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PURIFICATION OF BARLEY NITRATE REDUCTASE AND DEMONSTRATION OF NICKED SUBUNITS*

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; nitrate reductase; purification; proteolytic nicking; subunit MW.

Abstract—Nitrate reductase was purified from 90-hr-old, nitrate-treated barley shoots by the same four-step procedure under four sets of conditions (A, B, C, D). The conditions ranged from one where no attempt was made to protect the enzyme from proteolytic nicking (scheme A) to one which was likely to afford a high level of protection (scheme D). Each purified nitrate reductase sample, from the four different conditions, co-migrated as a single protein band in non-denaturing gels with nitrate reductase and nitroblue tetrazolium reductase activity. Specific gel staining showed that enzyme purified by scheme A and by scheme D co-migrated with haem. Both these samples migrated as a single homogeneous band in gels of different percentage polyacrylamide with a MW of *ca* 205 000. However, enzyme purified under scheme D where concerted attempts were made to protect against proteolytic attack had a specific activity which was 24 times that of enzyme purified under scheme A. After SDS gel electrophoresis of nitrate reductase purified by scheme A, a prominent band was seen at 59 000 MW, with others at 38 000 and 20 000 and a very faint band at 103 000 MW. By contrast, a prominent band at 103 000 MW with others at 59 000 38 000 and 20 000 MW was seen after SDS gel electrophoresis of nitrate reductase purified by scheme D. In both cases other protein bands were also present. We interpret these findings as showing that the *ca* 205 000 MW barley nitrate reductase purified under the conditions described in this paper is a dimer of *ca* 103 000 MW subunits which are particularly sensitive to proteinase attack. Even highly nicked molecules, such as those purified under scheme A, behave under certain conditions (non-denaturing gel electrophoresis) as though subunit integrity has been retained although their specific activity is very low.

INTRODUCTION

The assimilatory NADH nitrate reductase (NADH: nitrate oxidoreductase, EC 1.6.6.1) of higher plants is a haemomolybdoflavoprotein which catalyses the two-electron reduction of nitrate to nitrite [1, 2]. The MW of the spinach enzyme has been reported to be 197 000 [3] whilst that from barley has been reported to be 203 000 (cv Golden Promise) [4] or 221 000 (cv Steptoe) [5]. Flavin stoichiometry has not been determined due to the ease of FAD dissociation during purification but the haem to molybdenum ratio for the spinach enzyme is *ca* 2:1 [6]. The haem has the spectral properties of a cytochrome b_{557} with an oxidized Soret band at 412 nm [3]. The very limited biochemical and genetic evidence from higher plants is consistent with molybdenum being carried on a low MW pterin cofactor of the type identified in other molybdoenzymes [7–10].

In addition to the physiological reaction, the enzyme carries a dehydrogenase function (which allows the transfer of electrons from NADH to a variety of electron acceptors such as cytochrome *c*, nitroblue tetrazolium and dichlorophenolindophenol) and also possesses FMNH and reduced viologen dye nitrate reductase activity [1, 2]. Inhibitor studies suggest that the dehydrogenase function (assayed as NADH cytochrome *c* reductase activity) of the barley enzyme is catalysed by the initial portion of the electron transport chain [11]. It is not clear if haem is obligatorily involved in the expression of cytochrome *c* reductase activity in the spinach enzyme [12] but the haem of the *Neurospora crassa* NADPH nitrate reductase is reduced by NADPH only when FAD is also present [13]. Removal of molybdenum [14] or substitution by tungsten [11] abolishes all nitrate reductase activities but has no effect on cytochrome *c* reductase activity. Electron flow is generally accepted to be:

$$\text{NADH} \rightarrow \text{FAD} \rightarrow \text{Haem} \rightarrow \text{Mo} \rightarrow \text{NO}_3^-.$$

Nitrate reductase is unstable in cell-free extracts from a variety of plant species unless bovine serum albumin or casein is included in the extraction buffer [15–21]. More recently, we have shown that the MW 203 000 nitrate reductase is unstable in cell-free extracts from barley primary leaves and that loss of activity is accompanied by the appearance of smaller cytochrome *c* reductase species of MW *ca* 40 000 and 61 000 [4, 22]. Since bovine serum albumin both stabilized nitrate reductase activity in cell-free extracts and prevented appearance of these cytochrome *c* reductase species, we concluded that these species were probably proteolytic degradation products of nitrate reductase [22]. The demonstration that leupeptin, an inhibitor of the cysteine endoproteinase of barley [23], also stabilized nitrate reductase in cell-free extracts and prevented appearance of these cytochrome *c* reductase species supported this conclusion [24].

