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Identification of the Ferredoxin-Binding Site of a Ferredoxin-Dependent Cyanobacterial Nitrate Reductase

Anurag P. Srivastava^{*,†,ID,⊥}, Emily P. Hardy[†], James P. Allen[‡], Brian J. Vaccaro[§], Michael K. Johnson[§], and David B. Knaff^{†,||}

[†]Department of Chemistry and Biochemistry, Texas Tech University Lubbock, Texas 79409-1061, United States

[‡]School of Molecular Sciences, Arizona State University, Tempe, Arizona 85287-1604, United States

[§]Department of Chemistry and Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602-2556, United States

^{||}Center for Biotechnology and Genomics, Texas Tech University, Lubbock, Texas 79409-3132, United States

Abstract

An *in silico* model for the 1:1 ferredoxin (Fd)/nitrate reductase (NR) complex, using the known structure of *Synechocystis* sp. PCC 6803 Fd and the *in silico* model of *Synechococcus* sp. PCC 7942 NR, is used to map the interaction sites that define the interface between Fd and NR. To test the electrostatic interactions predicted by the model complex, five positively charged NR amino acids (Arg43, Arg46, Arg197, Lys201, and Lys614) and a negatively charged amino acid (Glu219) were altered using site-directed mutagenesis and characterized by activity measurements, metal analysis, and electron paramagnetic resonance (EPR) studies. All of the charge replacement variants retained wild-type levels of activity with reduced methyl viologen (MV), but a significant decrease in activity was observed for the R43Q, R46Q, K201Q, and K614Q variants when reduced Fd served as the electron donor. EPR analysis as well as the Fe and Mo analyses showed that loss of activity observed with these variants was not the consequence of perturbation of the Mo center or [4Fe-4S] cluster. Therefore, the loss of the Fd-linked specific activity observed with these variants can be explained only by invoking a role for Arg43, Arg46, Lys201, and Lys614 in Fd binding. The R43Q, R46Q, K201Q, and K614Q NR variants also showed a decreased binding affinity for Fd, compared to that of wild-type NR, supporting a key role of these four positively charged residues in the productive binding of Fd.

*Corresponding Author: Telephone: 847-578-3458. Fax: 847-578-3240. anurag.srivastava@rosalindfranklin.edu.

ORCID

Anurag P. Srivastava: 0000-0003-3493-1375

[⊥]Present Address: A.P.S.: Department of Biochemistry and Molecular Biology, Rosalind Franklin University of Medicine and Science, North Chicago, IL 60064.

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

Graphical Abstract

Nitrate serves as a readily available source of inorganic nitrogen for cyanobacteria and plants.¹ In natural environments, fixed inorganic nitrogen (ammonium, nitrate, nitrite, and nitrous oxide) is available as a result of human agricultural and industrial activities and biological nitrogen fixation.¹ Nitrifying bacteria convert most of the fixed inorganic nitrogen (ammonium and nitrite) into nitrate, which then serves as source of inorganic nitrogen for assimilation into amino acids or serves as an electron acceptor during respiration.² The pathway by which nitrate is converted into amino acids in plants and cyanobacteria is collectively known as nitrate or nitrogen assimilation. In cyanobacteria, nitrate assimilation requires three redox reactions.^{3, 4} The first step is catalyzed by nitrate reductase (NR) and involves the initial two-electron reduction of nitrate to nitrite. This is followed by the six-electron reduction of nitrite to ammonia, which is catalyzed by nitrite reductase. The ammonia then reacts with glutamate to form glutamine in a reaction catalyzed by glutamine synthetase. Glutamine formation requires ATP but does not involve any redox chemistry. However, in the final step of the reductive portion of the nitrate assimilation pathway, glutamine reacts with one molecule of 2-oxoglutarate to form two molecules of glutamate in the two-electron reduction reaction catalyzed by glutamate synthase.^{3, 4} Cyanobacteria are unique among oxygenic photosynthetic organisms in using reduced Fd as the physiological electron donor for all three of the reductant-requiring steps of the nitrate assimilation pathway.^{3, 4} While all other oxygenic phototrophs use reduced Fd as the electron donor for both the reduction of nitrite to ammonia and the reaction of glutamine and 2-oxoglutarate to form glutamate, the two-electron reduction of nitrate to nitrite utilizes NADPH as the electron donor.^{4, 5}

The Fd-dependent NR of the cyanobacterium *Synechococcus* sp. PCC 7942 is the product of the *narB* gene and is among the best characterized of the Fd-dependent nitrate reductases.⁶ The complete amino acid sequence, as deduced from the nucleotide sequence of the *narB* gene, indicates a 78 kDa protein, which contains one bis-molybdopterin guanine dinucleotide (MGD) Mo center and one [4Fe-4S]^{2+,+} cluster as prosthetic groups.^{2, 6} The redox and spectroscopic properties of these two prosthetic groups have been investigated by

