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Nitrate Reductase-Deficient Mutants in Barley[1]

IMMUNOELECTROPHORETIC CHARACTERIZATION

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ABSTRACT

Nitrate reductase-deficient barley (*Hordeum vulgare* L.) mutants were assayed for the presence of a functional molybdenum cofactor determined from the activity of the molybdoenzyme, xanthine dehydrogenase, and for nitrate reductase-associated activities. Rocket immunoelectrophoresis was used to detect nitrate reductase cross-reacting material in the mutants. The cross-reacting material levels of the mutants ranged from 8 to 136% of the wild type and were correlated with their nitrate reductase-associated activities, except for *nar* 1c, which lacked all associated nitrate reductase activities but had 38% of the wild-type cross-reacting material. The cross-reacting material of two *nar* 1 mutants, as well as *nar* 2a, Xno 18, Xno 19, and Xno 29, exhibited rocket immunoprecipitates that were similar to the wild-type enzyme indicating structural homology between the mutant and wild-type nitrate reductase proteins. The cross-reacting materials of the seven remaining *nar* 1 alleles formed rockets only in the presence of purified wild-type nitrate reductase, suggesting structural modifications of the mutant cross-reacting materials. All *nar* 1 alleles and Xno 29 had xanthine dehydrogenase activity indicating the presence of functional molybdenum cofactors. These results suggest that *nar* 1 is the structural gene for nitrate reductase. Mutants *nar* 2a, Xno 18, and Xno 19 lacked xanthine dehydrogenase activity and are considered to be molybdenum cofactor deficient mutants. Cross-reacting material was not detected in uninduced wild-type or mutant extracts, suggesting that nitrate reductase is synthesized *de novo* in response to nitrate.

Barley (*Hordeum vulgare* L.) NR[4] has several features in common with other eukaryotic NR. The mol wt of the barley NR holoenzyme is 221,000 (10), which is similar to NR from spinach (7), tobacco cell cultures (17), and *Neurospora crassa* (6). Barley NR consists of two identical 110,000 subunits (10, 11) presumably complexed with a small mol wt MoCo detected in other NR (7). More recently, the ability of FAD to stabilize barley NR in crude extracts (12), and the detection of a functional Cyt b_{557} (21) have led us to conclude that barley NR reduces nitrate using NADH as a physiological reductant via an electron transport chain similar to that proposed for the *N. crassa* enzyme (6). Furthermore, purified barley NR, like NR from other organisms, is capable of nitrate reduction using NADH, $FMNH_2$, and MVH, and also has NADH CR activity (10).

NR-deficient mutants have proven important tools for investigating genetic and biochemical aspects of this essential enzyme in the nitrate assimilation pathway. *N. crassa* (6) and *Aspergillus nidulans* (2) mutants deficient in NR have been isolated and characterized extensively on the basis of their NR-associated activities (NADPH NR, $FMNH_2$ NR, MVH NR, and NADPH CR), MoCo content, and inheritance. NR-deficient mutants isolated in higher plants have also been characterized in regard to NR-associated activities, MoCo content, and inheritance in the case of tobacco cells in culture (16, 18) and NR-associated activities and inheritance in barley (8, 20, 22, 23, 27). In the majority of the cases, the presence of defective NR protein in NR-deficient mutants has been determined by assaying the NR-associated activities.

Previous genetic analysis of 10 NR-deficient barley mutants isolated in our laboratory indicated that nine of the mutants were allelic (*nar* 1a–*nar* 1i), while one mutant represented a second NR gene (*nar* 2a) (8). Mutants Xno 18 and Xno 19 have been shown to be allelic to one another and nonallelic to *nar* 1 (20). The allelic relationships of Xno 29 have not been established. Based on the genetic data and the NR-associated activities of the *nar* mutants, it was postulated that the *nar* 1 locus was the NR structural gene while *nar* 2 was involved in MoCo function (8). Shumny and Tokarev (20) have suggested that Xno 29 also codes for the structural NR gene.

