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Complex formation between ferredoxin and *Synechococcus* ferredoxin:nitrate oxidoreductase

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Abstract

The ferredoxin-dependent nitrate reductase from the cyanobacterium *Synechococcus* sp. PCC 7942 has been shown to form a high-affinity complex with ferredoxin at low ionic strength. This complex, detected by changes in both the absorbance and circular dichroism (CD) spectra, did not form at high ionic strength. When reduced ferredoxin served as the electron donor for the reduction of nitrate to nitrite, the activity of the enzyme declined markedly as the ionic strength increased. In contrast, the activity of the enzyme with reduced methyl viologen (a non-physiological electron donor) was independent of ionic strength. These results suggest that an electrostatically stabilized complex between *Synechococcus* nitrate reductase and ferredoxin plays an important role in the mechanism of nitrate reduction catalyzed by this enzyme. Treatment of *Synechococcus* nitrate reductase with either an arginine-modifying reagent or a lysine-modifying reagent inhibited the ferredoxin-dependent activity of the enzyme but did not affect the methyl viologen-dependent activity. Treatment with these reagents also resulted in a large decrease in the affinity of the enzyme for ferredoxin. Formation of a nitrate reductase complex with ferredoxin prior to treatment with either reagent protected the enzyme against loss of ferredoxin-dependent activity. These results suggest that lysine and arginine residues are present at the ferredoxin-binding site of *Synechococcus* nitrate reductase. Results of experiments using site-specific, charge reversal variants of the ferredoxin from the cyanobacterium *Anabaena* sp. PCC 7119 as an electron donor to nitrate reductase were consistent with a role for negatively charged residues on ferredoxin in the interaction with *Synechococcus* nitrate reductase.

Keywords: Nitrate reductase; Ferredoxin-binding; Electrostatic interaction

1. Introduction

The assimilation of inorganic nitrogen compounds by oxygenic photosynthetic organisms begins (with the exception of nitrogen fixation) with the two-electron reduction of nitrate to nitrite, catalyzed by nitrate reductase [1]. Nitrite is then reduced to ammonia, in a six-electron reaction catalyzed by nitrite reductase [1]. The ammonia formed is incorporated into glutamine, in a reaction catalyzed by glutamine synthetase that requires ATP but does not involve an oxidation-reduction reaction. The next reaction in this pathway is the two-electron reductive conversion of glutamine plus 2-oxoglutarate to two molecules of glutamate, catalyzed by glutamate synthase [1]. In all oxygenic phototrophs, the physiological electron donor for the reactions catalyzed by nitrite reductase is the [2Fe–2S] cluster-containing protein, ferredoxin [1–3]. Although some cyanobacteria contain a pyridine nucleotide-dependent glutamate synthase [4], cyanobacteria also contain ferredoxin-dependent glutamate synthases and the chloroplast glutamate

Abbreviations: CD, circular dichroism; FNR, ferredoxin:NADP$^+$ oxidoreductase; FTR, ferredoxin:thioredoxin reductase; Tris, tris(hydroxymethyl)amino-methane

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synthases all use ferredoxin as the electron donor [1–3]. While the nitrate reductase-catalyzed reduction of nitrate to nitrite in photosynthetic eukaryotes (i.e., in algae and plants) utilizes NADPH as the electron donor [1,2], cyanobacterial nitrate reductases are unique among this class of enzymes in utilizing reduced ferredoxin as the electron donor [3].

Several ferredoxin-dependent enzymes, including nitrite reductase, glutamate synthase, ferredoxin:$NADP^+$ oxidoreductase (FNR), and ferredoxin:thioredoxin reductase (FTR) have been shown to form complexes with ferredoxin [2]. These enzymes, which exhibit high affinities for ferredoxin at low ionic strength, do not form complexes with ferredoxin at high ionic strength, suggesting that electrostatic forces play an important role in protein/protein interactions in these complexes [2]. Three-dimensional structures for two FNR/ferredoxin complexes, one involving the maize leaf proteins [5], and one involving the proteins from the cyanobacterium *Anabaena* sp. PCC 7119 [6] are available. These structures clearly show the existence of interactions between positively charged side chains on FNR and negatively charged side chains on ferredoxin at the protein/protein interface.

