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The role of lysine and arginine residues at the ferredoxin-binding site of spinach glutamate synthase

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Treatment of ferredoxin-dependent, spinach glutamate synthase with either the arginine-modifying reagent phenylglyoxal or the lysine-modifying reagents *N*-acetylsuccinimide and dansyl chloride resulted in a significant loss of enzymatic activity when the physiological electron donor, reduced ferredoxin, was used as the electron-donating substrate. In contrast, the reagents caused no inhibition of enzyme activity when the non-physiological reductant, reduced methyl viologen, was used as the electron donor. Formation of an electrostatically-stabilized complex between glutamate synthase and ferredoxin prior to exposure of the enzyme to phenylglyoxal or *N*-acetylsuccinimide protected the enzyme against the loss of ferredoxin-dependent activity caused by either modifying agent. Treatment of glutamate synthase with either reagent resulted in a loss of ferredoxin-binding capacity, as assayed by affinity chromatography, gel filtration, spectral perturbations and by the ability of the enzyme to form an active cross-linked complex with ferredoxin. Absorbance and circular dichroism spectra indicated that neither of the modifying reagents produced major conformational changes in the enzyme. These results have been interpreted in terms of a ferredoxin-binding site on glutamate synthase, similar to those found on other ferredoxin-dependent chloroplast enzymes, that contains both lysine and arginine residues.

Introduction

Ferredoxin-dependent glutamate synthase (EC 1.4.7.1), found in the chloroplasts of plants and algae, catalyzes the reductive conversion of glutamine *plus* 2-oxoglutarate to two glutamates and uses reduced ferredoxin to supply the two electrons required for this reaction [1]. A monomeric form of the enzyme, which is located in the chloroplast stroma [2], has been purified to homogeneity from spinach [3] and the green alga *Chlamydomonas reinhardtii* [4] and shown to contain one FMN, one FAD and one [3Fe-4S] cluster per approx. 160 kDa monomer [3,5–7]. In contrast, a 156 kDa, ferredoxin-dependent glutamate synthase has recently been purified from the cyanobacterium *Synechococcus* sp. PCC 6301 and shown to contain one FMN but no FAD [8]. The gene encoding the ferredoxin-dependent glutamate synthase of maize has been cloned and sequenced and a relative molecular mass of 165 298 has been calculated for the mature protein [9]. A flavin-binding domain can be recognized in the maize enzyme sequence [9] and the cysteine spacings in the deduced amino-acid sequence are those expected for a [3Fe-4S] cluster [6,9]. Work in our laboratory (Nalbantoglu, B., Moomaw, C., Hsu, J., Hirasawa, M., Nguyen, H.T., Knaff, D.B. and Allen, R.D., unpublished observations) has identified a 3.8 kb clone in a spinach cDNA library that codes for a large portion of the spinach ferredoxin-dependent glutamate synthase. Amino-acid sequencing of peptides derived from the purified spinach enzyme, plus DNA sequencing of this 3.8 kb clone and of a PCR product that contains a portion of the 5′ region of the gene, indicate that the spinach and maize enzymes are approx. 85% homologous at the amino-acid level.

Glutamate synthase, like several other ferredoxin-dependent enzymes located in plant chloroplasts, forms an electrostatically-stabilized complex with ferredoxin [1,10–12]. In the best-studied of these systems, involving complex formation between ferredoxin and ferredoxin:$NADP^+$ oxidoreductase (EC 1.18.1.2, hereafter abbreviated FNR), a variety of experimental techniques [1] and computer modeling studies [13,14] have established that ferredoxin supplies the negatively charged groups and FNR supplies the positively

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Abbreviations: CD, circular dichroism; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; FNR, ferredoxin:$NADP^+$ oxidoreductase; PMSF, phenylmethylsulfonyl fluoride; SDS sodium dodecylsulfate.