Some NR-deficient barley mutants have inactive NR which has been detected by the ability of inactive NR CRM to protect a known amount of active wild-type NR from antibody inhibition (9). Further improvements in NR purification have facilitated the production of antiserum monospecific for barley NR. This study reports the characterization of this NR antiserum and its use in rocket immunoelectrophoretic quantification and characterization of the NR CRM of 13 NR-deficient barley mutants.

MATERIALS AND METHODS

Plant Material and Growth. Wild-type and the NR-deficient barley (*Hordeum vulgare* L., cv Steptoe) *nar* mutants have been described by Kleinhofs *et al.* (8). Three other mutants, Xno 18 (chlo 18), Xno 19 (chlo 19), and Xno 29 (chlo 29) were isolated from the barley cv Winer (20, 22, 23) and supplied to us by Dr. B. I. Tokarev. Seedlings were grown in vermiculite under continuous light at 18°C and watered by subirrigation with deionized H_2O for 5 d. The seedlings were watered with nutrient solution con-

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[4] Abbreviations: NR, nitrate reductase; MoCo, molybdenum cofactor; FAD, flavin adenine dinucleotide; $FMNH_2$, reduced flavin mononucleotide; MVH, reduced methyl viologen; CR, Cyt *c* reductase; CRM, cross-reacting material; BIS, *N'*,*N'*-methylenebisacrylamide; PMSF, phenylmethylsulfonyl fluoride; XDH, xanthine dehydrogenase.

taining 15 mM NO_3^-, 5 mM NH_4^+, or zero N, 24 and 12 h before harvest on day 6 (8, 26).

Enzyme Assays. NADH and $FMNH_2$ NR, and NADH CR assays were performed as previously described (8). One unit enzyme activity equals μmol product min^{-1}.

Purification of NR and Production of Antiserum. NR was partially purified by blue A Sepharose (Amicon Blue A) affinity column chromatography (10, 11). Nitrate-induced 6-d-old barley seedlings were frozen in liquid N_2 and ground in a Waring Blendor containing liquid N_2. After the evaporation of the liquid N_2, the frozen powder was transferred to chilled extraction buffer (12) containing 10 μM leupeptin and 1% casein at a ratio of 1 g fresh weight to 4 ml buffer. The slurry was stirred for 15 min, filtered through Miracloth, and centrifuged at 13,700*g* for 15 min. The supernatant was adjusted to 50% saturation with solid $(NH_4)_2SO_4$, and the resulting pellets containing NR activity were stored at −16°C until purification. The frozen pellets were dissolved in column buffer (25 mM Tris-HCl [pH 8.2], 1 mM EDTA, 5 μM FAD, 1 μM Na_2MoO_4, 3 mM DTT, and 10 μg/ml BSA) and desalted on a G-25 Sephadex column equilibrated in the same buffer. The desalted extract was loaded onto a 2.5- × 20-cm blue A column and washed overnight with the column buffer. NR was eluted using a linear 0 to 200 μM NADH gradient. Fractions containing NR activity were dialyzed against chilled glycerol for 1 h and stored at −16°C.

NR was further purified by preparative slab polyacrylamide gel electrophoresis (4). Affinity-purified NR (0.54 mg; specific activity, 5.6 units/mg protein) was applied to a trough in a 1.5-mm slab gel. Both the stacking (2.5% acrylamide, 0.625% BIS) and resolving (7% acrylamide, 0.184% BIS) gels were poured using a final concentration of 378 mM Tris-HCl (pH 8.9). A small amount of bromophenol blue was added to the upper buffer reservoir, and the electrophoresis started at 10 mamp per slab gel. The current was increased to 15 mamp per slab after the tracker dye had entered the lower gel and was maintained until the tracker dye reached the bottom of the gel (10 cm). All steps of the purification were carried out at 0 to 4°C.