The ferredoxin-dependent nitrate reductase of the cyanobacterium *Synechococcus* sp. PCC 7942, the product of the *narB* gene [7,8], is among the best characterized of the ferredoxin-dependent nitrate reductases [8,9]. The complete amino acid sequence, deduced from the nucleotide sequence of the *narB* gene, is known for the 76 kDa protein, which has been shown to contain one molybdopterin cofactor (likely to be a *bis*-molybdopterin guanine dinucleotide) and one iron/sulfur cluster as prosthetic groups [9]. The native protein and a His-tagged variant, both of which are fully active, can be expressed in *Escherichia coli* and site-directed mutagenesis experiments have identified the four cysteines that are the likely ligands to the iron/sulfur cluster. Until now, no information has been available in the literature on the nature of the interaction between ferredoxin and any cyanobacterial nitrate reductase. We report below the first evidence that *Synechococcus* nitrate reductase and ferredoxin form an electrostatically stabilized complex that appears to share some general properties with the complexes formed between ferredoxin and other ferredoxin-dependent enzymes but to differ in some of the details of the interaction.

2. Materials and methods

Spinach leaf ferredoxin was isolated and purified as described previously [10]. After purification to $A_{422\ nm}/A_{277\ nm}$ ratios of at least 0.45, the protein was stored in 30 mM Tris–HCl buffer (pH 8.0) at −20 °C until used. Ferredoxin concentrations were estimated from the absorbance at 422 nm, using an extinction coefficient of 9.7 $mM^{-1}\ cm^{-1}$ [11]. Wild-type ferredoxin from the cyanobacterium *Anabaena* sp. PCC 7119 and its E94K, E95K and E94KE95K variants were prepared and their concentrations estimated as described previously [12]. The native form of *Synechococcus* sp. PCC 7942 nitrate reductase was expressed in *E. coli* and purified as described previously, using a ferredoxin affinity column for the final purification step [9]. The His-tagged variant of *Synechococcus* sp. PCC 7942 nitrate reductase was expressed in *E. coli* and purified, using a Ni^{2+} affinity column purchased from Amersham Bioscience, as described previously [9]. Both wild-type nitrate reductase and its His-tagged variant showed a single Coomassie blue-staining band after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The enzyme was stored at −80 °C in 25 mM potassium phosphate buffer (pH 7.5) containing 500 mM NaCl until used. Buffer exchange was accomplished using a Centricon concentrator (Amicon) with a 50 kDa cut-off membrane. Nitrate reductase concentrations were estimated from the protein concentration, measured according to the method of Bradford [13], using bovine serum albumin as a standard. Absorbance spectra were measured using a Shimadzu Model UV2401 spectrophotometer at 1.0 nm spectral resolution. Circular dichroism (CD) spectra were measured using an OLIS Model DMS-10 spectropolarimeter. Absorbance and CD difference spectra were obtained using computer subtraction. Binding curves for the interaction of nitrate reductase with ferredoxin are displayed with vertical error bars that correspond to two times the instrument noise level. Ferredoxin-linked and methyl viologen-linked enzyme activities were determined as described by Manzano et al. [14] and by Mikami and Ida [15], respectively. All activity measurements were carried out two or more times. One unit of activity corresponds to 1 μmol of nitrate reduced per minute. The average deviations for these replicate activity measurements ranged from 5% to 10%. Treatment of nitrate reductase with phenylglyoxal and *N*-acetylsuccinimide (both obtained from ICN Biomedicals), was carried out as described previously for spinach nitrite reductase [16,17] and glutamate synthase [18].

3. Results

As indicated above, interactions between ferredoxin and ferredoxin-dependent enzymes such as FNR, FTR, nitrite reductase and glutamate synthase contain a significant electrostatic component [2]. As electrostatic interactions weaken when the ionic strength of the solution increases [19], one might expect that the rate of electron transfer from reduced ferredoxin to nitrate, catalyzed by the *Synechococcus* nitrate reductase, would decrease with increasing ionic strength if electrostatic interactions between the two proteins played an important role in the enzyme mechanism. Fig. 1 shows that the rate of nitrate reduction, with reduced ferredoxin serving as the electron donor, declined dramatically as the ionic strength of the assay mixture was increased by adding NaCl to the buffer solution. Very similar results were obtained when the ionic

