

Update on Biochemistry

Nitrate Reductase Biochemistry Comes of Age¹

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NR (EC 1.6.6.1-3) was first isolated and characterized more than 40 years ago, and each decade of study of this key enzyme of nitrate assimilation has been associated with a new understanding of its structure and function. Briefly, NR is a homodimeric enzyme (native form = A₂) with each subunit containing a 100-kD polypeptide and one each of molybdate, Mo-pterin, Fe, heme, and FAD (Redinbaugh and Campbell, 1985). NR has two active sites, one where NADH donates electrons to FAD to begin the transport of electrons via the heme-Fe to the Mo/Mo-pterin in the second active site, where nitrate is reduced to nitrite. NR has two-site, ping-pong, steady-state kinetics, where the enzyme “pings and pongs” between oxidized and reduced forms, as NADH/NAD⁺ bind at the electron donor active site and nitrate/nitrite bind at the electron acceptor site. The most recent advances have resulted from the cloning of the NR gene (Campbell and Kinghorn, 1990; Solomonson and Barber, 1990; Rouze and Caboche, 1992). The deduced amino acid sequence of higher-plant NR showed that it contained about 900 residues with a predicted molecular size of approximately 100 kD. Although this size is a bit smaller than the NR polypeptide appears on SDS-PAGE gels (110–115 kD; Redinbaugh and Campbell, 1985), this may be explained by the runs of acidic residues near the N terminus of well-characterized and sequenced NR forms such as squash (Hyde et al., 1991).

In this general review, I will focus on recent advances in NR biochemistry. I last reviewed this topic in a general way in 1988 (Campbell, 1988). Reviews by Solomonson and Barber (1990), Rouze and Caboche (1992), and Crawford (1995) provide more detailed accounts of various aspects of this topic than will be presented here. Recently, Kaiser and Huber (1994) reviewed posttranslational regulation of NR in an *Update*.

NR STRUCTURE

The important discovery from the NR amino acid sequence was that the apparent cofactor-binding regions of NR were laid out linearly in the backbone (Campbell and Kinghorn, 1990; Solomonson and Barber, 1990; Rouze and Caboche, 1992). This was demonstrated by similarity of the

NR amino acid sequence to sequences of known proteins with defined functionality (Fig. 1). The N-terminal region of NR is similar in sequence to the Mo-pterin cofactor-binding region of mammalian SO (EC 1.8.3.1); the middle region of NR is similar to mammalian and plant Cyt *b*₅, a heme-Fe protein; and the C-terminal region of NR is similar to mammalian Cb₅R (EC 1.6.2.2), a FAD-containing enzyme (Fig. 1). In each of these similarity regions it appeared that virtually the entire sequence of the homolog was contained in the NR sequence without interruption, suggesting interesting things about the evolution of NR that will be discussed below in more detail. Of the 20 or so eukaryotic NR genes now cloned from higher plants, fungi, and algae, each contains these same regions of polypeptide sequence similarity for the cofactor-binding regions of the NR backbone. Three other regions of the NR amino acid sequence are defined by comparison of the various NR clones as being highly variable in sequence: a region containing runs of acidic residues at the N terminus varying in length from 30 to 100 residues, which appears to play a role in NR stability (Nussaume et al., 1995), and two “hinge” regions situated between the cofactor-binding regions (Fig. 1). Hinge 1 between the MC-NR and Cyt *b*, which is about 30 residues long and does not vary greatly in length among NR forms, contains a site where trypsin can access the backbone and cleave it in the native enzyme (Solomonson and Barber, 1990). Hinge 2 between the Cyt *b* and CbR regions of NR, which varies in length from about 15 to 30 residues, also may contain a protease site. The function of the hinge regions in NR and the reason that NR has its three cofactor-binding regions all in one polypeptide has not been established, although it is clear that regulation of NR expression is simplified by inclusion of all of its components in one polypeptide. Possible functions for the hinge regions in NR will be discussed below in relation to NR regulation.

