The Choice of Reducing Substrate Is Altered by Replacement of an Alanine by a Proline in the FAD Domain of a Bispecific NAD(P)H-Nitrate Reductase from Birch\textsuperscript{1,2}

Thomas Schöndorf and Wolfgang Hachtel\textsuperscript{*}
Botanisches Institut, Universität Bonn, Kirschallee 1, 53115 Bonn, Germany

Differences in the amino acid sequence between the bispecific NAD(P)H-nitrate reductase of birch (Betula pendula Roth) and the monospecific NADH-nitrate reductases of a variety of other higher plants have been found at the dinucleotide-binding site in the FAD domain. To pinpoint amino acid residues that determine the choice of reducing substrate, we introduced mutations into the cDNA coding for birch nitrate reductase. These mutations were aimed at replacing certain amino acids of the NAD(P)H-binding site by conserved amino acids located at identical positions in NADH-monospecific enzymes. The mutated cDNAs were integrated into the genome of tobacco by Agrobacterium-mediated transformation. Transgenic tobacco (Nicotiana tabacum) plants were grown on a medium containing ammonium as the sole nitrogen source to keep endogenous tobacco nitrate reductase activity low. Whereas some of the mutated enzymes showed a slight preference for NADPH, as does the nonmutated birch enzyme, the activity of some others greatly depended on the availability of NADH and was low with NADPH alone. Comparison of the mutations reveals that replacement of a single amino acid in the birch sequence (alanine\textsuperscript{871} by proline) is critical for the use of reducing substrate.

NR catalyzes the first step of the nitrate assimilation pathway in bacteria, fungi, algae, and higher plants. Three redox cofactors, FAD, heme, and a molybdenum cofactor, are bound to each monomer of the homo-dimeric plant NR and are used in that order for the catalytic transfer of electrons from NADH and NADPH, respectively, to nitrate. Each of these prosthetic groups is held in one of three catalytic domains. The heme domain occupies a central part, and the molybdenum-containing domain is located in the N-terminal half in the NR polypeptide. The FAD domain, including the NADH/NADPH-binding region, is completely C terminal (Hoff et al., 1992; Rouzé and Ca
cboche, 1992; Warner and Kleinhofs, 1992; Crawford and Arst, 1993).

In eukaryotes, three classifications of reduced pyridine nucleotide-dependent NRs are recognized (Rouzé and Ca
cboche, 1992). An NADP-specific NR (EC 1.6.6.3) occurs in fungi and mosses (Funaria) but has not been found in higher plants. Some algal species and most higher plants contain an NADH-specific NR (EC 1.6.6.1). A bispecific NAD(P)H-NR (EC 1.6.6.2) has been found in mosses (Sphagnum) and algae (Chlamydomonas) and in some higher plant species. Most species possessing NAD(P)H-NR also contain an NADH-NR. In a few cases, as in the birch tree Betula pendula (Friemann et al., 1991), the bi-specific enzyme is the sole NR.

The FAD domain of NRs is defined by overall homology with the hydrophilic domain of human and bovine CB5R (Yubisui et al., 1984). In this domain, two especially conserved segments in the proposed NADH/NADPH-binding region are noteworthy: a G-rich segment, suggestive of a pyrophosphate-binding loop found in virtually all nicotinamide-dependent enzymes, and a segment containing a Cys-Gly dipeptide present in all NRs and also in CB5R and related flavoprotein oxidoreductases (Breit et al., 1991; Friemann et al., 1991; Hyde et al., 1991; Porter 1991; Segal et al., 1992). Although a function has not been assigned unambiguously to this latter segment, the sulfhydryl of the Cys may be functionally important (Karplus et al., 1991; Shirabe et al., 1991).

It would be of special interest to pinpoint those residues that determine the choice of the reducing substrate. NADPH differs structurally from NADH only by the presence of an additional phosphate group esterified to the 2'-hydroxyl group of its AMP moiety. It is the possession of the extra phosphate group by NADPH that permits the reductases to make a distinction. This is a simple yet impressive example of the power of molecular recognition in biochemical reactions.

