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NITRATE REDUCTASE-DEFICIENT MUTANTS IN BARLEY: ENZYME STABILITY AND PEPTIDE MAPPING*

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; stability; peptide mapping; nitrate reductase; mutants.

Abstract—Thermal stability and pH optima of NADH-nitrate reductase-associated cytochrome *c* reductase and $FMNH_2$-nitrate reductase from wild type, cv Steptoe or Winer, and mutants *nar* 1d, *nar* 1g, *nar* 1h, Xno 18 and Xno 19 were compared to determine if structural differences in the nitrate reductase protein could be detected. Also, the nitrate reductase-associated cytochrome *c* reductase from *nar* 1d was purified and compared with the wild type by peptide mapping. The pH optimum for $FMNH_2$-nitrate reductase from Steptoe and *nar* 1h, and for NADH-cytochrome *c* reductase from Steptoe, *nar* 1d, *nar* 1g and *nar* 2a was 7.5. Thermal stabilities of the nitrate reductase-associated activities ($FMNH_2$-nitrate reductase or NADH-cytochrome *c* reductase) from *nar* mutants were less than the Steptoe wild type, while Xno mutants were equal to the Winer wild type. Cleveland peptide maps of *nar* 1d NADH-cytochrome *c* reductase and Steptoe nitrate reductase were identical when digested with endoprotease lys-C but were distinctly different in one peptide when digested with *Staphylococcus aureus* endoprotease V8. These results provide evidence that *nar* 1 gene codes for the nitrate reductase polypeptide.

INTRODUCTION

Higher plant mutants defective in nitrate reductase (NR) activity have been identified and described in *Arabidopsis thaliana* [1], *Nicotiana tabacum* [2], *Datura innoxia* [3], *Hordeum vulgare* [4–6] and *Pisum sativum* [7–9]. Only in *Nicotiana tabacum* [2, 10, 11] and *Hordeum vulgare* [5, 6, 12, 13] has an extensive investigation of the biochemical properties of the mutants been carried out.

We have characterized NR-deficient *Hordeum vulgare* mutants for their NADH-NR, the NR-associated catalytic activities [$FMNH_2$-NR and NADH-cytochrome *c* reductase (CR)], nitrite reductase (NiR) activity and their cross-reacting material (CRM) against specific barley NR antiserum [6, 12, 13]. Under nitrate-induced growth conditions, all NR-deficient mutants have elevated NiR activities, but possess different levels of NR-associated catalytic activities and antigenicity. These data support the concept that the NR-deficient mutants represent different mutation events at the loci regulating NR activity. The *nar* 1 locus has been postulated to encode the NR structural protein while the *nar* 2 locus is presumed to be involved with the molybdenum cofactor (MoCo) functions [6, 13]. In the present paper, properties of the NR-associated catalytic activities ($FMNH_2$-NR and NADH-CR) and peptide maps of NRs from the wild type and the mutant *nar* 1d are compared.

RESULTS

The $FMNH_2$-NR pH optimum was 7.5 for the wild type (cv Steptoe) and the mutant *nar* 1h (data not shown). The NADH-CR pH optimum was also identical for the wild type and all of the mutants tested. The peak was somewhat broad and centered around pH 7.5 (data not shown).

The *nar* 1h $FMNH_2$-NR was more sensitive to heat inactivation than the wild type Steptoe $FMNH_2$-NR (Figs. 1 and 2). The enzyme was 50% inactivated at 46° for the wild type and at 45° for *nar* 1h (Fig. 1). To obtain 50% inactivation of the $FMNH_2$-NR at 42° required 47 min and 72 min for the mutant *nar* 1h and Steptoe, respectively (Fig. 2). Decay of the $FMNH_2$-NR from both genotypes followed first-order kinetics.

