Probing the Determinants of Coenzyme Specificity in Ferredoxin-NADP+ Reductase by Site-directed Mutagenesis*

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On the basis of sequence and three-dimensional structure comparison between Anabaena PCC7119 ferredoxin-NADP+ reductase (FNR) and other reductases from its structurally related family that bind either NADP+/H or NAD+/H, a set of amino acid residues that might determine the FNR coenzyme specificity can be assigned. These residues include Thr-155, Ser-223, Arg-224, Arg-233 and Tyr-235. Systematic replacement of these amino acids was done to identify which of them are the main determinants of coenzyme specificity. Our data indicate that all of the residues interacting with the 2’-phosphate of NADP+/H in Anabaena FNR are not involved to the same extent in determining coenzyme specificity and affinity. Thus, it is found that Ser-223 and Tyr-235 are important for determining NADP+/H specificity and orientation with respect to the protein, whereas Arg-224 and Arg-233 provide only secondary interactions in Anabaena FNR. The analysis of the T155G FNR form also indicates that the determinants of coenzyme specificity are not only situated in the 2’-phosphate NADP+/H interacting region but that other regions of the protein must be involved. These regions, although not interacting directly with the coenzyme, must produce specific structural arrangements of the backbone chain that determine coenzyme specificity. The loop formed by residues 261–268 in Anabaena FNR must be one of these regions.

During the last decades, the understanding of protein function and, more specifically, the role of the individual amino acid residues involved in substrate binding and in the catalytic action have achieved considerable progress. Among the most relevant enzymes studied are those involved in electron transfer processes due to their practical importance. Now, the opportunity to design novel proteins is becoming more feasible, especially due to the increased detailed knowledge of the three-dimensional structure of many proteins. As a first step in this direction recent investigations have been aimed to redesign already existing proteins, so that they can produce a function different to that for which they were naturally synthesized (1, 2). Following this direction, a lot of effort is being made in the description of the determinants of coenzyme specificity for NAD(P)+/H-dependent redox enzymes (3–5). In biological systems NAD+/H is almost exclusively used by enzymes that catalyse oxidative exergonic reactions, whereas reductive endergonic reactions are generally catalyzed by enzymes that utilize NADP+/H (6). However, the only structural difference between them is the presence of a 2’-P group bound to the AMP moiety of the coenzyme in NADP+/H, and it is the presence or the absence of this phosphate group that permits the enzymes to make the distinction between these two coenzymes. Moreover, among the structurally related enzymes of the FNR family, members with preference either for NADP+/H or NAD+/H can be found. Crystallographic studies have demonstrated that discrimination between these coenzymes does not result from the presence of different structural domains in these enzymes (7–9).

We describe the introduction of point mutations in the coenzyme binding domain of ferredoxin-NADP+ reductase (FNR,1 EC 1.18.1.2) from the cyanobacterium Anabaena PCC7119 to probe the determinants of its coenzyme specificity and also as an initial attempt to alter the coenzyme specificity. This enzyme consists of a soluble single polypeptide chain that contains a noncovalently bound FAD group that is the cofactor involved in the redox reaction. Several points prompted us to choose FNR. During photosynthesis FNR accepts electrons from ferredoxin and uses them to convert NADP+ into NADPH (10). This process is highly specific for NADP+/H versus NAD+/H (11–13). Extensive biochemical characterization of FNR from different sources and, in particular, from Anabaena has been carried out (11–23), and several three-dimensional structures of FNR forms are available (24–26). Moreover, the structural arrangement of FNR has been proposed to be the prototype of a family of flavin oxidoreductases that interact specifically with either NADP+/H or NAD+/H (8, 25, 27). Finally, considering the high economical value of the reduced form of NADP+/H, the development of an in vitro system using FNR to generate NADPH is of high interest. Work is already

1 The abbreviations used are: FNR, ferredoxin-NADP+ reductase; DCPIP, 2,6-dichlorophenolindophenol; 2’-P, 2’-phosphate; WT, wild type.
under progress in this direction, and we foresee this development occurring in the near future (28). If we also achieve change of coenzyme specificity for FNR, generation of NADH could also be obtained with the same system. Moreover, due to the much cheaper value of NADH versus NADPH, the system could be used in the opposite direction, with NADH as reducing power, to produce reduced proteins (like ferredoxin, flavodoxin, hydrogenase, cytochrome P450 reductase, etc.).