The methods of site-directed mutagenesis provide the technology required to study recognition processes of this kind by switching the co-substrate specificity of an enzyme, for example from using NADPH to NADH. Favorable prerequisites for redesigning a protein in this way are the occurrence of differing dinucleotide specificity within a closely related family of enzymes and knowledge of the structural features possibly involved in the binding of the dinucleotides. These prerequisites are met by NRs. The well-conserved Cys-Gly dipeptide in the FAD domain is part of a consensus motive found in NADH-NRs from higher plants and in CB5R (see Friemann et al., 1991; Rouzé

\textsuperscript{1} This work was partially funded by a grant from the Deutsche Forschungsgemeinschaft to W.H. T.S. received a scholarship from the Land Nordrhein-Westfalen.

\textsuperscript{2} Dedicated to Professor Dr. Dieter Klambt (Universität Bonn, Germany) on the occasion of his 65th birthday.

\textsuperscript{*} Corresponding author; fax 49-228-228-735513.

Abbreviations: CB5R, NADH-Cyt b \textsubscript{5} reductase; FNR, Fd-NADP reductase; NOS, nitric oxide synthase; NR, nitrate reductase; PDR, phthalate dioxygenase reductase; SR, sulfite reductase.
and Caboche, 1992). The NAD(P)H-NR sequences from birch (Friemann et al., 1991) and barley (Miyazaki et al., 1991) and the NADPH-NR sequences from Aspergillus nidulans (Johnstone et al., 1990) and Neurospora crassa (Okamoto et al., 1991) all deviate in having only one or two Pro residues next to CG instead of three successive Pro's in the consensus sequence. The replacement of two Pro residues with Ala and Ser, respectively, in the birch NR is likely to be of considerable structural relevance for the dinucleotide-binding site and responsible for the ability of the birch NR to use both NADH and NADPH. In this paper, we describe the effect of replacing the tagged residues of the FAD domain of birch NR with residues conserved in NADH-NRs.

MATERIALS AND METHODS

Plant Material

Birch (Betula pendula Roth) seedlings were grown for 5 to 6 weeks in hydroponic culture (Ingestad, 1971) with medium containing 10 mM potassium nitrate. For transformation, Nicotiana tabacum was grown in a greenhouse under controlled conditions. To measure NR activities, N. tabacum was grown under sterile conditions on agar medium containing either ammonium nitrate or ammonium succinate as the sole nitrogen source.

Bacterial Strains and Vectors

pUC19 (Yanish-Perron et al., 1985) was used as cloning and sequencing vector. The vector pGA482 was used for plant transformation (An, 1986). Vectors were cloned either in Escherichia coli K-12 strains JM83 and JM105 (Viera and Messing, 1982) or in Agrobacterium tumefaciens LBA4404 harboring the helper plasmid pTiBo542 (An et al., 1985).

DNA Manipulations

Plasmid-DNA was prepared from E. coli either by a mini-preparation method (Holmes and Quigley, 1981) or by using the Quiagen-tip 100 (Quiagen, Hilden, Germany) system. DNA from A. tumefaciens was obtained by a mini-preparation method (Slusarenko, 1990). Restriction endonuclease digestion and modification of DNA, DNA electrophoresis, and Southern blotting were performed by standard procedures (Sambrook et al., 1989). For DNA-DNA hybridization, a digoxigenin-labeled pGA482-plasmid DNA. The infection of leaf discs from N. tabacum cv Xanthi with A. tumefaciens, the selection of transformed calli by use of kanamycin, and the regeneration of transgenic plants were performed as described (Horsch et al., 1988; Zhu, 1991).

A. tumefaciens-Mediated Transformation of Tobacco

Vectors derived from pGA482 were introduced into A. tumefaciens by the direct transformation method (Höfgen and Willmitzer, 1988). For selection of transformants, A. tumefaciens cells were grown on tetracyclin medium. Transformation was confirmed by Southern hybridization of the total A. tumefaciens DNA with digoxigenin-labeled pGA482-plasmid DNA. The infection of leaf discs from N. tabacum cv Xanthi with A. tumefaciens, the selection of transformed calli by use of kanamycin, and the regeneration of transgenic plants were performed as described (Horsch et al., 1988; Zhu, 1991).

