Competition between C-terminal Tyrosine and Nicotinamide Modulates Pyridine Nucleotide Affinity and Specificity in Plant Ferredoxin-NADP⁺ Reductase*

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Chloroplast ferredoxin-NADP⁺ reductase has a 32,000-fold preference for NADPH over NADH, consistent with its main physiological role of NADP⁺ photoreduction for de novo carbohydrate biosynthesis. Although it is distant from the 2'-phosphoryl group of NADP⁺, replacement of the C-terminal tyrosine (Tyr³⁰⁸ in the pea enzyme) by Trp, Phe, Gly, and Ser produced enzyme forms in which the preference for NADPH over NADH over NADH was decreased about 2-, 10-, 300-, and 400-fold, respectively. Remarkably, in the case of the Y308S mutant, the $k_{cat}$ value for the NADH-dependent activity approached that for the NADH-ferricyanide reaction. The data presented support the hypothesis that specific recognition of the 2'-phosphate group of NADP(H) is required but not sufficient to ensure a high degree of discrimination against NAD(H) in ferredoxin-NADP⁺ reductase. Thus, the C-terminal tyrosine enhances the specificity of the reductase for NAD(H) by destabilizing the interaction of a moiety common to both coenzymes, i.e. the nicotinamide.

Nicotinamide adenine dinucleotides (NAD(H) and NADP(H)) are coenzymes used by a very large class of dehydrogenases. The vast majority of these oxidoreductases show a strong preference for one of the pyridine nucleotides. In general, NADP(H)-dependent and NAD(H)-dependent dehydrogenases are involved in reductive anabolic pathways and oxidative catabolic pathways, respectively. Crystallographic studies have shown that many pyridine nucleotide-dependent dehydrogenases have similar dinucleotide binding domains known as a Rossmann fold (1). The structural determinants responsible for coenzyme specificity in these enzymes are thought to be those regions that recognize the distinct features of the two dinucleotides, i.e. the 2'-OH versus the 2'-phosphate group of the adenosine ribose. Attempts to redesign the coenzyme specificity of dehydrogenases by site-directed mutagenesis of the 2'-phosphate binding pocket have revealed that a single residue appears to be of primary importance but that several nearby residues are also involved in determining coenzyme preference (2–11). A discouraging reality is that the shift in specificity is often achieved at the expense of catalytic potency (i.e. the loss in the effectiveness with the natural substrate is much greater than the gain in effectiveness of the new substrate). Here, we identify a second structural feature distant from the 2'-phosphate binding region that is required for coenzyme discrimination in ferredoxin-NADP⁺ reductase (FRN; EC 1.18.1.2), the enzyme responsible for storing light energy as reducing equivalents of NADPH during photosynthesis (for reviews see Refs. 12 and 13).

The three-dimensional structures of FRN from spinach and the cyanobacterium Anabaena PCC 7119 have been described at high resolution (14–16). The flavoprotein molecule is made up of two structural domains, each containing approximately 150 amino acids. The C-terminal domain harbors the residues involved in NADP⁺ binding, whereas one molecule of the prothethetic group FAD is tightly bound to the N-terminal domain through a combination of noncovalent interactions (14–16). The two-domain structure of FRN can be regarded as the basic module for a large structural family of flavin-dependent electron transferases, with more than 20 nonequivalent members identified so far (17). The FRN family comprises both NADP⁺ and NAD-dependent oxidoreductases. In Fig. 1, the “fingerprint” sequence regions involved in pyridine nucleotide binding for selected members of the family are shown. A distinguishing feature of many members of the family is the presence of an aromatic residue (Tyr, Trp, or Phe), whose side chain lies close and parallel to the re-face of the isoalloxazine ring of flavin in the active site, preventing access to the nicotinamide ring of NAD(P)(H). Recently, replacement of the C-terminal Tyr of pea FNR (Tyr³⁰⁸) with Ser or Gly generated mutants displaying a highly enhanced affinity for NADP⁺. This allowed us to solve crystal structures of the complexes of FRN with NADP(H) (25), showing the productive binding of the nicotinamide ring in the active site (Fig. 2). The increased binding of NADP⁺ by these FRN mutants led us to hypothesize that removal of the aromatic side chain of the C-terminal residue would promote

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† The abbreviations used are: FNR, ferredoxin-NADP⁺ oxidoreductase (EC 1.18.1.2); 2'-AMP, 2'-phosphoadenosine-5'-monophosphate; 2'-ADP, 2'-diphosphoadenosine-5'-diphosphate; thionicotinamide adenine dinucleotide phosphate.
enhanced binding of NAD+. Here, we report that these mutants form stable complexes with NAD+ and have a greatly enhanced ability to use NADH as an electron donor for catalysis. Our results indicate that the aromatic C-terminal residue of FNR plays a key role in modulating pyridine nucleotide discrimination.