NR was identified by staining the slab gels for MVH NR activity (14) and excised from the gel. Segments (1 cm) of the gel slices containing NR activity were subjected to electrophoresis in the presence of SDS on 15% acrylamide, 0.075% BIS gels (13) to determine the purity of the preparations. A single protein band of 110,000 mol wt was observed, confirming that barley NR had been purified to homogeneity. To prepare the antigen for injection, MVH was eluted from the gel slices by washing in distilled H_2O for 1 h. The gel was frozen, lyophilized, and stored at −16°C until used. Pulverized gel (30 mg) was emulsified in 1.5 ml of 1:1 mixture of distilled H_2O and Freund's adjuvant using a Sorvall Omnimixer for 15 min at room temperature. Two French Lop rabbits were injected at multiple sites four times at 10-d intervals using NR purified from two slab gels (about 200 μg protein) per rabbit. The first injection was made with Freund's complete adjuvant while the following injections were with Freund's incomplete adjuvant. The rabbits were bled through an ear vein 10 d after the fourth injection and the blood allowed to clot for 1 h at room temperature followed by 5 h at 8°C. Antiserum was collected after centrifuging at 2,000*g* for 15 min, and the supernatant was stored at −16°C. No further treatment of the antiserum from either rabbit was required for this study. Control serum was taken from each rabbit before the first injection.

Antiserum Titration. Crude extracts were prepared from 6-d-old nitrate-induced or uninduced wild-type barley seedlings. One g of seedlings was ground in a chilled mortar and pestle in 6 ml of extraction buffer containing 250 mM Tris-HCl (pH 8.2), 1 mM EDTA, 3 mM DTT, 5 μM FAD, 1 μM Na_2MoO_4, 2 μM antipain, 1 μg/ml pepstatin, 1 mM PMSF, and 3% BSA. The brei was centrifuged at 30,000*g* for 20 min, and the supernatant was used as the enzyme source.

Different dilutions of antiserum and control serum were made in 100 μl of 50 mM K-phosphate buffer (pH 7.5) and added to 1 ml of wild-type NR extract in 1.5-ml Eppendorf microcentrifuge tubes. The samples were incubated for 1.5 h at 4°C, centrifuged at 12,000*g* for 5 min, and supernatants were assayed for NADH NR, $FMNH_2$ NR, and NADH CR activities.

Immunoelectrophoresis. Enzyme extraction for immunoelectrophoresis and polyacrylamide gel electrophoresis was similar to that for antiserum titration except that 1 g fresh weight of seedling tissue was homogenized in 1 ml of 500 mM Tris-HCl (pH 8.6), 1 mM EDTA, 3 mM DTT, 25 μM FAD, 5 μM Na_2MoO_4, 10 μM antipain, 5 μg/ml pepstatin, 5 mM PMSF, and 3% BSA.

Crossed immunoelectrophoresis (25) was performed on wild-type barley extracts and partially purified NR. Antigen samples were electrophoresed in the first dimension in 1% w/v agarose (BioRad) gels. The separated antigens were immunoelectrophoresed in the second dimension in 0.8% w/v agarose gels containing antiserum.

For rocket immunoelectrophoresis, agarose gels (0.8% w/v) containing 0.4% v/v rabbit antiserum were poured into 1.5- × 70- × 100-mm molds using Tris-barbital buffer (pH 8.6) for both the gel and electrophoresis buffers (25). Sample wells 3 mm in diameter were cut across the cathode end of the gel, and 9 μl of barley extract were applied to each well. Rocket immunoelectrophoresis was conducted for 20 h at 1 v/cm, and the gels were maintained at 4°C during the run. Mutant NR CRM were quantified by comparison to a standard curve developed using nitrate-induced wild-type extracts diluted to different extents with extraction buffer.

Staining Procedures. Immunoprecipitates were stained for protein using Coomassie Brilliant Blue R-250 after the gels were deproteinized and dried onto gel bond film (Marine Colloids). The immunoprecipitates were also stained for MVH NR activity (14) and NADH diaphorase activity (1). XDH was detected in polyacrylamide electrophoresis gels of enzyme extracts (15).