protein film voltammetry and electron paramagnetic resonance (EPR) spectroscopy.² Titrations, using EPR spectroscopy to monitor the cofactor redox states, have shown that the oxidized form of the [4Fe-4S]²⁺ cluster is EPR silent, while one-electron reduction results in an EPR signal characteristic of an $S = 1/2$ [4Fe-4S]⁺ cluster with an E_m value of -190 mV.^{2, 7} The Mo center of the enzyme was also characterized by EPR spectroscopy,^{2, 7} and an E_m value of -150 mV was estimated for a Mo⁶⁺ to Mo⁵⁺ transition by EPR-monitored redox titrations.² Protein film voltammetry suggested that during the catalytic cycle nitrate binds to a two-electron reduced form of the enzyme in which the cluster is in its [4Fe-4S]⁺ form and the molybdopterin is in the Mo⁵⁺ state.² A [2Fe-2S] Fd serves as the electron donor for this catalytic reaction and forms a high-affinity 1:1 complex with NR at a low ionic strength.⁷⁻⁹ Jepson et al.² proposed a pathway for electron transfer from reduced Fd to *Synechococcus* sp. PCC 7942 NR in which Fd first reduces the [4Fe-4S] cluster, which in turn reduces then Mo center, the site in NR where nitrate is bound and reduced. As Fd is a one-electron carrier and NR contains only one Fd-binding site, this process requires two separate, sequential electron transfers from Fd to the enzyme.⁹ The first reduced Fd transfers its electron to the [4Fe-4S] cluster, which subsequently reduces Mo, from Mo⁶⁺ to Mo⁵⁺ in the first step and from Mo⁵⁺ to Mo⁴⁺ in the second step.^{2, 5} A two-electron transfer from Mo⁴⁺ to bound nitrate completes the process, producing nitrite and returning the enzyme to its original state.^{2, 5} Flash photolysis experiments on *Synechococcus* sp. PCC 7942 NR showed that the enzyme completes one full catalytic cycle under the conditions favoring two sequential electron transfers from the reduced Fd to the NR.¹⁰

A combination of sequence alignments and site-directed mutagenesis identified four conserved cysteine residues in *Synechococcus* sp. PCC 7942 NR, i.e., Cys9, Cys12, Cys16, and Cys56, that most likely serve as ligands of the [4Fe-4S] cluster that is located at the N-terminus of the enzyme.⁶ Kinetic studies, substrate binding studies, computer modeling, and amino acid replacements have been used to investigate the molecular mechanism of the reaction catalyzed by this enzyme.^{7, 9} Site-directed mutagenesis study in our laboratory identified three highly conserved basic amino acids, i.e., Lys58, Arg70, and Lys130 in *Synechococcus* sp. PCC 7942 NR, that are essential for its full catalytic activity.⁷ This study also presented a computer-based *in silico* model of the three-dimensional structure of *Synechococcus* sp. PCC 7942 NR that has provided many details of the possible mechanism of action of this enzyme.⁷ As there is no three-dimensional structure available for any Fd-dependent NR, the *in silico* model of *Synechococcus* sp. PCC 7942 NR was calculated on the basis of the known structure of the dissimilatory NR from *Desulfovibrio desulfuricans*.¹¹ On the basis of the *in silico* model⁷ and studies of the active sites in related enzymes,¹¹⁻¹⁷ our recent characterization of site-directed variants of *Synechococcus* sp. PCC 7942 NR identified five amino acids, Cys148, Met149, Met306, Asp163, and Arg351, that are essential for activity and are involved in either nitrate binding, prosthetic group binding, or catalysis.⁹

In addition to characterizing amino acids likely to be present at or near the active site⁹ and highly conserved basic amino acids,⁷ we investigated the possible locations for the Fd-binding domain on *Synechococcus* sp. PCC 7942 NR. To map the amino acid residues forming the interface for Fd docking to the NR, an *in silico* model for the 1:1 Fd-NR complex was constructed, using the known structure of *Synechocystis* sp. PCC 6803 Fd

[Protein Data Bank (PDB) entry 1OFF, ref 18] and the *in silico* model of *Synechococcus* sp. PCC 7942 NR.^{7,9} This *in silico* model of the Fd–NR complex provided specific details about the interaction sites that are involved in the formation of the Fd–NR complex. In this work, site-directed mutagenesis was used to test this model of the complex and to identify specific amino acids of NR involved in the formation of the complex with Fd.

MATERIALS AND METHODS

Both recombinant wild-type Fd from the cyanobacterium *Synechocystis* sp. PCC 6803 and recombinant, C-terminally Histagged *Synechococcus* sp. PCC 7942 NR were expressed in *Escherichia coli* and purified as described previously.^{7,9,10} The QuikChange (QC) site-directed mutagenesis kit (Stratagene) was used to generate site-specific replacement variants of NR as described previously.^{7,9} Polymerase chain reaction (PCR) was used to generate site-specific replacement variants of NR using mutagenic primers listed in Table 1. The same expression and purification procedure was followed for the variants of NR and for the wild-type enzyme.^{7,9} The purity of all variants described in this study was judged to be >90% based upon a visual assessment analysis of Coomassie Brilliant Blue-stained SDS–PAGE.

Ultraviolet–visible (UV–vis) absorbance spectra and difference absorbance spectra for wild-type NR and its variant forms were recorded using a Shimadzu model UV-2401PC spectrophotometer with a spectral resolution of 0.5 nm. An OLIS model DSM-10 UV–vis CD spectrophotometer was used to record circular dichroism (CD) spectra for the wild-type enzyme and the variants at a spectral resolution of 1.0 nm. The Fd concentration was measured using an extinction coefficient of 9.7 mM^{−1} cm^{−1} at 420 nm.¹⁹ The Bradford method was used to measure NR protein concentrations, using bovine serum albumin as a standard.²⁰ X-Band (~9.6 GHz) EPR spectra were recorded using a Bruker ESP-300E EPR spectrometer equipped with an ER-4116 dual-mode cavity and an Oxford Instruments ESR-9 liquid helium flow cryostat. Spin quantifications were performed under non-power saturation conditions using a 1 mM Cu²⁺ EDTA standard as described by Aasa and Vänngård.²¹ EPR spectra were recorded for *Synechococcus* sp. PCC 7942 NR samples as purified aerobically and reduced anaerobically with a 10-fold excess of dithionite. The iron (Fe) and molybdenum (Mo) contents were estimated using inductively coupled plasma (ICP) mass spectrometry in the School of Molecular Sciences at Arizona State University, as described previously.^{7,9}

