxin, in which one aromatic amino acid (phenylalanine) is replaced by another aromatic amino acid (tryptophan), supports the reaction catalyzed by spinach glutamate synthase with the same v_{max} as the wild-type *Anabaena* ferredoxin (Table 1). The binding of both the F65I and F65W mutants of *Anabaena* ferredoxin to spinach glutamate synthase, as measured by spectral perturbation titrations (not shown), was similar to that observed with wild-type *Anabaena* ferredoxin, so the tighter binding to the enzyme observed for the F65A ferredoxin mutant appears to be unique to this specific amino acid substitution.

In addition to its use in investigating a possible role for a specific aromatic amino acid in electron transfer from ferredoxin, site-directed mutagenesis has also been used to provide evidence that the glutamate residue at position 94 of *Anabaena* ferredoxin is essential for maximal rates of electron transfer from this ferredoxin to *Anabaena* FNR [16,18,36], to the ferredoxin-dependent *Anabaena* nitrite and nitrate reductases [36] and to the ferredoxin-dependent *Synechocystis* glutamate synthase [37]. Experiments with site-directed mutants of *Synechocystis* ferredoxin revealed a similar requirement for a glutamate at the corresponding position (E92) for optimal electron transfer to *Synechocystis* glutamate synthase [37]. The requirement for a glutamate appears to be quite specific for this position, as replacement of the adjacent E95 in *Anabaena* ferredoxin has no effect on the ability of ferredoxin to serve as an electron donor to FNR [16,18] and E94K/E95K and E94Q/E95Q [36] double [16,18] mutants of *Anabaena* ferredoxin behaved very much like the single E94K and E94Q ferredoxin mutants, respectively, in so far as electron donation to FNR and nitrite reductase were concerned. Although qualitatively similar results were obtained with *Anabaena* nitrate reductase and glutamate synthase as target enzymes, the E94Q/E95Q mutant of *Anabaena* ferredoxin gave slightly lower rates than when the E94Q single mutant as an electron donor to these two enzymes [36,37]. Similarly, a E92Q/E93Q double mutant of *Synechocystis* ferredoxin supports glutamate synthase activity at a rate only slightly lower than that observed with the E92Q single mutant of *Synechocystis* ferredoxin (E93 in *Synechocystis* ferredoxin corresponds to E95 in *Anabaena* ferredoxin). Substitution of E92 in spinach ferredoxin (isoform 1), the position equivalent to E94 in *Anabaena* ferredoxin and E92 in *Synechocystis* ferredoxin, also results in decreased rates of electron flow from reduced ferredoxin to spinach FNR [38]. Recent evidence also suggests that the corresponding amino acid in ferredoxin from the green alga *C. reinhardtii*, E91, plays an important role in electron transfer from ferredoxin to FNR, nitrite reductase, glutamate synthase and ferredoxin:thioredoxin reductase [39]. Here again, the presence of a glutamate is required at a specific position, as replacement of E92 in the *C. reinhardtii* ferredoxin had no effect on electron transfer to thioredoxin reductase [39].