charged groups involved in complex formation. Chemical modification [10] and immunological studies [11,12,15,16] indicate that the ferredoxin-binding sites on FNR, glutamate synthase and ferredoxin:nitrite oxidoreductase (EC 1.7.7.1, hereafter referred to as nitrite reductase) are chemically similar. It has been shown that treatment of both FNR [17–21] and nitrite reductase [22] with reagents that modify either lysine or arginine residues inhibits ferredoxin binding by these two enzymes. Thus, it was of interest to determine whether treatment of glutamate synthase with these reagents would also affect the capacity of this third ferredoxin-dependent enzyme to bind its physiological, electron donating substrate. We report below that both the arginine-modifying reagent phenylglyoxal and the lysine-modifying reagents *N*-acetylsuccinimide and dansyl chloride diminish ferredoxin-binding by spinach glutamate synthase, apparently without affecting either binding of the other substrates of the enzyme or catalysis. These results have been interpreted in terms of the presence of at least one arginine and one lysine residue at the ferredoxin-binding site of glutamate synthase.

Materials and Methods

Spinach (*Spinacia oleracea*) leaves were purchased from local markets during the 1992 growing season. Spinach leaf ferredoxin ($A_{422\ nm}/A_{277\ nm} = 0.45$) was purified as described previously [23] and stored in 30 mM Tris-HCl buffer (pH 8.0) at liquid nitrogen temperature until used. Ferredoxin concentrations were estimated from the absorbance at 422 nm using an extinction coefficient of 9.7 $mM^{-1}\ cm^{-1}$ [24]. Spinach leaf glutamate synthase was purified as described previously [11]. The purified protein was concentrated using an Omega-cell ultrafiltration membrane (molecular mass cut-off = 100 kDa) and stored in 100 mM Tricine-KOH buffer (pH 7.5) containing 200 mM NaCl, 1 mM 2-oxoglutarate and 0.1% β-mercaptoethanol at liquid nitrogen temperature. The enzyme appeared to be homogeneous, as indicated by the presence of a single Coomassie blue-staining band (160 kDa) after polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS) and a single Coomassie Blue-staining band with $pI = 5.2$ after isoelectric focusing, and had a specific activity [3] of 90 units/mg protein. Antibodies raised against spinach glutamate synthase were prepared as described previously [11]. Antibodies raised against spinach ferredoxin were a gift from Dr. Richard Malkin (University of California, Berkeley). Western blots and Ouchterlony immunodouble diffusion experiments were performed as described previously [11,12].

Dansyl chloride, phenylglyoxal, 2,3-butanedione and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were purchased from Sigma Chemical Co. *N*-Acetylsuccinimide was purchased from ICN Biochemicals. Molecular mass standards for gel filtration and polyacrylamide gel electrophoresis were purchased from Bio-Rad and Sigma Chemical Co., respectively. Isoelectric focusing standards ($pI = 2.80$ to 6.55) were purchased from Pharmacia-LKB. Ouchterlony immunodouble diffusion plates were purchased from Cooper Biomedical. All other reagents were of the highest purity available.

Glutamate synthase activities with either reduced ferredoxin or reduced methyl viologen as the electron donor were measured as described previously [3]. Protein concentrations were determined according to the method of Bradford [25], using bovine serum albumin as a standard. Absorbance and circular dichroism (CD) spectra were measured at ambient temperature using a Shimadzu UV/V 2100 spectrophotometer and a JASCO Model J-20 spectropolarimeter, respectively. Difference spectra were obtained as described previously [26]. Molecular masses were determined under non-denaturing conditions by gel filtration on an Ultrogel AcA 34 column (1.5 × 80 cm) in 100 mM Tricine-KOH buffer (pH 7.5) containing 200 mM NaCl, 1 mM 2-oxoglutarate and 0.1% β-mercaptoethanol, according to the method of Andrews [27]. The affinity chromatography matrix with ferredoxin-coupled to Sepharose 4B was prepared as described by Shin and Oshino [28]. Gel electrophoresis in the absence or presence of SDS was performed by modifications of the procedures of Davis [29] and Laemmli [30], respectively, on a Pharmacia Phastsystem using directions supplied by the vendor. Isoelectric focusing was performed on the Pharmacia Phastsystem, using a modification of the procedure of Righetti and Drysdale [31].