NR activities with either reduced MV or reduced Fd as the electron donors were measured as previously described.^{7,9} Fits to the Michaelis–Menten equation were obtained for all plots of NR activity as a function of nitrate concentration at a saturating Fd concentration and as a function of Fd concentration at a saturating nitrate concentration. Kinetic parameters were determined using GraphPad Prism 6 by fitting the data (i.e., initial velocity vs substrate concentration) to the Michaelis–Menten equation. Formation of a complex between Fd and NR was measured using the spectral perturbation method as described previously.^{7–9} Formation of a complex of wild-type NR with Fd was monitored at 420 nm minus 600 nm. However, variations were observed in the maxima of the difference spectra for different NR variants upon binding of Fd. Consequently, to maximize the magnitude of

the absorbance changes used for Fd dissociation constant (K_d) determinations, the following wavelength pairs were used to monitor the binding of Fd to the NR variants: R43Q, 460 nm minus 520 nm; R43K, 450 nm minus 650 nm; R46Q, 420 nm minus 600 nm; R46K, 450 nm minus 600 nm; R197Q, 410 nm minus 470 nm; K201Q, 400 nm minus 650 nm; K201R, 410 nm minus 470 nm; E219Q, 500 nm minus 650 nm; K614R and K614Q, 500 nm minus 600 nm.

The docking of the Fd from *Synechocystis* sp. PCC 6803 (PDB entry 1OFF, ref 18) to the previously published *in silico* model^{7, 9} was performed using three programs, ZDOCK, ClusterPro, and Rosetta.^{22–26} These programs dock two structures by balancing a number of protein–protein interactions, including electrostatic interactions, van der Waals interactions, hydrogen bonds, solvation energies, and hydrophobic interactions. A fundamental assumption of these programs is that the backbone conformations of proteins typically do not change upon association; therefore, these programs treat the models as static and do not accommodate any flexibility of the proteins. Each program generates and scores thousands of different random positions. The most favorable models from each program were then evaluated on the basis of the relative positions and orientations of the two docked proteins.

RESULTS

Figure 1 shows the results of an *in silico* search for the energetically most favorable docking site(s) for Fd on *Synechococcus* sp. PCC 7942 NR. As there are no three-dimensional structures known for any *Synechococcus* Fd, the structure of the Fd from the cyanobacterium *Synechocystis* sp. 6803 was used in this model of the complex. Given the close similarity in tertiary structures of “plant-type” Fds,^{27, 28} the high degree of primary sequence homology of *Synechocystis* and *Synechococcus* Fds (Figure 2), and the essentially identical NR activities using the *Synechocystis* and *Synechococcus* Fds as electron donors,⁷ the use of *Synechocystis* Fd in this modeling exercise should not introduce any significant uncertainties (Figure 2). The two most favorable structures predicted by the modeling (one colored light red and one dark red), which used the known structure of Fd (PDB entry 1OFF, ref 18) and our previously published^{7, 9} *in silico* model for the enzyme, share a common binding site for Fd on the enzyme but predict somewhat different orientations for the bound Fd (Figure 1A). Of these two models, we have focused on the complex that has the Fd docked into a niche on the surface of NR, where a pocket is formed by a number of α -helices and β -strands of NR (Figure 1B). Fd slips into this site, assisted by interactions with several loops located near the [2Fe-2S] cluster of Fd. In the model, the [2Fe-2S] cluster of the Fd is 11.9 Å from the [4Fe-4S] cluster of NR. Also included are the locations of the [2Fe-2S] cluster of Fd and the two prosthetic groups of the enzyme. The model predicts specific amino acid residues to be present at the binding site, involving electrostatic interactions of ionizable side chains along with hydrophobic contributions from NR and Fd. As the surface of *Synechocystis* sp. PCC 6803 Fd would be negatively charged at physiological pH values^{27, 28} and because the predicted binding interface for NR on Fd is largely composed of negatively charged side aspartates and glutamates, the positively charged amino acids predicted to be present on the Fd-binding interface of NR are lysines and arginines.

We assessed the involvement of individual amino acids on NR in binding Fd by mutagenic replacement of the positively charged side chains of Arg43, Arg46, Arg197, Lys201, and Lys614 and the negatively charged Glu219 that are modeled as being present at the NR–Fd complex interface. Activity was measured using both reduced Fd and the nonphysiological donor, reduced MV, as the electron donor.³ On the basis of the structural models, none of these selected residues are close to either of the enzyme's prosthetic groups (Table 2). Site-specific variants at these positions were generated using mutagenic primers (see Table 1) by site-directed mutagenesis. PCR amplification with these primers was performed with the method as described previously.^{7,9} The mutated plasmids were sequenced, using sequence-specific primers to confirm the mutations. The variants were expressed in *E. coli*, and their expression level was comparable to that of the wild-type enzyme. The yields of the variants of NR were approximately 8 mg of purified protein per liter of *E. coli* culture. Coomassie Brilliant Blue-stained SDS–PAGE confirmed the purity of these variants. SDS–PAGE analysis indicated that all of the enzyme samples used in this study were at least 90% pure and had an approximate molecular mass of 78 kDa. UV–vis absorption spectra for the wild-type enzyme and the variants were recorded over the spectral range from 250 to 700 nm. The absorbance spectra of the variants were similar to those of wild-type NR and showed a characteristic spectral feature (broad shoulder) at 320, 400, and 450 nm, consistent with the spectral features observed with the wild-type enzyme (data not shown). Circular dichroism (CD) spectra were recorded in the UV region (200–300 nm) for each of the variants. Comparison with the CD spectra of wild-type NR shows that none of the mutational replacements results in a significant conformational change. This is illustrated in Figure 3, which shows the UV–CD spectra of the wild-type enzyme and two of the variants used in this study, i.e. R46Q and K614Q.