**EXPERIMENTAL PROCEDURES**

NADP(H), NADH, and NADP+ analogs (2′-P-AMP, 2′-P-ADP-ribose, and thio-NADP+) were obtained from Sigma. All other chemicals were of analytical grade. Procedures employed for the expression, mutagenesis, and purification of recombinant pea FNR forms from *Escherichia coli* cells have been reported elsewhere (25, 26). The FNR-Y308S binds NADP+ so tightly that this recombinant enzyme was isolated from *E. coli* lysates in complex with the nucleotide, whereas FNR-Y308G was isolated as a mixture of NADP+-bound and ligand-free forms. Removal of NADP+ from both the purified FNR-Y308G and FNR-Y308S was carried out according to Ref. 25. Recombinant spinach FNR was purified as described in Ref. 27.

**Spectral Analyses**—Absorption spectra recordings were performed on Hewlett-Packard 8452A or 8453 diode array spectrophotometers. Extinction coefficients of the FNR forms were determined by resolving the FAD from the apoprotein by treatment with 0.2% (w/v) sodium dodecyl sulfate and spectrophotometrically quantitating the released FAD (27). To determine difference spectra and dissociation constants of the FNR complexes with NAD(P)+ and analogs, the various reductase mutants were spectrophotometrically titrated with the corresponding ligands. FNR samples were diluted in 10 mM Tris-HCl, pH 7.7 (at 25 °C) to a final concentration of ~20 μM. Absorption spectra were recorded after successive additions of nucleotide. Difference spectra were computed by subtracting from each spectrum, the spectrum recorded in the absence of the ligand, taking dilution into account. Absorption values at several different wavelengths were plotted and analyzed as described in Ref. 27.

**RESULTS**

**Pyridine Nucleotide Specificity of Pea FNR Mutants**—The *Km* for the FNR reductive half-reaction because for the wild-type reductase the rate-limiting step in this reaction is the reduction of FAD by NADP+ (27, 29). We therefore determined the kinetic parameters of the Tyr308 mutants in both the NADP+- and NADH-ferricyanide reductase reactions assayed under the same conditions (Table I). With the exception of FNR-Y308F, all mutants showed a decrease in *kcat* in the NADP+-dependent activity, with FNR-Y308G and FNR-Y308S displaying the lowest values. With regard to the *km* of the enzyme for NADPH, the Phe and Trp replacements produced moderate decreases (about 5-fold), whereas the Gly and Ser replacements led to 25- and 55-fold decreases, respectively (Table I). All mutants showed a significantly higher catalytic efficiency (*kcat/Km*) with respect to wild-type pea FNR. The most efficient enzyme was the FNR-Y308F with a 6-fold enhancement in its catalytic efficiency (Table I).