Different crystallographic approaches on a variety of FNR forms from different sources have provided a picture of how the NADP$^+$ substrate must bind to FNR (24–26). Thus, the studies of spinach and Anabaena FNR revealed the importance of the side chains of residues Arg-100, Ser-223, Arg-224, Arg-233, Tyr-235, and Gln-237 (Anabaena FNR numeration) in the stabilization of the complex with NADP$^+/H$ by making contacts to its adenine ring, its 2'-P, and its 5'-phosphoryl (24, 25). The negative charge of the 2'-P group is apparently stabilized by the lateral chains of two positive-charged arginine residues, Arg-224 and Arg-233 (Arg-235 and Lys-244 in the spinach enzyme). The 2'-P of NADP$^+/H$ might also form hydrogen bonds with Ser-223 and a Tyr-235 residue (A and B). In the case of NAD$^+/H$ enzymes (C) these residues are not conserved. The region interacting with the pyrophosphate of the coenzyme presents a different consensus sequence depending on the coenzyme, NADPH (D and E) or NADH (F). In NADP$^+/H$-dependent enzymes Thr-155 (Pro), Thr-157, and Ala-160 (Anabaena FNR numbering) are always conserved (D and E). In those interacting with NAD$^+/H$, the equivalent residues are Gly, X, and Thr (F). The FAD cofactor and the coenzyme (if present) are drawn as sticks. Flavin cofactor are colored orange, and NADP$^+/H$ analogues are colored yellow. This figure was drawn using MOLSCRIPT (61) and RENDER (62).
conservative substitutions in all the NADP⁺/H-dependent enzymes (Fig. 1B, Table I). However, in the NAD⁺/H-dependent enzymes, these residues are not conserved, which interrupts the stabilization of 2’-P group and probably modifies the stacking interaction with the adenine moiety of NADP⁺/H by changing Tyr-235 by Phe (Fig. 1D). Sequence and structure analysis suggest that other regions of the protein might also account for FNR coenzyme specificity (Table I, Fig. 1, D–F). Thus, most of the members of the FNR family that bind NADP⁺/H show the sequence T(P)/GTGXAP (residues 155–161 in Anabaena FNR); whereas in those interacting with NAD⁺/H, the corresponding sequence is GGXGTXP (Table I). These residues form the loop between β1 and α-helix A as well as the first residues of this α-helix, whose N-terminal end might stabilize an interaction with the negative pyrophosphate of the coenzyme (Fig. 1D). A similar motif has also been shown to exist in the case of the flavoenzyme glutathione reductase, which is not a member of the FNR family and to be involved in coenzyme specificity (3).

To confirm the importance of the interactions with the 2’-P group of NADP⁺ and of the TGTGXAP FNR motif in Anabaena FNR coenzyme specificity, the T155G, S223G, S223D, R224Q, R233A, Y235F, and Y235A Anabaena FNR mutants have been constructed and characterized by a variety of techniques. The choice of the introduced mutations has been made taking into account the residues that occupy the equivalent positions in the NAD⁺/H-dependent members of the FNR family and trying to simulate a potential change in cofactor specificity (Table I). The rest of the mutations have been analyzed as controls. Moreover, after a careful analysis of the three-dimensional structure recently reported for a complex between FNR and ferredoxin (29), none of these residues is expected to be involved in ferredoxin binding or electron transfer.

### EXPERIMENTAL PROCEDURES

**Oligonucleotide-directed Mutagenesis—Anabaena FNR mutants were prepared using a construct of the petH gene previously cloned into the expression vector pTrc99a as a template (30). The FNR mutants T155G, S223D, S223G, R224Q, R233A, Y235F, and Y235A were produced using the Transformer site-directed mutagenesis kit from CLONTECH in combination with suitable synthetic oligonucleotides. The pTrc99a vectors with the desired mutation were used to transform the Escherichia coli Pasteur collection strain 0225 (17).**

**Purification of the FNR Mutants—**FNR mutants were purified from isopropyl-1-thio-β-D-galactopyranoside-induced LB cultures as described previously (17, 30). Some of the mutants were not retained by the Bicharcon blue gel and were purified by fast protein liquid chromatography using a Mono-Q column. UV-visible spectra and SDS-polyacrylamide gel electrophoresis were used as purity criteria.

**Spectral Analysis—**Ultraviolet-visible spectral analyses were carried out either on a Hewlett-Packard diode array 8452 spectrophotometer, a Kontron Uvikon 860 spectrophotometer, or a Kontron Uvikon 942 spectrophotometer. Circular dichroism was carried out on a Jasco 710 spectropolarimeter at room temperature in a 1-cm path length cuvette. Protein concentrations were 0.7 μM for the far UV and 3 μM for the aromatic and visible regions of the spectrum. Photodestruction of different FNR forms was performed at room temperature in an anaerobic cuvette containing 32–65 μM FNR samples and 3 μM 5-deazariboflavin in 50 mM Tris/HCl buffer, pH 8. The solutions were made anaerobic by repeated evacuation and flushing with O₂-free Ar. The spectra were recorded in a HP8452 diode array spectrophotometer before and after irradiating the samples with a 300-W light source for different times. Dissociation constants of the complexes between oxidized FNR mutants and either NAD⁺ or NAD⁺ were measured by differential spectroscopy using a double beam spectrophotometer at 25 °C as previously described (12, 17).