Although the cloning of NR resulted in the informative amino acid sequence comparisons modeled in Figure 1, this tells us virtually nothing about the anatomy of the NR's 3-D shape and the details of the cofactor- and the substrate-binding sites. For its structure to be “seen,” NR must be

¹ The research in the author's laboratory on nitrate reductase was supported by grants from the National Science Foundation and the U.S. Department of Agriculture.

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Abbreviations: CbR, Cyt *b* reductase fragment of NR; Cb₅R, Cyt *b*₅ reductase; CcR, Cyt *c* reductase fragment of NR; FNR, Fd NADP⁺ reductase; MC-NR, molybdenum-containing nitrate-reducing fragment of NR; NR, nitrate reductase; SO, sulfite oxidase; 3-D, three-dimensional.

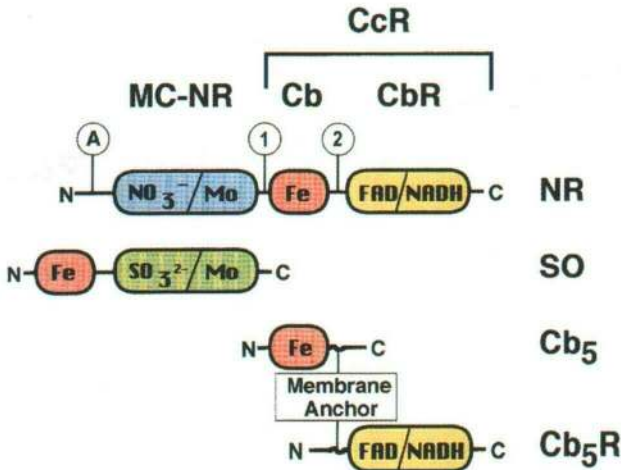


Figure 1. Model of the NR amino acid sequence showing its relationship to other eukaryotic enzymes and proteins with similar sequences. This model is based on amino acid sequence comparisons and alignments (Campbell and Kinghorn, 1990). The N and C termini of the sequence models are designated N- and -C, respectively. The major fragments of NR with functionality as catalysts and stability as structural units are designated at the top: MC-NR, Cb (Cyt *b* fragment or domain), CbR, and CcR, which comprises the Cb domain, hinge 2, and the CbR fragment. The designations for the enzymes/proteins are shown along the side: NR, SO, Cb₅ (Cyt *b*₅), and Cb₅R. In the model for NR, the circled A, 1, and 2 designate the acidic N-terminal region and the two hinge regions of variable sequence that join the major functional/structural fragments of the enzyme. Within the fragments themselves are shown the cofactors and substrates of the enzymes/proteins: NO₃⁻, nitrate-binding and reduction site; Mo, molybdate/molybdenum-pterin binding site; Fe, heme-Fe binding site; FAD, FAD-binding site; NADH, NADH-binding and reaction site; and SO₃²⁻, sulfite-binding and oxidation site. NR and SO are soluble enzymes, NR in the cytosol and SO in the inner membrane fluid of mitochondria, whereas Cb₅ and Cb₅R are membrane bound on the ER, the cytosolic face of the outer membrane of mitochondria and the cytosolic face of the plasma membrane; the positions of the membrane anchor sequences of the latter two proteins are shown. Soluble forms of Cb₅ and Cb₅R also exist in red blood cells that are identical in sequence to the soluble portions of the membrane-bound forms.