To account for the production of these species, we speculated that the FAD and haem components may be organized in the subunit as separate functional domains,

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whilst an additional domain may be involved in the binding of the molybdenum cofactor. The domains were envisaged as being linked by hinge regions which are hypersensitive to proteinase attack [22]. The MW 40 000 cytochrome *c* reductase species might represent a fragment of the subunit carrying the NADH binding site and probably FAD, since FAD is needed for cytochrome *c* reductase activity [13].

In this paper, we show that enzyme purified to electrophoretic homogeneity has a subunit MW of *ca* 103 000 in our hands. This subunit is very sensitive to proteolytic attack and can be nicked to protein species of MW 59 000, 38 000 and 20 000, amongst others. Preliminary accounts of this work have been presented [25, 26].

RESULTS

Purification of nitrate reductase from 4-day-old barley shoots

In order to demonstrate that barley nitrate reductase is susceptible to proteolysis we have sought to purify the enzyme from barley shoots under conditions which might be expected to affect enzyme integrity to different extents.

In scheme A no attempt was made to stabilize nitrate reductase during purification and enzyme was purified from 4-day-old barley shoots which had been grown in the presence of half-Hoagland's nutrient solution containing 15 mM KNO_3 as sole nitrogen source. Shoots were ground in a mortar and pestle with buffer I (0.05 M potassium phosphate buffer, pH 7.5, containing 0.1 mM EDTA, 10 μM FAD and 1 mM cysteine) and after streptomycin sulphate treatment, the 0–45% ammonium sulphate fraction was applied to a Biogel A1.5m column. The major peak of enzyme activity which eluted (Fig. 1a) was pooled and after concentration by ammonium sulphate (45%) precipitation was applied to a blue-dextran Sepharose column. After washing off unadsorbed protein, nitrate reductase was eluted with the same buffer containing 5 μM NADH (Fig. 1b) and the pooled fractions were concentrated with an Amicon thin-channel concentrator.

This scheme (scheme A) was modified as summarized in the legend to Table 1 to give three other schemes (B, C and D) which were used to purify nitrate reductase from 4-

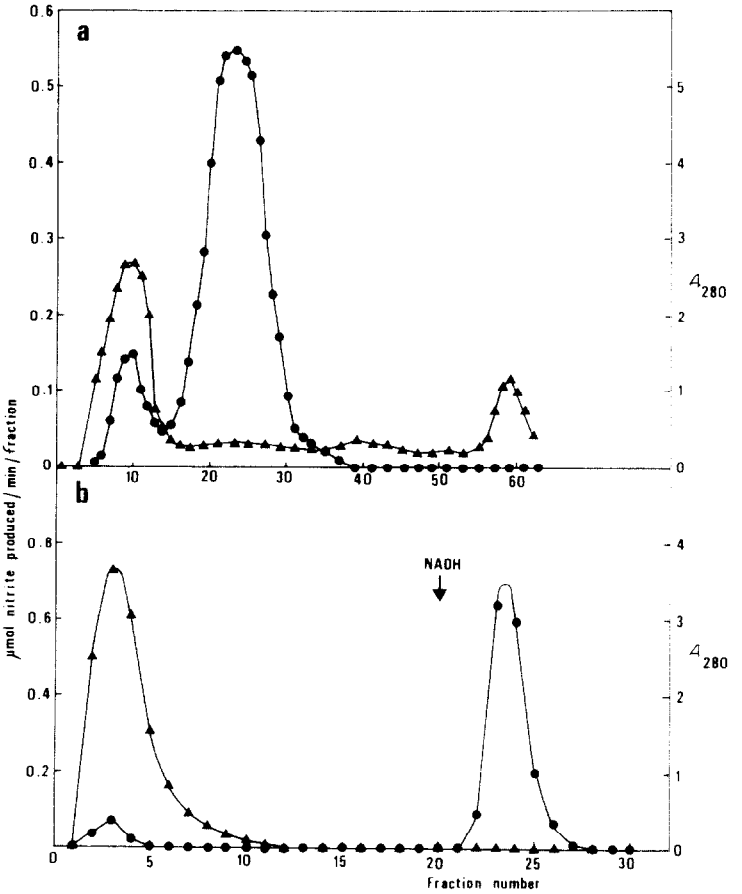

Fig. 1. (a) Distribution of NADH nitrate reductase activity (●) and protein (▲) after Biogel A1.5m gel filtration (4.1 cm × 108 cm column) of a 0–45% $(NH_4)_2SO_4$ fraction from 90 hr-old barley shoots. Elution buffer was buffer I and 16 ml fractions were collected. (b) Distribution of NADH nitrate reductase activity (●) and protein (▲) after blue-dextran Sepharose affinity chromatography (2 × 9 cm column) of pooled fractions containing NADH nitrate reductase from Biogel A1.5m. Elution buffer was buffer II (0.02 M potassium phosphate buffer, pH 7.5, containing 0.1 mM EDTA, 10 μM FAD and 1 mM cysteine) and 5 ml fractions were collected. The arrow denotes the start of elution of nitrate reductase with buffer II containing 5 μM NADH.