**Enzymatic Assays—**Diaphorase activity, assayed with DCPIP as artificial electron acceptor, was determined for all the FNR mutants as described previously (17). Both NADPH and NADH were assayed as coenzyme electron donors to each of the different FNR mutants. Unless otherwise stated, all the measurements were carried out in 50 mM Tris/HCl, pH 8.0. In all measurements, direct reduction of DCPIP by the coenzyme was subtracted from that of the enzyme-coenzyme mixture. The kinetic results obtained from the diaphorase activity were analyzed by using the Michaelis-Menten kinetic model and the enzyme concentrations used in the diaphorase reactions studied using NADH, high enzyme concentrations (0.5–9 μM) were required to detect and follow their activity. Therefore, in some of these cases the enzyme concentration used was only 100 times higher than that of the corresponding enzyme. This was also the case for the S223D FNR form with NADPH, where the enzyme concentration in the cuvette was 1 μM. When assaying the reaction of the other FNR enzymes with NADH, enzyme concentrations ranging from 3 to 25 μM were used.

**Stopped-flow Kinetic Measurements—**Fast electron transfer processes between NADPH or NADH and the different FNR samples were studied by stopped-flow methodology under anaerobic conditions using an Applied Photophysics SX17.7MV spectrophotometer interfaced with an Acorn 5000 computer using the SX.17MV software of Applied Photophysics. The absorbance (h₅₄₅) were calculated by fitting the data to a mono- or bi-exponential equation. Samples were made anaerobic (in specially designed tumblers that fit the stopped-flow apparatus) by successive evacuation and flushing with O₂-free Ar in 50 mM Tris/HCl, pH 8.0. Final FNR concentrations were kept between 6 and 11 μM, whereas, unless otherwise stated, NADPH and NADH final concentrations were in the range of 160–200 μM, and in some cases concentrations of the artificial electron acceptor were used at 0.5 or at 2.5 mM. The same methodology was also applied to the study of the reduction of NAD⁺ by T155G FNR samples. The time course of the reactions was followed at 460 nm, although other wavelengths were also analyzed (340 and 600 nm).

**Crystal Growth, Data Collection, and Structure Refinement—**Crystals of the T155G FNR mutant were grown by the hanging drop method. The 3.5-μl droplets consisted of 2 μl of 25.9 mg of protein/ml of solution.
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Expression and Purification of the Different FNR Mutants—

The level of expression in E. coli of all the mutated FNR forms was judged to be similar to that of the recombinant WT. All the mutants were obtained in homogeneous form and in amounts suitable to perform the demanding characterization studies described herein. Mutants at the position of Ser-223 interacted weakly with the Cibacron blue column, which binds specifically those proteins with a NAD(P)H interaction site, requiring the use of a fast protein liquid chromatography Mono-Q column for purification.

Spectral Properties—No major differences were detected in the UV-visible absorption and CD spectra of any of the FNR forms (Fig. 2). Therefore, no major structural perturbations appear to have been introduced by the mutations, and the extinction coefficient of *Anabaena* WT FNR (9.4 mm⁻¹ cm⁻¹ at 458 nm) (38) has been assumed herein for all the FNR mutants. Illumination of the FNR forms in the presence of 5-deazariboflavin caused the reduction of the protein to the neutral FNR semiquinone form with maxima in the range of 520 and 588–595 nm for all the FNR mutants (Fig. 2B). As for the WT enzyme, isosbestic points are also detected around 364 and 507 nm for the oxidized-semiquinone transition for all the mutants. Under the assayed conditions WT FNR stabilizes only 22% semiquinone. However, although a similar amount of semiquinone form is stabilized by most of the mutants, S223G and S223D showed an unexpected increment in the proportion of the radical stabilization (34 and 43%, respectively).

Steady-state Kinetics of the Different FNR Forms—The steady-state kinetic parameters of the different FNR mutants were analyzed for the DCP/IP-diaphorase reaction using either NADPH or NADH as electron donor (Table III). T155G, R224Q, and Y235F yielded similar values for $k_{cat}$ in the NADPH-dependent reaction to the WT enzyme. This parameter decreased by a factor of 4, 2, and 4 for S223G, R233A, and Y235A, respectively, and was up to 200 times smaller in the case of the S223D FNR form. With regard to the $K_m$ value for NADPH, T155G, R224Q, and Y235F showed moderated increments with regard to the WT enzyme value (about 4-, 13-, and 7-fold), whereas S223G, R233A, and Y235A yielded much higher $K_m$ values for NADPH (about 50-, 36-, and 67-fold, respectively), with S233D being the mutant with the highest $K_m$ value (about 125-fold larger) (Table III). Taking into account the kinetic parameters obtained, all the mutants showed a significantly decreased catalytic efficiency ($k_{cat}/K_m$) with regard to the WT FNR. The introduction of an aspartic acid residue at position 223 was the mutation that most affected the catalytic efficiency of the enzyme with NADPH (Fig. 3A).