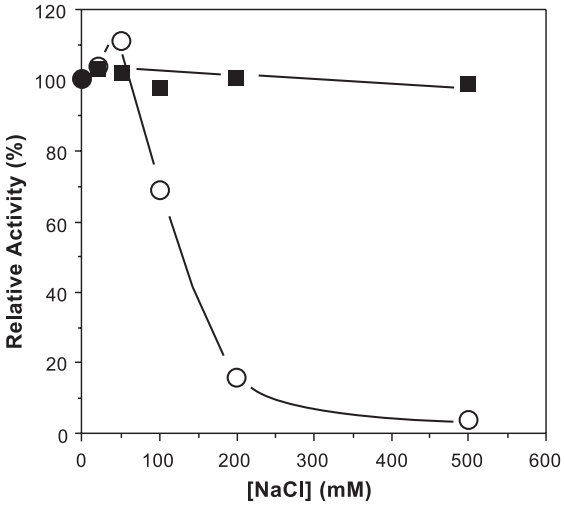

Fig. 1. The ferredoxin-dependent activity (open circles) and methyl viologen-dependent activity (closed squares) of the effect of ionic strength on the activity of *Synechococcus* nitrate reductase. The ferredoxin-dependent activity (open circles) and methyl viologen-dependent activities of the His-tagged enzyme were assayed as described in Materials and methods. For the ferredoxin-dependent assay, 100% activity corresponds to 0.8 units (40 μg of enzyme) per 1.0 ml of assay mixture. For the methyl viologen-dependent assay, 100% activity corresponds to 0.2 units (10 μg of enzyme) per 1.0 ml of assay mixture.

strength was increased by adding KCl or $MgSO_4$, suggesting that inhibition of the nitrate reductase-catalyzed reaction is due to the increase in ionic strength and does not arise from a specific ion effect. As ferredoxin from *Synechococcus* sp. PCC 7942 was not available, spinach ferredoxin was used for these activity measurements (as well as for the spectral perturbation measurements described below). Plots of initial rates of the nitrate reductase-catalyzed reaction versus the concentration of spinach ferredoxin were hyperbolic, with a K_m value of 50 μM (data not shown). This K_m value is similar to values between 15 and 75 μM reported previously for cyanobacterial ferredoxins serving as electron donors to ferredoxin-dependent, cyanobacterial nitrate reductases [3,20]. These observations, and the fact that ferredoxins from oxygenic phototrophs show high degrees of sequence homology [21], suggest that results obtained with spinach ferredoxin can be used to model the interaction between a cyanobacterial nitrate reductase and its physiological electron-donating substrate.

Fig. 1 also shows that, in contrast with the effects seen when reduced ferredoxin was the electron donor for nitrate reduction, the rate of the nitrate reductase-catalyzed reaction was essentially independent of ionic strength when the non-physiological electron donor, reduced methyl viologen, was used to support nitrate reduction. It should be pointed out that while the ferredoxin-dependent activities of cyanobacterial nitrate reductases have pH optima near 8.5 (the pH used to obtain the data of Fig. 1), little activity with methyl viologen as the electron donor is observed under the conditions used in this study, unless the assays are conducted at much more alkaline pH values [14,15,20,22]. Thus, methyl viologen-dependent activity data was obtained at pH 10.2 and, because the assays with the two electron donors were carried out at two quite different pH values, the two curves shown in Fig. 1 were obtained under different experimental conditions. Nevertheless, it can be concluded that the inhibitory effect of increasing ionic strength on the catalytic activity of *Synechococcus* nitrate reductase apparently does not represent inhibition of the enzyme's intrinsic catalytic ability but rather represents an effect on the interaction between the enzyme and ferredoxin.

Complex formation between ferredoxin and ferredoxin-dependent enzymes often produces changes in the absorbance or CD spectra (or both) of the enzyme and/or ferredoxin [10,23–31]. The spectral perturbations that result from protein/protein interactions in these ferredoxin/enzyme complexes can be used to monitor complex formation and, in some cases, measure K_d values and protein/protein stoichiometries for the complexes [10,23–31]. Fig. 2 shows the difference spectrum that resulted from mixing *Synechococcus* nitrate reductase with an equimolar amount of oxidized spinach ferredoxin at low ionic strength (i.e., this is the spectrum that resulted from subtracting the sum of the separate spectra of nitrate reductase and ferredoxin from the spectrum of a 1:1 mixture of the two proteins). The only readily discernable feature in this difference spectrum is the negative feature centered near 280 nm. Although this feature is of relatively small amplitude, it and the low-amplitude positive offset at wavelengths above 300 nm are completely reproducible. Mixing the two proteins in high ionic strength buffer (i.e., in the presence of 250 mM NaCl) produced no spectral perturbation, suggesting that the complex formation between the proteins that produces the changes in the spectra of one or both proteins does not occur at high ionic strength.