The mutants used in this study all possess substantial nitrate inducible NADH-CR activity. The NADH-CR thermal stability of the *nar* mutants was compared with the parent cv Steptoe and the Xno mutants were compared with the parent cv Winer. There were distinct differences in the NADH-CR thermal stabilities among the *nar* mutants tested. Within the *nar* 1 locus, the allele *nar* 1g had the most unstable NADH-CR while the *nar* 1d NADH-CR was intermediate in its thermal stability when compared with the wild type NADH-CR. The *nar* 2a NADH-CR was almost as unstable as the *nar* 1g NADH-CR. The ranking of the mutants based on the NADH-CR thermal stability was the same when determined by either time or temperature for 50% inactivation (Table 1). The

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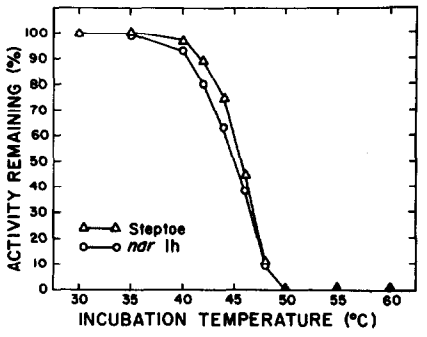

Fig. 1. Effect of incubation temperature on thermal stabilities of wild type (cv Steptoe) and mutant *nar* 1h $FMNH_2$-NR activity. Aliquots were incubated separately in a water bath at the temperatures indicated for 10 min and assayed for $FMNH_2$-NR at 30°.

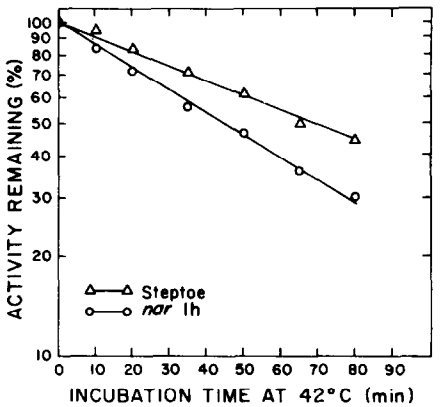

Fig. 2. Effect of incubation time at 42° on thermal stabilities of wild type (cv Steptoe) and mutant *nar* 1h $FMNH_2$-NR activity. Extracts were incubated in a 42° water bath. Aliquots were withdrawn at indicated intervals, cooled in an ice bath and assayed for $FMNH_2$-NR at 30°.

Table 1. Heat inactivation of cytochrome *c* reductase in *nar* mutants

Genotype	Time for 50% inactivation at 38° (min)	Temperature for 50% inactivation in 10 min (°)
Wild type (cv Steptoe)	41	43
nar 1d	28	41.5
nar 2a	15	41
nar 1g	10	39.5

Xno mutants displayed similar NADH-CR thermal stability to the wild type cv Winer (data not shown).

NADH-CR isolated from the mutant *nar* 1d was peptide-mapped and compared with the wild type Steptoe NADH-NR. Cleveland mapping [14] using endoprotease lys-C showed identical peptide patterns for the Steptoe NADH-NR and the *nar* 1d NADH-CR (Fig. 3). Similar experiments using *Staphylococcus aureus* protease V8, specific for glutamic acid residues, resulted in peptide patterns for Steptoe and *nar* 1d NR proteins which were identical except for one peptide in the 20 kd region (Fig. 4). The Steptoe NADH-NR peptide map had one additional polypeptide that migrated slightly faster (i.e. smaller) than 20 kd which was not present in the *nar* 1d NADH-CR peptide map (Fig. 4, arrow 1). The *nar* 1d NADH-CR peptide map had one additional polypeptide that migrated slightly slower (i.e. larger) than 20 kd and was not present in the Steptoe NADH-NR peptide map (Fig. 4, arrow 2). To substantiate this observation, the two proteins were digested in the same gel slot. Again, both Steptoe NADH-NR and *nar* 1d NADH-CR had identical peptide patterns except for the difference around 20 kd (Fig. 5, slots 1 and 3). In the gel slot which contained both Steptoe NADH-NR and *nar* 1d NADH-CR proteins, all the peptides common to the two proteins were seen, including the two polypeptides by which they differ (Fig. 5, slot 2).