To probe the hypothesis that these mutants would show less discrimination against NAD(H), we measured the ferricyanide reductase activity of the Tyr308 mutants using NADH as electron donor. Interestingly, the FNR variants with the lower *kcat* values in the NADP+-dependent reaction turned out to be the ones displaying the highest NADH-dependent activities (Table I). In the latter reaction, the FNR-Y308S showed a *kcat* value 2 orders of magnitude higher than that of the wild-type enzyme and comparable with the *kcat* of the wild-type FNR with...
NADPH as a substrate. This observation strongly supports the idea that the decrease in $k_{cat}$ for the NADPH-dependent dephosphorylation reaction of the Gly and Ser mutants was caused by tight binding of the NADP$^+$ product during enzyme turnover (25). The FNR-Y308G displayed kinetic parameters similar to those of the Ser mutant, whereas the FNR-Y308F, which was very efficient as an NADPH-dependent catalyst, also showed a remarkably high $k_{cat}$ in the NADH-dependent reaction. As foreseen, the $K_a$ of FNR for NADH was indeed affected by mutations of Tyr$^{308}$ with a pattern similar to that observed for NADPH. Whereas substituting Phe for Tyr produced only a 3-fold change in the $K_a$ value for NADH, replacement with Gly or Ser caused a 20- and 35-fold decrease, respectively, as compared with the wild-type value (Table I). Thus, the catalytic efficiency for the NADH-dependent reaction was increased by about 3-, 80-, 900-, and 1700-fold in the Trp, Phe, Gly, and Ser mutants, respectively. The most convenient measure of the discrimination of an enzyme between two competing substrates is the ratio of the catalytic efficiencies ($k_{cat}/K_m$) with each substrate (30). This ratio is very high (about 32,000) for wild-type spinach or pea FNR, indicating strong preference for NADPH, but declines sharply with nonaromatic mutations of Tyr$^{308}$ to reach a value of just 77 in the case of FNR-Y308S (Table I).

**Interaction of Pea FNR Mutants with NADP$^+$ and Analogs**

All amino acid replacements of Tyr$^{308}$, including the conservative Tyr to Phe substitution, resulted in significant changes in the near ultraviolet and visible spectra of the bound flavin (data not shown, but see Ref. 25), indicative of perturbations in the FAD microenvironment. The interaction of these mutants with various nucleotide analogs was investigated by monitoring changes in the visible region of the flavin absorption spectrum. The intensity of the peak at about 510 nm in the difference spectra of the complexes correlates with the nicotinamide ring stacking on the re-face of the isalloxazine (25, 27–29). Therefore, titration experiments could provide information not only on the affinity of the various FNR forms for the ligands but also on the extent of nicotinamide positioning in the active site near the isalloxazine ring. The Tyr$^{308}$ mutants were thus titrated with NADP$^+$, NAD$^+$, thio-NADP$^+$, 2'-P-AMP, and 2'-P-ADP-ribose. Selected difference spectra for complexes between various FNR mutants and ligands are displayed in Fig. 3 (B–E) and compared with those of the wild-type enzyme (Fig. 3A). NADP$^+$ binding elicited difference spectral changes that were similar in shape in all FNR variants but more intense in the mutants (Fig. 3). Thio-NADP$^+$ causes spectral changes of higher intensity than NADP$^+$ upon binding to wild-type FNR (Fig. 3A and Ref. 28), presumably reflecting a greater active site occupancy of the thionicotinamide ring as compared with nicotinamide. The same behavior was observed, although to a lower extent, with both the FNR-Y308W (Fig. 3B) and the FNR-Y308F (Fig. 3C). On the contrary, thio-NADP$^+$ elicited essentially the same spectral perturbations as NADP$^+$ upon binding to the FNR-Y308S, indicating that the nicotinamide ring of NADP$^+$ already interacts at full occupancy in the active site in the complex of this mutant (Fig. 3E). The FNR mutants were also tested with NAD$^+$, and, interestingly, binding of this nucleotide elicited difference spectral changes similar to those of the NADP$^+$ complexes (Fig. 3, C–E). Assuming that the FNR-Y308S complex with NAD$^+$ has 100% occupancy of the productive nicotinamide binding mode and that the spectral changes induced by productive nicotinamide binding are not dependent on the mutations or the cofactor used, then the occupancies of all complexes can be estimated by comparison with the $\Delta A_{510}$ of the FNR-Y308S-NADP$^+$ complex (Table II). The nicotinamide occupancies for the NAD$^+$ complexes of FNR-Y308G (79%), FNR-Y308S (78%), and FNR-Y308F (26%) correlate well with the $k_{cat}$ values of these mutants in the NADH-ferricyanide reductase activity (Table I).