RESULTS AND DISCUSSION

Mutant Enzyme Activities. NR-deficient mutants were assayed for NADH NR, $FMNH_2$ NR, and NADH CR activities (Table I). The Xno mutants isolated from the barley cv Winer exhibited NADH NR activities that were similar to the *nar* Steptoe mutants. Furthermore, like all *nar* mutants, except *nar* 1h, the Xno mutants were deficient in $FMNH_2$ NR. The Xno mutants had NADH CR

Table I. *NADH NR, $FMNH_2$ NR, and NADH CR Activities in Extracts of Nitrate-Induced NR-Deficient Mutant and Wild-Type Barley Seedlings*

Selection No.	Gene Designation	NR		CR
		NADH	$FMNH_2$	
		μmol g^{-1} fresh wt h^{-1}		
Az 12	*nar* 1a	1.9	0	16
Az 13	*nar* 1b	2.9	0	142
Az 23	*nar* 1c	2.0	0	16
Az 28	*nar* 1d	1.4	0	240
Az 29	*nar* 1e	2.8	0	20
Az 30	*nar* 1f	2.7	0	20
Az 31	*nar* 1g	1.7	0	118
Az 32	*nar* 1h	0.8	18	29
Az 33	*nar* 1i	1.9	0	110
Az 34	*nar* 2a	3.2	0	126
Xno 18		1.0	0	146
Xno 19		0.7	0	106
Xno 29		3.1	0	70
Steptoe	Wild type	40.6	25	211
Winer	Wild type	29.5	29	252