Table 3 summarizes the effects of mutagenic replacements at each of the selected NR docking site amino acids with the polar, but uncharged, amino acid glutamine on the kinetic properties of enzyme, with both reduced MV and *Synechocystis* sp. PCC 6803 Fd serving as the electron donors. Figure 4 shows representative plots of the enzyme activities of the wild-type enzyme and two variants (R46Q and R197Q) as a function of Fd concentration (at a saturating nitrate concentration) and nitrate concentration (at a saturating Fd concentration) with best fits to the Michaelis–Menten equation. All of the variants of *Synechococcus* sp. PCC 7942 NR retain wild-type levels of activity when reduced MV serves as the electron donor, but R43Q, R46Q, K201Q, and K614Q variants show activity that is substantially decreased compared to that of the wild type when reduced Fd serves as the electron donor. Specifically, the R43Q variant retains 91% of wild-type activity with reduced MV but only 16% of wild-type activity with reduced Fd. Similarly, R46Q retains 97% of wild-type activity with reduced MV and 24% of the Fd-dependent activity of the wild-type enzyme. With reduced MV as the electron donor, the K201Q and K614Q variants exhibit 99 and 98% of the wild-type enzyme activity, respectively, but show a dramatic loss of activity compared to that of the wild type with Fd as the electron donor, i.e., 85 and 88% loss of activity for K201Q and K614Q, respectively. In contrast, nonconservative replacement of Arg197 and Glu219 with glutamine has no effect on either MV- or Fd-dependent activity. Moreover, the R197Q and E219Q variants have K_M values for both Fd and nitrate that are similar to that of the wild-type enzyme. The R46Q variant has inhibited Fd-dependent activity and exhibits a

K_M for Fd and nitrate that is 9-fold greater than the measured values for the wild-type enzyme. The rates observed with the R43Q, K201Q, and K614Q variants were too low to allow an accurate determination of the K_M value for its substrates. These observations are consistent with those expected if Arg43, Arg46, Lys201, and Lys614 all play important roles in binding and orienting Fd for productive electron transfer.

To investigate if arginine at positions 43 and 46 and lysine at positions 201 and 614 are specifically required for effective electron donation by Fd, or whether it is simply a positively charged side chain that is required at these positions, the R43K, R46K, K201R, and K614R charge-conserving variants were prepared and characterized. The charge-conserving variants of Arg43, Arg46, Lys201, and Lys614 retain essentially full wild-type activity with MV as the electron donor (Table 3). With Fd as the electron donor, these variants also exhibit levels of activity that are generally close to the wild-type level and much higher than those measured for the glutamine-substituted variants (i.e., 91% of wild-type activity for the R43K variant, 98% of wild-type activity for the R46K variant, 92% of wild-type activity for the K201R variant, and 63% of wild-type activity for the K614R variant). The activity with Fd as the electron donor was sufficiently high to allow determinations of the K_M values for both nitrate and Fd for the reaction catalyzed by these charge-conserving variants (Table 3). The K_M values for both Fd and nitrate obtained with R43K, R46K, K201R, and K614R variants are quite similar to those observed for the wild-type enzyme [i.e., 38 μM for Fd and 6.0 μM for nitrate (see Table 3)]. These results suggest that the presence of a positive charge at these positions is the most important factor for productive Fd binding and that there is not a strict requirement for arginine or lysine per se.

In principle, the significant loss of Fd-dependent activity observed for the R43Q, R46Q, K201Q, and K614Q variants could also arise from a decrease in Fd binding affinities. This possibility was addressed by measuring the Fd dissociation constant (K_d) of the wild type and variants using a spectral perturbation method developed previously for the wild-type enzyme.⁸ Table 4 summarizes the Fd K_d values obtained for the wild-type and variant enzymes. Figure 5 shows representative plots of difference absorption versus Fd concentration and the fits that were used to calculate Fd K_d values for the wild-type, R43Q, R46Q, R46K, K201Q, and K614Q enzymes. Spectral perturbation measurements showed decreased Fd binding affinities for the R43Q (5-fold), R46Q (2.5-fold), and K201Q (3-fold) variants. No spectral perturbations were observed for K614Q, which prevented assessment of the K_d value. The R197Q and E219Q variants that retained wild-type level activity with both electron donors showed almost the same binding affinities for Fd as the wild-type enzyme. The conservative replacement variants generally exhibited enhanced Fd binding affinities compared to those of the equivalent nonconservative replacement variants. Wild-type Fd binding affinities were observed for R46K and K201R, and the Fd binding affinity for R43K was enhanced 2.5-fold compared to that of R43Q. In contrast to the K614Q variant in which no spectral perturbations were detected anywhere in the visible region when Fd was mixed with the enzyme, the K614R variant showed spectral changes after mixing with Fd that made it possible to estimate the binding affinity of the variant for Fd. In fact, this NR variant showed an affinity for Fd (>5-fold) significantly lower than that of the wild-type enzyme. These observations are consistent with the hypothesis that the side chains of Arg43, Arg46, Lys201, and Lys614 play an important role in Fd binding.