Cross-linking of ferredoxin to glutamate synthase was carried out using the procedure described by Privalle et al. for cross-linking ferredoxin to nitrite reductase [32], modified as described previously [12]. The activity of the cross-linked complex was assayed, in the absence of free ferredoxin, as described previously [12]. Binding studies using membrane ultrafiltration were conducted using an Amicon 10 ml-capacity stirred cell and an Omega Series membrane with a 100 kDa cut-off, essentially as described previously [11].

Results

Fig. 1A shows that treating glutamate synthase with the arginine-modifying reagent phenylglyoxal at 4°C for 80 min resulted in an approx. 70% inhibition of enzyme activity when reduced ferredoxin was used as the electron donor, without any detectable inhibition of activity when reduced methyl viologen was used as the electron donor. Longer incubation times, up to 18 h, produced only a small additional inhibition of the ferredoxin-linked activity but did not affect the methyl

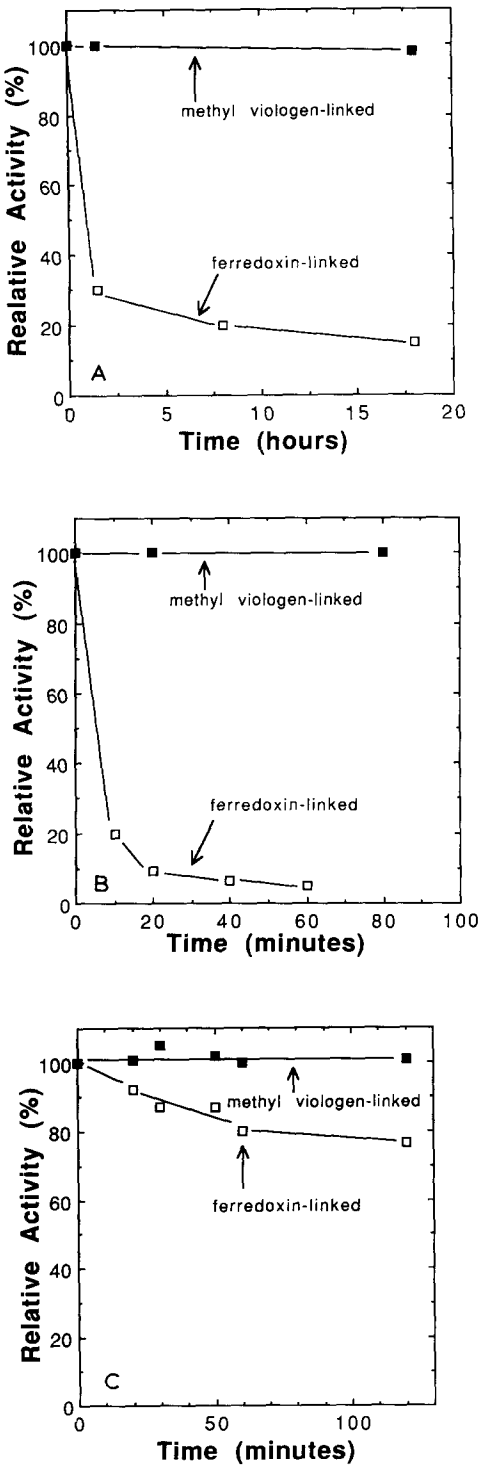

Fig. 1. The effect of chemical modification on the activity of spinach glutamate synthase. (A) Glutamate synthase (final concentration = 500 μM) was mixed with phenylglyoxal (final concentration = 40 mM) in 10 mM Tricine-KOH buffer (pH 7.5) containing 200 mM NaCl, 1 mM 2-oxoglutarate and 0.1% β-mercaptoethanol. At the indicated times, aliquots were diluted approx. 1000-fold into the same buffer and subsequently assayed for activity as described in Materials and Methods. (B) Conditions were as in (A), but *N*-acetylsuccinimide replaced phenylglyoxal. (C) Conditions were as in (A), but dansyl chloride replaced phenylglyoxal. 100% corresponds to specific activities of 90 units/mg for the ferredoxin-linked activity and 14.1 units/mg for the methyl viologen-linked activity.