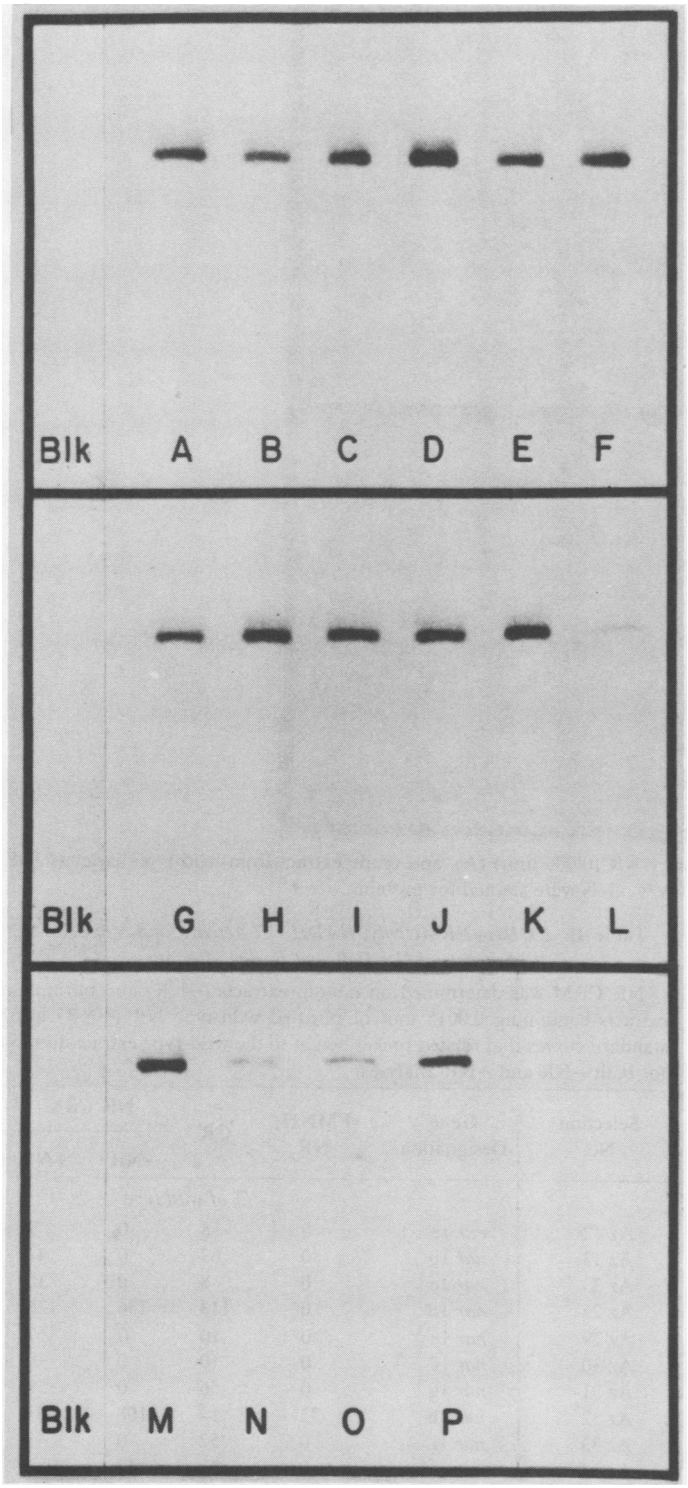

FIG. 1. XDH activity-stained polyacrylamide electrophoresis gels of crude extracts from ammonia-grown NR-deficient mutant and wild-type barley seedlings. Blank (Blk) gels were stained without hypoxanthine. Gel slots are labeled as follows: Steptoe (A), *nar* 1a (B), *nar* 1b (C), *nar* 1c (D), *nar* 1d (E), *nar* 1e (F), Steptoe (G), *nar* 1f (H), *nar* 1g (I), *nar* 1h (J), *nar* 1i (K), *nar* 2a (L), Winer (M), Xno 18 (N), Xno 19 (O), and Xno 29 (P). Extracts were prepared as for rocket immunoelectrophoresis, except that 0.1% BSA was added to the extraction buffer and the extracts were centrifuged 100,000*g* at 4°C for 1 h. Electrophoresis was as described for enzyme purification, and 50 μl of extract were applied per sample well.

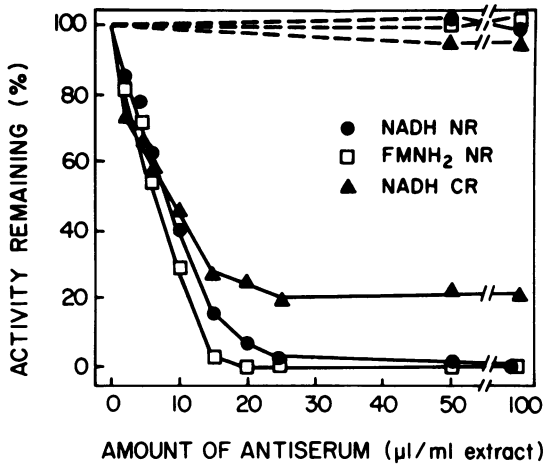

FIG. 2. Inhibition of NR-associated activities in crude extracts of nitrate-induced Steptoe seedlings by control serum (- - -) and antiserum directed against nitrate reductase (——). Initial enzyme activities of the Steptoe extract were 0.092 unit/ml NADH NR, 0.082 unit/ml $FMNH_2$ NR, and 0.641 unit/ml NADH CR.

levels similar to *nar* 1b, *nar* 1g, *nar* 1i, and *nar* 2a. The high level of NADH CR of *nar* 1d was unique to that allele.

Since the initial characterization of the *nar* mutants (8), we have improved NR stability in crude extracts by modifying buffer components (12) and by adding protease inhibitors (29). Re-examination of the *nar* mutant activities (Table I) revealed some differences from previously published data (8). The NADH NR activities of the mutants tended to be slightly higher compared with Steptoe. This is probably due to the improved stability of the NAD(P)H-bispecific NR present in the mutant seedlings (3). Another difference was that the NADH CR and $FMNH_2$ NR activities of the mutants *nar* 1d and *nar* 1h were considerably lower than previously reported (8). This may be due to the 24 h nitrate induction period used in this study, whereas in previous studies the seedlings were grown on nitrate for the entire experiment. The 24-h nitrate induction may have been insufficient for these mutants to synthesize maximum levels of NR. The $FMNH_2$ NR activity of both Steptoe and *nar* 1h was lower than the Steptoe NADH NR activity, which suggests that the extraction buffer interfered with the $FMNH_2$ assay.

