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NADH NITRATE REDUCTASE AND RELATED NADH CYTOCHROME *c* REDUCTASE SPECIES IN BARLEY

IAN S. SMALL and JOHN L. WRAY

Department of Biochemistry and Microbiology, University of St. Andrews, North Street, St. Andrews, Fife, KY16 9AL, U.K.

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; nitrate reductase; cytochrome *c* reductase species; sedimentation coefficient; Stokes radius; MW.

Abstract—Nitrate and nitrate-less barley (*Hordeum vulgare* cv Golden Promise) shoot extracts were examined by Sephadex G200 gel filtration and sucrose density gradient analysis and the MWs of NR and CR species present were determined from their Stokes radii and sedimentation coefficients by the method of Siegel and Monty. Nitrate-less plant extracts possessed a CR species of MW 27 800 whilst nitrate-plant extracts possessed CR species of MW 203 000, 61 000, 40 000 and 27 800. The MW 203 000 CR species was associated with NADH–NR, FMNH–NR and MV°–NR activities and represents the NR complex. The MW 40 000 and 61 000 CR species were shown to be derived from the NR complex. We suggest that the MW 40 000 and 61 000 CR species represent either subunits of the NR complex or domains cleaved from the intact NR complex by endogenous proteinases.

INTRODUCTION

Treatment of barley plants with nitrate leads to the induction of the NADH–NR complex and to the increase in activity of a small CR species which is also seen in nitrate-less plants [1]. Other workers have subsequently reported the existence of a small CR species in rice [2], spinach [3] and in maize and mustard [4]. One of us has speculated that the small CR species may be a dissociation product of the NR complex or a precursor protein [1].

In this paper we present information on some physical properties of the CR species present in extracts from nitrate-treated and nitrate-untreated barley plants and discuss possible relationships between these species. Preliminary accounts of some of this work have been published [5, 6].

RESULTS AND DISCUSSION

Spinach NR is the best characterized higher plant NR at present [7]. This enzyme contains FAD [7], cytochrome b_{557} [8] and molybdenum [9]. These properties are probably also shared by the NR complex from barley and other higher plants. However, no higher plant NR has yet been purified sufficiently to assess the number of moles of prosthetic group per mole of enzyme.

Similarly little is known about the subunit composition of the enzyme, the subunit location of the prosthetic groups and the role of subunits in the expression of the partial activities. These problems, together with others concerning the mechanism and regulation of subunit assembly/disassembly and the role that nitrate plays in the formation of the subunits represent some of the more intriguing problems in the biochemistry of higher plant nitrogen metabolism.

One of us has previously reported that both nitrate-less and nitrate-plant extracts possess a CR species which sediments at 3.7 S and which is distinct from the NR associated CR species [1]. We suggested that this CR species might be a dissociation product of the NR complex or perhaps a precursor protein [1]. However, no supporting evidence was put forward and the suggestion was based exclusively on the assumption that the small CR species from both plant types were identical because they both possessed a sedimentation coefficient of 3.7 S [1].

In this present investigation the use of larger gradients, and comparison with the reference proteins catalase, alcohol dehydrogenase and myoglobin, has enabled us to make a more accurate measurement of the sedimentation coefficient of this, and other, CR species than was previously possible [1]. We have also determined the Stokes radius of NR and CR species by Sephadex G200 gel filtration and have calculated the MW and frictional ratio [10] and axial ratio [11] of these species from these data.

Abbreviations: NR: nitrate reductase; CR: NADH cytochrome *c* reductase; MV°: reduced methyl viologen; SDG: sucrose density gradient; nitrate plant: plants grown in the presence of nitrate; nitrate-less plant: plants grown in the absence of nitrate.