To assess if any changes in Fd-linked activity for the variants compared to that of wild-type *Synechococcus* NR were a consequence of loss or perturbation of the Mo or [4Fe-4S] centers, EPR studies as well as Fe and Mo analyses were performed for each variant. The analytical data in Table 4 show that the wild type and essentially all variants investigated contained approximately one [4Fe-4S] cluster and one Mo center per nitrate reductase monomer. The only exceptions were the R46Q and R197Q variants, which were approximately 25% deficient in both the [4Fe-4S] cluster and the Mo center. The effects of the K614Q, K614R, R46Q, R46K, R43Q, R43K, R197Q, K201Q, K201R, and E219Q mutations on the EPR properties of the Mo and [4Fe-4S] centers are shown in Figure 6. At 70 K, air-oxidized samples of all nine mutants exhibit the same high-*g* proton-split $S = 1/2$ Mo⁵⁺ resonance ($g_1 = 2.023$, $g_2 = 1.998$, and $g_3 = 1.993$; $A_1 = 6.8$ G, $A_2 = 7.5$ G, and $A_3 = 6.8$ G) accounting for 20–30% of the Mo content, as previously observed for the wild-type enzyme^{2, 7, 9} (see Figure 6A). On the basis of crystallographic and density functional theory studies,^{16, 29, 30} the structure of this Mo⁵⁺ species is proposed to be a bis-MGD Mo center ligated by cysteinate and a terminal sulfido, with the nonexchangeable proton hyperfine splitting originating from the cysteine β -CH₂ protons.^{30, 31} After dithionite reduction, the spectra of the wild-type enzyme and all nine variants recorded at 10 K exhibit the characteristic fast-relaxing rhombic resonance ($g_1 = 2.06$, $g_2 = 1.95$, and $g_3 = 1.91$) associated with the $S = 1/2$ [4Fe-4S]⁺ cluster and accounting for 90–100% of the Fe content^{2, 7, 9} (see Figure 6B).

EPR studies of dithionite-reduced samples at 70 K facilitated characterization of the slower-relaxing Mo⁵⁺ species in isolation (see Figure 6C). Two distinct types of Mo⁵⁺ species have been observed for the dithionite-reduced wild-type enzyme, and we have yet to define the specific conditions required to elicit each type of resonance. One is termed the high-*g* proton-split Mo⁵⁺ signal ($g_1 = 1.997$, $g_2 = 1.990$, and $g_3 = 1.982$; $A_1 = 6.5$ G, $A_2 = 6.0$ G, and $A_3 = 5.0$ G) that accounts for 0–20% of the Mo in wild-type samples.^{2, 9} The other is termed the low-potential Mo⁵⁺ signal ($g_1 = 2.017$, $g_2 = 1.988$, and $g_3 = 1.962$ with negligible proton splitting) that accounts for 0–10% of the Mo content in wild-type samples.^{7, 9} The observation of two distinct Mo⁵⁺ species in dithionite-reduced wild-type samples illustrates the plasticity of the Mo active site and suggests facile interconversion between these two Mo⁵⁺ species. The high-*g* proton-split Mo⁵⁺ signal is proposed to comprise a bis-MGD Mo center ligated by a side-on η^2 -cysteine persulfide with a partial disulfide bond. The nonexchangeable proton hyperfine splitting originates from the cysteine β -CH₂ protons.^{9, 16, 29–31} Because the low-potential Mo⁵⁺ signal lacks observable proton splitting, this suggests dissociation of the cysteine or conversion of the η^2 -cysteine persulfide ligand into a terminal cysteine persulfide ligand, which requires cleavage of only one cysteine S–Mo bond.⁹ Figure 6C shows that the dithionite-reduced K614Q and K614R samples exhibit weak low-potential Mo⁵⁺ resonances accounting for 4% of the Mo. All the other variants investigated, i.e., R46Q, R46K, R43Q, R43K, R197Q, K201Q, K201R, and E219Q, exhibit high-*g* proton-split Mo⁵⁺ resonances accounting for 3–20% of the Mo. While the proton hyperfine splitting in the high-*g* proton-split Mo⁵⁺ resonances shown in Figure 6C cannot be observed because of the spectra being recorded with a 6.3 G modulation amplitude, they are observed in spectra recorded with a 2.9 G modulation amplitude. In each case, the spectra

recorded with a 2.9 G modulation amplitude exhibited proton splittings identical to those reported for the wild type (see ref 9).

On the basis of the analytical data and the EPR properties of air-oxidized and dithionite-reduced samples, we conclude that the Mo and [4Fe-4S] prosthetic groups remain intact and essentially unperturbed compared to the wild type in all the *Synechococcus* NR mutants investigated in this work. While there are significant differences in the magnitude of the high-*g* proton-split Mo⁵⁺ resonances (3–20% of Mo), these differences do not correlate with either the reduced MV-dependent or the reduced Fd-dependent specific activities listed in Table 3. Hence, the dramatic decrease in the Fd-linked specific activity that is observed for the R46Q, K614Q, R43Q, and K201Q variants can be interpreted only in terms of changes in the Fd-binding region.