Fig. 3 shows the dependence of the magnitude of the spectral change at 280 nm minus 330 nm, associated with

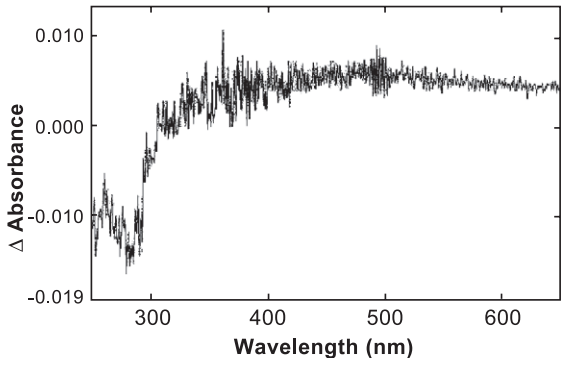

Fig. 2. The difference spectrum resulting from complex formation between *Synechococcus* nitrate reductase and spinach ferredoxin. His-tagged nitrate reductase and ferredoxin, both present in the oxidized form, were present at concentrations of 16 μM in 10 mM potassium phosphate buffer (pH 7.5). The optical pathlength was 1.0 cm.

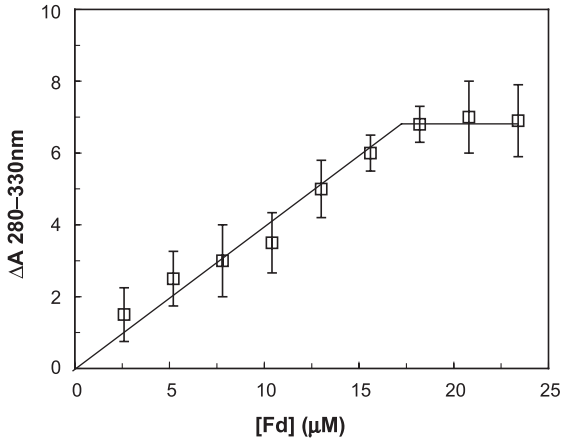

Fig. 3. The binding isotherm for complex formation between *Synechococcus* nitrate reductase and spinach ferredoxin, monitored by changes in the absorbance spectrum in the ultraviolet-region. Conditions were as in Fig. 2, except that the ferredoxin concentration was varied as indicated. The *y*-axis scale is a relative one, with the maximal absorbance change corresponding to 0.020 absorbance units.

complex formation between ferredoxin and nitrate reductase at low ionic strength, on the ratio of ferredoxin to nitrate reductase. Difference spectra obtained at all ferredoxin/nitrate reductase ratios were identical to that shown in Fig. 2. Identical difference spectra and titration curves were obtained regardless of whether wild-type nitrate reductase or its His-tagged variant was used. The data of Fig. 3 are consistent with the formation of a 1:1 complex of very high affinity between the two proteins and we have plotted the data on the assumption that there is in fact no significant deviation from the straight line expected for a 1:1 ferredoxin/nitrate reductase stoichiometry of infinitely high affinity for the complex [32]. However, the low amplitude of the changes in absorbance resulting from complex formation between the two proteins and the resulting low signal/noise ratio (indicated by the vertical error bars) does not allow one to exclude the possibility that the binding curve has the hyperbolic shape that would be expected for a binding affinity lower than that assumed. If the binding affinity were substantially lower than we have estimated, the conclusion that the complex has a 1:1 stoichiometry could also be in error. However, the best fit for the data obtained from a ferredoxin titration of the spectral perturbation is most consistent with a 1:1 stoichiometry and indeed this is the stoichiometry found for most of the previously studied ferredoxin/enzyme complexes [2]. Fig. 4 shows a similar plot of the magnitude of the change in the CD spectra that occurred when oxidized spinach ferredoxin and *Synechococcus* nitrate reductase were mixed at low ionic strength versus the ferredoxin concentration. The fact that this profile is essentially identical to that of Fig. 3 provides additional evidence for the formation of a high-affinity complex between the enzyme and ferredoxin, with the most probable protein/protein stoichiometry being 1:1. As was the case for the absorbance changes in absorbance spectra shown in Figs. 2 and 3, no changes in CD spectra were observed if the proteins were mixed at high ionic strength, suggesting that the protein/protein interactions that produce the changes in CD spectra are electrostatic in nature.