Fig. 5 shows that v_{max} for the *Anabaena* E94K/E95K mutant ferredoxin is only 15% of that observed with wild-type *Anabaena* ferredoxin. The low activity makes it difficult to accurately determine the concentration of the E94K/E95K mutant required for half-maximal activity but, as can be seen from Fig. 5, this parameter appears to be quite similar for the wild-type and the E94K/E95K mutant *Anabaena* ferredoxins. Direct binding measurements (not shown), using spectral perturbation titrations, indicate little difference in binding affinity to spinach glutamate synthase between wild-type and E94K/E95K *Anabaena* ferredoxins. These results, i.e., a large decrease in v_{max} and little change in the ferredoxin concentration dependence of activity or binding, are similar to those reported previously for the E94Q mutant of *Anabaena* ferredoxin and the E92Q mutant of *Synechocystis* ferredoxin as electron donors to the *Synechocystis* glutamate synthase [37]. Table 1 also provides evidence that the requirement for a ferredoxin glutamate residue for optimal rates of the reaction catalyzed by spinach glutamate synthase is specific for position 94 (*Anabaena* numbering). While a E95K mutant of *Anabaena* ferredoxin supports the glutamate synthase-catalyzed reaction with kinetic parameters that are indistinguishable from those of wild-type *Anabaena* ferredoxin, an E94Q mutant ferredoxin can support the spinach glutamate synthase-catalyzed reaction with a v_{max} only 12% of that observed with wild-type *Anabaena* ferredoxin. It thus appears that it is not the introduction of a positive charge at position 94 that results in the large decrease in rate, as the rate observed for the E94Q mutant is slightly lower than that observed for the E94K mutant. Spectral perturbation binding studies (not shown) indicate that the E95K and E94Q *Anabaena* ferredoxins bind to spinach glutamate synthase with approximately the same affinity characteristic of the wild-type ferredoxin. In an attempt to determine whether a negative charge at position 94 of *Anabaena* ferredoxin is sufficient for wild-type rate, the properties of a E94D mutant of *Anabaena* ferredoxin, a mutation that should retain the negative charge at position 94, were examined. While spectral perturbation titrations showed no effect of this conservative substitution on ferredoxin binding to glutamate synthase, the v_{max} obtained with the E94D *Anabaena* ferredoxin was only 33% of the value obtained with wild-type ferredoxin (Table 1). At a comparable ionic strength (approximately 180 mM), the E94D mutant is approximately 50% as active as WT *Anabaena* ferredoxin in electron transfer activity with *Anabaena* FNR [18].

4. Discussion

Treatment of spinach glutamate synthase with NBS inhibits this ferredoxin-dependent enzyme in a manner quite similar to that observed previously for two other ferredoxin-dependent, spinach chloroplast enzymes, nitrite reductase [13] and FNR [14]. The results reported above for glutamate synthase support the idea that NBS is specific for tryptophan modification under the conditions used and that other glutamate synthase amino acids are not modified, to any detectable degree, under these conditions. The time course observed for the inhibition of glutamate synthase is very similar to that observed for the modification of two tryptophan residues on the enzyme (Fig. 1). While it has been demonstrated that a total of 2 mol of tryptophan are modified by NBS per mol of enzyme, it is not yet known whether this represents complete NBS modification of two residues or partial modification of more than two tryptophan residues. Distinguishing between these two possibilities would require analysis of tryptophan-containing peptides derived from glutamate synthase—a difficult task for a $M_r = 162$ kDa protein containing 16 tryptophan residues (a complete sequence of the mature form of spinach glutamate synthase has recently been obtained in our laboratory and no tryptophans are present, other than the 16 found in the partial sequence reported in Ref. [3]). It should be further noted that the data presented above do not allow one to distinguish between the possibility that the 85% inhibition caused by NBS arises entirely from the modification of a single tryptophan residue, with the modification of an additional tryptophan (or partial modification of additional tryptophans) with identical kinetics being a fortuitous coincidence, or whether this extent of inhibition requires modification of two tryptophans. Despite these uncertainties, the very similar time courses demonstrated for inhibition and tryptophan modification, and the observation that

ferredoxin protects (at low, but not at high ionic strength) the enzyme against both the inhibition and the tryptophan modification that result from exposure to NBS, are clearly consistent with the hypothesis that tryptophan modification is the cause of the inhibition of glutamate synthase by NBS and that the modified tryptophan residues are located at the ferredoxin binding site(s) of glutamate synthase.