DISCUSSION

In addition to providing a basis for designing mutagenic replacements that can provide information about the interaction domains involved in the formation of the complex between Fd and NR, NR–Fd complex models also provide information about the distances between and orientations of the electron transfer prosthetic groups involved in the reaction catalyzed by this Fd-dependent NR. In the model shown in Figure 1, the distance from the Fd [2Fe-2S] cluster to the NR [4Fe-4S] cluster is 11.9 Å. In the lowest-energy, most favorable models, this distance ranged from 10 to 17 Å, suggesting that a reasonable estimate for this distance predicted by the modeling is 13.5 ± 3.5 Å. It is this distance that is likely to play an important role in determining the rate of electron transfer from reduced Fd to NR. The most probable mechanism for electron transfer from Fd to NR involves an initial electron transfer from the Fd [2Fe-2S]⁺ cluster to the NR [4Fe-4S]²⁺ cluster, followed by intramolecular electron transfer from the [4Fe-4S]⁺ cluster to the Mo center of NR.²

The structural model for the Fd–NR complex presented above not only provides estimates of distances between electron-carrying prosthetic groups but also identifies specific amino acids at the protein–protein interaction interface. Five positively charged amino acids (Arg43, Arg46, Arg197, Lys201, and Lys614) and a negatively charged amino acid (Glu219) were chosen to test the *in silico* NR–Fd complex model. Charge replacement variants (R43Q, R46Q, K201Q, and K614Q) retained wild-type levels of activity with MV, but substantial losses of activity were found when reduced Fd served as the electron donor. These observations, along with the higher K_M observed for Fd with the R46Q variant when compared to that of wild-type NR, indicate that Arg43, Arg46, Lys201, and Lys614 play an important role in binding Fd to facilitate the electron transfer to the Mo active site via the [4Fe-4S]^{2+,+} cluster. R43Q, R46Q, and K201Q variants also showed decreased binding affinity for Fd, when compared to that of wild-type NR, which further supports the role of Arg43, Arg46, and Lys201 in Fd binding. As the K614Q variant did not show any spectral perturbations upon being titrated with Fd, no conclusions about its ability to bind Fd could be drawn. Charge-conserving replacement mutations at Arg43, Arg46, Lys201, and Lys614 (i.e., R43K, R46K, K201R, and K614R, respectively) yielded Fd-dependent activities much higher than those of the charge-eliminating replacements. In addition to the increase in Fd-dependent activity, these four charge-conserving replacement variants showed increased

binding affinities for Fd and K_M values for both of substrates similar to those observed for the wild-type enzyme, indicating that the presence of a positive charge at these positions is the most important factor for productive Fd binding and electron transfer.

The involvement of Arg43, Arg46, Lys201, and Lys614 in ferredoxin binding is also consistent with amino acid sequence alignments of Fd-dependent cyanobacterial and non-Fd-dependent photosynthetic nitrate reductases (Figure 7). The sequence alignments show that all four of the positively charged amino acids at positions 43, 46, 201, and 614 are conserved in ferredoxin-dependent cyanobacterial NRs, but only one (Arg43) is conserved in non-Fd-dependent photosynthetic NRs. The sequence alignments also show that the amino acids at positions 197 and 219 in *Synechococcus* sp. PCC 7942 NR are not conserved in either cyanobacterial or other photosynthetic NRs. This is in agreement with the observation that variants at positions 197 and 219 showed wild-type levels of activity with ferredoxin as the electron donor.

The mutagenesis results presented in this work provide convincing support for the prediction based on the NR–Fd complex model that the four positively charged amino acids (Arg43, Arg46, Lys201, and Lys614) of NR are involved in binding Fd. While there are a number of other interactions that would stabilize the complex, for example, polar interactions involving Fd residues Tyr38, Ser39, and Ser63 and a hydrophobic interaction involving hydrophobic residue Phe64 of Fd, the results presented herein demonstrate that electrostatic interactions are the crucial element. The *in silico* NR–Fd complex model shown in Figure 8 allowed identification of the specific negatively charged Fd amino acids (Asp12, Asp35, Glu31, Glu93, and Glu94) that are putatively involved in electrostatic interactions with the four positively charged amino acid residues of NR. The specific interactions are R43–D12, R46–D35, K201–E31, K614–E93, and K614–E94, where the pairs are residues of NR and Fd, respectively. All five of these negatively charged Fd amino acid residues are highly conserved (Figure 2). The exception of D12, which is equivalent to A12 in *Synechococcus* sp. PCC 7942, suggests that R43 may interact with a different amino acid residue, probably D35 after small rotations of the side chains upon binding. The largest impact on K_d for binding of Fd to NR was the alteration of K614, which may be due to the loss of two electrostatic interactions involving K614.

The results presented herein are consistent with earlier reports that the negatively charged residues of Fd play an important role in efficient electron transfer from Fd to the Fd-dependent enzymes.^{8, 32, 33} Data for the ionic strength dependence of the stability constants for these complexes led to the proposal that they were stabilized predominantly by electrostatic forces, with Fd supplying the negatively charged groups and the enzymes supplying the positively charged groups involved in complex formation.^{3, 34} Chemical modification studies have been used to explore the effects of eliminating these negative charges on the ability of Fd to interact productively with target enzymes, and the results of these studies support the hypothesis that Fd does indeed contribute negative charges to the stabilization of these complexes.³⁵ Additionally, site-directed mutagenesis has also provided evidence that negatively charged Fd amino acids of *Anabaena* sp. PCC 7120³⁶ and spinach³³ are crucial for electron transfer from Fd to ferredoxin:NADP⁺ reductase (FNR) and involved in the formation of an electrostatic complex with positively charged amino acids of FNR.

Chemical modification studies have yielded results supporting the proposal that positive charges on the target enzymes, e.g., FNR,³⁷ glutamate synthase,³⁸ and nitrite reductase,³⁹ play an important role in the formation of the complex with Fd.