crystallized and the crystals analyzed by x-ray diffraction. The limited quantities of pure NR available and, perhaps to some extent, NR's complex structure and large size have prevented this goal from being achieved. Another approach is to use recombinant protein expression technology to provide the large quantities of protein needed for crystallization and x-ray analysis. Unfortunately, to date no one has succeeded in producing by recombinant expression holo-NR in an active form in sufficient quantities for structural analysis. On the other hand, recombinant expression of fragments of NR has been achieved, which turns out to be possible because of the linear arrangement of the NR fragments in both the polypeptide and the gene or cDNA (Fig. 1). The proof that the sequence comparisons were valid came when redox active and functional fragments of NR were produced by expression in *Escherichia coli* of gene fragments for corn NR's CbR (originally called NR's flavin or FAD domain) and *Chlorella* NR's Cyt *b* (Hyde and Campbell, 1990; Cannons et al., 1991). The recombinant CbR of

corn NR, a 30-kD protein, is an active NADH:ferricyanide reductase that is also a partial activity of holo-NR, depending only on the enzyme's FAD. Subsequently, the Cyt *b* and CbR fragments of corn NR and the CcR fragment of NR were recombinantly produced as a 58-kD protein, having NADH:Cyt *c* reductase activity, which is also a property of holo-NR, depending on both the FAD and the heme-Fe (Campbell, 1992). Recently, a fusion of mammalian Cyt *b*₅ and spinach NR's CbR fragment was recombinantly produced, which also catalyzes NADH-dependent Cyt *c* reduction (Quinn et al., 1994). More recently, my laboratory has produced CcR fragments of spinach NR with the 42-kD minimum size predicted for the region of the enzyme in *E. coli* and *Pichia pastoris*, a methylotrophic yeast that produces very high levels of recombinant proteins (N. Shiraishi and W.H. Campbell, unpublished observations).

Since the recombinant corn CbR was a soluble protein produced in large quantities and easily purified (Hyde and Campbell, 1990), it was crystallized and its 3-D structure was determined (Lu et al., 1994). It had been predicted by amino acid sequence comparisons that NR's CbR fragment was a member of a family of flavin-containing oxidoreductases that reduce cytochromes (Hyde et al., 1991; Karplus et al., 1991). This family is known as the FNR family of flavoproteins because FNR was the first member to have its 3-D structure determined (Karplus et al., 1991). The family consists of FNR (EC 1.18.1.2), NR, nitric oxide synthase (EC 1.14.13.39), Cb₅R, Cyt P450 reductase (EC 1.6.2.3), and some other enzymes containing FAD or FMN, and differs from the other well-known flavoproteins whose structures have been determined, namely glutathione reductase (EC 1.6.4.2) and glycolate oxidase (EC 1.1.3.15) (Karplus et al., 1991; Lu et al., 1994). This is one of the first times that plant proteins have taken a leading role in establishing a new structural family of enzymes, which exists in many different eukaryotic and prokaryotic species (Correll et al., 1992). Subsequently, crystals of NR's CbR were produced with the inhibitor ADP bound to the apparent NADH-binding site, and an active site mutant called C242S was also crystallized (Lu et al., 1995). In addition, it has been possible to model the CcR fragment of NR, since NR's Cyt *b* domain is highly similar in sequence to mammalian Cyt *b*₅, which is of known 3-D structure (Fig. 2; Mathews et al., 1971; Lu et al., 1995).

NR's CbR fragment has two domains: one for binding FAD at its N terminus and one presumed to be for binding NADH at its C terminus, with the two domains joined by a short linker sequence (Fig. 2). There is a central cleft between the two domains, which is the active site where NADH transfers electrons to FAD. Reduced FAD in turn reduces ferricyanide in the recombinant fragment or the internal Cyt *b* of NR in the holoenzyme and recombinant CcR fragment. The FAD domain of CbR is a six-stranded, β -barrel structure with a single α helix important in FAD binding. NR's FAD domain has virtually the same shape as FNR's FAD domain, confirming the amino acid sequence prediction that these two key plant enzymes are closely related in structure while differing significantly in sequence and function. The apparent NADH-binding do-