In Vitro Assays of NR

Crude leaf extracts and partially purified NR were prepared as described earlier (Friemann et al., 1992). NR assays were performed in vitro in the presence of NADH and/or NADPH in saturating concentrations (0.7 mM) by measuring the amount of nitrite formed in NR assay buffer at 25°C within 1 h after the addition of NR preparations (Hageman and Reed, 1980). To verify NADPH-dependent NR activity, parallel assays were run in the presence of pyruvate and lactate dehydrogenase to oxidize any NADH in the NADPH-containing assay system (Dailey et al., 1982). In immunochemical inhibition tests (Meyer et al., 1987), aliquots of crude extracts were each incubated with a sample containing the monoclonal antibody 96-6-25 against maize NR (Chérel et al., 1985, 1986) that was diluted between 1:50 and 1:6400.

Antibodies and Western Blotting

To raise polyclonal antibodies against birch NR, we synthesized a 17-mer oligopeptide identical to the amino acid sequence of the birch NR between Lys924 and Gin1040 that is hydrophilic and less conserved between birch and tobacco (Friemann et al., 1991). For oligopeptide synthesis, a Biosyn apparatus (Pharmacia) and instructions provided by the manufacturer were used. The oligopeptide was coupled to snail hemocyanin and injected into rabbits.

Samples of leaf protein obtained by ammonium sulfate (30–60%) fractionation of crude extracts were separated by SDS-PAGE and transferred to nitrocellulose. The blot was incubated with an IgG fraction from the obtained rabbit antisera. Then, a conjugate of goat anti-rabbit IgG/alkaline phosphatase was applied, and NR protein-antibody complexes were identified by a color reaction (Timmons and Dunbar, 1990).

ELISA

The amount of NR protein in leaf extracts was determined by a two-sided ELISA (Chérel et al., 1986). The monoclonal antibody 96–9–25 against maize NR (Chérel et al., 1985) and the polyclonal antibody preparation S6 against tobacco (Chérel et al., 1986) were used. To concentrate the polyclonal antibodies prior to application, S6 was mixed with an extract from the NR-deficient mutant E23 of Nicotiana plumbaginifolia (Chérel et al., 1990), and the non-
specific antibody-protein complexes were pelleted. The ELISA was quantified using a standard extract from tobacco with a known concentration of NR protein determined densiometrically (M. Caboche and T. Moureaux, personal communication). NR-inhibition tests demonstrated identical affinities for NR from tobacco and birch.

### RESULTS

#### Construction of a Chimeric Gene Encoding Mutated Birch NR Protein

A cDNA encoding the complete amino acid sequence of birch NR has been cloned into the plasmid pUC19 (cDNA-clone BCNR1) (Friemann et al., 1991). This cDNA was fused with the constitutive cauliflower mosaic virus 35S promoter (Odell et al., 1985) and the polyadenylation signal of the nos gene (Bevan et al., 1983). A SphI-Sacl fragment comprising the C-terminal portion of the coding region of this cDNA was subcloned into pUC19 and used for site-directed mutagenesis (Ho et al., 1989; Horton et al., 1989). The SphI-Sacl fragment of the original expression construct was then replaced by the mutated SphI-Sacl fragment to construct the mutant expression plasmids. The mutations that were introduced into the putative NAD(P)H-binding site of the birch NR are listed in Table I. Each of the mutations was confirmed by sequence determination using both pUC primers and primers that specifically allowed sequencing of the mutated site. For correct identification, about 40 nucleotides both upstream and downstream from the mutation were determined.

#### Transformation of Tobacco and Screening of Transformants

The original and the mutated expression constructs were excised from the pUC-derived vectors by XbaI restriction, and each was inserted into the XbaI site of the agrobacterial transfer vector pGA482. Transformed agrobacteria harboring one of these vectors, as identified by selective growth on tetracyclin medium and by Southern hybridization of total A. tumefaciens DNA, were used to inoculate tobacco leaf discs. Tobacco plants were regenerated from leaf calli on a kanamycin-containing medium, and transformants were screened by two different tests.

First, to identify the chimeric birch NR-gene in transgenic plants, genomic DNA was extracted from leaves and amplified by PCR using a pair of primers, one binding to the 35S promoter and the other binding to the 5’ end of the coding sequence. For a Southern blot analysis of these PCR products, a digoxigenin-labeled probe was prepared from the transfer vector containing the nonmutated chimeric sequence (plasmid tpNBO) by PCR using the same primers described above. As expected, the probe hybridized to a 0.6-kb fragment specifically amplified from the DNA from tobacco transformants. No hybridization was observed in untransformed control tobacco leaf DNA (Fig. 1).