Table 1. Purification of NADH nitrate reductase from 4-day-old barley shoots

Scheme	Purification step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
A	Streptomycin sulphate (5 mg/g) supernatant	1125	3713	1.93	0.00052	100	—
	0–45% $(NH_4)_2SO_4$ fraction	30	1950	1.33	0.00068	68.9	1.3
	Pooled Biogel A1.5m peak	170	126	0.91	0.0072	47.2	13.8
	0–45% $(NH_4)_2SO_4$ fraction	2.3	60.7	0.89	0.0146	46.1	28.1
	Pooled and concentrated blue dextran Sepharose peak	2.0	0.49	0.134	0.273	6.9	525
B	Streptomycin sulphate (5 mg/g) supernatant	930	4042	6.24	0.0015	100	—
	0–45% $(NH_4)_2SO_4$ fraction	27.5	1425	9.05	0.0063	145	4.2
	Pooled Biogel A1.5m peak	225	206.3	3.00	0.0145	48.1	9.7
	0–45% $(NH_4)_2SO_4$ fraction	4.5	185.3	4.46	0.0241	71.5	16.1
	Pooled and concentrated blue dextran Sepharose peak	2.0	0.95	0.75	0.789	12.0	526
C	Streptomycin sulphate (5 mg/g) supernatant	830	3255	12.7	0.0039	100	—
	0–45% $(NH_4)_2SO_4$ fraction	25	1062	18.93	0.0178	149.1	4.5
	Pooled Biogel A1.5m peak	125	147.5	6.73	0.045	52.9	11.5
	0–45% $(NH_4)_2SO_4$ fraction	5	140.4	7.45	0.053	58.7	13.6
	Pooled and concentrated blue dextran Sepharose peak	2.9	0.41	1.36	3.34	10.7	856
D	Streptomycin sulphate (5 mg/g) supernatant	950	3166	24.43	0.0074	100	—
	0–45% $(NH_4)_2SO_4$ fraction	20	1235	32.87	0.0266	140.3	3.6
	Pooled Biogel A1.5m peak	144.	179.3	11.80	0.0658	50.4	8.9
	0–45% $(NH_4)_2SO_4$ fraction	3.7	173.5	13.19	0.0760	56.3	10.3
	Pooled and concentrated blue dextran Sepharose peak	2.0	0.35	2.26	6.48	9.6	876

Nitrate reductase was purified from 350–400 g of 4-day-old barley shoots by the same purification steps, as indicated above and in the Experimental, but conditions of plant growth and purification buffers were altered to give four schemes, A–D. Scheme A: Plants grown with nutrient solution containing 15 mM KNO_3 as sole nitrogen source and purification carried out in buffer I; Scheme B: Plants grown with nutrient solution containing 10 mM KNO_3/30 mM NH_4Cl as sole nitrogen source and purification carried out in buffer I; Scheme C: Plants grown as in scheme B and purification carried out in buffer I containing 10 μM leupeptin; Scheme D: Plants grown as in scheme B and purification carried out in pH 8.5 buffer containing 10 μM leupeptin. Buffer I consists of 0.05 M potassium phosphate buffer, pH 7.5, containing 0.1 mM EDTA, 10 μM FAD and 1mM cysteine [4]. The pH 8.5 buffer consists of 0.25 M Tris–HCl, pH 8.5, containing 3 mM DTT, 5 μM FAD, 1 μM sodium molybdate and 1 mM EDTA [27]. One unit of enzyme activity is defined as 1 μmol nitrite produced per min.

day-old barley shoots.

In scheme B, nitrate reductase was purified with buffer I as in scheme A but plants were grown in the presence of 30 mM NH_4Cl and 10 mM KNO_3 as nitrogen source rather than 15 mM KNO_3. Nitrate reductase is much more stable in cell-free extracts from *in vitro* tobacco callus cultures (Mendel, R.-R., personal communication) or barley plants (Wray, J. L., unpublished results) grown with ammonium nitrate as nitrogen source rather than potassium nitrate, although the underlying reason is, as yet, unclear.

In scheme C, plants were grown in the presence of 30 mM NH_4Cl and 10 mM KNO_3 as nitrogen source and nitrate reductase was purified with buffer I containing 10 μM leupeptin. Leupeptin is extremely effective in stabilizing nitrate reductase in cell-free extracts [24].