The DCP/IP-diaphorase activity of the WT and all the FNR mutants was also assayed with NADH as electron donor (Table III). The data indicate that, in terms of both $k_{cat}$ and $K_m$, NADH is a very poor reductant for WT FNR ($k_{cat}$ decreased 38-fold, and $K_m$ for the coenzyme increased by a factor of 133 with respect to NADPH). Therefore, *Anabaena* FNR has a very low catalytic efficiency with NADH (Table III), and the specificity of the enzyme for NADPH, expressed as the ratio between the catalytic efficiency for NADPH and NADH, was found to be 67,000 times higher (Fig. 3). Although all the mutants presented a $k_{cat}$ value for the NADH-dependent reaction within a factor of 10 with regard to that of WT FNR, R224Q, R233A, and Y235F show a clear increase of this value (4–7-fold), whereas T155G and Y235A a decrease (about 7–8-fold) (Table III). It is noteworthy that these two mutants, T155G and Y235A, are the only ones showing a decrease in the $K_m$ value for NADH (about 4- and 2-fold respectively), whereas the rest of the mutants show an increment for this parameter to within a factor of 4 of the WT value. As is also shown in Table III, the catalytic efficiency of all these mutants with NADH is within a factor of 10 that of the WT enzyme with this coenzyme and for all of them is considerably smaller than that observed with NADPH (Fig. 3). However, it is noteworthy that when Ser-223 is replaced by an aspartic acid, the catalytic efficiency with NADPH approximates that obtained with NADH. Thus, the S223D single mutation decreases the enzyme specificity for NADPH from 67,000 times in the WT to only 8 times in the mutant (Fig. 3A).

Interaction of FNR Mutants with NADP⁺ and NAD⁺—The interaction of the different FNR forms with either NADP⁺ or NAD⁺ was investigated by differential spectroscopy (Fig. 4). When NADP⁺ binds to oxidized WT FNR, the visible spectrum

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**Table II**

<table>
<thead>
<tr>
<th>r.m.s., root mean square.</th>
<th>Data collection</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (K)</td>
<td>291</td>
<td></td>
</tr>
<tr>
<td>Source</td>
<td>Rotating anode</td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>P65</td>
<td></td>
</tr>
<tr>
<td>Cell a, b, c (Å)</td>
<td>88.13, 88.13, 97.25</td>
<td></td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>40.1–2.4</td>
<td></td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>15,728</td>
<td></td>
</tr>
<tr>
<td>Completeness of data (%)</td>
<td>99.5</td>
<td></td>
</tr>
<tr>
<td>All data</td>
<td>97.2</td>
<td></td>
</tr>
<tr>
<td>$R_{sym}$</td>
<td>0.10</td>
<td></td>
</tr>
</tbody>
</table>

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| r.m.s. deviation | 9.0–2.4 |
| Free R-factor   | 0.16    |
| Bond lengths (Å) | 0.014   |
| Bond angles (degree) | 1.4    |

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Buffered with 10 mM Tris/HCl, pH 8.0, 1 μl of unbuffered β-octylglucoside at 5% (w/v), and 2 μl of reservoir solution containing 18% (w/v) polyethylene glycol 6000, 20 mM ammonium sulfate, and 0.1 mM sodium acetate, pH 5.0. The droplet was equilibrated against 1 ml of reservoir solution at 20 °C. Under these conditions crystals grew within 1–7 days up to a maximum size of (0.8 × 0.4 × 0.4 mm) in the presence of a phase separation caused by the detergent.

These crystals were mounted in glass capillaries and screened on a Mar Research (Germany) image plate area detector for intensity, resolution, and mosaic spread using graphite-monochromated CuKα radiation generated by an Enraf-Nonius rotating anode generator. X-ray data for the T155G FNR were collected at 20 °C to a maximum resolution of 2.4 Å. Crystals belong to the P6₅, hexagonal space group with the following unit cell dimensions: a = b = 88.13 Å and c = 97.25 Å. The $V_M$ is 3.0 Å³/Da with one FNR molecule in the asymmetric unit and 60% solvent content. The x-ray data set was processed with MOSFLM (31) and scaled and reduced with SCALA from the CCP4 package (32).

The T155G structure was solved by molecular replacement using the program AmoRe (33) on the basis of the 1.9 Å resolution native FNR model (24) without the FAD cofactor. An unambiguous single solution for the rotation and translation functions was obtained. This solution was refined by the fast rigid-body refinement program FITTING (34). The model was subjected to alternate cycles of conjugate gradient refinement with the program X-PLOR (35) and manual model building with the software package O (36). The crystallographic $R$ and $R_{free}$ (37) values converged to values of 0.16 and 0.24, respectively for reflections between 9.0- and 2.4-Å resolution (Table II). The final model contains 2335 nonhydrogen protein atoms and 1 FAD, 1 SO₄²⁻, and 267 solvent molecules. The atomic coordinates of the T155G FNR mutant have been deposited in the Protein Data Bank (code 1bqe).
of the bound flavin undergoes a perturbation, yielding the difference spectrum shown in Fig. 4A. This has been shown in the case of the *Anabaena* FNR to be due to the interaction of the 2,5'-ADP moiety of the cofactor with the reductase (12). The spectral perturbations observed for oxidized R224Q, R233A, and Y235F FNRs upon NADP⁺ binding were weaker but very similar in shape to those observed for the WT FNR, and only minor displacements of the minima (around 392 and 502 nm) and maxima (around 354, 458, 480, and 522 nm) were detected (Fig. 4, A and B). The difference spectra obtained at different coenzyme concentrations allowed the determination of the dissociation constants and binding energies for the corresponding complexes (Fig. 4F, Table IV) (12). Thus, Y235F, R224Q, and R233A bind NADP⁺ 35-, 95-, and 210-fold weaker than the WT FNR. Therefore, although binding of NADP⁺ to either Y235F, R224Q, or R233A FNRs produce equivalent structural perturbations around the flavin ring as those observed for the WT, apparently the positive side chains of Arg-224 and Arg-233 play an active role in positioning the coenzyme by making direct contacts with it. Interestingly, binding of NADP⁺ to T155G FNR elicited spectral changes at different wavelengths than those reported for WT FNR (Fig. 4D), with minima at 396 and 440 nm and maxima around 476 and 512 nm, whereas the binding was estimated to be only 17-fold weaker than that of the WT (Table IV). This result indicates that, although Thr-155 might not be directly involved in the interaction with the coenzyme, the replacement of Thr-155 by Gly produced slight structural changes in the protein that have an important influence in the arrangement of the flavin environment when the coenzyme is bound. Finally, when oxidized S223D, S223G, and Y235A FNR forms were titrated with NAD⁺, no difference spectra were detected in the flavin region of the spectra as shown in Fig. 4E for Y235A. These mutants were only characterized by a loss of absorbance peaking in the 334–338 nm range, which allowed estimation of very high values for the dissociation constants in the case of these complexes (Table IV), indicating the importance of positions Ser-223 and Tyr-235 in coenzyme recognition and binding.