SDG analysis of nitrate-less plant extracts

SDG analysis of nitrate-less plant extracts revealed the presence of a major CR species sedimenting in the 3S region (Fig. 1a) and probably equivalent to CR species C reported previously [1]. From an examination of 5 experiments the sedimentation coefficient of this CR species was determined to be 2.71 ± 0.05 S. Traces of a second CR species (probably CR species A [1]) were detected at the bottom of the gradient (see below).

Gel filtration on Sephadex G200 of nitrate-less plant extracts

Gel filtration, on Sephadex G200, revealed the presence of two CR species (Fig. 2a). The first species eluted just after the void volume and is probably equivalent to the high MW CR species A which sediments to the bottom of sucrose density gradients [1]. By comparison with the reference proteins catalase, alcohol dehydrogenase and serum albumin it is possible to determine the Stokes radius of the CR species using the correlation of Porath [12]. The first eluted CR species had a Stokes radius of 91Å and the second eluted CR species had a Stokes radius of 22 Å. SDG analysis of this 25 Å species showed that it possessed a sedimentation coefficient of 2.7 S (data not shown).

SDG analysis of nitrate-plant extracts

SDG analysis of nitrate-plant extracts revealed the presence of 3 CR species (Fig. 1b). Since no sucrose cushion was used in this investigation the heaviest species is equivalent to CR species A reported previously [1]. Its level in the plant is independent of the presence of nitrate and it is not considered to be related to either of the other two CR species seen in Fig. 1b [1]. The CR species of intermediate size cosedimented with NADH–NR activity, FMNH–NR activity (data not shown) and MV°–NR activity (Fig. 1b),

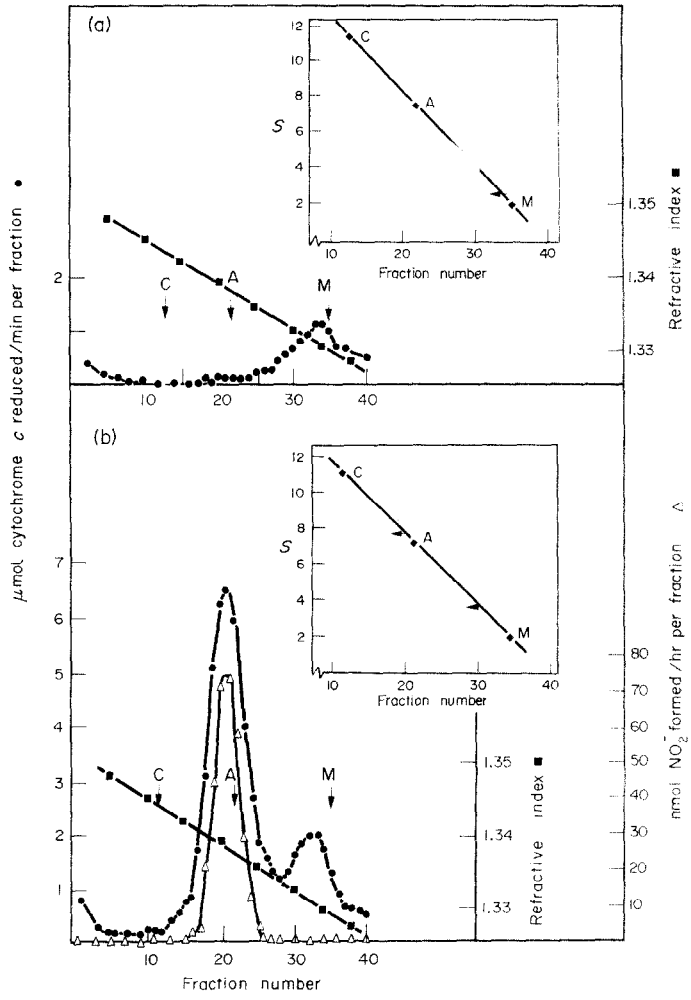

Fig. 1. Sucrose density gradient analysis of MV°–NR and CR species present in (a) nitrate-less plant extracts and (b) nitrate-plant extracts. Conditions of centrifugation and extraction of plant material are described in Experimental. C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation. *Inset*: Relationship between sedimentation coefficient of reference proteins and their position after centrifugation. Intersecting arrows denote the CR and MVH–NR species present.