Xanthine Dehydrogenase. Wild-type levels of XDH activity were observed in zymograms of extracts of all *nar* 1 alleles and Xno 29 (Fig. 1). The mutants *nar* 2a, Xno 18, and Xno 19 stained very faintly for XDH activity.

XDH (EC 1.2.1.37) is a molybdoenzyme which catalyzes the oxidation of hypoxanthine via xanthine to uric acid (2). Based on the observation that certain NR-deficient mutants in *Aspergillus nidulans* also lacked XDH activity, Pateman *et al.* (19) hypothesized that the MoCo of XDH is common to several enzymes, including NR. Consequently, mutants affecting the activity of two or more molybdoenzymes are presumed to be MoCo mutants. Therefore, mutants *nar* 2a, Xno 18, and Xno 19 are classified as MoCo mutants. It is noteworthy that these mutants are not absolutely deficient in XDH activity (Fig. 1). This may be due to leaky mutations. Certainly *nar* 2a is known to be somewhat leaky for NADH NR activity (8) (Table I) and even mutants Xno 18 and Xno 19 are not absolutely deficient in NADH NR activity. Perhaps totally deficient MoCo mutants are lethal and only leaky MoCo mutants can be recovered in intact plants.

Antiserum Characterization. Antiserum produced against purified barley NR inhibited the *in vitro* NADH NR activity of extracts from nitrate-induced wild-type seedlings (Fig. 2). The associated $FMNH_2$ NR and NADH CR activities of the extract