Electrostatic interactions have also been found to be important for interactions of [2Fe-2S] Fd with mammalian and bacterial enzymes. For example, mitochondrial cytochrome P450 (P450) and Fd reductase have also been shown to form an electrostatic complex with Fd, in which Fd provides negative charges and P450s and Fd reductases provide the positive charges.⁴⁰ Site-directed mutagenesis identified the specific negatively charged amino acids of human Fd that are important for forming a complex with Fd reductase and mitochondrial cytochrome P450_{scc} (CYP11A1).⁴¹ In addition to the Fd amino acids, site-directed mutagenesis has also shown that positively charged amino acids of mammalian mitochondrial P450s [P450_{scc} (CYP11A1) and P450_{c27} (CYP27A1)] are important for the formation of a complex with adrenodoxin, a [2Fe-2S] Fd in adrenal glands.⁴² In bacterial systems, electrostatic interactions were also found to be crucial for the formation of the complex between Fd and cytochrome P450s. Positively charged amino acid residues in cytochrome P450 (CYP199A2) from *Rhodopseudomonas palustris* were found to be important for the formation of a complex with the [2Fe-2S] Fd *palustris*redoxin.⁴³

On the basis of the studies described above and the fact that plant-type Fds typically have isoelectric points well below 7.0 and would thus exhibit net negative surface charges at physiological pH values, it seemed logical to assume that Fd would supply the negative charges and NR would provide the positive charges. Indeed, this was shown to be the case with *Synechococcus* sp. PCC 7942 NR in which positively charged amino acids were shown to be essential for the formation of the complex with Fd.⁸ Chemical modification of *Synechococcus* sp. PCC 7942 NR, in which positive charges were eliminated from NR with either phenylglyoxal or *N*-acetylsuccinimide, was found to interfere with the formation of the complex between NR and Fd.⁸ The data presented in this study are consistent with the observations made by Hirasawa et al.⁸ that the positively charged residues of NR are involved in the formation of the complex with Fd and provide specific details about the positively charged amino acids of *Synechococcus* sp. PCC 7942 NR that are involved in the formation of the complex with Fd.

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DEDICATION

This work is dedicated to the memory of David B. Knaff (1941–2016).

ABBREVIATIONS

CD	circular dichroism
EPR	electron paramagnetic resonance
Fd	ferredoxin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICP	inductively coupled plasma
IPTG	isopropyl β -D-thiogalactopyranoside
LB	Luria-Bertani
MGD	molybdopterin guanine dinucleotide
MV	methyl viologen
PCR	polymerase chain reaction
PMSF	phenylmethanesulfonyl fluoride
SDS–PAGE	sodium dodecyl sulfate–poly-acrylamide gel electrophoresis
UV	ultraviolet

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Figure 1.

(A) *In silico* model for two possible Fd orientations in the NR-Fd complex. The structure of NR is shown as a surface model (blue) and two of the possible associations of Fd (light and dark red). (B) *In silico* model for the NR-Fd complex. Shown are the backbone models of Fd (purple) and nitrate reductase (wheat). Fd (purple) with its Fe-S cofactor (atom type) with the nitrate reductase cofactors (atom type).

Figure 2.

Amino acid sequence alignment of cyanobacterial ferredoxins. Both N-terminal and C-terminal regions of the protein are included. The following cyanobacterial ferredoxins are shown: PCC 6803, *Synechocystis* sp. PCC 6803; Anabaena, *Anabaena cylindrica*; P13C2, *Limnithrix* sp. P13C2; Lyngbya, *Lyngbya aestuarii*; NIES-3709, *Geminocystis* sp. NIES-3709; PCC 7942, *Synechococcus* sp. PCC 7942. Negatively charged amino acids at position 12 are highlighted in red, at position 31 in turquoise, at position 35 in green, at position 93 in magenta, and at position 94 in yellow. Hyphens indicate gaps introduced to optimize alignments. Asterisks denote fully conserved residues.

Figure 3. Circular dichroism spectra of wild-type nitrate reductase and its R46Q and K614Q variants. Spectra were measured at ambient temperature in a 1.0 cm cell using solutions of either wild-type nitrate reductase or the indicated variant. All samples contained 0.75 μ M nitrate reductase in 10 mM potassium phosphate buffer (pH 7.5).

Figure 4. Michaelis-Menten plots of the activity of wild-type, R46Q, and R197Q nitrate reductase as a function of Fd concentration (at a saturating nitrate concentration) and nitrate concentration (at a saturating Fd concentration). The solid lines are best fits to the Michaelis–Menten equation (data presented here are from a single measurement). Values of K_M for nitrate and Fd are listed in Table 3.

Figure 5. Measurement of ferredoxin dissociation constants (K_d) by difference UV-vis absorption measurements for wild-type, R43Q, R46Q, R46K, K201Q, and K614R nitrate reductases. Solid lines show best fits using the K_d values listed in Table 4. Titrations were conducted in samples containing 10 μM nitrate reductase in 25 mM HEPES buffer (pH 8.0).

Figure 6.

EPR spectra of wild-type *Synechococcus* sp. PCC 7942 nitrate reductase and the R43Q, R43K, K614Q, K614R, R46Q, R46K, R197Q, K201Q, K201R, and E219Q variants. (A) Air-oxidized samples recorded at 70 K and a microwave power of 2 mW. (B) Dithionite-reduced samples recorded at 20 K and 1 mW. (C) Dithionite-reduced samples recorded at 70 K and 2 mW. All spectra were recorded at 9.60 GHz with a modulation amplitude of 6.3 G. The intensities of all spectra have been normalized with respect to the wild-type enzyme, to correct for differences in the spectrometer gain and the sample protein concentration.