It has already been shown that NAD⁺ is not able to produce any spectral perturbation in the flavin absorption range when added to WT FNR, presumably due to the absence of the 2'-P group, which is essential for NADP⁺ binding to *Anabaena* FNR (12). When the mutants were titrated with NAD⁺, only T155G elicited a weak difference spectrum in the flavin region with maxima at 420 and 505 nm (Fig. 4C). These data indicate that replacement of Thr-155 by Gly produced some changes in the protein that allow NAD⁺ to perturb the FAD environment of FNR. No difference spectra were obtained with any other FNR mutant in the flavin region of the spectra.

### Fast Kinetic Studies of the Reduction of FNR Mutants by NADPH and NADH

The fast kinetic reaction of oxidized *Anabaena* FNR forms with either NADPH or NADH was de-
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Table IV

Dissociation constants and free energy for complex formation of different Anabaena PCC 7119 FNR forms in the oxidized state with NADP⁺ and NAD⁺

<table>
<thead>
<tr>
<th>FNR form</th>
<th>(K_d^{NADP⁺})</th>
<th>(ΔG^{NADP⁺})</th>
<th>(K_d^{NAD⁺})</th>
<th>(ΔG^{NAD⁺})</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>5.7 ± 0.3 †</td>
<td>−7.10 ± 0.05 †</td>
<td>ND ‡</td>
<td>ND ‡</td>
</tr>
<tr>
<td>T155G</td>
<td>99 ± 10</td>
<td>−5.50 ± 0.06</td>
<td>ND ‡</td>
<td>ND ‡</td>
</tr>
<tr>
<td>S223G</td>
<td>&gt;8000</td>
<td>ND ‡</td>
<td>ND ‡</td>
<td>ND ‡</td>
</tr>
<tr>
<td>S223D</td>
<td>ND ‡</td>
<td>ND ‡</td>
<td>ND ‡</td>
<td>ND ‡</td>
</tr>
<tr>
<td>R224Q</td>
<td>535 ± 20</td>
<td>−4.44 ± 0.02</td>
<td>ND ‡</td>
<td>ND ‡</td>
</tr>
<tr>
<td>R233A</td>
<td>1200</td>
<td>−4.2</td>
<td>ND ‡</td>
<td>ND ‡</td>
</tr>
<tr>
<td>Y235F</td>
<td>200 ± 20</td>
<td>−5.00 ± 0.04</td>
<td>ND ‡</td>
<td>ND ‡</td>
</tr>
<tr>
<td>Y235A</td>
<td>ND ‡</td>
<td>ND ‡</td>
<td>ND ‡</td>
<td>ND ‡</td>
</tr>
</tbody>
</table>

† Data from Medina et al. (17).
‡ No difference spectra were detected in the flavin wavelength range.
§ Spectral perturbations were too weak even at high coenzyme concentration.

In the case of the T155G FNR form, the kinetics of reoxidation of the enzyme by NADP⁺ has also been studied by stopped-flow. An increase in absorbance at 460 nm due to FNR reoxidation and an increase at 340 nm due to NADPH formation were observed (Fig. 4). The traces were well fit by a single exponential having a rate constant of at least 350 s⁻¹. This behavior is similar to that reported for the WT enzyme for which a \(k_{obs}\) greater than 550 s⁻¹ has been reported when analyzed under similar conditions (17).