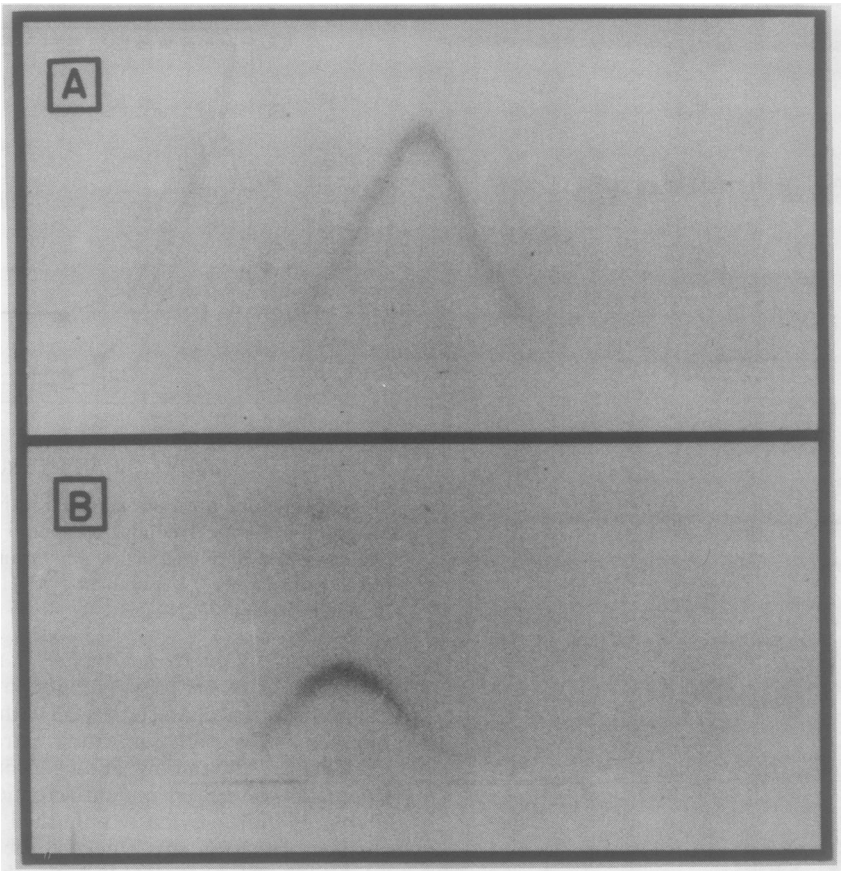

FIG. 3. Crossed immunoelectrophoresis of blue A Sepharose-purified barley NR (0.006 unit) (A), and crude extract from wild-type barley (0.005 unit) (B). Antiserum concentration of gel A was 0.3% v/v and gel B was 0.4% v/v. Gels were stained for protein.

were also inhibited by the antiserum. The $FMNH_2$ NR activity of the wild-type enzyme was inhibited with slightly less antiserum than the NADH NR of the same extract. Even at very high antiserum concentrations, 21% of the NADH CR activity in the extract remained active. This NADH CR activity was presumably not related to NR since a similar level of CR was present in uninduced wild-type seedlings and was not inhibited by the antiserum (data not shown). The presence of NADH CR that are not related to NR has been demonstrated in extracts of wild-type barley (28). Therefore, it was concluded that the antiserum inhibited the three NR associated activities similarly which is consistent with the action of *Chlorella* NR antiserum on the *Chlorella* enzyme (5).

Monospecificity of the antiserum was demonstrated by the formation of a single immunoprecipitate when either purified NR or wild-type seedling crude extracts were subjected to crossed immunoelectrophoresis and the gels stained for protein (Fig. 3, A and B). The single immunoprecipitate seen in these gels also stained positively for MVH NR and NADH diaphorase activity (data not shown), indicating that the immunoprecipitates were formed by the specific reaction of the antibodies with NR.

Rocket Immunoelectrophoresis of Mutant CRM. NR-deficient mutant seedling NR CRM was quantified by comparison with the NR CRM found in nitrate-induced wild-type seedlings. Undiluted wild-type extract exhibited a rocket height of about 20 mm, while dilutions of this extract resulted in a linear relationship between rocket height and the degree of dilution of wild-type extract (data not shown). To further increase the sensitivity of the analysis and to detect modified NR CRM, a fixed amount of partially purified wild-type NR was added to each sample and to the standard curve. The relationship between rocket height and NR content was linear over a broad range of enzyme concentration. The rocket immunoelectrophoretic determinations of NR CRM were expressed on a relative basis since we were unable to purify sufficient homogeneous NR for accurate protein calibration of the rocket heights.

Table II. *$FMNH_2$ NR Activity, NADH CR Activity, and NR CRM in Extracts of NR-Deficient Barley Mutants*

NR CRM was determined on mutant extracts (−NR) and on mutant extracts containing 0.0015 unit of purified wild-type NR (+NR) using standard curves that related rocket height to the wild-type extract dilution for both −NR and +NR analyses.

Selection No.	Gene Designation	$FMNH_2$ NR	CR	NR CRM	
				−NR	+NR
		% of wild type			
Az 12	*nar* 1a	0	8	0	9
Az 13	*nar* 1b	0	67	0	41
Az 23	*nar* 1c	0	8	0	38
Az 28	*nar* 1d	0	113	136	126
Az 29	*nar* 1e	0	10	0	7
Az 30	*nar* 1f	0	10	0	9
Az 31	*nar* 1g	0	56	0	58
Az 32	*nar* 1h	72	14	108	101
Az 33	*nar* 1i	0	52	0	43
Az 34	*nar* 2a	0	60	41	56
Xno 18		0	58	33	49
Xno 19		0	42	25	36
Xno 29		0	35	13	30

Rocket immunoelectrophoresis of the nitrate-induced mutant seedling extracts without added NR protein resulted in rockets from only 6 of the 13 mutants analyzed (Table II). Two mutants,

nar 1d and *nar* 1h, exhibited greater amounts of NR CRM than the wild type, while *nar* 2a, Xno 18, Xno 19, and Xno 29 contained considerably less CRM than the wild type. These results indicate that the NR CRM of these mutants was structurally similar to wild-type NR.