As ferredoxin is a very acidic protein that contains several highly conserved acidic residues [21] and has a large net negative surface charge at pH values near and above 7.0 [33,34], one might expect that ferredoxin provides a substantial number of the negative charges involved in electrostatic complex formation with *Synechococcus* nitrate reductase. Site-directed mutagenesis studies with ferredoxins from a number of different oxygenic phototrophs have shown that replacement of one, but not the other, of two conserved glutamates found near the C-termini of ferredoxins results in a marked loss of steady-state activity with the ferredoxin-dependent enzymes FNR, FTR, nitrite reductase and glutamate synthase, as well as in decreased rates of electron transfer to FNR in flash photolysis experiments [12,34–41]. One example of this approach is illustrated by results from our earlier work which showed that substituting a lysine residue for Glu94 in the ferredoxin from the cyanobacterium *Anabaena* sp. PCC 7119 resulted in a large decrease in the steady-state rate of the reaction catalyzed by the ferredoxin-dependent spinach chloroplast glutamate synthase while, in contrast, activity with a E95K variant of this *Anabaena* ferredoxin was essentially identical to that observed with the wild-type ferredoxin [41]. In these experiments, it was also demonstrated that a E94K, E95K doubly mutated *Anabaena* ferredoxin behaved in a fashion identical to the E94K variant of *Anabaena* ferredoxin, providing additional support for the hypothesis that the negative charge contributed by Glu95 in the wild-type *Anabaena* ferredoxin does not play an important role in the protein's interaction with spinach glutamate synthase [41].

Table 1 summarizes the results of experiments designed to test the effect of charge-reversal replacements of the

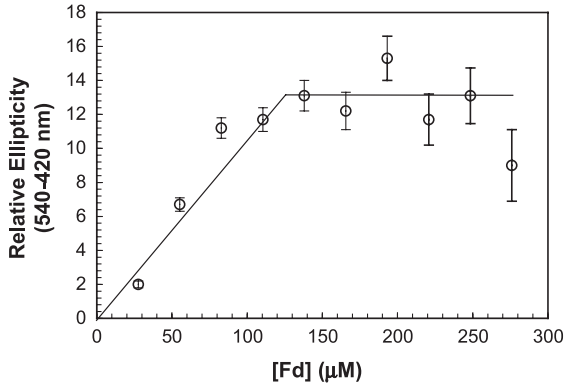

Fig. 4. The binding isotherm for complex formation between *Synechococcus* nitrate reductase and spinach ferredoxin, monitored by changes in the CD spectrum. Conditions were as in Fig. 2, except that the concentration of His-tagged nitrate reductase was 135 μM.

Table 1
The effect of glutamate/lysine substitutions on the ability of *Anabaena* ferredoxin to serve as an electron donor for nitrate reductase

Anabaena ferredoxin	Relative activity
Wild-type	100
E94K	19
E95K	38
E94KE95K	10

All activities were measured using a ferredoxin concentration of 700 μg/ml. 100% activity corresponds to 450 units/mg protein.

glutamate residues at positions 94 and 95 of *Anabaena* ferredoxin on its ability to serve as an electron donor to *Synechococcus* nitrate reductase in steady-state assays. Unlike the results obtained previously, using these same ferredoxins as electron donors to spinach glutamate synthase [41], E94K and E95K charge-reversal variants of *Anabaena* ferredoxin were both much impaired in their ability to serve as electron donors to *Synechococcus* nitrate reductase (Table 1), even though the effect with the E94K variant (with a relative activity of 19% of the wild-type control) was somewhat greater than that observed with the E95K variant (with a relative activity of 38% of the wild-type control). Furthermore, as can also be seen in Table 1, the E94K, E95K doubly mutated *Anabaena* ferredoxin shows significantly lower activity with *Synechococcus* nitrate reductase than does either of the singly mutated variants, a result that also differs from those obtained with glutamate synthase [41]. It should be pointed out that Schmitz and Böhme [37] had previously demonstrated that an E94Q, E95Q doubly mutated *Anabaena* ferredoxin variant was less active as an electron donor to *Anabaena* nitrate reductase than a E94Q singly mutated variant, while the singly mutated E94Q and the doubly mutated E94Q, E95Q variants of *Anabaena* ferredoxin were equally active as electron donors to *Anabaena* nitrite reductase. Unfortunately, Schmitz and Böhme [37] did not report any results with an *Anabaena* ferredoxin variant mutated only at Glu95, so no direct comparison of the effects of charge elimination at these two conserved glutamate residues could be made in this earlier study.