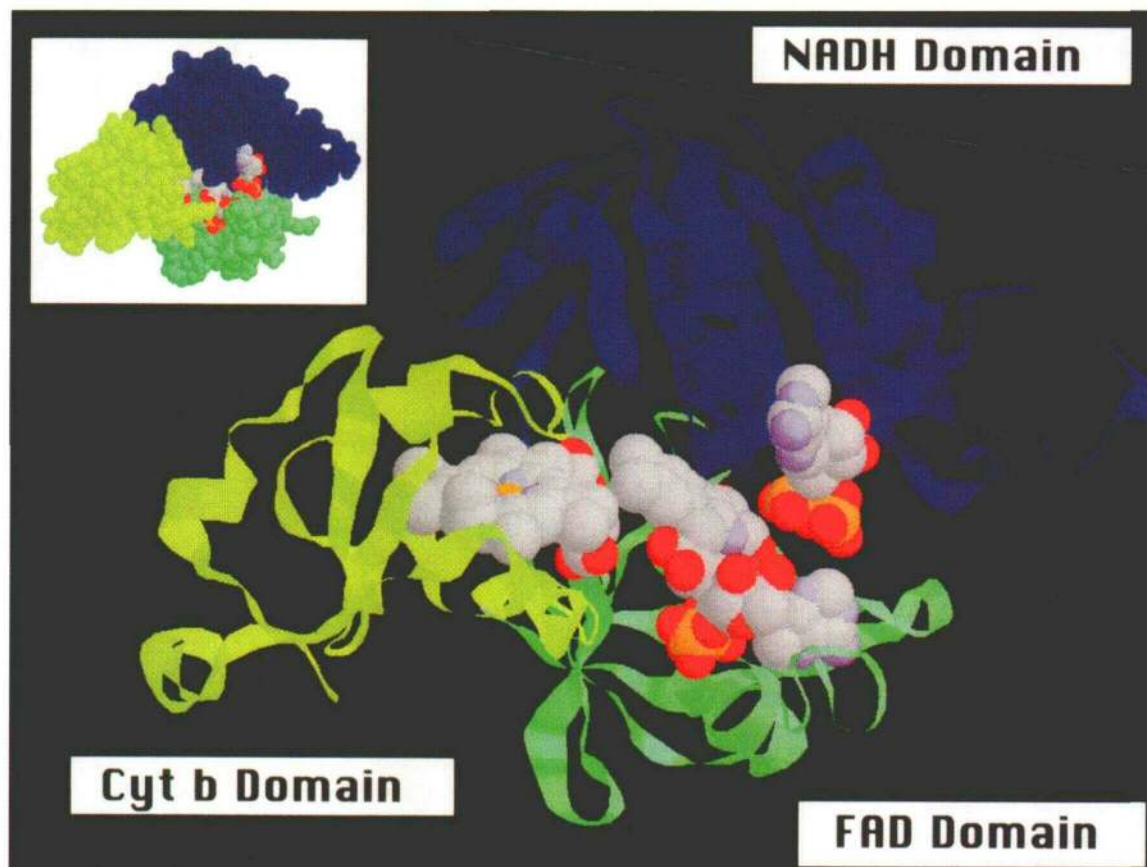


Figure 2. Model of the Cyt *c* reductase fragment of NR. This model was derived by docking the 3-D structure of mammalian Cyt *b*₅ to the 3-D structure of corn NR's CbR using electrostatic calculations as a guide in positioning the two protein models relative to one another (Lu et al., 1995). In the ribbon model, the NADH domain of the CbR fragment of NR with bound ADP (shown as space filling model) is blue, the FAD domain of CbR with bound FAD (space filling model) is green, and mammalian Cyt *b*₅ with bound heme-Fe (space filling model—central Fe atom) is yellow. The 3- β strand linker between the FAD and NADH domains of NR's CbR is also blue in the ribbon model; it is to the left of the main portion of the NADH domain, with its fusion to the green ribbon of the FAD domain hidden behind Cyt *b*₅. Hinge 2 is not modeled here; however, it would most probably extend along the surface of Cyt *b*₅ from its C terminus to the N terminus of the FAD domain of CbR, which is visible just below one of the α helices of Cyt *b*₅. In the cameo inset, the same color scheme is used and space-filling representations for the major domains of the Cyt *c* reductase model are shown with the cofactors and ADP inhibitor bound, to provide a better sense of the size and shape of the CcR fragment of NR.