Second, expression of the chimeric birch NR gene was detected in transgenic plants by western blot analysis. We used antibodies against a synthetic 17-mer oligopeptide with the amino acid sequence of birch NR between Lys624 and Glu640 that is hydrophilic and less conserved between birch and tobacco. As expected, transgenic plants produced proteins of about 125 kD that cross-reacted with these antibodies (Fig. 2). No cross-reaction of the birch-specific NR antibodies with leaf extracts from untransformed tobacco plants grown on either ammonium (Fig. 2) or nitrate-containing medium (not shown) was observed.

#### NR from Transgenic Tobacco Plants

For the determination of NADH/NADPH specificity of NR expressed from chimeric birch NR genes in transgenic tobacco, it is essential to repress the endogenous tobacco NR. This repression was achieved by growing tobacco plants for 2 weeks on agar medium containing ammonium succinate as the sole nitrogen source. NADH-dependent NR activity has decreased by a factor of 9 in leaves of tobacco plants grown on NH₄⁺-succinate compared to

---

**Table I. Mutations introduced into the putative NAD(P)H-binding site of birch NR**

<table>
<thead>
<tr>
<th>Mutant Designation</th>
<th>Mutant Transfer Plasmid</th>
<th>Changed Amino Acid Position(s)</th>
<th>Codon Change</th>
<th>Amino Acid Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>tpAP</td>
<td>871</td>
<td>GCC → Ala</td>
<td>Pro</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CCC</td>
<td></td>
</tr>
<tr>
<td>SP</td>
<td>tpSP</td>
<td>873</td>
<td>TCA → Ser</td>
<td>Pro</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CCA</td>
<td></td>
</tr>
<tr>
<td>EF</td>
<td>tpEF</td>
<td>877</td>
<td>GAA → Glu</td>
<td>Phe</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TTT</td>
<td></td>
</tr>
<tr>
<td>LP</td>
<td>tpLP</td>
<td>881</td>
<td>CTA → Leu</td>
<td>Pro</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CCA</td>
<td></td>
</tr>
<tr>
<td>APEF</td>
<td>tpAPEF</td>
<td>871, 877</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APLP</td>
<td>tpAFLP</td>
<td>871, 881</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APSPLP</td>
<td>tpAPSPLP</td>
<td>871, 873, 881</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOB</td>
<td>tpNOB</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
tobacco grown with nitrate (Table II). By an ELISA, about 2.5 ng of NR protein g\(^{-1}\) fresh weight of leaves was determined for wild-type tobacco grown with ammonium succinate and about 100 ng g\(^{-1}\) fresh weight for wild-type tobacco grown with nitrate. In the experiments with transgenic tobacco, therefore, we transferred the plants 2 weeks prior to the extraction of NR from the regeneration medium to a NH\(_4\)-succinate medium.

In vitro activity of NR extracted from birch and from transgenic tobacco containing the nonmutated birch sequence depended on the presence or absence of NADH and NADPH, respectively, in a similar fashion (Table II). Nonmutated birch NR from transgenic tobacco utilized both NADH and NADPH for nitrate reduction. The maximum activity obtained with NADPH was found to exceed the NADH-dependent activity by about 80%. This result is in contrast to the data obtained with wild-type N. tabacum, which show a rather low NADPH-dependent NR activity. NADPH-dependent nitrate reduction by birch NR was not due to the activity of a phosphatase in the leaf extracts, possibly leading to conversion of NADPH to NADH, since the NADPH-dependent activity did not decrease when lactate dehydrogenase and pyruvate were added to the assay mixtures to oxidize any NADH. To examine the contribution of the NADH-specific tobacco NR to the total activity of NR from transgenic tobacco plants, NADH was added to NR assays containing NADPH in saturating amounts. Because NADH did not stimulate the nitratereducing activity in these assays, there is no indication of significant amounts of additional NADH-NR (Table II).

Figure 2. Confirmation of expression of the chimeric birch NR gene in transgenic tobacco plants by western blotting of protein extracted from leaves and separated by SDS-PAGE and detection with antibodies against a synthetic oligopeptide corresponding to the C terminus of birch NR. Lane a, Protein from birch. Lanes b to e, Protein from tobacco transformed with tpAPLP (b), tpNBO (c), tpLP (d), and tpAP (e). Lane f, Protein from control tobacco plants grown on ammonium. M, Protein mass (kD).