We have previously used the arginine-modifying reagent phenylglyoxal and the lysine-modifying reagent *N*-acetylsuccinimide to demonstrate that these amino acids are involved in ferredoxin binding by spinach nitrite reductase [16,17] and spinach glutamate synthase [18]. Fig. 5 shows the effect of treating *Synechococcus* nitrate reductase with a 115-fold excess of *N*-acetylsuccinimide on the ferredoxin-dependent and methyl viologen-dependent activities of the enzyme. At short incubation times with *N*-acetylsuccinimide, a reagent that eliminates the positive charge on the side chain amino group of accessible lysine residues [42], approximately 80% of the ferredoxin-dependent activity was lost while only 5–10% of the methyl viologen dependent activity was lost. These data are consistent with the hypothesis that one or more nitrate reductase lysine residues are involved in binding ferredoxin to the enzyme. Fig. 5 also shows that addition of ferredoxin prior to addition of *N*-acetylsuccinimide, under the low ionic strength conditions shown to favor formation of the 1:1 ferredoxin/enzyme complex, protected the enzyme against the effect of *N*-acetylsuccinimide. The protection suggests that the lysine residue(s) modified is (are) at the ferredoxin-binding site of *Synechococcus* nitrate reductase.

Fig. 6 shows that treatment of *Synechococcus* nitrate reductase with a 76-fold excess of phenylglyoxal, a reagent that eliminates the positive charge normally found on the guanidino side chain group of arginine residues [43], also results in a very substantial loss of the enzyme's ferredoxin-dependent activity with relatively little effect on its methyl viologen-dependent activity. Formation of the 1:1 ferredoxin/enzyme complex prior to the addition of phenylglyoxal results in almost complete protection against the inhibitory effect of this compound, consistent with the hypothesis that the arginine residue(s) being modified is (are) at the ferredoxin-binding site of *Synechococcus* nitrate reductase. It should be pointed out that although the pH values at which ferredoxin-dependent activity (pH 8.5) and at which methyl viologen-dependent activity (pH 10.2) were measured differ, the modification of the enzyme was always carried out at the pH 7.5. The absorbance spectrum of the enzyme, which was essentially identical to that reported previously [9], was

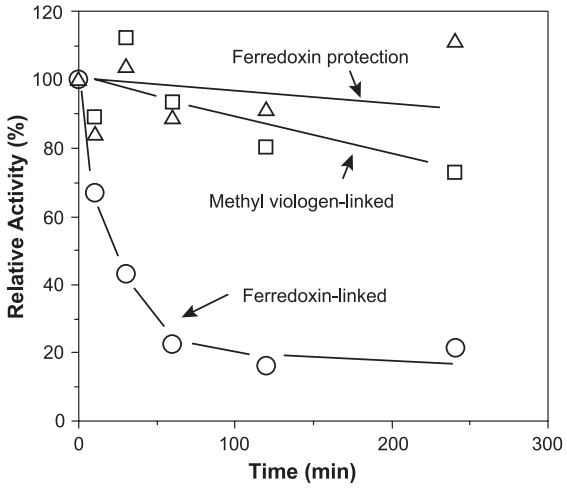

Fig. 5. The effect of *N*-acetylsuccinimide treatment on the activity of *Synechococcus* nitrate reductase. His-tagged nitrate reductase, in the presence (triangles) or absence (circles and squares) of equimolar ferredoxin, was incubated with a 115-fold excess of *N*-acetylsuccinimide for the times indicated in 10 mM potassium phosphate buffer (pH 7.5). The reaction was terminated by dilution and aliquots assayed for ferredoxin-dependent activity (circles and triangles) or methyl viologen-dependent activity (squares). 100% activity corresponds to 0.3 units (15 μg of enzyme) per 1.0 ml of assay mixture for the methyl viologen-dependent activity and 0.6 units (30 μg of enzyme) and 0.5 units (25 μg of enzyme) for the ferredoxin-dependent activities for nitrate reductase treated with *N*-acetylsuccinimide in the absence and presence, respectively, of equimolar ferredoxin.

unaffected by either *N*-acetylsuccinimide or phenylglyoxal at the concentrations used for the modification experiments shown in Figs. 5 and 6.