viologen-linked activity (Fig. 1A). Treatment of glutamate synthase with the lysine-modifiying reagent *N*-acetylsuccinimide for only 10 min at 4°C produced approx. 80% inhibition of the ferredoxin-linked activity of the enzyme without affecting the methyl viologen-linked activity (Fig. 1B). Increasing the incubation time of the enzyme with *N*-acetylsuccinimide increased the extent of inhibition of ferredoxin-linked activity to approx. 95% (with no detectable inhibition of the methyl viologen-linked activity), despite the limited stability of the modifying reagent in aqueous solutions [33]. Treatment of glutamate synthase with 2,3-butanedione, an arginine-modifying reagent that inhibits ferredoxin-binding by FNR [19], affected neither the ferredoxin-linked nor the methyl viologen-linked activities of glutamate synthase (data not shown). Treatment of glutamate synthase with dansyl chloride, a lysine-modifying reagent that inhibits ferredoxin-binding by FNR [17,18], produced a small, but reproducible and significant, inhibition (typically near 20%) in the ferredoxin-linked activity of glutamate synthase without affecting the methyl viologen-linked activity of the enzyme (Fig. 1C). As the effects on the ferredoxin-linked activity of glutamate synthase obtained by treatment with phenylglyoxal and *N*-acetylsuccinimide were substantially greater than those observed after dansyl chloride treatment, the dansyl chloride-treated enzyme was not characterized further.

The observation that treatment of glutamate synthase with either phenylglyoxal or *N*-acetylsuccinimide produced substantial inhibition of the ferredoxin-linked activity of the enzyme without affecting activity when the non-physiological electron donor, reduced methyl viologen, replaced ferredoxin suggested that treatment with both of these reagents modified amino acids at the ferredoxin-binding site of glutamate synthase. Evidence in support of this hypothesis comes from the observation that preincubation of glutamate synthase with ferredoxin at low ionic strength, which results in complex formation between the two proteins [1,10,23], completely protected the enzyme against the loss of ferredoxin-linked activity caused by subsequent treatment with either phenylglyoxal or *N*-acetylsuccinimide (data not shown). In contrast, preincubation of glutamate synthase with the substrate 2-oxoglutarate produced no protection against the loss of ferredoxin-dependent activity of glutamate synthase that results from treatment with either phenylglyoxal or *N*-acetylsuccinimide. As it is likely that 2-oxoglutarate and ferredoxin bind at different sites on the enzyme, the substrate-protection effect appears to be specific to the ferredoxin-binding site.

In order to test more directly whether treatment of glutamate synthase with the two modifying agents affects the ferredoxin-binding site of the enzyme, the capacity of the phenylglyoxal-modified and *N*-acetyl-