As expected, mutations at position 308 highly increased the affinity of the enzyme for NADP$^+$ and analogs (Table III). FNR-Y308S was the only mutant for which reliable $K_d$ values for NADP$^+$ and thio-NADP$^+$ could be obtained. Complexes of
The physiological role of leaf FNR is to store the photosynthetic energy as NADPH, and it is clearly tailored to avoid reduction of NAD\(^+\). Indeed, for wild-type FNR, NAD(H) is a very poor substrate, and much of this is due to its low affinity (33). Previous analyses of NADPH/NAD(P) specificity of NAD(P)-dependent dehydrogenases have quite reasonably focussed exclusively on the pocket that binds the 2'-phosphate group, i.e. the distinguishing trait between the two pyridine nucleotides (2–11). However, based on considerations of the bipartite binding model (Equation 1), we hypothesized that the pea FNR Tyr\(^{308}\) mutants would show enhanced binding of NAD\(^+\), because the increased affinity for the nicotinamide portion of the coenzyme would drive the binding of the whole dinucleotide. The expectation was borne out dramatically because even the most conservative mutant, FYR-N308F, deprived only of the hydroxyl group on the phenyl ring, showed a 10-fold decrease in coenzyme specificity, with the nonaromatic mutants reaching up to a 400-fold decrease. Interestingly, this specificity drop is entirely due to a large increase in catalytic efficiency with NADH as electron donor, because in vitro catalytic efficiency with NADPH was slightly but significantly increased in all mutants. This increase in the \(k_{cat}/K_m\) values for NADPH is due to a decrease in the \(K_m\) for the nucleotide consistent with the mutations nearly exclusively affecting binding affinity rather than orientation. Indeed, the effect of the various mutations on the \(K_m\) values parallels that on \(K_{cat}\) of the corresponding FYR-NAD\(^+\) complexes.

We have previously shown that positioning of the nicotinamide ring of NAD(P)H is critical for hydride transfer (27–29). Mutations at position 308 facilitate nicotinamide-isoalloxazine stacking and are then expected to increase hydride transfer rate rather than to inhibit it. Indeed, the high NADH-dependent diaphorase activities shown by the Gly and Ser mutants confirm that hydride transfer is not impaired. Thus, the negative effect of the Gly and Ser mutations on \(k_{cat}\) must be explained by a large decrease in product release rate because of a greatly enhanced affinity of these mutants for NAD\(^+\). As a consequence, the rate-limiting step of the reductive half-reaction changes from hydride transfer to NAD\(^+\) release.

The impressive increase in the catalytic efficiencies for NADH oxidation displayed by the Tyr\(^{308}\) mutants as compared with wild-type FNR (up to 1,700-fold), is due to a simultaneous increase in \(k_{cat}\) and a decrease in \(K_m\) values. As in the case of NADPH, the decreased \(K_m\) values of the FYR mutants correlate well with their increased affinity for NAD\(^+\). We interpret the progressive increase in \(k_{cat}\) observed when replacing Tyr\(^{308}\) with Trp, Phe, Gly, and Ser, in terms of a progressive increase in the nicotinamide ring occupancy, as measured by difference spectroscopy on the NAD\(^+\) complexes of these mutants (Fig. 3 and Table II). The >1,000-fold lower affinity for NAD\(^-\) exhibited by all FYR forms prevents product release from becoming rate-limiting and thus from negatively affecting \(k_{cat}\).

A result that deserves further discussion is the low \(k_{cat}\) value of wild-type FNR in the NADH-ferricyanide reductase activity and the fact that replacing Tyr\(^{308}\) can increase this value. It was not possible to obtain a direct measure of nicotinamide occupancy in the complex between wild-type FNR and NAD\(^-\), because of the exceedingly high \(K_f\) of this complex. However, the trend observed in the case of the mutants for which the information is available (Table II) suggests a very low occupancy level for the NAD\(^+\) complex of wild-type FNR. Thus, the interaction with the 2'-phosphate of the coenzyme is required not only for \(K_m\) values compatible with catalysis but also for anchoring the substrate in an orientation that enhances proper nicotinamide positioning in the active site, as is required for rapid hydride transfer. In the absence of this anchor, we pos-

### Table II

Degree of nicotinamide ring occupancy of the binding site as calculated from the extinction coefficient values at the peak near 510 nm of the difference spectra elicited by pyridine nucleotide binding to the various pea FYR forms.

| FYR form | Nicotinamide occupancy* |
|----------|-------------------------|
|          | NAD\(^+\) complex | Thio-NAD\(^+\) complex | NAD\(^+\) complex |
| Wild type | 14 | 37 | ND* |
| Y306W | 45 | 56 | ND* |
| Y306F | 85 | 103 | 26 |
| Y306G | 84 | 78 | ND* |
| Y308S | 104 | 79 | |

* Percentage of nicotinamide ring binding site occupancy, with 100% referring to NAD\(^+\) occupancy of FYR-Y308S (\(\Delta \varepsilon_{315} = 3.99 \text{ mM}^{-1} \text{cm}^{-1}\)).