When analyzing the reaction of WT FNR with NADH by stopped-flow a very slow decrease in the absorption at 460 nm was observed that was best fit by two processes having \(k_{obs}\)
values of 0.35 (−60% amplitude) and 0.005 (−40% amplitude) s⁻¹ (Fig. 7A, Table V). Reaction of NADH with R224Q, R233A, and Y235F took place with $k_{abs}$ values that were within a factor of 2–5 that obtained for the WT enzyme (Table V, Fig. 7B). However, although R224Q and R233A were best fit to a mono-exponential process, two processes were observed for the Y235F mutant, which shows an important increase in the exponential process, two processes were observed for the R224Q and R233A. Thus far, as was the case for the reactions with NADPH at long time scales, we do not have a good explanation for all the observed processes. Surely the observed changes in absorbance would reflect the association and interconversion of the NAD(P)⁺/H-FNR forms, and further work along these lines is under way and will be reported elsewhere. However, comparison of the data obtained for the different FNR forms, including the WT enzyme, provides valuable information in the present study.

**DISCUSSION**

**Effect of the Single Mutations at the 2'-Phosphate Interaction Site of NADP⁺** —The importance of a stabilizing interaction between the side chain of Ser-223 and the 2'-P of NADPH has been proven by replacement of this residue by a Gly, which lacks the OH group and, therefore, the hydrogen binding capability, and by an Asp, the residue that is highly conserved at the equivalent location in NADH-dependent enzymes (Table I; Fig. 1, A and C). Replacement of Ser-223 by either Gly or Asp produced enzymes that had only 0.5 and 0.005% of the catalytic efficiency of the WT FNR with NADPH (Table III, Fig. 3A), and the $k_{abs}$ values for the reaction with NADPH were at least 30- and 1500-fold, respectively, slower than that of the WT enzyme when analyzed by stopped-flow (Fig. 5, Table V). However, no gross differences were detected for the catalytic parameters with NADH when compared with those of the WT enzyme (Fig. 3B). Thus, steady-state and fast kinetic studies suggest that removal of the Ser-223, and/or introduction of a negatively

![FIG. 5. Time course of the anaerobic reactions of the different FNR forms with NADPH observed at 460 nm. A, 6 μM WT FNRox (bold line) reacted with 170 μM NADPH, 9 μM T155G FNRox (closed squares) reacted with 160 μM NADPH, 9 μM R224Q FNRox (dotted line) reacted with 190 μM NADPH, 6 μM Y235F FNRox (open circles) reacted with 170 μM NADPH. B, 9 μM S223G FNRox (open circles) reacted with 240 μM NADPH, 11 μM S223D FNRox (open squares) reacted with 160 μM NADPH, 6.5 μM R233A FNRox (closed squares) reacted with 2.5 mM NADPH, 15 μM Y235A FNRox (bold line) reacted with 240 μM NADPH. Reactions were carried out in 50 mM Tris/HCl, pH 8.0, at 13 °C. Final concentrations are given. U.A., units of absorbance.

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charged residue at this position produces an enzyme that lacks its ability to discriminate between NADP⁺/H and NAD⁺/H. This behavior must be mainly due to the low affinity of both Ser-223 mutants for either NADP⁺/H or NAD⁺/H, as suggested from the fact that neither of them showed a difference spectrum in the presence of any of the oxidized coenzymes (Table IV). Thus, our studies confirm that Ser-223 side chain is a crucial determinant of FNR coenzyme specificity for NADPH. The very low degree of interaction between S223G and the coenzyme suggests that stabilization of NADPH binding must be due to a hydrogen bond or a charge-dipole interaction between the Ser-223-OH group and the 2'-P of NADP⁺/H. Such interactions cannot be formed in this mutant. Moreover, introduction of a negative charge at position 223 almost completely prevents the FNR-NADP⁺/H interaction. As mentioned above, the NAD⁺/H-dependent enzymes of the FNR family have an aspartate residue at this position (Table I, Fig. 1). Several studies have already established the importance of such a negatively charged residue in NAD⁺/H-dependent enzymes from different families as well as the strong alteration of the affinity for NAD⁺/H when hydrogen bonds between this carboxylate group and the adenosine 2'- and 3'-hydroxyl positions of the coenzyme are not able to be formed (9, 40–42). It has also been shown that the introduction of such a negative charge at the residue that occupies the position spatially similar in some NADP⁺/H-dependent enzymes prevents the interaction with the 2'-P of the coenzyme and might even enhance NAD⁺/H affinity (43, 44). However, this latest effect has not been shown by our S223D mutant (Fig. 3B, Table V). Thus, it can be proposed that in the case of Anabaena FNR, the introduction of a negatively charged residue at position 223 produces a change in the catalytic behavior of the enzyme that is mainly due to the electrostatic repulsion between the negative side chain and the negatively charged 2'-P of NADP⁺/H, which prevents its binding rather than allowing a favorable interaction of the Asp-233 with the 2'-OH group of NAD⁺/H.