DISCUSSION

Previous studies have shown that some of the barley NR-deficient mutants, although unable to catalyse the reduction of nitrate, retain a protein which can cross-react with NR-specific antiserum and can catalyse some of the reactions associated with NR such as $FMNH_2$-NR and NADH-CR [6, 12, 13]. The $FMNH_2$-NR and NADH-CR activities provide a means of assaying for the presence of altered forms of the NR protein in these mutants. A study of some of the physical parameters of these mutant proteins presented here establishes that the *nar* 1 gene

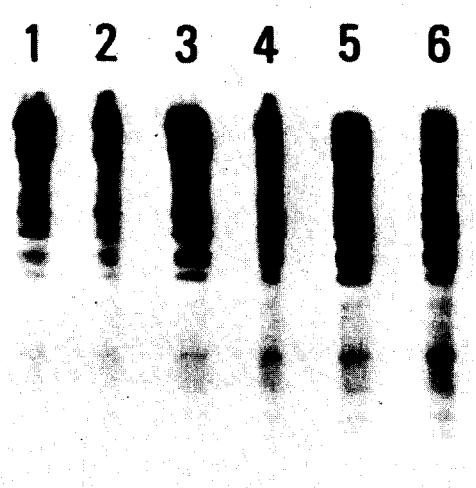

Fig. 3. Cleveland peptide maps using protease lys-C digestion of the wild type NADH-NR and the mutant *nar* 1d NADH-CR. Gel slots 1, 3 and 5 contained *ca* 10 μg of ^{14}C-labeled *nar* 1d NADH-CR subunit protein. Slots 2, 4 and 6 contained approximately the same amount of ^{14}C-labeled Steptoe NADH-NR subunit protein. Protease used was 0.25 μg/slot in 1 and 2, 0.5 μg/slot in 3 and 4, and 1.0 μg/slot in 5 and 6.

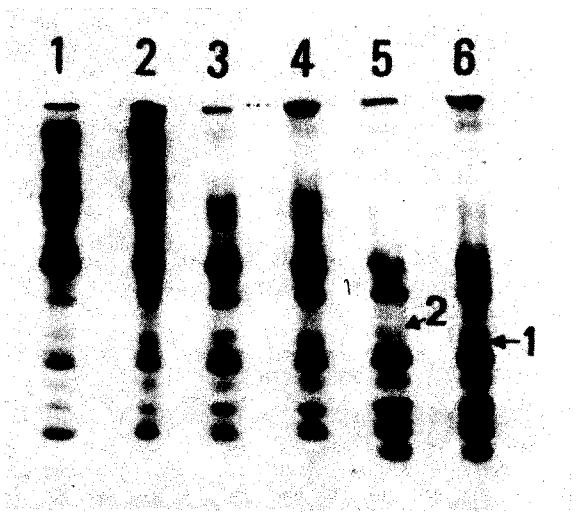

Fig. 4. Cleveland peptide maps using *Staphylococcus aureus* protease V8 digestion of the wild type NADH-NR and the mutant *nar* 1d NADH-CR. Gel slots 1, 3 and 5 contained *ca* 10 μg of ^{14}C-labeled *nar* 1d NADH-CR subunit protein. Slots 2, 4 and 6 contained approximately the same amount of ^{14}C-labeled Steptoe NADH-NR subunit protein. Protease used was 0.01 μg/slot in 1 and 2, 0.04 μg/slot in 3 and 4, 0.1 μg/slot in 5 and 6.