### Utilization of NADH and NADPH by Mutant Birch NR

The effects of the various mutations introduced into the birch NR sequence now could be examined directly by measuring NR activity in extracts from transgenic tobacco. In Figure 3, NR activity values obtained with NADPH and with NADH-NADPH are presented as a percentage of the values obtained with NADH (= 100%). The calculation of percent values was a suitable means to level out the considerable variation among different plants containing the same mutant gene when in vitro NR activity was measured as nmol NO\(_2\) \( \text{formed g}^{-1}\) fresh weight min\(^{-1}\). Birch NR mutants SP, LP, and EF utilize NADH and NADPH in a fashion almost identical to that of the wild-type birch enzyme; the preference of these mutant enzymes for NADPH in less pronounced compared to the birch NR expressed in transgenic tobacco. The NR mutants AP, APLP, APEF, and APSPLP preferentially use NADH; the activity of these mutant enzymes in the presence of NADPH as the sole reducing substrate is only about one-third of the NADH-dependent activity.

Using a two-sided ELISA we were able to determine the amount of NR protein in leaf extracts and to correlate these values with NR activity. The specific NR activity (nmol NO\(_2\) \( \text{formed g}^{-1}\) NR protein h\(^{-1}\)) obtained with NADH and/or NADPH for each mutant enzyme is shown in Table III. Again, all enzymes harboring the mutation Ala to Pro exhibited a dramatic alteration in the choice of the reducing substrate compared to the nonmutated birch NR.

### Table II. In vitro activity of NR from leaves of birch (B. pendula), wild-type tobacco (N. tabacum), and transgenic tobacco containing a chimeric gene encoding nonmutated birch NR (NBO)

<table>
<thead>
<tr>
<th>Plant</th>
<th>+NADH (b)</th>
<th>+NADPH</th>
<th>+NADH + LDH</th>
<th>+NADPH + LDH</th>
<th>+NADH + LDH</th>
<th>+NADPH + LDH</th>
<th>+NADH + LDH</th>
<th>+NADPH + LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. pendula(^a)</td>
<td>15.0</td>
<td>18.4</td>
<td>15.8</td>
<td>1.5</td>
<td>17.7</td>
<td>17.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. tabacum(^d)</td>
<td>16.6</td>
<td>2.2</td>
<td>15.1</td>
<td>1.5</td>
<td>2.8</td>
<td>2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH(_4)NO(_3)</td>
<td>1.9</td>
<td>1.9</td>
<td>3.3</td>
<td>0.6</td>
<td>0.6</td>
<td>1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH(_3)-succinate</td>
<td>31.3</td>
<td>55.8</td>
<td>38.3</td>
<td>2.7</td>
<td>50.2</td>
<td>45.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Mean values from three independent plants. 
\(^b\) Activity assays were performed in the presence of either NADH, NADPH, NADH + NADPH, NADH + lactate dehydrogenase (LDH) + pyruvate, NADPH + LDH + pyruvate, or NADH + NADPH + LDH + pyruvate.
\(^c\) Birch plants were grown hydroponically on a nitrate-containing medium.
\(^d\) Tobacco plants were grown on agar medium containing either ammonium nitrate (wild type) or ammonium succinate (wild-type and transgenic plants).
DISCUSSION

Expression of Birch NR in Transgenic Tobacco Plants

Polyclonal antibodies against an oligopeptide of birch NR identified specifically one major and two minor protein bands on western blots of extracts obtained from transgenic tobacco plants (Fig. 2). Two protein bands and also patterns showing three or even more bands have been obtained by SDS-PAGE of purified NR from a number of species (see Moureaux et al., 1989). The smaller bands are commonly interpreted as being derived from a larger band by proteolytic modification during enzyme preparation. The occurrence of two NR isoforms has been reported for Sinapis alba (Schuster et al., 1989). In this case, the isoforms were detected by anion-exchange chromatography, and the monomers obtained from the isoforms were not distinguishable in size by SDS-PAGE. In birch, the origin of the multiple protein bands and the NR activity associated with each of these bands remain to be determined.