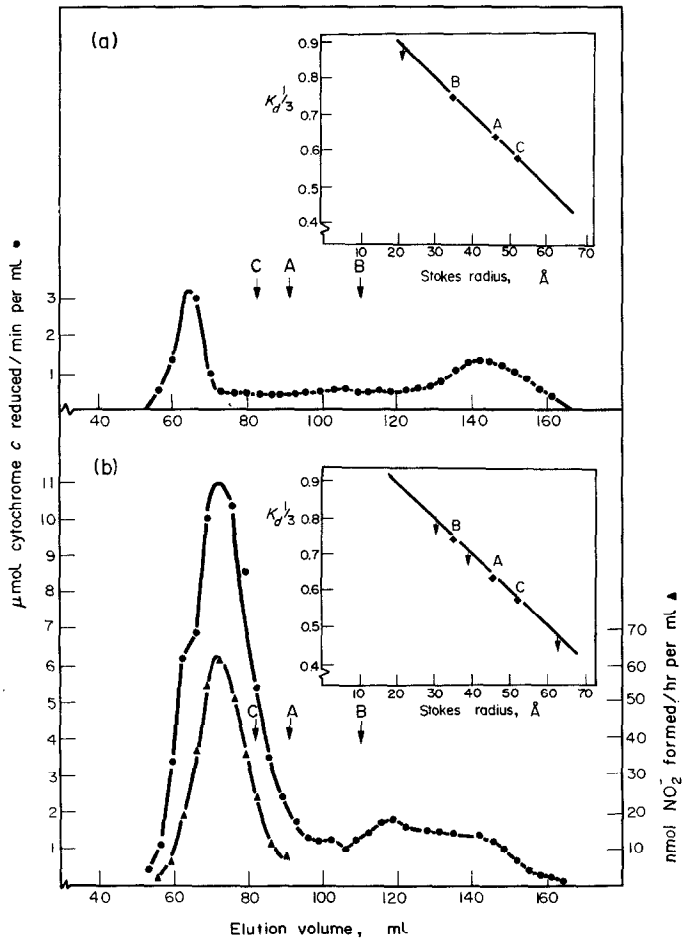

Fig. 2. Sephadex G200 gel filtration analysis of NADH–NR and CR species present in (a) nitrate-less plant extracts and (b) nitrate-plant extracts. Conditions of gel filtration and extraction of plant material are described in Experimental. C, A and B denote the positions at which the reference proteins catalase, alcohol dehydrogenase and bovine serum albumin are eluted. *Inset*: Relationship between the elution volume of the reference proteins and their Stokes radius after the correlation of Porath [14]. Intersecting arrows denote CR and NADH–NR species present.

and is equivalent to CR species B (the NR complex) reported previously [1]. The third CR species seen in Fig. 1b is probably equivalent to CR species C [1] and has a sedimentation coefficient of *ca* 3 S.

From an examination of 11 experiments the sedimentation coefficient of the NR-associated CR species (the NR complex) was found to be 7.69 ± 0.06 S. The profile of CR activity was always symmetrical.

In contrast, the *ca* 3 S CR species always exhibited an asymmetrical profile (Fig. 1b) which differed in different experiments. An examination of 9 experiments showed that the sedimentation coefficient of this CR species was 3.05 ± 0.10 S. Its activity profile differed in 3 respects from the activity profile exhibited by the *ca* 3 S CR species from nitrate-less plant extracts. Firstly the level of CR activity in the *ca* 3 S region was *ca* twice that found in nitrate-less plants. Secondly, the CR profile was always asymmetrical whereas the profile associated with the *ca* 3 S CR species from nitrate-less plants was always symmetrical. Thirdly, the sedimentation coeffcient of 2.71 S determined for the CR profile from nitrate-less plants was lower than the lowest value found for the CR profile from nitrate-plants (range 2.9–3.1 S).