In scheme D, plants were grown in the presence of 30 mM NH_4Cl and 10 mM KNO_3 as nitrogen source and nitrate reductase was purified in Kuo's buffer [27] containing 10 μM leupeptin. Nitrate reductase is very stable in cell-free extracts from barley shoots prepared in this buffer, which consists of 0.25 M Tris-HCl, pH 8.5, 3 mM DTT, 5 μm FAD, 1 μM sodium molybdate and 1 mM EDTA. Purification by schemes B, C and D produced elution profiles from Biogel A1.5 m and blue-dextran Sepharose columns which were essentially the same as those shown in Figs. 1a and 1b. Details of the purification schemes are summarized in Table 1. Yields of active nitrate reductase ranged from 4 to 12% whilst extent of purification ranged between 525- and 850-fold.

Electrophoretic homogeneity, MW and specific activity of purified nitrate reductase

From measurements of Stokes' radius and of sedimentation in sucrose gradients we have previously calculated that barley nitrate reductase present in cell-free extracts has a MW of 203 000 [4]. In an attempt to confirm this value, nitrate reductase purified by schemes A and D was subjected to electrophoresis in non-denaturing gels of different percentage composition. A single protein band was observed in both cases after staining gels with Coomassie blue [Fig. 2a, data for scheme D, and Fig. 3a (A and D)], and comparison with migration of reference protein gave a MW of 205 000 (Fig. 2b) for nitrate reductase purified by scheme D. The same MW was obtained after electrophoresis of a sample of nitrate reductase from scheme A.

Nitrate reductase purified by schemes B and C was also electrophoretically homogeneous [Fig. 3a (B and C)], and the single protein band comigrated with methyl viologen-nitrate reductase and a single nitroblue tetrazolium reductase activity [Figs. 3b (scheme A) and 3c (scheme D)]. However, although nitrate reductase purified by all four schemes was electrophoretically homogeneous in non-denaturing gels, there was a 24-fold difference in specific activity between enzyme prepared by scheme A and that prepared by scheme D. Specific activity of enzyme purified by schemes B and C was intermediate (Table 1).

SDS gel electrophoresis of nitrate reductase purified by schemes A to D

SDS gel electrophoresis of the homogeneous nitrate reductase purified by scheme A, in which no attempt was made to stabilize nitrate reductase, is shown in Fig. 4a(A). Several protein bands were apparent including a major one of MW 59 000 and minor bands of MW 38 000 and