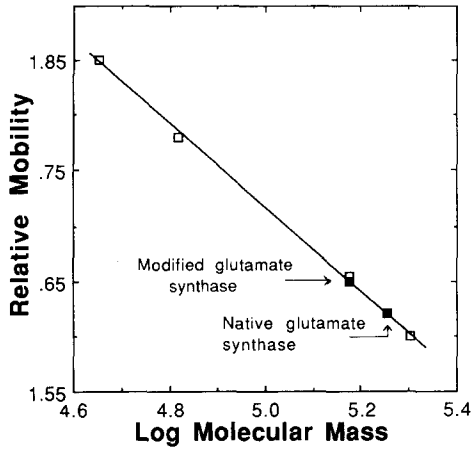

Fig. 2. Gel filtration chromatography of native and *N*-acetylsuccinimide-treated glutamate synthase in the presence of ferredoxin. Gel filtration was performed on an Ultrogel AcA 34 column (1.5 × 80 cm) equilibrated with 10 mM Tricine-KOH buffer (pH 7.5) containing 1 mM 2-oxoglutarate and 0.1% β-mercaptoethanol. The closed squares represent experiments in which 50 nmol of ferredoxin was chromatographed with 25 nmol of either unmodified glutamate synthase or enzyme that had been treated with *N*-acetylsuccinimide for 4 h at 4°C. The open squares represent molecular mass standards: Ovalbumin (45 kDa); bovine serum albumin (66 kDa); alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa).

succinimide-modified enzymes to bind ferredoxin was tested using a number of different ferredoxin-binding assays. It was first demonstrated that neither the phenylglyoxal-treated enzyme nor the *N*-acetylsuccinimide-treated enzyme was retained at low ionic strength by an affinity matrix containing ferredoxin covalently coupled to Sepharose 4B (data not shown), although we have confirmed the observation [3] that native glutamate synthase does bind to the ferredoxin affinity matrix under these conditions. Fig. 2 shows that glutamate synthase modified by treatment with *N*-acetylsuccinimide does not co-migrate with ferredoxin during gel filtration chromatography at low ionic strength. The apparent molecular mass of the *N*-acetylsuccinimide-modified glutamate synthase during gel filtration chromatography in the presence of ferredoxin at low ionic strength is 160 kDa, indistinguishable from that of glutamate synthase itself. Results similar to those observed with the *N*-acetylsuccinimide-modified enzyme were obtained with the phenylglyoxal-modified enzyme, which also did not co-chromatograph with ferredoxin at low ionic strength (data not shown). In contrast, as had been demonstrated previously [10], the unmodified enzyme does co-migrate with ferredoxin under these conditions. Fig. 2 shows that the apparent molecular mass of native glutamate synthase during gel flitration chromatography in the presence of ferredoxin at low ionic strength is 180 kDa, consistent with co-chromatography of glutamate synthase and ferredoxin as a 2:1 complex. We had previously demonstrated that complex formation between ferredoxin and glutamate synthase can be monitored by an ultrafiltration assay [11] and by perturbations in the absorbance and CD spectra of the two proteins that result from their interaction [10,11]. Both the phenylglyoxal-treated enzyme and the *N*-acetylsuccinimide-treated enzyme fail to bind ferredoxin in the ultrafiltration assay and produced only extremely small spectral changes, less than 10% of those observed with native glutamate synthase, when mixed with ferredoxin at low ionic strength (data not shown). Treatment of glutamate synthase with either reagent completely eliminated the changes in CD spectra, indicative of complex formation, observed when the native enzyme is mixed with ferredoxin at low ionic strength (data not shown). The simplest interpretation of all these observations is that treatment of glutamate synthase with either *N*-acetylsuccinimide or phenylglyoxal does, in fact, eliminate or greatly diminish the ability of the enzyme to bind ferredoxin.