These results suggest that the CR activity seen in the *ca* 3 S region in extracts from nitrate-less plants is not the same as the CR activity seen in the *ca* 3 S region from nitrate plants. Extracts from nitrate-less plants possess a single small CR species which sediments at 2.71 S. The variable *ca* 3 S CR profile seen in nitrate-plant extracts may be explained by the presence of other CR species, somewhat heavier than 2.71 S, in addition, perhaps, to the 2.71 S CR species. The activity profile in the *ca* 3 S region seen in nitrate-plant extracts would then be the result of the summation of the activity profiles of individual CR species possessing closely similar sedimentation coefficients.

Gel filtration on Sephadex G200 of nitrate-plant extracts

Gel filtration on Sephadex G200 of nitrate-plant extracts revealed the presence of the expected major CR species associated with NR activity and of three smaller CR species (Fig. 2b). The major NR-associated

CR species (the NR complex) had a Stokes radius of 64 Å. The shoulder on the leading edge of this peak is equivalent to the first eluted CR species in Fig. 2a since in other experiments where it is eluted as a discrete peak it was also shown to have a Stokes radius of 91 Å.

The three smaller CR species eluted at 102, 118 and 142 ml. They were consistently observed to be present in nitrate-plant extracts, although the species eluting at 102 ml was always present in very small amounts, and we have assigned Stokes radii of 39, 31 and 25 Å to these species. The 25 Å species is also present in nitrate-less plant extracts, whereas the 39 and 31 Å CR species are present only in nitrate-plant extracts. These 3 CR species are probably responsible for the variable CR profile seen in the *ca* 3 S region after SDG analysis of nitrate plant extracts (Fig. 1b).

The 39 and 31 Å CR species are derived from the NR complex

Since the 39 and 31 Å CR species are present only in nitrate-plant extracts it is likely that they are related in some way to the NR complex which itself possesses CR activity and is present only in nitrate-plant extracts (Figs. 1b and 2b). These species could be newly synthesized NR precursor subunits destined ultimately for assembly into the functional NR complex or they could be CR species derived from the NR complex. Alternatively they may be present for both these reasons.

We attempted to see if these two CR species were derived from the NR complex by subjecting a fraction from the leading edge of the NR peak in Fig. 2b to SDG analysis. However the relatively low CR activity present in the sample meant that no CR activity could be recovered from the gradients.

We circumvented this problem by using Biogel A 1.5 m instead of Sephadex G200 to separate the CR species present in the tissue extract. Biogel A 1.5 m allows fractionation to be carried out on the same basis as with Sephadex G200 but the very much faster flow rate allowed the use of a larger column and the application of a larger enzyme sample. Fractions obtained after elution could then be applied directly to sucrose density gradients and the distribution of CR species determined.

Elution of nitrate-plant extract, prepared as described in the Experimental, on Biogel A 1.5 m revealed the presence of 3 peaks of CR activity (Fig. 3). The first peak eluted just after the void volume and is considered to be equivalent to the shoulder on the major CR peak in Fig. 2b. The second peak co-eluted with NADH–NR activity and represents the NR complex. SDG analysis of the third peak showed that the trailing edge consisted exclusively of a 3.1 S CR species whilst the leading edge also possessed a 3.8 S CR species (data not shown).

SDG analysis of fraction 48 taken from the leading edge of the major CR peak in Fig. 3 gave the CR profile seen in Fig. 4. In addition to the major NR-associated CR peak a smaller heterogeneous CR peak was also present which appears to consist of two CR species. Sedimentation coefficients of 3.8 and 3.1 S CR have been assigned to these species. The amount of these CR species relative to the 7.7 S CR species is much greater than could be accounted for by contamination of fraction 48 and we conclude that the 3.8 and 3.1 S CR species seen in Fig. 4 are derived from the NR complex.