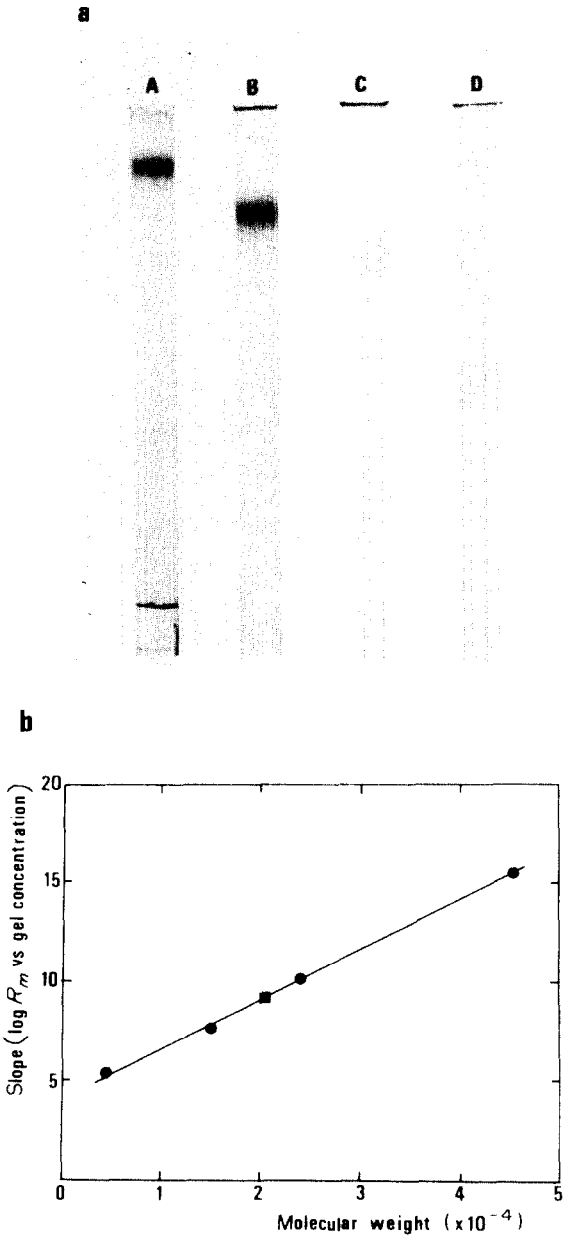

Fig. 2. (a) Electrophoresis of 30 μg NADH nitrate reductase purified by scheme D in (A) 12%, (B) 10%, (C) 7% and (D) 4.5% polyacrylamide gels followed by protein staining with Coomassie blue stain. The gels formed from the two lower percentage acrylamide concentrations showed much greater swelling in the stain/destain solutions than the other two gels, resulting in a fainter protein band. Re-insertion of the gels into glass tubes prior to photography accentuated this. (b) Relationship between the slope of net migration vs differing gel concentration and MW of protein standards (●) and nitrate reductase (■), electrophoresed at the same time as in (a). Protein standards were ovalbumin, 47 000 MW; lactate dehydrogenase, 135 000 MW; catalase, 240 000 MW; and ferritin, 450 000 MW (ref. [52]).

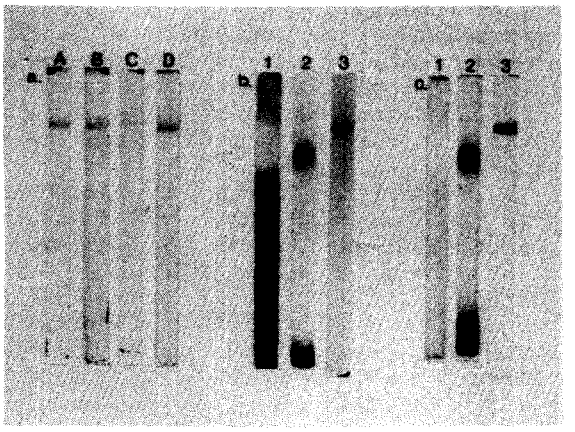

Fig. 3. Homogeneity of NADH nitrate reductase samples as judged by electrophoresis under non-denaturing conditions in 6% polyacrylamide gels. The letters A, B, C and D refer to samples from the relevant purification scheme (Table 1) stained for (a) protein after electrophoresis, (b) sample from scheme A and (c) sample from scheme D after staining specifically for: 1, methyl viologen nitrate reductase activity; 2, nitroblue tetrazolium reductase activity; 3, haem. The single protein species in all nitrate reductase samples had an R_f of 0.23, which also corresponded to the position of the bands revealed by specific gel staining. The specific stain for methyl viologen nitrate reductase activity (modification of the nitrite reductase activity stain in ref. [49]) results in an achromic band against an initially blue transient background which is made a permanent pink by counter-staining with 2,3,5-triphenyltetrazolium chloride. The dark band at the bottom of some gels is the bromophenol blue tracking dye.

20 000. There was also a faint band of MW 103 000 and others of *ca* 71 000, 69 000, 54 000 and 48 500. In contrast, nitrate reductase purified by a scheme (D) likely to minimize proteolytic attack had a major protein band at MW 103 000 although other bands, including those at MW 59 000, 38 000 and 20 000, were also present [Fig. 4a(D)]. SDS gel electrophoresis of nitrate reductase purified by schemes B and C had protein band patterns with intermediate amounts of the MW 103 000 band [Fig. 4a (B and C)].

Presence of haem in barley nitrate reductase

Nitrate reductase purified by schemes A and D contained a haem moiety as judged by specific staining of gels for haem after non-denaturing electrophoresis of nitrate reductase samples (Figs. 3b and 3c).

DISCUSSION

Nitrate reductase purified from the barley cultivar Golden Promise by scheme A, in which no attempt was made to stabilize the enzyme, and by scheme D, in which concerted attempts were made to stabilize the enzyme (by conditions of plant growth and extraction in Kuo's buffer [27] containing leupeptin [24]), was homogeneous as judged by electrophoresis in non-denaturing polyacrylamide gels (Figs. 2 and 3a). Nitrate reductase purified by