Previous studies suggest that the positively charged side chains of Arg-224 and Arg-233 might be involved in FNR coenzyme recognition (15, 16, 24–26, 45–47). To test this hypothesis, the R224Q and R233A FNR mutants were produced by removing the positive charge. These enzymes were found to have 7.5 and 1.5%, respectively, the catalytic efficiency of the WT FNR with NADPH (Table III, Fig. 3A), and these low efficiencies are mainly due to the high $K_{m}$ values for NADPH for these mutants. When analyzing the catalytic parameters with NADH for these mutants, both of them showed only a moderate increment in the $k_{cat}$ value with regard to the WT FNR. Combination of these two factors produced a slight increment in their catalytic efficiencies with NADH (Table III, Fig. 3B). Both of these mutants elicited changes in their visible spectra upon NADP⁺ addition (Fig. 4), indicating coenzyme binding. R224Q and R233A were estimated to bind NADP⁺ 90- and 210-fold weaker than the WT FNR (Table IV). However, no affinity for NAD⁺ was detected (Table IV). When analyzing the fast kinetic parameters of the reaction of NADPH with R224Q and R233A, different behaviors were detected for both mutants. R224Q shows a pattern of behavior similar to that of the WT enzyme but had $k_{obs1}$ and $k_{obs2}$ values at least 3 and 10 times smaller, respectively (Fig. 5, Table V). However, the reaction with R233A is more than 500-fold slower than that of the WT enzyme, even at high NADPH concentration. Finally, when these mutants were analyzed for the reaction with NADH, both reactions were best fit to monoeponential processes, with the observed rate constants for both of the mutants slightly higher than those observed for the WT enzyme (Table V). The obtained results indicate that replacement of Arg-224 by a Gln produces an enzyme that is still able to form a productive complex with NADPH, although considerably weaker, and to accept electrons efficiently from the coenzyme. This indicates that the function played by the guanidinium group of Arg-224 in stabilizing an interaction with the 2'-P could be also supported in a considerable degree by the Gln side chain. Moreover, a Gln residue at this position also slightly improves some of the processes studied with NADH. Analyzing sequence homology at this position allows the behavior observed for the R224Q mutant to be easily understood. As expected, all the NADP⁺/H-dependent enzymes of the FNR family possess a positively charged residue at this position.
Moreover, it is noteworthy that this is also the case for some of the NAD<sub>H</sub>/H-dependent enzymes, and it is evident that in those enzymes that do not have negatively charged residues at this position, residues having side chains (Gln or His) capable of forming hydrogen bonds or dipole-charge interactions with the 2'-P are located at the equivalent position. Therefore, we can conclude that, although Arg-224 might be involved in an electrostatic interaction with the 2'-P that allows tighter binding to the substrate, it must be mainly involved in stabilization of the coenzyme binding through hydrogen bonds or charge-dipole interactions. However, this residue is not involved in determining FNR specificity for NADP<sup>+</sup>/H versus NAD<sup>+</sup>/H. Replacement of Arg-233 by an Ala produces a more drastic effect on the interaction between the enzyme with its natural coenzyme, NADP<sup>+</sup>/H, which therefore affects the fast electron transfer process. This indicates that the side chain of the Ala is not able to provide some interactions that apparently allow an appropriate orientation of the coenzyme in its FNR binding region to allow subsequent electron transfer. Also, taking into account the conservation of a positively charged residue in all the NADP<sup>+</sup>/H-dependent enzymes, and the fact that none of the NAD<sup>+</sup>/H-dependent enzymes have a positively charged side chain at this position, our results suggest that the Arg-233 side chain provides stabilization of the 2'-P group and allows optimal NADP<sup>+</sup>/H binding. However, removal of this interaction only slightly improves the affinity of the FNR for NAD<sup>+</sup>/H. Positively charged residues equivalent to the aforementioned have already been shown to play a role in determining NADP<sup>+</sup>/H specificity in other enzymes, such as cytochrome P-450 reductase or isocitrate dehydrogenase (48, 49).

The three-dimensional structural analysis of the complexes of FNR with NADP<sup>+</sup> also indicates a close interaction between the adenine moiety of the NADP<sup>+</sup> and the aromatic side chain of Tyr-235, which is conserved as a Tyr in all NADP<sup>+</sup>/H-dependent enzymes and as a Phe in the NAD<sup>+</sup>/H-dependent enzymes of the family (Fig. 1, Table I) (24, 26). The residue at this position of *Anabaena* FNR has been replaced either by a
Phe or by an Ala. The diaphorase activities with NADPH for the Y235F and Y235A FNR enzymes were, respectively, 28% and just 0.3% of the WT catalytic efficiency (Table III, Fig. 3A). These results are mainly due to a 7-fold increment in the $K_m$ of the Y235F form and to a 70-fold for the Y235A mutant, which was also accompanied by a 4-fold decrease of its $k_{cat}$. A slightly increment in the $k_{cat}$ value of Y235F with NADH was also observed (Table III, Fig. 3B). On the contrary, Y235A was 4-fold less efficient with NADH than the WT. Moreover, whereas Y235F binds NADP$^+$ 35-fold weaker than the WT FNR (Fig. 4), no binding of this coenzyme was detected for Y235A. None of the mutants elicited a difference spectrum in the presence of NAD$^+$, indicating a very low affinity for this coenzyme (Table IV). Fast kinetic parameters of the reactions of either NADPH or NADH with Y235F and Y235A also show different behaviors for both mutants. Although Y235F behaved similarly to the WT enzyme, with only slightly slower observed rate constants (Figs. 5 and 7, Table V), the destabilizing effect introduced by the Ala at position 235 clearly resulted in a drastic decrease of the observed rate constant values of the processes. Therefore, our results clearly indicate that replacing Tyr-235 for a nonaromatic residue noticeably reduced the ability of FNR to interact efficiently with NADP$^+$H, and it even produced a decrease in the already low affinity of the enzyme for NAD$^+$H. This confirms that the stacking interaction between the aromatic residue at position 235 of Anabaena FNR and the adenine ring of NAD(P)$^+$H is required to provide an adequate orientation between the protein and the coenzyme, which places the nicotinamide ring in a position capable of accepting electrons from the isoalloxazine ring. Our data also show that a Tyr at position 235 is more efficient than a Phe for NADP$^+$H binding and electron transfer, confirming the importance of the hydrogen bond between the Tyr-OH and the 2$'$P of NADP$^+$H in complex formation and orientation. The lack of this hydrogen bond in the Y235F mutant still allows NADP$^+$H binding but results in a weaker and less productive complex. On the other hand it is noticeable that this latter mutant appears to accommodate NAD$^+$H more efficiently. This might also be expected, taking into account that the NADH-dependent members of the enzyme have a Phe at this position (Table I, Fig. 1D).