The seven mutants which failed to form rockets in the absence of wild-type NR exhibited from 9 to 58% of the wild-type NR CRM in the presence of purified NR protein. The presence of wild-type NR in extracts of mutants *nar* 1d, *nar* 1h, *nar* 2a, Xno 18, Xno 19, and Xno 29 did not significantly alter the amounts of CRM measured. After the addition of wild-type NR to the mutant extracts, the levels of NR CRM detected by rocket immunoelectrophoresis were correlated with the NR-associated activities of the mutants except for *nar* 1c. The mutant *nar* 1c was the only mutant which exhibited substantial NR CRM (38%) that did not have any NR-associated activity.

The NR CRM in some *nar* 1 mutants required the presence of wild-type NR to form rocket immunoprecipitates, indicating that these mutant NR CRM have an altered three-dimensional structure. Thermally denatured or 4 M urea-denatured barley NR also required the addition of wild-type NR for rocket formation (data not shown) indicating that denaturation of NR reduces the number of antigenic determinants of the enzyme. The modified antigen antibody complexes formed with denatured NR are most likely soluble and migrate out of the immunoelectrophoresis gels, as was demonstrated with the NR CRM of the *N. crassa* mutant *nit*-1 (1). The NR CRM of *nit*-1 mutant is dissociated into 4.5 to 5.2S subunits (6) which may account for its altered immunoelectrophoretic properties (1). Presumably, the added wild-type NR forms an immunoprecipitate to which the mutant NR CRM may add to increase the rocket height even though it is conformationally different from the wild-type enzyme. The similarity of the immunoelectrophoretic properties of the seven barley *nar* 1 NR CRM and *N. crassa* mutant *nit*-1 CRM suggests that these barley mutant NR may also be dissociated into subunits.

The nitrate-dependent induction of NR CRM in the barley mutants was similar to the wild types. When mutant and wild-type seedlings were watered with ammonia or nitrogen-free nutrient solutions, no NR CRM was detected by rocket immunoelectrophoresis even after the addition of purified wild-type NR protein to the extracts (data not shown). Therefore, the nitrate induction of barley NR is different from that observed in *Chlorella* which involves the activation of a precursor NR (5). It also appears that under the growth conditions of this study, none of the NR mutants are constitutive for NR CRM and that NR is synthesized *de novo* in response to nitrate. In the wild-type *N. crassa*, NR is synthesized *de novo* (1, 6), but in certain *nit*-1 and *nit*-3 alleles, the NR CRM is constitutively regulated (24).

An inactive NR precursor found in ammonia-grown *Chlorella* extracts also exhibited immunoelectrophoretic properties similar to these structurally modified barley mutant NR CRM (5). Further characterization of this precursor led to the conclusion that it was rapidly turning over and, therefore, present in extracts in a partially degraded form which accounted for its modified cross-reaction (5). Proteolytic degradation of the barley mutant NR may also account for their reduced cross-reaction; however, inclusion of protease inhibitors in the extraction buffer should reduce *in vitro* degradation of the NR CRM. Further study is required to determine if *in vivo* proteolytic degradation of the NR CRM resulted in the altered immunoelectrophoretic character of certain mutants.

In conclusion, rocket immunoelectrophoresis was used to quantify the NR CRM levels of 13 NR-deficient barley mutants. The NR CRM of some but not all *nar* 1 alleles exhibited different immunoelectrophoretic properties than NR CRM of the wild type, indicating that structural differences in NR CRM occur within the same allelic group. This observation, considered with the fact that the *nar* 1 alleles possess different NR partial activity phenotypes and have wild-type XDH activity, strongly supports the hypothesis that *nar* 1 codes for the NR structural gene (8). The deficiency of XDH activity in Xno 18, Xno 19, and *nar* 2a confirms that these NR-deficient mutants lack a functional MoCo.

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