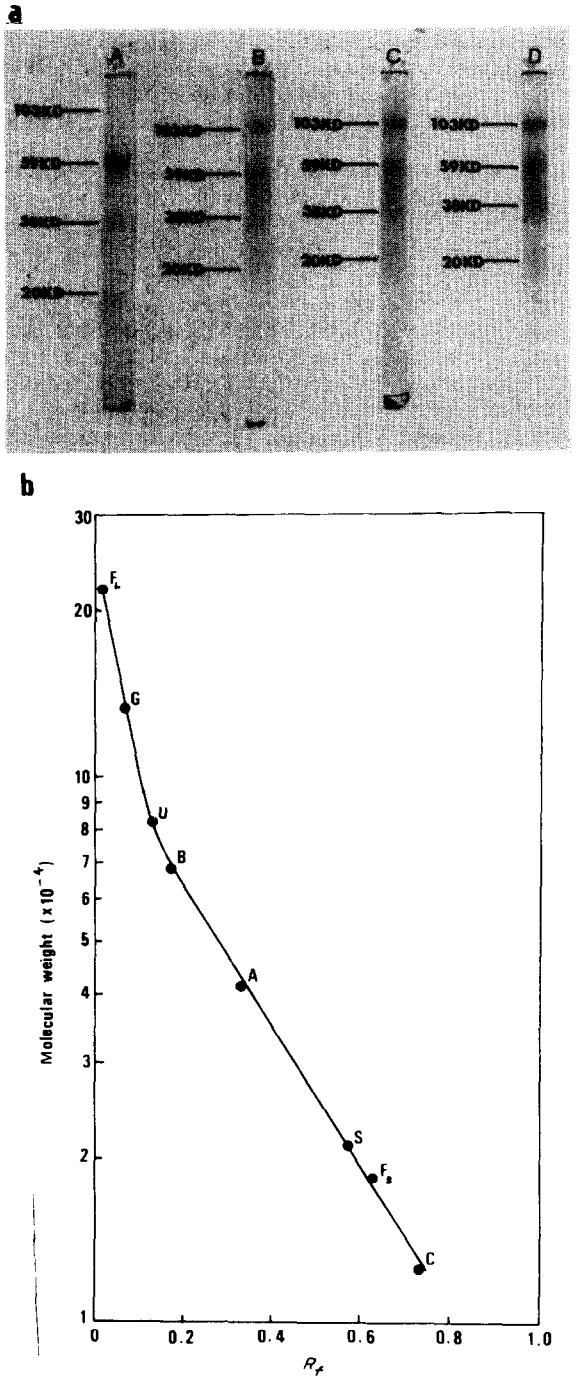

Fig. 4. (a) SDS-PAGE of nitrate reductase purified by scheme A (A), scheme B (B), scheme C (C) and scheme D (D) followed by Coomassie blue staining for protein. MWs (kilodaltons) were determined by reference to protein standards, run in separate gels at the same time as the nitrate reductase samples, after scanning in a Vitatron gel scanner. (B) Relationship between the relative mobility of protein standards in SDS gels and their MW. Letters refer to protein standards as follows: F_L, ferritin large sub-unit (220 000 MW); G, β-galactosidase (133 000 MW); U, urease (83 000 MW); B, bovine serum albumin (68 000 MW); A, alcohol dehydrogenase (41 000 MW); S, soya trypsin inhibitor (21 000 MW); F_S, ferritin small sub-unit (18 500 MW); C, cytochrome *c* (12 500 MW).

schemes B and C, in which intermediate attempts were made to stabilize the enzyme, was also electrophoretically homogeneous (Fig. 3a). The enzyme contained haem (Figs. 3b and 3c) and had a MW of 205 000 (Fig. 2b). Whilst this is in good agreement with our previous estimated value of 203 000 [4] it is smaller than the value of 221 000 determined for the enzyme from the barley cultivar Steptoe [5]. Whether this discrepancy is due to technique, different MWs of the enzymes from the two barley cultivars, or to proteolytic nicking of the cultivar Golden Promise enzyme is unclear. However, the MW of nitrate reductase from *Aspergillus nidulans* [28], *Penicillium chrysogenum* [29] and spinach [6] has been reported to be around 200 000, whilst that from *Neurospora crassa* [30] and the yeast *Rhodotorula glutinis* [31] has been reported to be around 230 000.

Very recently Kuo *et al.* [32] have reported the purification of nitrate reductase from the barley cultivar Steptoe by a procedure very similar to that used in scheme D except that the Biogel A1.5m step was omitted (allowing a more rapid purification), rabbit serum albumin was included in the extraction buffer and plants were grown at a much lower temperature. Enzyme purified by this procedure had a specific activity similar to that of enzyme purified by scheme D but contained additional abundant protein species of MW 60 000 and 41 000 amongst others (Fig. 1A of ref. [32]) as judged by electrophoresis in non-denaturing gels. SDS gel electrophoresis revealed a major band at MW 110 000 (the subunit of nitrate reductase) together with these additional species. Whilst these data are very similar to those reported here, peptide mapping of the MW 60 000 and 41 000 species with *S. aureus* protease V8 and endoproteinase lys-C [33] was interpreted to show no homology with the MW 110 000 subunit. They concluded that these smaller protein species, co-isolated with nitrate reductase from the affinity gel column, are not related to it and are contaminants, rather than components, of nitrate reductase.