main of CbR is a six-stranded β sheet that resembles the "Rossmann" fold of dehydrogenases (Lu et al., 1994). NR's NADH domain differs in some minor aspects when compared to the NADP⁺ domain of FNR, which is not surprising, since different pyridine nucleotides are bound to these structures; but it is clear that they are closely related. A difference between NR's CbR and FNR is that the FAD domain is a bit more separated from the NADH domain in NR than the corresponding domains are in FNR (Karplus et al., 1991; Lu et al., 1994). Although the biochemical significance of this difference is not clear, it also has been found in the 3-D model recently reported for Cb₅R, which is much more closely related in sequence and functionality to NR's CbR than it is to FNR (Nishida et al., 1995).

The model of the CcR fragment of NR illustrates how the heme-Fe of the Cyt *b* domain may approach the FAD of the CbR fragment to facilitate electron transfer (Fig. 2; Lu et al., 1995). It is generally predicted that the isoalloxazine ring of

FAD should be in the same plane as the heme-Fe for optimum electron transfer, and this optimum alignment is only partially achieved in the model. The model of NR's CcR also predicts amino acid side chains that may be important at the interface of the enzyme's Cyt *b* and FAD domains, which are charged side chains of amino acids, and one interaction between a carboxylic acid on the heme and a His of the FAD domain (Lu et al., 1995). These predictions can now be tested by site-directed mutagenesis of NR's recombinantly expressed CcR fragment. Of course, this model of CcR only provides an idea of how NR really looks, and current experiments working toward crystallization of the recombinant CcR fragment of spinach NR will possibly provide a more definitive look at this part of NR's structure. The elusive prize of "seeing" NR's MC-NR fragment and the overall shape of the native holo-NR dimer remain for future investigations. Recently, Su et al. (1996) succeeded in expressing low levels of active holo-NR in *P.*

pastoris using an Arabidopsis NR cDNA, which may be the breakthrough needed to propel structural studies of holo-NR.

NR FUNCTIONALITY

When all the available NR sequences are compared, only two Cys residues remain as invariant and absolutely conserved residues in the sequence: one in the MC-NR fragment and one in the CbR fragment. Tomsett and co-workers (Garde et al., 1995) carried out site-directed mutagenesis on the invariant Cys residue in the MC-NR fragment of *Aspergillus* NADPH:NR to change it to an Ala residue and found that this mutant had no NR activity. This strongly supports the concept that this Cys is essential for NR functionality, especially since no other mutation generated by this group resulted in complete loss of all nitrate-reducing activities, including activities driven by reduced dyes (Garde et al., 1995). It has been suggested that this Cys residue of NR is involved in binding the Mo-pterin cofactor to the MC-NR fragment and holding it in the nitrate-reducing active site of the enzyme (Solomonson and Barber, 1990). NADPH:NR activity was lost in an *Aspergillus* NR mutant where one of the reputed His ligands of the heme-Fe in the Cyt *b* domain was changed to an Ala, and reduced bromphenol blue:NR activity was retained (Garde et al., 1995). A similar mutant of a His ligand of the heme-Fe was found among tobacco plants lacking NADH:NR activity when the mutant NR gene was sequenced (Meyer et al., 1991). This NR mutant also retained reduced bromphenol blue:NR activity, but lacked reduced methyl viologen:NR activity, suggesting that these reduced dyes donate electrons to different sites in NR. This is an important result, since it was long thought that methyl viologen donated electrons directly to the Mo/Mo-pterin of NR, which is a functionality that must now be assigned to reduced bromphenol blue. Reduced methyl viologen:NR activity clearly depends on the Cyt *b* domain in some way; most probably this dye donates an electron to the heme-Fe, which transfers it to the Mo/Mo-pterin in the usual manner for nitrate reduction.