One simple, attractive hypothesis that would explain the data obtained in this study would place one essential tryptophan residue at each of two ferredoxin-binding sites on glutamate synthase. However, it should be pointed out that the question of whether glutamate synthase contains one or two high-affinity binding sites for ferredoxin is a subject of some disagreement in the literature. Although ultrafiltration and cross-linking experiments carried out in our laboratory are most consistent with the presence of two ferredoxin-binding sites on the spinach enzyme [8,9], data obtained from cross-linking experiments with the very similar *Synechocystis* glutamate synthase have been interpreted in terms of a single binding site for ferredoxin on the enzyme [37]. Furthermore, all of the other ferredoxin-dependent enzymes for which this question has been investigated, i.e., FNR, nitrite reductase and thioredoxin reductase, contain only a single high-affinity binding site for ferredoxin [1]. Nevertheless, the sigmoidal binding curves observed for complex formation between ferredoxin and spinach glutamate synthase presented in this study (Fig. 3) are more consistent with the presence of two binding sites for ferredoxin on the enzyme than for one binding site. An attempt to identify two putative ferredoxin-binding sites on spinach glutamate synthase using sequence comparisons between tryptophan-containing portions of spinach glutamate synthase failed to identify any two tryptophan-containing regions with significant sequence homologies to one another. Attempts to find sequence homologies between tryptophan-containing portions of spinach glutamate synthase and the tryptophan-containing regions of spinach FNR and nitrite reductase were also unsuccessful.

The detailed comparison of native and NBS-modified glutamate synthase, reported above, makes it unlikely that the inhibition caused by NBS arises from any large conformational change in the enzyme. Oxidation–reduction titrations make it unlikely that the inhibition caused by NBS arises from a modification in either the 1-electron E_m value of the enzyme's [3Fe–4S] cluster or the 2-electron E_m value of the FMN group of the enzyme (E_m values are not available for either of the two 1-electron processes involving the FMN semiquinone oxidation state for either the native or the NBS-treated enzyme). NBS-treated glutamate synthase does show a slightly increased affinity for oxidized ferredoxin, compared to that of the native enzyme, and thus, it is possible that the inhibition caused by NBS treatment could arise from a less favorable release of the oxidized ferredoxin product from the NBS-modified enzyme. However, the size of the increase in the affinity of glutamate synthase for product, caused by NBS treatment, is rather small when compared to the 85% decrease in turnover number seen with the NBS-treated enzyme.

The possibility that aromatic amino acids, such as tryptophan, can play a role in facilitating electron transfer in proteins has been extensively discussed [40] and it is possible that the loss of enzyme activity (with either reduced ferredoxin or reduced methyl viologen serving as the electron donor) which accompanies the tryptophan modification caused by NBS treatment, arises from an effect on electron transfer per se. The contrast between the effect of NBS, which inhibits the ferredoxin-linked and methyl viologen-linked activities of the enzyme equally, to that observed with lysine-modifying and arginine-modifying reagents, which inhibit only the ferredoxin-linked activity [9], is consistent with such an interpretation. Furthermore, the flash photolysis experiments reported above, which reveal an effect of NBS treatment on the kinetic stability of the semiquinone state of the enzyme's FMN prosthetic group, raise the possibility that a glutamate synthase tryptophan residue may play some as yet undefined role in an electron transfer reaction involving the FMN prosthetic group of the enzyme. However, given our present incomplete knowledge of the detailed mechanism by which glutamate synthase functions and the absence of any information on the tertiary structure of the enzyme, it is clearly premature to ascribe an obligatory role for tryptophan in a direct electron transfer step of the reaction.

Only modest decreases in v_{max} for the reaction catalyzed by spinach glutamate synthase were observed with F65A and F65I mutants of *Anabaena*