We think it unlikely that the MW 60 000 and 41 000 protein species seen by Kuo *et al.* [32] as pronounced protein contaminants after non-denaturing electrophoresis are the same as the MW 59 000 and 38 000 protein species seen by us only after SDS gel electrophoresis of purified nitrate reductase (cf. Figs. 2a and 3a with Fig. 4a). Our purified nitrate reductase behaves as a single protein band in non-denaturing gels which co-migrates with the single nitrate reductase and NBT-reductase (diaphorase) activity band (Fig. 3). SDS gel electrophoresis of the enzyme purified by scheme A revealed two major protein species of MW 59 000 and 38 000 which accounted for over 90% of the dye binding (Fig. 4a). These species are clearly derived from the nitrate reductase band and we believe are related to it. The alternative explanation, i.e. that they are unrelated to nitrate reductase, but fortuitously migrate with it in non-denaturing gels, is unlikely since the preparation was electrophoretically homogeneous in gels of different percentage acrylamide and migrated as a protein of MW 205 000 (Fig. 2). Protein species of MW 59 000 and 38 000 would have been excluded by the gel filtration step that we used, but which was omitted by Kuo *et al.* [9].

As increasing efforts were made (from scheme A through to scheme D) to stabilize nitrate reductase during purification, the specific activity of the purified nitrate reductase increased 24-fold (Table 1). This was not due to differences in extent of purification since all preparations were electrophoretically homogeneous (Fig. 3). Rather, enzyme purified by scheme D was around 24-fold as active as enzyme purified by scheme A. Increasing specific activity of the purified nitrate reductase was accompanied by increasing amounts of the MW 103 000 band seen after SDS gel electrophoresis although other protein species were always present (Fig. 4a).

Thus the presence of leupeptin in the extraction or purification buffers not only prevents the loss of nitrate reductase in cell-free extracts and the formation of smaller cytochrome *c* reductase species of MW 61 000 and 40 000 [24] but also increases the specific activity of, and the relative amounts of a MW 103 000 protein species to smaller MW protein species in SDS gel electrophoresis profiles of, purified nitrate reductase [Table 1 and Fig. 4a (B and C)]. Purification in Kuo's buffer [27] containing 10 μM leupeptin was even more effective in this respect [Table 1 and Fig. 4a(D)]. Leupeptin also prevents loss of several enzyme activities in cell-free extracts of castor bean endosperm, the degradation of water-soluble and crystalloid protein of castor bean protein-bodies by the partially purified castor bean cysteine endoproteinase [34, 35] and stabilizes nitrate reductase in cell-free extracts from aseptically grown tobacco callus [36] and cell cultures (Buchanan, R. J. and Wray, J. L., unpublished results).

The data presented in this paper are consistent with the suggestion that the MW 205 000 nitrate reductase from the barley cultivar Golden Promise is a dimer of MW 103 000 subunits which can be proteolytically nicked to smaller protein species of MW 59 000, 38 000 and 20 000 amongst others. Nitrate reductase molecules purified under conditions which do not limit proteolysis contain only a small amount of intact subunits and as a consequence have a low specific activity (Table 1). Preparations purified under conditions which do limit proteolysis contain a larger proportion of intact subunits and consequently have a higher specific activity (Table 1). Clearly even preparations of nitrate reductase molecules purified in this laboratory by scheme D still contain a large number of nicked subunits but behave under certain conditions (non-denaturing gel electrophoresis) as though subunit integrity has been retained.

The phenomena reported here have their parallels elsewhere. Proteolytically nicked rat liver pyruvate kinase has only one-third the specific activity of native enzyme although it behaves in non-denaturing gels like the native enzyme. SDS gels revealed the presence not only of the native subunit but also of a smaller protein species representing the nicked subunit [37]. Purification of the yeast pyruvate dehydrogenase complex in the presence of proteinase inhibitors increased both specific activity (4- to 5-fold) and stability over enzyme purified in their absence [38–40]. Yeast tryptophan synthase purified under conditions which limited proteolysis had a 10-fold higher specific activity than enzyme purified under conditions which allowed proteolysis [41]. Proteolytic modification of the α-subunit [42] or of the isolated β-subunit [43] resulted in fragments which, given the correct renaturation conditions and the other subunit, will form a nicked protein with identical physical and biochemical characteristics to the native protein. We have already speculated [22] that a similar phenomenon may be responsible for the formation of NADH nitrate reductase activity when molybdenum cofactor, released from acid-treated spinach nitrate reductase, and spinach 3–4S NADH cytochrome *c* reductase species are co-incubated [44].

Whilst proteolytic nicking of nitrate reductase occurs under our growth, extraction and/or purification conditions, and may explain the complex pattern seen after SDS gel electrophoresis of purified spinach nitrate reductase [6] and the presence of protein species of MW 35 000 and of MW 40 000 and 60 000 in purified preparations of squash [45] and tobacco [46, 47] nitrate reductase respectively, it does not necessarily follow that it occurs during all growth, extraction and purification procedures. The relationship of the MW 61 000 and 40 000 cytochrome *c* reductase species seen in cell-free extracts of barley [4, 22, 24] to the MW 59 000 and 38 000 protein species seen only after SDS gel electrophoresis of purified nitrate reductase (Fig. 4) is presently not clear.