Although it seemed likely that the inhibitory effects of phenylglyoxal and *N*-acetylsuccinimide on the ferredoxin-dependent activity of *Synechococcus* nitrate reductase resulted from the elimination of positive charges involved in ferredoxin binding, it was of interest to determine whether these reagents did in fact affect ferredoxin binding by the enzyme. Addition of spinach ferredoxin to nitrate reductase that had been treated with *N*-acetylsuccinimide to the point where 80% of the enzyme's ferredoxin-linked activity had been lost produced no detectable spectral changes. Thus, treatment of *Synechococcus* nitrate reductase with this lysine-modifying reagent either increases K_d to the point where significant complex formation between ferredoxin and the enzyme does not occur or it affects the protein/protein interactions in a manner so that complex formation does not produce any detectable spectral perturbation. Mixing spinach ferredoxin with *Synechococcus* nitrate reductase that had been treated with phenylglyoxal to the point where 60% of the enzyme's ferredoxin-linked activity had been lost produced a difference spectrum essentially identical to that obtained for the untreated enzyme. However, the shape of the binding curve observed for the binding of spinach ferredoxin to the phenylglyoxal-modified was hyperbolic and was quite different from the intersecting straight lines obtained for the untreated enzyme, indicating weaker binding than that observed for the unmodified enzyme. A double-reciprocal plot of the binding data (Fig. 7) gave a good fit to the straight line expected for a single ferredoxin-binding site with $K_d = 2.0$ μM. Thus, modification of enzyme arginine residues causes a significant decrease in the binding affinity of the enzyme for ferredoxin.

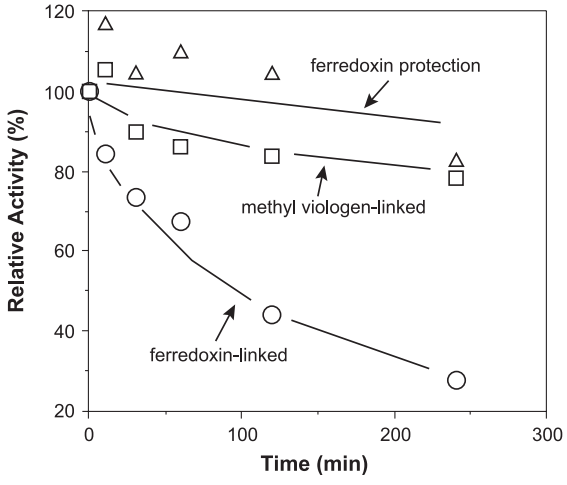

Fig. 6. The effect of phenylglyoxal treatment on the activity of *Synechococcus* nitrate reductase. His-tagged nitrate reductase, in the presence (triangles) or absence (circles and squares) of equimolar ferredoxin, was incubated with a 76-fold excess of phenylglyoxal for the times indicated in 10 mM potassium phosphate buffer (pH 7.5). The reaction was terminated by dilution and aliquots assayed for ferredoxin-dependent activity (circles and triangles) or methyl viologen-dependent activity (squares). 100% activity corresponds to 0.3 units (15 μg of enzyme) per 1.0 ml of assay mixture for the methyl viologen-dependent activity and 0.4 units for the ferredoxin-dependent activities for nitrate reductase treated with phenylglyoxal in either the absence or presence of equimolar ferredoxin.

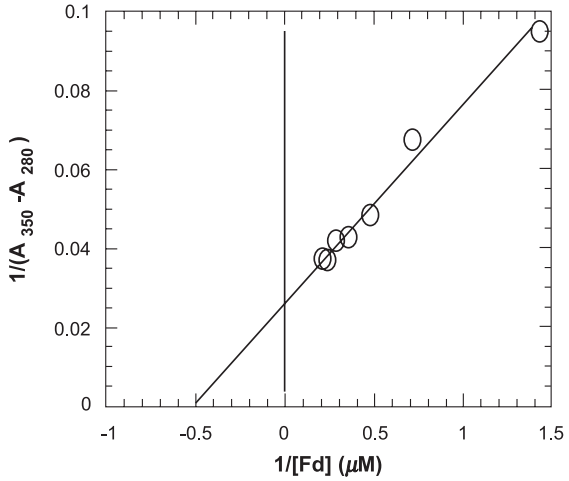

Fig. 7. The effect of phenylglyoxal treatment of *Synechococcus* nitrate reductase on the ferredoxin-binding affinity of the enzyme. Reaction conditions were as in Fig. 2 except that His-tagged nitrate reductase, which had been treated with phenylglyoxal for 2.5 h, was present at a concentration of 5.3 μM.