The other invariant Cys of all NR forms has been studied by site-directed mutagenesis of the recombinant CbR fragment of corn NADH:NR (Dwivedi et al., 1994). There are five Cys residues in recombinant corn CbR, each of which was converted to a Ser residue and the mutant enzyme forms purified from the *E. coli* extracts by affinity chromatography. Only when the invariant Cys, which is residue 242 in the recombinant protein, thus is called C242S, was mutated did CbR largely lose its ferricyanide reductase activity while retaining its native shape, as indicated by visible and circular dichroism spectra being identical to those of the wild type (Dwivedi et al., 1994; Ratnam et al., 1995). Inhibitor studies using *p*-hydroxymercuribenzoate, which reacts with thiol groups, showed that only the C242S mutant of the corn CbR was resistant to inhibition, whereas all other Cys to Ser mutants, as well as the wild type, lost their activity in the presence of the inhibitor. Kinetic studies indicated that CbR's V_{max} for ferricyanide reduction by NADH and the rate of reduction of CbR's FAD by NADH,

assayed by pre-steady-state methods, were decreased by more than 5-fold in the C242S mutant compared to wild type. The NADH K_m and NADH binding constant were almost unchanged (Dwivedi et al., 1994; Ratnam et al., 1995). Thus, the longstanding suggestion that this Cys thiol in the NADH active site of NR has a role in binding NADH was largely disproved, and it now can be convincingly stated that the role of this invariant Cys thiol is in assisting electron transfer from NADH to FAD in the active site of the CbR.

Clearly, since CbR retains some NADH:ferricyanide reductase activity in its C242S mutant, the invariant Cys is not absolutely required for catalytic activity. So, this Cys residue is not redox active in the electron transfer process and it is not involved in the chemistry of FAD reduction by NADH. The much greater efficiency of electron transfer in the wild type versus the C242S mutant of CbR indicates that the Cys thiol is "essential" for making a highly efficient NR catalyst, suggesting that this is sufficient evolutionary pressure for retaining this residue in all NR forms so far cloned as well as in all other members of the FNR structural family of flavoenzymes (Dwivedi et al., 1994; Ratnam et al., 1995). The crystal structure of the C242S mutant of CbR helps to explain the role of the invariant Cys thiol in efficient electron transfer (Lu et al., 1995). In wild-type CbR's 3-D structure, the C242 thiol projects into the active-site cleft, where it may interact with the carboxamide side chain on the pyridine ring of NADH, whereas in the C242S mutant of CbR, the Ser hydroxyl group is hydrogen bonded to the backbone of the protein at residue 147, which leaves a large void in the active site (Lu et al., 1995). Thus, the invariant Cys's thiol may be for positioning NADH in the active site for efficient electron transfer to FAD.

Another aspect of NR's functionality brought into better focus by the availability of the 3-D structure of its CbR fragment is how pyridine nucleotide specificity is determined. NR exists in three forms: NADH specific (EC 1.6.6.1), NAD(P)H bispecific (EC 1.6.6.2), and NADPH specific (EC 1.6.6.3). The 3-D structure of the recombinant CbR of corn NR with the inhibitor ADP bound in the apparent NADH binding site shows that the negatively charged side chain of the Asp residue at position 205 (D205) is within bonding distance of the 2' hydroxyl group of the ADP's Rib (Lu et al., 1995). No other polar amino acid side chains of CbR are in the vicinity of the 2' hydroxyl group of the ADP Rib. Since most NADH-specific NR forms have either an aspartic acid or Glu residue at their position corresponding to D205 of corn NR's CbR, we have suggested that the negative charge of this side chain probably plays a role in excluding the negatively charged 2' phosphate of NADPH from the active site of these NR forms (Lu et al., 1994). Higher-plant NAD(P)H:NR and fungal NADPH:NR forms have either a Ser or a Thr in the position corresponding to that of corn CbR's D205 when the amino acid sequences are aligned. Thus, the lack of a negatively charged side chain here may allow NADPH into the active site. However, this simple theory cannot completely explain why some NR forms are highly specific for NADPH and others are bispe-

cific and accept electrons from both NADH and NADPH. Recent results for birch NAD(P)H:NR suggest that residues more remote from the actual ligands to the 2' hydroxyl/2' phosphate of NADH/NADPH (as judged by the 3-D structure of ADP bound to corn's CbR) also play a role in determining pyridine nucleotide specificity (Schondorf and Hatchtel, 1995). Pyridine nucleotide specificity of NR is currently under more complete investigation.