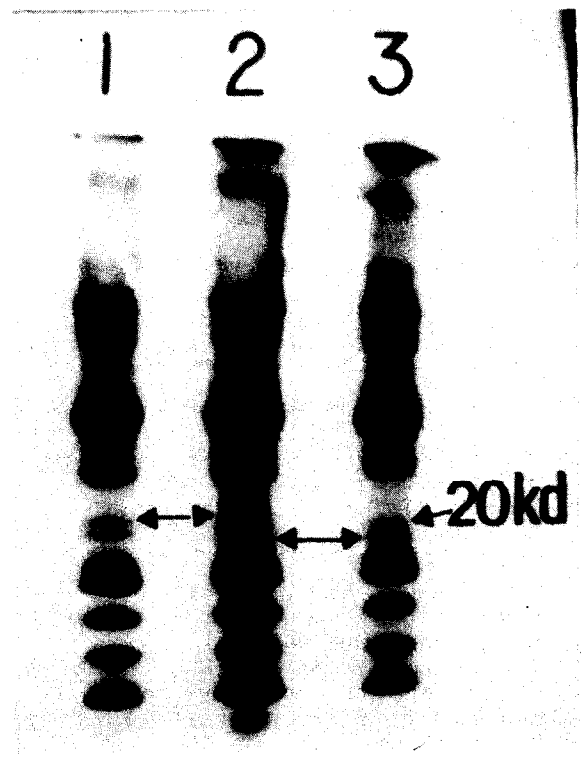

Fig 5. Cleveland peptide maps using *Staphylococcus aureus* protease V8 resulting from protease digestion of wild type NR subunit protein (slot 3), mutant *nar* 1d NADH-CR protein (slot 1) and a mixture of the two proteins (slot 2). Protease used was 0.04 μg/slot in 1 and 3, and 0.08 μg/slot in 2.

codes for the polypeptide structure of the barley NR. That conclusion is based on the following observations: (1) decreased thermal stability of the $FMNH_2$-NR in *nar* 1h and NADH-CR in *nar* 1d and *nar* 1g; (2) discretely altered peptide pattern of *nar* 1d NADH-CR when compared to the wild type NADH-NR; and (3) altered NR CRM in several of the *nar* 1 alleles [13]. Altered thermal stability of a protein is usually a good indication of an altered primary structure of the protein. The use of crude extracts for the thermal stability determinations in these studies may reduce the strength of the conclusion somewhat. However, the use of a NR stabilizing buffer and a protease inhibitor and other supporting evidence [13] make the conclusion of altered protein structure the most probable interpretation of the data.

The Cleveland peptide mapping procedure is suitable for the detection of differences or similarities of protein primary structures [14]. Nevertheless, the detection of a single amino acid change would not be expected unless the altered amino acid was involved in the recognition site of the protease being used for the digestion. This expectation was confirmed when the endoprotease lys-C, specific for lysine residues, was used. The data clearly demonstrated that the NADH-CR isolated from *nar* 1d and the NADH-NR isolated from the parent cv Steptoe are identical as far as the lys-C protease is able to discriminate. This result supports the conclusion reached previously by others [15] that the nitrate inducible NADH-CR is the same enzyme as the NADH-NR in barley. The results obtained with *Staphylococcus aureus* protease V8, specific for glutamic acid residues, showed that the NADH-CR from *nar* 1d differs from the NADH-NR from Steptoe by a glutamic acid residue (Figs. 4 and 5). From the data it appears that a glutamic acid residue in the wild type NR protein has been changed by mutation to some other amino acid which is no longer recognized by the *S. aureus* protease V8. Thus, the peptide generated by the proteolytic digestion of the mutant NR protein is somewhat larger than the wild type counterpart (Figs. 4 and 5).

The *nar* 1 alleles constitute the majority, but not all of the NR-deficient mutants currently known in barley. Other mutants, such as *nar* 2a, Xno 18, Xno 19, are not allelic to *nar* 1 [6, 16]. These mutants are also different from *nar* 1 in that they show a pleiotropic effect upon xanthine dehydrogenase activity and are, therefore, presumed to be MoCo mutants [13]. The results showed that the *nar* 2a NADH-CR was more thermolabile than the wild type control, while the Xno 18 and Xno 19 NADH-CR thermal stability was comparable to the wild type control. These data suggest that the *nar* 2 gene product also affects the structural stability of the NR protein. In *Aspergillus nidulans*, the *cnx* H MoCo mutant has been implicated as contributing to the structure of the NR protein [17]. Unfortunately, no further characterization of this component has been carried out and we have not been able to isolate sufficient NADH-CR from *nar* 2a for more rigorous tests. Thus the role of the *nar* 2 locus in the barley NR structure remains unresolved. The mutants Xno 18 and Xno 19 are allelic and represent yet another locus designated *nar* 3 [16, 18]. This locus does not appear to affect the barley NR structure directly.