### Table III

Dissociation constants for the complexes of pea FYR forms with pyridine nucleotides.

| FYR form | \(K_d\) \(\mu\text{M}\) |
|----------|------------------|
| Wild type | 9.3 ± 2.9 | 3.90 ± 0.2 | ND* |
| Y306W | 5.5 ± 0.7 | 0.62 ± 0.38 | ND* |
| Y306F | <0.2b | <0.2b | 8600 ± 790 |
| Y306G | <0.2b | ND* | 500 ± 30 |
| Y308S | <0.2b | ND* | 190 ± 7 |

* ND, not determined.

### DISCUSSION

Mutants of the C-terminal tyrosine of FNR were originally generated to obtain functional information about the role of this residue (26, 31). With the realization that these mutants might show enhanced nicotinamide binding, the FYR-Y308S was recently used to obtain the three-dimensional structure of highly stabilized NADPH complexes, providing a comprehensive structural picture of the interaction between FYR and its substrate (Fig. 2 and Ref. 25). Structural and difference spectroscopy data implied that NADPH binds to FYR in a bipartite manner, i.e. that the two moieties of the dinucleotide can bind to the enzyme in a partially independent way, according to the following equation.

\[
\text{FNR} + \text{NADPH} \rightarrow \text{FNR-NADPH}^+ (AN) \quad \text{(Eq. 1)}
\]

where \((AN)\) represents the binary FYR-NADPH\(^+\) complex with the dinucleotide bound only through the 2'-P-AMP portion and \((AN)\) represents the complex involving also the interaction of the NMN moiety (25). In wild-type FNR, the enzyme-NADPH\(^+\) complex is mainly stabilized by interactions involving the adenosine moiety of the dinucleotide, whereas the nicotinamide ring does not contribute significantly to the binding energy (32). Thus, in the NADPH\(^+\) complex of wild-type FNR, the adenosine moiety fully occupies its binding site, whereas the nicotinamide moiety binds at a very low occupancy, i.e. \((AN) \rightarrow (AN)\) (28). The latter complex is destabilized because the energetic cost of displacing the Tyr\(^{308}\) side chain outweighs the energetic gain due to nicotinamide binding (25).
ulate that the AMP portion of the NADH molecule binds in an orientation that does not allow facile entry of the nicotinamide into the active site. The observation that the Gly and Ser mutants display NADH-dependent $k_{cat}$ values approaching those of wild-type FNR with NADPH as electron donor can be explained on the basis of the fact that when the nicotinamide becomes the dominant binding moiety, it is no longer being misplaced by the interaction of the AMP portion of the dinucleotide.

In conclusion, the results reported here illustrate a most interesting example of how enzymes could solve the problem of discriminating between very similar substrate analogs such as NADP(H) and NAD(H). In the case of FNR, discrimination benefits from the establishment of strong, specific contacts with the characteristic groups of each substrate (the 2'-OH or the 2'-phosphate of the adenosine ribose) and from weakening of the interactions with those traits common to both nucleotides, such as the nicotinamide ring. Both mechanisms contribute to developing a decisive preference for NADP(H) in this group of electron transferases and providing an additional rationale to the conservation of an aromatic residue in positions equivalent to Tyr308 in many members of the FNR structural family that use NADP(H) as the preferred or exclusive coenzyme (Fig. 1 and Refs. 17 and 34). Furthermore, what is distinct about these results is that the specificity change is due to enhancement of the activity with NADH while making no impact on the catalytic efficiency with NADPH. It is likely that by combining the Y308S mutation with additional amino acid replacements at the 2'-phosphate-binding site itself (in particular S228D) (11, 14), it will be possible to produce an NAD$^+$-specific ferredoxin reductase with an activity approaching the NADP$^+$-specific ferredoxin reductase activity of wild-type FNR. As a more general conclusion, the data here presented lead to the suggestion that when engineering proteins to discriminate between two similar ligands, it is even reasonable to target those interactions that are common to both.

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