NR REGULATION

Control of NR activity can be achieved in two ways: altering the activity level of existing enzyme or controlling the amount of enzyme by synthesizing new enzyme and degrading old enzyme. De novo synthesis of new NR, stimulated by nitrate, was established as a mechanism for controlling enzyme level when combined with NR protein degradation (Remmler and Campbell, 1986; Campbell, 1988; Solomonson and Barber, 1990). After NR clones became available, it was shown that the NR gene is transcribed in response to nitrate application to plants, leading to increased levels of NR mRNA, which is the underlying mechanism for nitrate stimulation of de novo NR synthesis (Solomonson and Barber, 1990; Rouze and Caboche, 1992). The signals triggering NR protein degradation in the presence of nitrate, even in the light, have not been identified. However, it is likely that NR degradation begins by attack either in the readily available hinge regions or the N-terminal region and eventually leads to total degradation of NR. NR activity level can be controlled by a posttranslational mechanism involving phosphorylation of the NR protein and binding of Mg^{2+} or another divalent cation and an inhibitor protein, which was recently reviewed here (Kaiser and Huber, 1994). These observations describe the end result of a signal transduction pathway where the triggering signals are light/dark transitions, as well as other environmental factors impacting dominant plant processes such as carbon assimilation. The mediators of this signal transduction pathway from the triggering signal(s) have just begun to be identified by the recent isolation of a calcium-dependent protein kinase catalyzing the phosphorylation of spinach NR in vitro (Bachmann et al., 1995).

Most recently, the NR protein kinase was used to identify the apparent key Ser residue in spinach NR, which is involved in activity regulation (Bachmann et al., 1996). Two approaches were used in this study. One involved the recombinant fragments of spinach NR, where the CcR fragment was produced with and without the hinge-1 region. Since the CcR-hinge-1 fragment was phosphorylated by the NR protein kinase and the CcR without hinge 1 was not, the only conserved Ser (residue 543 in spinach NR) in the hinge-1 region was the likely site of phosphorylation. Subsequently, synthetic peptides containing hinge-1 sequences were prepared and used to establish that Ser⁵⁴³ was indeed the phosphorylation site (Bachmann et al., 1996). Based on the tentative location of the target Ser for the NR protein kinase in hinge 1, a site-directed mutant of Arabidopsis NR (Ser⁵³⁴-Asp⁵³⁴; Arabidopsis NR is 9 residues shorter in length than spinach NR at the N terminus) was prepared and expressed in *Pichia* (Su et al., 1996). This mutant was

not inactivated in vitro by treatment with ATP and extracts of Arabidopsis leaf (source of the NR protein kinase and inhibitor protein), but the wild type was, which is consistent with Ser⁵³⁴ being the site of regulatory phosphorylation in Arabidopsis *Nia2*. Since Arg⁵⁴⁰ in hinge 1 of spinach NR has been identified as the target site for trypsin cleavage of the native enzyme (Campbell and Kinghorn, 1990), it can be concluded that this region in hinge 1 of NR is at the surface of the 3-D structure. This surface location allows the NR protein kinase to recognize and bind the sequence LKRTAS (residues 538-543 of spinach NR hinge 1), leading to the catalytic transfer of phosphate from ATP to form phospho-Ser⁵⁴³ (Bachmann et al., 1996). After this Ser residue is phosphorylated, presumably Mg^{2+} (or another divalent cation) will bind to NR and the complex with the NR inhibitor protein will form, resulting in loss of NR activity. It seems likely that other parts of NR beside the phosphorylation site may be involved with the interaction of NR and the inhibitor protein, but these are yet to be identified. In this regard, an NR mutant found in an NR-deficient Arabidopsis plant, where an invariant Gly residue in the MC-NR fragment of NR is converted to an acidic residue, renders the NR both inactive and without phosphorylation (LaBrie and Crawford, 1994). Thus, the overall 3-D shape of NR may be important in controlling phosphorylation, and further understanding of how phosphorylation of NR at the regulatory Ser leads to binding of the inhibitors and activity loss may not be gained until the 3-D shape of NR is known.