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Figure 8. Fd-NR complex *in silico* model showing specific Fd amino acids that are putatively involved in electrostatic interactions with Arg43, Arg46, Lys201, and Lys614 of *Synechococcus* sp. PCC 7942 nitrate reductase.

Table 1

Primers Used To Introduce Mutations into the pCSLM85 Nitrate Reductase Expression Plasmid^a

primer name	sequence (5'–3')
R43Q_F	CCGATCTGGCAGATT C AGGGCGATCGGCAACAT
R43Q_R	ATGTTGCCGATCGCC T GAATCTGCCAGATCGG
R43K_F	CCGATCTGGCAGATTAAAGGCGATCGGCAACAT
R43K_R	ATGTTGCCGATCGCCTTTAATCTGCCAGATCGG
R46Q_F	CAGATTCGGGGCGAT C AGCAACATCCCTCCAGT
R46Q_R	ACTGGAGGGATGTT G CTGATCGCCCCGAATCTG
R46K_F	CAGATTCGGGGCGAT AA CAACATCCCTCCAGT
R46K_R	ACTGGAGGGATGTT GTTT ATCGCCCCGAATCTG
R197Q_F	CTCTTCAATCGCTATCAGAAGCGCCATAAACAG
R197Q_R	CTGTTTATGGCGCTTCTGATAGCGATTGAAGAG
K201Q_F	TATCGCAAGCGCCATCAACAGGGTGGCACCAAC
K201Q_R	GTTGGTGCCACCCTGTTGATGGCGCTTGCATA
K201R_F	TATCGCAAGCGCCATAGACAGGGTGGCACCAAC
K201R_R	GTTGGTGCCACCCTGTCTATGGCGCTTGCATA
E219Q_F	TGCACACCAACGGCTCAGGTTGCCGATCTCCAC
E219Q_R	GTGGAGATCGGCAACCTGAGCCGTTGGTGTGCA
K614Q_F	ACCGGCCGCATCGAC CA AATCAACAAGCTGCAT
K614Q_R	ATGCAGCTTGTGATT T TGGTCGATGCGGCCGGT
K614R_F	ACCGGCCGCATCGAC AGA AATCAACAAGCTGCAT
K614R_R	ATGCAGCTTGTGATT CT GTGTCGATGCGGCCGGT

^aF indicates the forward direction primer and R the reverse direction primer. The mutagenic base replacements are shown in bold.

Table 2Distances from Amino Acid Residues Altered by Mutagenesis to the FeS and Mo Centers^a

residue	distance to the Fe-S cluster (Å)	distance to Mo (Å)
R43	9.4	28.7
R46	11.8	25.5
R197	12.5	23.1
K201	17.7	28.9
E219	18.8	23.9
K614	14.1	22.2

^aDistances are measured from the end of each side chain to the closest point of each cofactor.

Table 3

Kinetic Properties of Wild-Type Nitrate Reductase and Its Docking Site Variants

enzyme	MV-linked specific activity ^a	Fd-linked specific activity ^a	K_M with nitrate (μM)	K_M with Fd (μM)
wild type	790 (100%)	30.5 (100%)	6.0 ± 1.0	38.0 ± 3.7
R43Q	721 (91.2%)	5.0 (16.4%)	ND ^b	ND ^b
R43K	734 (92.9%)	27.8 (91%)	8.1 ± 0.7	48.6 ± 3.9
R46Q	768 (97.2%)	7.4 (24.3%)	55 ± 20	364 ± 94
R46K	783 (99.1%)	29.8 (97.7%)	6.6 ± 1.0	49.6 ± 16.7
R197Q	738 (93.4%)	27.9 (91.8%)	7.0 ± 0.7	41.9 ± 3.8
K201Q	779 (98.6%)	4.5 (14.8%)	ND ^b	ND ^b
K201R	775 (98.11%)	29.4 (91.8%)	9.6 ± 1.0	45.6 ± 3.1
E219Q	749 (94.8%)	29.3 (96.1%)	4.5 ± 0.4	42.2 ± 5.1
K614Q	771 (97.6%)	3.5 (11.5%)	ND ^b	ND ^b
K614R	761 (96.3%)	19.3 (63.3%)	4.2 ± 0.5	44.5 ± 16.2

^aIn units of micromoles of nitrite produced per minute per milligram of enzyme.^bNot determined (because of the very low activity).

Table 4

Prosthetic Group Content and Ferredoxin Binding Affinities of Wild-Type Nitrate Reductase and Its Docking Site Variants

enzyme	iron ^a	molybdenum ^a	K _d with Fd (μM)
wild type	3.6 ± 0.45	0.93 ± 0.04	7.8 ± 2.1
R43Q	3.6 ± 0.20	0.95 ± 0.10	38.6 ± 14
R43K	3.7 ± 0.20	0.95 ± 0.10	20.2 ± 1.1
R46Q	2.8 ± 0.3	0.70 ± 0.10	19.8 ± 5.1
R46K	4.0 ± 0.1	0.80 ± 0.30	3.7 ± 0.3
R197Q	3.0 ± 0.20	0.70 ± 0.10	7.5 ± 1.9
K201Q	4.0 ± 0.20	1.0 ± 0.10	26.3 ± 12
K201R	3.9 ± 0.20	0.95 ± 0.10	4.2 ± 1.4
E219Q	4.0 ± 0.20	1.0 ± 0.10	8.3 ± 3.0
K614Q	3.8 ± 0.34	1.04 ± 0.18	<i>b</i>
K614R	3.8 ± 0.39	1.11 ± 0.14	39.8 ± 17.6

^aIn units of moles per mole of enzyme.

^bCannot be detected by spectral perturbations.