ferredoxin (v_{max} values were, 60% and 89%, respectively, compared to those obtained with wild-type *Anabaena* ferredoxin). This observation, and the fact that a conservative mutant that retains an aromatic amino acid at this position (F65W) shows kinetic and binding parameters indistinguishable from those of the wild-type protein, suggest that while an aromaticity at this position may perhaps contribute slightly to an optimal overall rate, there is certainly no absolute requirement for an aromatic amino acid at this ferredoxin position in the ferredoxin-dependent reaction catalyzed by glutamate synthase. The observation that the substitution of the non-aromatic alanine (but not isoleucine) for phenylalanine at this position in ferredoxin causes decreases in the ferredoxin concentrations required for both half-maximal activity and half-maximal binding to glutamate synthase by approximately one order of magnitude was unanticipated, given earlier evidence that ferredoxin binding to the enzyme appeared to be largely electrostatic in nature [1]. It is possible that the size of the amino acid side chain at position 65 may play a significant role in electron transfer, with a small side chain decreasing the overall rate by decreasing k_{off} for oxidized ferredoxin. Van der Waal's volumes for the side chain groups of phenylalanine (135 Å^3), isoleucine (124 Å^3), and tryptophan (163 Å^3), which are reasonably similar to each other but are considerably larger than that of alanine (67 Å^3), are consistent with such an interpretation. The observation that the oxidized form of the F65A ferredoxin mutant (but not the F65I mutant) binds more tightly to glutamate synthase than does wild-type *Anabaena* ferredoxin is also consistent with the hypothesis that a steric effect that increases the binding affinity of oxidized ferredoxin for the enzyme could lower the overall rate by decreasing a rate-limiting product release step. However, a larger series of position 65 mutants would have to be examined before any definitive conclusion about steric effects can be reached. The results reported above are qualitatively similar to those observed when the F65I mutant of *Anabaena* ferredoxin and the F63A mutant of *Synechocystis* ferredoxin were used as electron donors in the reaction catalyzed by *Synechocystis* glutamate synthase [37], although in the study of the *Synechocystis*, enzyme rates were measured only at a single ferredoxin concentration (F.J. Florencio and H. Böhme, personal communication) so that it was not possible to unambiguously separate effects on v_{max} from those on K_m.

The results reported above for the effects of mutations that eliminate the glutamates at positions 94 and 95 on *Anabaena* ferredoxin on the ability of this ferredoxin to serve as an electron donor for spinach glutamate synthase strongly suggest that a glutamate at position 94, but not at position 95, is required for optimal electron transfer to the enzyme. The conservative mutation, E94D, caused similar decreases in activity toward spinach glutamate synthase (a 67% decrease in v_{max}) and *Anabaena* FNR (a 50% decrease in electron transfer activity, Ref. [18]). Experiments with E/D ferredoxin mutants have not been carried out with systems other than *Anabaena* ferredoxin/*Anabaena* FNR and *Anabaena* ferredoxin/spinach glutamate synthase, so it is not yet known whether the spatial requirements for the location of the negative charge at the position equivalent to position 94 in *Anabaena* ferredoxin will be similar for any other ferredoxin-dependent enzymes to those for glutamate synthase and FNR.

The results obtained from all studies to date using *Anabaena* ferredoxin mutants point to the importance of a glutamate residue at position 94 for optimal electron transfer to a number of ferredoxin-dependent enzymes ([16–18,36,37]). Furthermore, the glutamate at the equivalent positions in ferredoxins from the cyanobacterium *Synechocystis* [38], from the green alga *C. reinhardtii* [39] and from spinach chloroplasts [38] also appears to be important for optimal electron transfer to ferredoxin-dependent enzymes such as FNR, nitrite reductase, glutamate synthase and thioredoxin reductase. Non-conservative mutations at this position can increase the E_m value of spinach [41] and *Anabaena* [42] ferredoxin significantly, and this change in E_m may account, at least in part, for the effect of mutations on the rate of FNR-catalyzed electron transfer from NADPH to ferredoxin [36,38,41]. However, a detailed analysis of the properties of a large number of *Anabaena* ferredoxin mutants suggests that the change in E_m does not appear to cause the decreases in the rate of electron transfer from ferredoxin to FNR observed for [42].

What is perhaps somewhat surprising, considering the widely accepted view that negative charges on ferredoxin are involved in stabilizing the electrostatic complexes between ferredoxin and the enzymes for

which ferredoxin serves as the electron donor [1], are the observations that removing this negative charge on ferredoxin has much greater effects on the turnover number for electron transfer than it has on binding affinity or on the ferredoxin concentration required for half-maximal activity [16,18,37–39,42]. These observations would suggest that this negative charge plays a role in protein–protein orientation within the transient electron-transfer complex [42,43], rather than in complex stabilization.

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