We had previously demonstrated that ferredoxin could be cross-linked to glutamate synthase by EDC and that the cross-linked complex (which appears to contain two ferredoxins covalently linked to glutamate synthase) is enzymatically active [12]. If the action of EDC is simply to produce covalent linkages between carboxyl groups on ferredoxin and amino groups on glutamate synthase that are involved in formation of the electrostatically stabilized (presumably physiological) noncovalent complex, one might expect that the phenylglyoxal-modifiable arginine(s) and the *N*-acetylsuccinimide-modifiable lysine(s) at the ferredoxin-binding site of the native enzyme would be inaccessible to modification in the cross-linked ferredoxin/glutamate synthase complex. Furthermore, if, as argued above, the only effect of treating glutamate synthase with either phenylglyoxal or *N*-acetylsuccinimide is to modify groups involved in binding ferredoxin, it would be expected that these modifying reagents would have no effect on the cross-linked complex which contains ferredoxin already covalently anchored at its physiological binding site. These predictions appear to be correct, as neither reagent had any appreciable inhibitory effect on the activity of the cross-linked complex (Fig. 3). Furthermore, pre-treatment of glutamate synthase with either phenylglyoxal or *N*-acetylsuccinimide eliminated the ability of the enzyme to form a cross-linked adduct with ferredoxin in the presence of EDC. This is illustrated for *N*-acetylsuccinimide in Fig. 4, which shows that the apparent molecular mass of glutamate synthase that had been treated with *N*-acetylsuccinimide prior to incubation with ferredoxin and EDC is 150 kDa, a value identical, within the experimental uncertainties, to that of glutamate synthase itself. In contrast, as we had demonstrated previously [12], the apparent molecular mass after incubation of the native enzyme with ferredoxin

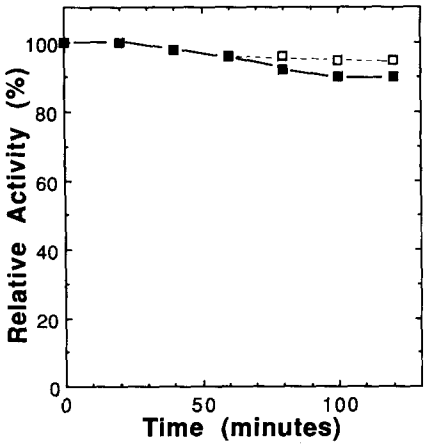

Fig. 3. The effect of *N*-acetylsuccinimide and phenylglyoxal on the cross-linked ferredoxin/glutamate synthase complex. The cross-linked complex, prepared as described in Materials and Methods, was mixed with a 200-fold excess of either *N*-acetylsuccinimide (open squares) or phenylglyoxal (closed squares) and incubated at 4°C. At the times indicated, aliquots were withdrawn and the activity of the cross-linked complex assayed in the absence of free ferredoxin as described in Materials and Methods.

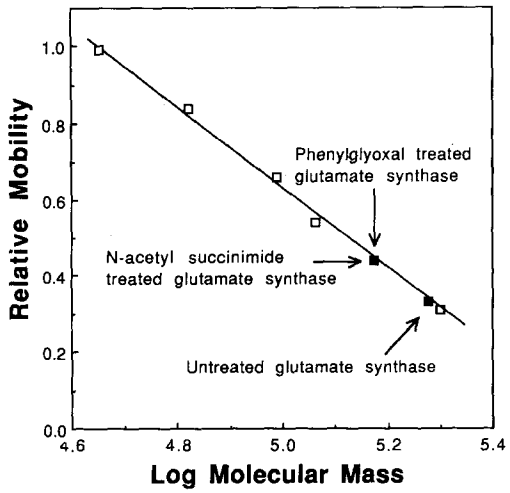

Fig. 4. Gel electrophoresis of cross-linking mixtures of ferredoxin with either native or modified glutamate synthase. Glutamate synthase was incubated with either *N*-acetylsuccinimide or phenylglyoxal for 4 h at 4°C and then centrifuged in an Amicon Centricon tube (cut-off 30 kDa) to remove excess modifying reagent. Native glutamate synthase and the two modified enzymes, all at concentrations of 5 μM, were then incubated with 20 μM ferredoxin and 5 mM EDC in 10 mM potassium phosphate buffer (pH 7.0) for 18 h. The cross-linking reaction mixtures were then subjected to electrophoresis and stained for protein with Coomassie Blue (closed squares). Molecular mass standards used (open squares) were: ovalbumin (45 kDa); bovine serum albumin (66 kDa); phosphorylase *b* (97.4 kDa); β-galactosidase (116.3 kDa) and myosin (200 kDa).

and EDC is 180 kDa (Fig. 4), the value expected for a 2:1 cross-linked complex of ferredoxin and glutamate synthase. We have confirmed our previous observations [12], using Western blots, that this 180 kDa cross-linked product is recognized by an antibody raised against glutamate synthase and have also demonstrated, using Western blots, that the cross-linked complex is recognized by an antibody raised against ferredoxin (data not shown). The results of these cross-linking experiments provide further evidence that the groups on glutamate synthase modified by phenylglyoxal and *N*-acetylsuccinimide are essential for ferredoxin binding by the enzyme.