NR GENES AND EVOLUTION

NR genes, like many other eukaryotic genes, contain introns. There are basically two theories to explain the common existence of introns in eukaryotic genes encoding proteins and their absence from similar prokaryotic genes: (a) introns were present in ancestral genes of all species and selectively lost during the evolution of prokaryotes, probably for reasons of "economics of survival," but were retained during evolution of eukaryotes, which have more energy to spare for maintaining less useful DNA and a greater need for the complex regulation and manipulation of the genome made possible by intervening DNA sequences in genes; and (b) introns were introduced into eukaryotic genes after the split in evolution of prokaryote and eukaryote lineages (Alberts et al., 1994). Although I do not wish to enter into the dispute over these two possibilities, it is interesting to look at intron locations in NR genes, because the regions encoding the functionalities of the enzyme are so clearly laid out in a linear array in the gene, which along with the sequence similarities to independent enzymes suggests that the NR gene was formed by fusion of existing exons (Fig. 1). Introns occur at one or all of three locations in higher-plant NR genes, although a fourth intron has been found in a bean NR gene (Campbell and Kinghorn, 1990; Jensen et al., 1994). Fungal NR genes have from 1 to 6 introns and for the most part the introns are found at different locations in the sequence than those in higher plants (Campbell and Kinghorn, 1990). Perhaps the most interesting NR gene is from *Volvox carteri*, a green

alga, which has 10 introns dividing the coding sequence into 11 exons (Gruber et al., 1992). The *Volvox* NR gene has 6 introns in the MC-NR fragment, with the 7th exon containing the hinge-1 region; exon 8 contains the core of the Cyt *b* domain, exon 9 contains hinge 2 and the first part of the FAD domain, exon 10 contains the end of the FAD domain and the beginning of the NAD(P)H domain, and exon 11 contains the remainder of the sequence beginning just before the key pyridine nucleotide-specificity-determining amino acid residues described above. Thus, the *Volvox* NR gene illustrates a way the ancestral NR gene may have formed from groups of exons representing primitive forms of a Mo-containing protein, a Cyt *b*-type heme-Fe protein, and a flavin-containing reductase enzyme.

Codon usage is another aspect of genome evolution well illustrated in NR genes (Campbell and Gowri, 1990). Since NR is a very large gene, all 61 codons of the genetic code are used in these genes. However, NR genes reflect the codon usage in the species from which they were isolated rather than following a pattern representative of the gene. Thus, monocot NR genes are highly biased toward codons ending in the nucleotides G and C, like many other monocot genes, whereas dicot NR genes have the more evenly distributed codon usage typical of dicot genes (Campbell and Gowri, 1990). This fine tuning of genes during the evolution of species is a fascinating aspect of the evolution of individual species and families of genera, which to my knowledge has no known mechanism. To make the nature of the puzzle clear, it can be restated in this way: the amino acid sequences of NR proteins have been more conserved in evolution of the NR gene than the codons used to encode these amino acid sequences. One supposes that codon usage in a species' translated genes was fine tuned to the abundance of the various tRNA molecules available for protein synthesis and perhaps also to optimize mRNA stability. But why plant families differ so greatly in codon usage or tRNA abundance, if that is the underlying driving force, remains mysterious. Furthermore, how in evolution were individual codons in a gene fine tuned to arrive at the distinct codon usage patterns found today in plant species?

Received February 9, 1996; accepted February 20, 1996.
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