Gel filtration of fraction 48 on Sephadex G200 produced the CR profile shown in Fig. 5. In addition to the major NR-associated CR peak there are two other minor CR peaks which elute at 102 and 118 ml and which possess Stokes radii of 39 and 31 Å, respectively. These values are the same as those possessed by the two small CR species which are present only in

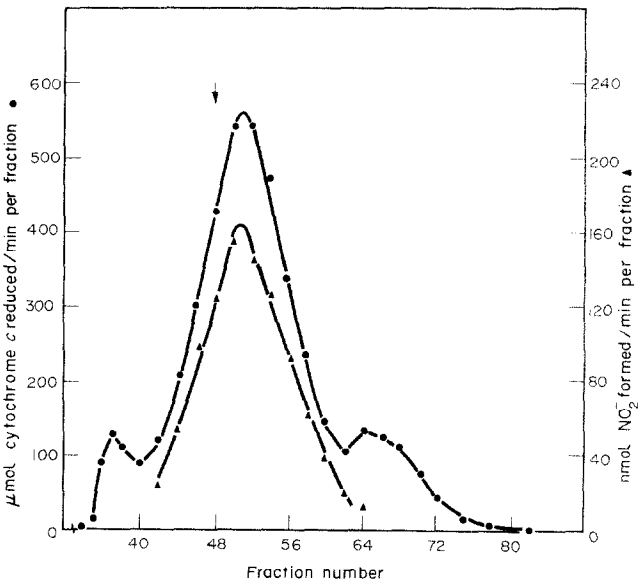

Fig. 3. Biogel A 1.5 m gel filtration analysis of CR and NADH–NR species present in nitrate-plant extracts. Conditions of gel filtration and extraction of plant material are described in Experimental. The arrow denotes fraction 48 which was further analysed by SDG centrifugation (Fig. 4) and G200 gel filtration (Fig. 5).

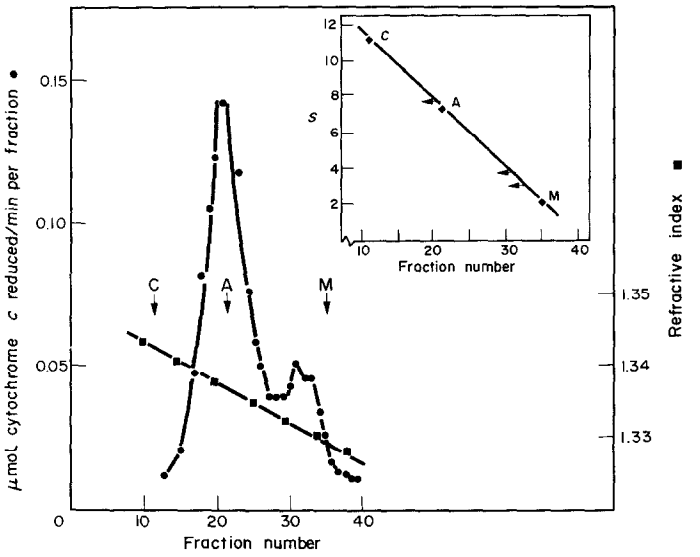

Fig. 4. CR species detected after SDG analysis of an NR sample from fraction 48 in Fig. 3. A sample from fraction 48 of the Biogel A 1.5 m gel filtration profile in Fig. 3 was subjected to SDG analysis as described in Experimental and the distribution of CR species determined. C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation. *Inset*: Relationship between sedimentation coefficient of reference proteins and their position after centrifugation. Intersecting arrows denote the CR species present.

nitrate-plant extracts. We may conclude, therefore, that at least part, and perhaps all, of the 39 and 31Å CR species present in nitrate-plant extracts are derived from the NR complex.