Western blots and Ouchterlony immunodouble diffusion experiments showed that an antibody raised against glutamate synthase recognized both the phenylglyoxal-modified enzyme and the *N*-acetylsuccinimide-

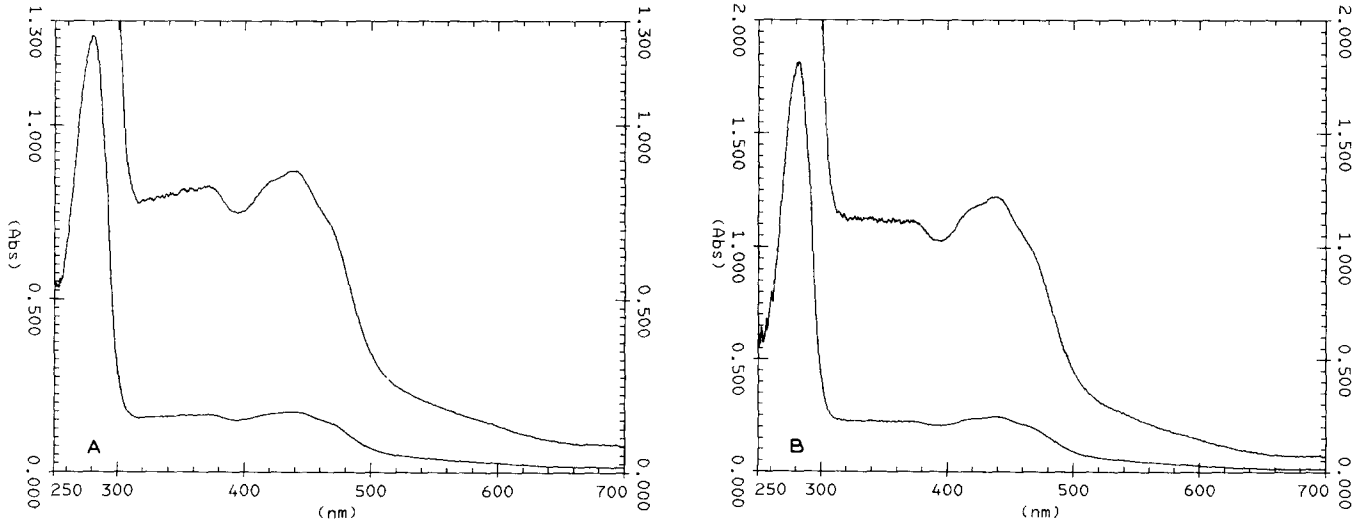

Fig. 5. Absorbance spectra of chemically-modified glutamate synthase. Glutamate synthase was incubated with a 200-fold excess of either *N*-acetylsuccinimide (A) or phenylglyoxal (B) for at 4°C in 100 mM Tricine-KOH buffer containing 200 mM NaCl, 1 mM 2-oxoglutarate and 0.1% β-mercaptoethanol. After 4 h, the reaction mixtures were diluted 100-fold with the same buffer and centrifuged in an Amicon Centricon tube (cut-off 30 kDa) to remove excess modifying reagent. Spectra were recorded vs. buffer blanks at enzyme concentrations of 3.1 μM (A) and 4.5 μM (B). The upper traces in both (A) and (B) show the spectra at 5-fold higher sensitivity.