By an ELISA we were able to quantify NR protein in an ammonium sulfate fraction (30–60%) of leaf extracts. The amount of NR protein from transformed tobacco varied among different plants in a broad range between 5 and 302 ng g\(^{-1}\) fresh weight (data not shown). This is in the order of magnitude observed for wild-type tobacco (about 100 ng NR protein g\(^{-1}\) fresh weight) and birch (about 20 ng NR protein g\(^{-1}\) fresh weight). NR protein was very low (2.5 ng g\(^{-1}\) fresh weight) when nontransformed tobacco was

| Table III. Specific activity of NR from leaves of transgenic tobacco plants\(^a\) containing a chimeric gene encoding nonmutated (NBO) and mutated\(^b\) birch NR |
|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Mutant  | +NADH\(^c\) | +NADPH +LDH | +NADPH +LDH +NADH | +NADPH +LDH +NADH | +NADPH +LDH +NADH | +NADPH +LDH +NADH |
| NBO    | 36.7  | 67.3  | 50.8  | 3.5   | 60.2  | 54.7  |
| AP     | 12.8  | 4.6   | 12.3  | 3.5   | 2.1   | 4.0   |
| SP     | 9.0   | 10.8  | 8.3   | 0.7   | 9.7   | 9.7   |
| LP     | 50.0  | 57.4  | 47.0  | 5.9   | 57.4  | 55.9  |
| EF     | 37.8  | 47.8  | 41.0  | 4.3   | 44.1  | 42.2  |
| APLP   | 31.5  | 20.9  | 30.9  | 5.8   | 16.1  | 19.6  |
| APEF   | 71.4  | 20.1  | 73.6  | 17.7  | 27.9  | 35.6  |
| APSLP  | 17.0  | 4.6   | 15.3  | 2.4   | 1.4   | 3.7   |

\(^a\) Plants were grown on agar medium containing ammonium succinate as the sole nitrogen source.  
\(^b\) For designation of mutants, see Table I.  
\(^c\) Mean values from three independent plants.  
\(^d\) See footnote b in Table II.
grown under conditions that were identical to those used for the cultivation of transgenic plants (this is on a medium containing ammonium succinate as the sole nitrogen source). The great variability in NR protein content in transgenic tobacco plants is reflected by a similar variation in enzyme activity per gram fresh weight. A large variability in the levels at which introduced foreign genes are expressed in tobacco has often been observed, but the reason for this is not known (see Hooykaas and Schilperoort, 1992).

**The NADH/NADPH-Binding Region**

NRs are members of a class of structurally related flavoprotein oxidoreductases that bind FAD as a cofactor and use NADH and/or NADPH (Hyde et al., 1991; Karplus et al., 1991). These enzymes are involved in a number of widely differing biochemical pathways: Ch5R (Yubisui et al., 1984), FNR (Karplus et al., 1991), NADPH-Cyt P450 reductases (Porter, 1991), NADPH-SR flavoproteins (Ostrowski et al., 1989), NOS (Bredt et al., 1991), NADPH-oxidase (Segal and Abo, 1993), and PDR (Correll et al., 1993). Although the NRs and related flavoenzymes have striking functional similarities, for the more distantly related pairs the overall sequence similarities are low. However, some well-conserved key residues have been revealed that function in pyridine dinucleotide binding and the catalytic center. An invariant motive is a CG-dipeptide (Fig. 4). A thiol that is thought to play an essential role in dinucleotide binding and catalysis has been localized to this Cys residue close to the C terminus of the FAD domain of NR (Barber and Solomonson, 1986) and Ch5R (Hackett et al., 1986). A site-directed approach with Ch5R (Shirabe et al., 1991) as well as three-dimensional structure modeling of FNR (Karplus et al., 1991), PDR (Correll et al., 1993), and the FAD-containing fragment of corn NR (Lu et al., 1994) suggest that, indeed, the Cys residue of the conserved dipeptide is in close contact with the nicotinamide and thus may be functionally important for facilitating catalysis by providing improved efficiency of electron transfer.