EXPERIMENTAL

Plant growth. Barley seedlings (*Hordeum vulgare* L.) were grown in vermiculite in a controlled growth chamber at 18° with

continuous illumination (300 $\mu E/m^2$ per sec) for 6–7 days and watered daily by sub-irrigation with a modified Hoagland soln containing nitrate [19]. The mutants *nar* 1d, *nar* 1g, *nar* 1h and *nar* 2a [6] used in this study were all selected from the parent cv Steptoe. Mutants Xno 18 and Xno 19, induced with ethyl methanesulfonate and selected from the parent cv Winer, were acquired from Dr. B. I. Tokarev [4].

Enzyme extraction and assay. Crude extracts were prepared and NADH-NR assays conducted as previously described [12] except that 1.5% casein [20] and 10 μM leupeptin [21] were included in the extraction buffer. $FMNH_2$-NR was assayed according to ref. [22]. Crude extracts for NADH-CR assay were made in the same manner, except the concn of dithiothreitol (DTT) in the extraction buffer was reduced to 1 mM to minimize interference with the CR assay. NADH-CR was determined according to ref. [15]. A buffer containing 50 mM each of Tris, KPi and maleic acid (TPM), adjusted to the indicated pH with KOH, was used for the determination of the pH optima of both NADH-CR and $FMNH_2$-NR. *In vitro* stabilities of $FMNH_2$-NR and NADH-CR were determined by incubating the crude extracts at indicated temps. and assaying aliquots at the standard assay temp. (30°) at indicated time points. Alternatively, crude extracts were incubated for 10 min at indicated temps. and transferred to a 0° ice bath until assays were conducted. Temps. used for these expts were selected based on preliminary data. The activities were expressed as per cent remaining, relative to the activity measured at zero incubation time for the fixed temp. expts or relative to the activity measured at the 30° incubation temp. for the fixed time expts. All expts were repeated at least twice and all assays were carried out in triplicate.

Preparation of subunit protein and peptide mapping. The wild type NR was isolated from barley (cv Steptoe) leaves as described [23] except that 10 μM leupeptin was included in the extraction buffer and a blue A-Sepharose (Amicon) affinity column matrix was used. NR eluted from a blue A-Sepharose column was lyophilized. The lyophilized affinity-purified NR (10.15 μkat) was dissolved in 0.4 ml 0.15 M Tris–HCl (pH 8.8), 8 M urea, 1 mM DTT and incubated with 25 μCi of [2-^{14}C]iodoacetic acid at 37° for 1.5 hr [24]. The mixture was then diluted by adding 1.2 ml of stacking gel buffer containing 10% glycerol, 0.001% bromophenol blue, 5% SDS and 5% β-mercaptoethanol and subjected to a preparative SDS-polyacrylamide slab gel electrophoresis using the buffer system described in ref. [25]. A 1.5 mm thick 15% resolving gel and 6% stacking gel were prepared from a stock soln of 30% acrylamide–1.15% *N,N'*-methylenebisacrylamide. Electrophoresis was initiated at 25 mA per slab and increased to 50 mA after the tracking dye had completely entered the resolving gel, and terminated when the tracking dye had just migrated out of the resolving gel. After brief staining, destaining and extensive rinsing with cold glass-distilled H_2O, a gel strip (*ca* 3 mm wide) containing the MW 110 000 NR subunit protein band was excised horizontally from the slab gel. The gel strip containing ^{14}C-labeled NR subunit protein was cut into 5 mm wide pieces, equilibrated with stacking gel buffer containing 1 mM EDTA [14] and stored at −16° until used.

The ^{14}C-labeled subunit protein from mutant *nar* 1d was isolated by the procedures described for isolating the wild type subunit protein, except that during affinity chromatography the mutant NR complex was monitored by NADH-CR activity instead of NADH-NR.

Peptide mapping was carried out in 1.5 mm thick slab gels according to the procedures described in ref. [14], except that the 16.5% resolving and 4% stacking gels were made from a stock soln of 30% acrylamide–0.4% *N,N'*-methylenebisacrylamide. Proteases used were *Staphylococcus aureus* V8 (Miles) and endoprotease lys-C (Boehringer-Mannheim). After electrophoresis, the gel was washed with agitation for *ca* 25 min with two changes of destaining soln to remove excess SDS before brief staining and destaining were carried out to reveal standard MW markers. Fluorographs of the peptide maps were developed by the procedure described in ref. [26].

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