Physical parameters of NR and the CR species present in barley plant extracts

From a knowledge of the sedimentation coefficient, S, and Stokes radius, a, and by assuming a partial specific volume, $\bar{v}$, of 0.725 cm^3/g, it is possible to calculate the MW of some of the CR species described above and also some other physical parameters (Table 1). The NR complex, which possesses both NR activity and CR activity and which is present only in nitrate-plant extracts has a sedimentation coefficient of 7.7 S, a Stokes radius of 64 Å and a calculated MW of 203 000. It has a frictional ratio f/f_0, of 1.65 and is grossly asymmetrical with an axial ratio, r_1/r_2, of 11:1 (Table 1). These data clearly show the great similarity, in terms of several physical parameters, between the barley NR and the NRs from spinach, the fungi *Aspergillus nidulans* and *Neurospora crassa*, and the yeast *Rhodotorula glutinis* (Table 2).

The other two CR species of interest are present only in nitrate-plant extracts, and are derived, at least in part, from the NR complex. These two CR species possess sedimentation coefficients of 3.8 and 3.1 S, Stokes radii of 39 and 31 Å and calculated MWs of 61 000 and 40 000, respectively. Their frictional ratios and axial ratios are presented in Table 1.

The CR species which is present in extracts from both nitrate and nitrate-less plants has a sedimentation coefficient of 2.7 S, a Stokes radius of 25 Å and a calculated MW of 27 800. We consider that this CR species, seen both in nitrate- and nitrate-less plant extracts, is unrelated to the NR complex for three reasons, viz. it is not formed when the NR complex breaks down (Fig. 4 and 5), it is present in the same amounts (Fig. 2a and b) in both nitrate and nitrate-less plant extracts and it approximates more closely to a globular protein than either the MW 61 000 and 40 000 CR species which are derived from the asymmetrical NR complex (Table 1).

The sedimentation coefficient of MV^0–nitrate reductase was found to be 4.2 S (data not shown) and we conclude that none of the CR species discussed above represents a partial activity of this protein.

Relationship of the MW 40 000 and 61 000 CR species to the NR complex

We have previously suggested that these two CR species may represent subunits (or subunit associations) of the higher plant NR complex, [5, 6]. However studies on the purified enzymes from *Neurospora crassa* [16] and *Rhodotorula glutinis* [17] suggest that they contain two identical subunits whilst the larger NR from *Chlorella vulgaris* appears to contain three identical subunits [18, 19]. If barley NR fits this pattern then it would be expected to contain two MW 100 000 subunits, each of which carry FAD and cytochrome b_{557}.

An alternative possibility therefore is that the two CR species represent domains [20], that is independent or semi-independent structural regions of the NR subunits, formed as a consequence of proteolytic cleavage during the extraction procedure. It is well known that NR can be attacked by proteinases [21–23]. The tryptic cleavage of the haemomolybdoprotein, sulphite oxidase, has recently been reported [24, 25] and it is known that catalytically functional domains of *S. cerevisiae* cytochrome b_2 [26] and *E. coli* pyruvate dehydrogenase [27] can be produced from the intact enzyme by endogenous proteinases.