EXPERIMENTAL

Chemicals. Cysteine–HCl, FAD (grade III), NAD^+ (yeast, grade IV), NADH (yeast, grade III), dithiothreitol, cytochrome *c* (horse heart, type III), nitroblue tetrazolium (grade III), streptomycin sulphate and β-mercaptoethanol were obtained from the Sigma (London) Chemical Co. Ltd. Acrylamide and *N,N'*-methylene bisacrylamide (electrophoretic grades) were purchased from BDH Chemicals Ltd., Poole, Dorset. CNBr activated Sepharose 4B and blue dextran 2000 were obtained from Pharmacia (Great Britain) Ltd., Hounslow, Middlesex. Biogel A1.5 m was obtained from Biorad Laboratories Ltd., Watford, Herts. All other chemicals were of the highest grade available from the usual commercial sources.

Growth of plant material. Plants of *Hordeum vulgare* cv Golden Promise were grown in trays of vermiculite as previously described [4] with half-Hoagland's nutrient soln containing either 15 mM KNO_3 or 10 mM KNO_3/30 mM NH_4Cl as sole N source.

Preparation of blue-dextran Sepharose. This was prepared from blue dextran 2000 and CNBr-Sepharose 4B essentially by the method of ref. [48]. 11.7 mg of blue dextran was bound per ml of swollen gel.

Purification of nitrate reductase. Nitrate reductase was purified by a combination of the methods described previously [4, 22]. 90-hr-old barley shoots (350–400 g) were ground in a chilled mortar and pestle with either buffer I (0.05 M KPi buffer, pH 7.5, containing 0.1 mM EDTA, 10 μM FAD and 1 mM cysteine), buffer I containing 10 μM leupeptin or the buffer described by Kuo *et al.* [27] (0.25 M Tris–HCl, pH 8.5, 3 mM DTT, 5 μM FAD, 1 μM Na molybdate and 1 mM EDTA) containing 10 μM leupeptin (3 ml buffer/g tissue). The brei was squeezed through muslin and streptomycin sulphate was added to the extract (5 mg/g tissue). After stirring for 5 min, precipitated nucleic acid material was removed by centrifugation at 33 000 *g* for 20 min. The supernatant was adjusted to 45% saturation with solid $(NH_4)_2SO_4$ and precipitated protein was collected by centrifugation and dissolved in the same buffer. The sample was passed through a Biogel A1.5m column (4.1 × 108 cm) equilibrated in the same buffer and fractions containing NR activity were pooled and adjusted to 45% saturation with $(NH_4)_2SO_4$. The precipitated protein was collected by centrifugation, dissolved in the same buffer, adjusted to 40% (v/v) with glycerol and stored overnight at −70°. The protein was again precipitated with saturated $(NH_4)_2SO_4$ in the same buffer to remove glycerol, dissolved in a minimum of buffer II (0.02 M KPi buffer, pH 7.5, 0.1 mM EDTA, 10 μM FAD and 1 mM cysteine) without (schemes A and B) or with (schemes C and D) 10 μM leupeptin and applied to a blue-dextran Sepharose column (2 × 9 cm). The column was washed with buffer II until the absorbance of the eluate at 280 nm was less than 0.02 and NR was eluted with buffer II containing 5 μM NADH. The peak fractions containing NR activity were pooled and concd to *ca* 2 ml in an Amicon thin-channel concentrator with a PM-10 membrane. Any subsequent concn of the sample for gel electrophoresis was effected by dialysis against polyethylene glycol.

Electrophoretic techniques. Electrophoresis was carried out in 5% acrylamide gels prepared in 0.2 M Tris–HCl, pH 8.5. Running buffer was 0.08 M Tris–HCl, pH 8.5. Protein was detected in gels by staining with Coomassie blue, methyl viologen nitrate reductase was detected by the method of ref. [49] except nitrate was substituted for nitrite, NBT reductase activity by the method of ref. [50] and haem by the method of ref. [51]. The native MW of nitrate reductase was determined in gels of differing polyacrylamide concn by reference to standard proteins by the method of ref. [52]. For SDS gels, samples and standard proteins were boiled in 0.02 M Pi, pH 7.0, containing 1.25% SDS and 2.5% β-mercaptoethanol and electrophoresis was carried out by the method of ref. [53].

Enzyme assays. NADH nitrate reductase was assayed as previously described [11]. Protein was assayed according to the method of ref. [54] using bovine serum albumin as a standard.

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