The molecular basis for selective co-substrate recognition in NRs has been discussed (Lu et al., 1994) on the basis of comparisons between FNR (Karplus et al., 1991) and PDR (Correll et al., 1993). It seems that NADPH-dependent NRs have a positively charged residue in the vicinity of the 2' phosphate-binding site, as in FNR, and that NADH-specific NRs have negatively charged residues at that site, as in PDR (which is also NADH dependent). Bispecific NRs seem to avoid the negative charge close to the 2' phosphate-binding site. However, in the absence of any bound pyridine nucleotide from the obtained crystals of the FAD-containing fragment of corn NR (Lu et al., 1994), a more definitive assignment of the factors determining pyridine nucleotide specificity in NR will have to await the results of structural studies of this fragment in complex with NADH and site-directed mutagenesis experiments.

In the work presented in this paper, site-directed mutagenesis was used to identify amino acid side chains in the NR from birch that confer bispecificity for the co-substrate. Systematic replacement of a number of amino acids, all of which occur in a motif adjacent to the invariant Cys-Gly dipeptide in the dinucleotide-binding domain, converts the enzyme into one displaying a marked preference for NADH. In particular, this conversion is achieved by changing Ala871 to Pro. Further support for a functional relevance of the tagged residues is provided by comparing the sequences of this peptide segment in NRs and related flavoproteins (Fig. 4). Three Pro residues next to the Cys-Gly dipeptide are conserved in the NADH-NRs from higher plants, with only one deviation in the squash sequence, and in Ch5R (Hackett et al., 1986). The NAD(P)H-NRs from birch (Friemann et al., 1991) and barley (Miyazaki et al., 1991), and the NADP-NRs from *A. nidulans* (Johnstone et al., 1990) and *N. crassa* (Okamoto et al., 1991), have only one or two of the three Pro's. In addition, Pro's next to Cys-Gly are completely missing in NADP(H)-dependent bacterial SRs (Ostrowski et al., 1989), NOS (Bredt et al., 1991), and NADPH-oxidase (Segal and Abo, 1993).

The three-dimensional structure of the C-terminal FAD-containing fragment of corn NADH-NR (Lu et al., 1994) reveals an overall structural similarity of the pyridine dinucleotide-binding domain in NR and FNR (Karplus et al., 1991). Replacing Ala871 in the birch sequence by structurally inflexible Pro might affect the topology of the binding domain in a manner that makes the specific pocket occupied by the 2' phosphate group less accessible and thus allows no proper binding of NADPH. This conclusion can be accurate, however, only for the birch NR, since the barley NAD(P)H-NR and the *Aspergillus* and *Neurospora* NADP-NRs have a Pro in the Ala871 position. This is indicative of a role of additional residues in affecting the structure of the binding site with regard to co-substrate specificity. The third Pro is replaced by Ala in the barley NAD(P)H-NR and in the *Aspergillus* NADPH-NR and by

---

**Figure 4.** Sequence alignment of a peptide segment containing residues that may be functionally important for binding of NADH and/or NADPH by NRs and related flavoprotein oxidoreductases. CP450R, Cyt P450 reductase. The conserved Cys-Gly dipeptide and Pro residues are shown in boldface. Ala871 of birch NR is indicated. A dash designates a gap. (All sequence data are from the SwissProt data base.)
Gly in the Neurospora NADPH-NR. An Ala or Gly instead of Pro in position 873 might have a similar effect as an Ala instead of Pro in position 871. In fact, it would be very interesting to examine the effect of the Ala<sup>871</sup> → Pro, Ser<sup>873</sup> → Ala double mutation, one that would be comparable to the barley NAD(P)H-NR sequence, to see whether the Ala-to-Pro conversion effect at position 871 might be canceled by the Ser-to-Ala conversion at position 873.

ACKNOWLEDGMENTS

We are grateful to Michel Caboche for monoclonal antibody 96-9-25 and polyclonal antibody S6. We are indebted to Andreas Friemann, André de Kathen, Michel Caboche, Thérèse Moureaux, Thomas Münster, Dieter Klambt, and Rolf Nimzyk for help and advice. The technical assistance of Heidi Geithmann, Klaus Bahr, and Doris Lemke is gratefully acknowledged.

Received November 1, 1994; accepted January 29, 1995.

LITERATURE CITED


Hoff T, Stummann BM, Henningken KW (1992) Structure, function and regulation of nitrate reductase in higher plants. Physiol Plant 84: 616-624


Ingestad T (1971) A definition of optimum nutrient requirements in birch seedlings. Physiol Plant 24: 118-125