However, the evidence presented in this paper does

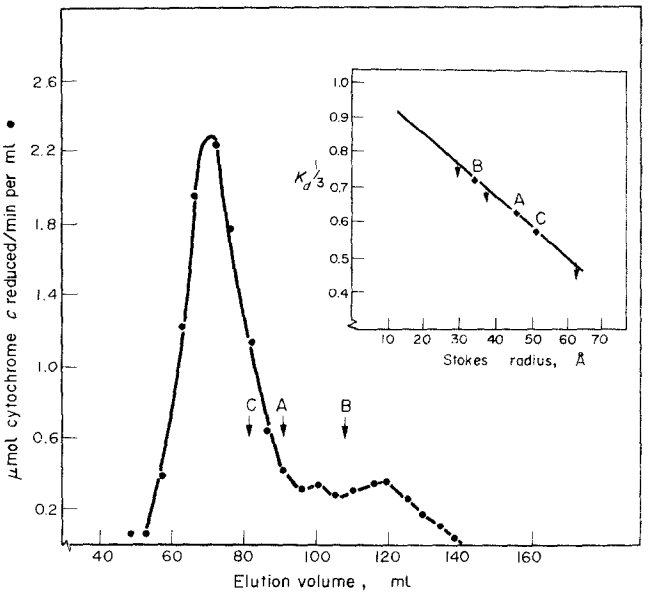

Fig. 5. CR species detected after Sephadex G200 gel filtration of an NR sample from fraction 48 in Fig. 3. A sample from fraction 48 of the Biogel A 1.5 m gel filtration profile in Fig. 3 was subjected to analysis by Sephadex G200 gel filtration as described in Experimental and the distribution of CR species determined. C, A and B denote the positions at which the reference proteins catalase, alcohol dehydrogenase and bovine serum albumin are eluted. *Inset*: Relationship between the elution volume of the reference proteins and their Stokes radius after the correlation of Porath [14]. Intersecting arrows denote CR species present.

not allow us to distinguish between these two possibilites at present.

EXPERIMENTAL

Barley (*Hordeum vulgare* cv. Golden Promise) seeds were thickly sown in trays in Vermiculite, watered with H_2O placed in darkness at 28° to germinate. At 24, 48, and 72 hr, after sowing, seeds were treated with half strength Hoagland nutrient soln (15 mM KNO_3) and at 68 hr, when the coleoptiles were 1 cm long plants were transferred to continuous light, supplied by 3 Grolux fluorescent tubes, at 26° Plants were usually harvested 90 hr after sowing when shoots were 4 cm long. For nitrate-less plants growth conditions were as above but the half strength Hoagland nutrient soln lacked nitrate.

Enzyme extraction. For expts involving Sephadex G200 gel filtration and sucrose density gradient analysis, barley shoots (2 g) were ground in a chilled mortar and pestle with 6 ml cold 50 mM K-Pi buffer containing 1 mM cysteine, 0.1 mM EDTA and 10 μM FAD (pH 7.5) (buffer I). After centrifugation at 38 000 g for 20 min at 3° an aliquot of the resulting supernatant was used. In the case of gel filtration on Biogel A 1.5 m, barley shoots (150 g) were harvested and ground in a mortar and pestle with buffer I (3 ml buffer/g barley shoots). The brei was squeezed through a double layer of muslin and the filtrate centrifuged at 40 000 g for 1 hr at 3°. Streptomycin sulphate (0.75 g) in buffer I (15 ml) was added dropwise at 4°

Table 1. Physical parameters of NADH–cytochrome *c* reductase species present in extracts from nitrate-treated and untreated barley shoots

Treatment	Stokes radius (Å)	Sedimentation coefficient (S)	MW	Frictional ratio (f/f_0)	Axial ratio (r_1/r_2)
Nitrate-treated	91	ND	—	—	—
	64*	7.7	203 000	1.65	11:1
	39	3.8	61 000	1.50	8:1
	31	3.1	40 000	1.38	6:1
	25	ND	—	—	—
Untreated	91	ND	—	—	—
	25	2.7	27 800	1.25	4:1

Stokes' radii and sedimentation coefficients were determined as described in Experimental and the other parameters were calculated from these values by the methods of Oncley [11] and Siegel and Monty [10]. ND = not determined.
*The NADH–nitrate reductase complex.