4. Discussion

We have been able to demonstrate that the ferredoxin-dependent nitrate reductase from *Synechococcus* sp. PCC 7942 forms a high-affinity, 1:1 complex with ferredoxin at low ionic strength. As complex formation cannot be detected at high ionic strength, it appears likely that electrostatic forces play an important role in stabilizing the complex between the two proteins. Experiments utilizing variants of *Anabaena* ferredoxin, in which the negative charges normally present at positions 94 and 95 have been eliminated, as electron donors to *Synechococcus* nitrate reductase suggest that these two negatively charged ferredoxin residues may both contribute to productive interactions between ferredoxin and the enzyme. The chemical modification studies reported above suggest that the positive charges contributed by some of the 44 arginine and 23 lysine residues on *Synechococcus* nitrate reductase make significant contributions to these electrostatic binding interactions. However, it is also possible that the effects of these chemical modifiers arise from steric hindrance, in which the modifying groups interfere with ferredoxin docking because of their size, and do not arise from elimination of the positive charges on arginine and lysine.

Complex formation between *Synechococcus* nitrate reductase and ferredoxin exhibits significant similarity to the

previously characterized interactions between ferredoxin and other ferredoxin-enzymes such as FNR, FTR, nitrite reductase and glutamate synthase [2]. However, the observation that replacing the negative charge arising from Glu95 of *Anabaena* ferredoxin has almost as large an effect in decreasing the ability of this ferredoxin to serve as an electron donor to *Synechococcus* nitrate reductase as does replacing the negative charge on Glu94 is quite different from the behavior most often observed with other ferredoxin-dependent enzymes, where elimination of the negative charge at the position corresponding to Glu95 in *Anabaena* ferredoxin usually has little or no effect on activity [12,34–41]. Thus, the binding site on ferredoxin for nitrate reductase appears to differ in some details from the site(s) on ferredoxin involved in binding these other enzymes. However, it should be pointed out that our observations with these ferredoxin variants and *Synechococcus* nitrate reductase do not represent the first reports of this type of behavior. For example, charge elimination and charge reversal variants of the ferredoxin from the green alga *Chlamydomonas reinhardtii* at Glu91 and Glu92 exhibited approximately equal decreases in activity, compared to wild-type *C. reinhardtii* ferredoxin, with *C. reinhardtii* nitrite reductase and glutamate synthase (Glu91 and Glu92 in *C. reinhardtii* ferredoxin correspond to Glu94 and Glu95 in *Anabaena* ferredoxin) [44]. Furthermore, evidence exists supporting the hypothesis that the binding site on spinach ferredoxin for spinach FTR differs in some details from that for spinach FNR [45] and that this is also the case for the binding sites on maize chloroplast ferredoxin III for maize chloroplast FNR and sulfite reductase [46]. Questions have also been raised as to whether Glu93, the residue in spinach ferredoxin I and maize leaf ferredoxin III that corresponds to Glu94 in *Anabaena* ferredoxin, participates directly in binding chloroplast FNR [5,6,33].

It should be noted that the apparent high affinity of *Synechococcus* nitrate reductase for ferredoxin is comparable to that reported previously for spinach FTR [30] and FNR [24,32] and is considerably greater than the affinities reported previously for spinach nitrite reductase [10] and spinach glutamate synthase [29]. The description of the interaction between ferredoxin and nitrate reductase in this report derive from measurements made under one specific set of conditions and thus does not take into account factors such as the effects of pH, concentrations of specific ions and the redox states of the proteins. The effect of these parameters on the relative affinities of ferredoxin for different ferredoxin-dependent enzymes will no doubt be important in any attempt to understand the distribution of electrons derived from reduced ferredoxin to different target enzymes [2]. This is of particular interest in an organism like *Synechococcus* sp. PCC 7942, in which photosynthetically reduced ferredoxin is used in a wide number of reactions, including those catalyzed by FNR, FTR, nitrate reductase, nitrite reductase, glutamate synthase and sulfite reductase.

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