modified enzyme, indicating that neither of the modifying reagents affects the major antigenic epitope(s) of glutamate synthase. Treatment of glutamate synthase with phenylglyoxal or *N*-acetylsuccinimide at 4°C for 2 h had no effect on the p*I* of the enzyme. In both cases the modified enzymes exhibited single Coomassie Blue-staining bands with p*I* = 5.2, the same value observed for the native enzyme. These results suggest that relatively few arginine groups and lysine groups on glutamate synthase are modified by phenylglyoxal and *N*-acetylsuccinimide, respectively, under conditions where only the ferredoxin-linked activity of the enzyme is inhibited. Prolonged incubation of glutamate synthase with *N*-acetylsuccinimide converted most of the enzyme to a form with p*I* = 3.9, while prolonged exposure to phenylglyoxal produced forms of the enzyme with p*I* = 4.3 and 4.8, in addition to a p*I* = 5.3 form. Treatment of glutamate synthase with *N*-acetylsuccinimide, even for periods as long as 4 h, had virtually no effect on the absorbance spectrum of the enzyme. The absorbance spectrum of the *N*-acetylsuccinimide-treated enzyme, shown in Fig. 5A, is essentially identical to that of the native enzyme [3]. Treatment of glutamate synthase with phenylglyoxal for 4 h at 4°C did produce a very small bleaching in the absorbance feature at 360 nm (Fig. 5B). The CD spectra of glutamate synthase treated with either phenylglyoxal or *N*-acetylsuccinimide for 4 h at 4°C both exhibited positive features at 339, 388 and 435 nm and negative features at 206, 216, 485 and 555 nm, essentially identical in magnitude and wavelength to those seen in the CD spectrum of the native enzyme [11], indicating that treatment with either modifier under conditions where only the ferredoxin-linked activity of the enzyme is affected does not produce major conformational changes in the enzyme.

Discussion

Chemical modifications of enzymes have been widely used in elucidating various aspects of enzyme mechanisms [34] and have proven useful in identifying amino acids likely to be involved in ferredoxin binding by ferredoxin-dependent plant enzymes [1,22,35]. Previous chemical modification studies and immunological data all support the hypothesis that at least three chloroplast enzymes that use reduced ferredoxin as an electron donor (i.e., FNR, nitrite reductase and glutamate synthase) have antigenically related, positively charged binding sites for ferredoxin [1,22,35]. Site-directed mutagenesis is becoming an increasingly powerful tool for identifying amino acids essential to specific enzyme functions and has been applied successfully to the ferredoxin-dependent enzyme FNR [36]. While site-directed mutagenesis has an advantage of specificity, compared to chemical modification, both phenylglyoxal [37] and *N*-acetylsuccinimide [38] have been demonstrated to show good selectivity in exclusively modifying arginine and lysine, respectively [34]. In any event, for enzymes such as the ferredoxin-linked glutamate synthase from spinach, for which the gene has not yet been fully characterized, chemical modification remains a useful alternative to site-directed mutagenesis in conducting structure/function investigations. We have reported evidence (see above), based on chemical modification procedures, that provides considerable support for the presence of at least one arginine and one lysine residue at the ferredoxin-binding site of glutamate synthase. We do not yet have evidence for the number of lysine and arginine residues, respectively, that are modified under conditions where treatment with *N*-acetylsuccinimide or phenylglyoxal inhibits ferredoxin-binding by glutamate synthase without apparently affecting other enzyme functions. However, the observation that the p*I* values of the phenylglyoxal-modified and *N*-acetylsuccinimide-modified enzymes did not differ appreciably from that of the native enzyme (see above) suggests that relatively few residues were modified, despite the fact that the modifying reagents were used at 80-fold to 200-fold excesses over glutamate synthase. Once the complete DNA sequence of the gene encoding the ferredoxin-linked, spinach glutamate synthase has been determined (see above) and its amino-acid sequence deduced, experiments can be designed to determine the location of the lysine and arginine residues modified under conditions where ferredoxin binding by the enzyme is specifically affected. We are currently in the process of completing the sequencing and designing such experiments.

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