**Effect of the Single Mutation T155G—Thr-155 of Anabaena FNR** was replaced by a Gly, the residue present at the equivalent position in all NAD$^+$H -dependent members of the family (Fig. 1, Table I), to test if this residue and the region that contains it is involved in coenzyme specificity and if NAD$^+$H binding could be induced. This mutation produced only a slight effect on the catalytic properties of the enzyme when its reactivity was assayed with NADPH either by steady-state or by fast kinetic methods (Table III and V, Fig. 3). The $K_m$ for NADPH and $K_d$ for the T155G-NADP$^+$ complex suggest that these changes must be due to a decrease in the affinity for the cofactor (Tables III and IV). The latest analysis also indicates that this mutant accommodates the NADP$^+$H coenzyme in a different orientation with regard to the flavin ring than the WT (Fig. 4). Analysis of the T155G FNR three-dimensional structure indicates that the introduced mutation produces an alteration of a hydrogen bond network (Fig. 9), which produces a slight displacement of the backbone in the NADP$^+$H binding domain (Fig. 8). In particular, important structural differences are observed in the loop comprising residues 261–268. Interestingly, the three-dimensional structural analysis of this region in the different members of the FNR family shows that in the case of NAD$^+$H-dependent enzymes a different organization of the hydrogen bond network (Fig. 9) and a hairpin-like region rich in proline residues (Table I, Fig. 10) are present. This analysis also shows that the conformation of such a region rich in proline residues in the NAD$^+$H members would not be compatible with the presence of a Thr (or Pro) at the position equivalent to 155 of Anabaena FNR in the NADP$^+$H members due to steric hindrance (Fig. 10). Replacement of Thr-155 in Anabaena FNR by a Gly produces a slight retraction of the loop that might explain some of the behaviors observed for this mutant such as the increase in its affinity for NAD$^+$H, as
indicated by the decrease observed in the $K_e$ for NADH and by the fact that all of the FNR forms assayed, T155G was the only one that showed a difference spectra upon NAD$^+$ binding (Fig. 4C). However, the mutant did not enhance reactivity with NADH, as shown by the very small values obtained by $k_{cat}$ and $k_{obs}$ when studied by steady-state and by fast kinetic methods (Table III and V). This indicates that, although the structural modifications induced by a Gly at position 155 enhance the enzyme affinity for NAD$^+/H$, the resulting complex does not provide an orientation conducive to electron transfer. This was expected, since the other regions of the protein that determine the coenzyme specificity are involved in NADP$^+/H$ recognition.

General Conclusions—The analysis of the determinants of coenzyme specificity in *Anabaena* FNR indicates that all the residues interacting with the 2'-P group of NADPH, Ser-223, R224, Arg-233, and Y235, are not involved to the same extent in determining coenzyme affinity and specificity. Thus, the side chain of Ser-223 is crucial in determining NADP$^+/H$ binding, with the presence of a negatively charged residue at this position preventing its binding. Although the presence of a positively charged residue at positions 224 and 233 of *Anabaena* FNR are not crucial in determining coenzyme specificity, these residues are involved in providing a stronger interaction between the enzyme and NADP$^+/H$. The importance of an aromatic residue at position 235 of *Anabaena* FNR for the interaction with both coenzymes, NADP$^+/H$ and NAD$^+/H$, has been demonstrated, as has the fact that a hydrogen bond between the coenzyme and the OH group of Tyr-235 is also involved in determining the NADP$^+/H$ versus NAD$^+/H$ specificity in FNR.

Finally, our results also indicate that the determinants of coenzyme specificity of FNR are not only situated in the 2'-P binding region, but that other regions of the protein must be involved. Thus, the arrangement of the backbone chain in the coenzyme binding domain around the loop that connects strand 4 and the α-helix D in *Anabaena* FNR must be one of the regions which determines coenzyme specificity.

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