Table 2. Comparative physical parameters of nitrate reductase species from barley and from other eukaryotes

Organism	Stokes radius (Å)	Sedimentation coefficient (S)	MW	Frictional ratio (f/f_0)	Axial ratio (r_1/r_2)	Reference
Barley	64	7.7	203 000	1.65	11:1	—
Spinach	60	8.1	197 000	1.55	10:1	8
Aspergillus nidulans	64	7.8	206 000	(1.64)	(11:1)	13
	63	7.6	197 000	1.7	(11:1)	14
Neurospora crassa	70	7.9	228 000	(1.73)	(13:1)	15, 16
Rhodotorula glutinis	70.5	7.9	230 000	(1.74)	(13:1)	17

The Stokes radius and sedimentation coefficient of the barley enzyme were determined as described in Experimental and the other parameters were calculated from these values by the methods of Oncley [11] and Siegel and Monty [10]. Figures in brackets denote values calculated by us from the data presented by the authors.

to the supernatant and the soln was stirred at 4° for 20 min, followed by centrifugation at 40 000 g for 20 min to remove precipitated nucleic acid. The resulting supernatant was adjusted to 60% satn with $(NH_4)_2SO_4$ by the addition of 1.5 vols. of saturated $(NH_4)_2SO_4$ (pH 7.5). After stirring for 30 min at 4°, precipated protein was collected by centrifugation at 20 000 g for 20 min and dissolved in 36 ml of buffer I. After desalting at 4° on a column (4.1×41 cm) of Sephadex G25 previously equilibrated in buffer I, fractions containing protein were pooled, adjusted to 40% satn with respect to glycerol and stored at −70°. Fractions stored under these conditions were found to have lost 10% of their original NR activity after 10 days. Prior to futher use, half of the above sample (equivalent to 75 g of barley shoots) was thawed and protein was again precipitated at 4° with $(NH_4)_2SO_4$ at 60% satn to remove glycerol which markedly reduces flow rates during subsequent gel filtration. The protein was collected by centrifugation at 20 000 g for 20 min and dissolved in buffer I lacking cysteine (buffer II) to facilitate assay of CR. An aliquot of this sample was applied to the Biogel A 1.5 m column.

SDG centrifugation. Enzyme soln (0.4 ml) was layered on top of the 18 ml linear 2–18% (w/v) sucrose gradient. The sucrose solns were prepared in 0.1 M K-Pi buffer containing 1 mM EDTA and 10 μM FAD (pH 7.5). After centrifugation at 94 000 g for 24 hr at 2° the gradients were fractionated from the bottom of the tube into 41 fractions of 15 drops each. The sedimentation coefficient of enzymes was determined by reference to the standard markers catalase (bovine liver, 11.3 S), alcohol dehydrogenase (yeast, 7.4 S) and myoglobin (whale skeletal muscle, 2 S) run together with the enzyme sample. Gradient linearity was confirmed with a Bellingham and Stanley Abbé-type refractometer.

Sephadex G200 gel filtration. Enzyme soln (1 ml) was applied to a column (2×52 cm) of Sephadex G200 equilibrated in buffer II. The enzyme soln was then eluted with buffer II and fractions were collected at 12 ml/hr. The Stokes radius of NR and CR species was determined by comparison with the reference proteins catalase (bovine liver, 52 Å), alcohol dehydrogenase (yeast, 46 Å) and albumin (bovine serum, 35 Å) run together with the enzyme sample. All column operations were carried out at 4°.

Biogel A 1.5 m gel filtration. A 9 ml sample of enzyme, prepared as above, was applied to a column (4.1×67 cm) of Biogel A 1.5 m 100–200 mesh equilibrated in buffer II. Enzyme was eluted with buffer II and 14 ml fractions were collected. All column operations were carried out at 4°.

Enzyme assays. NADH–NR, FMNH–NR and CR were assayed by the method of ref. [1]. MV°–NR was assayed in the following way: 0.6 ml K-Pi buffer, pH 7.5, 0.1 ml 1 mM methyl viologen, 0.1 ml 0.1 M KNO_3 and 0.1 ml enzyme were pipetted into the incubation tubes. 0.1 ml of Na dithionite (10 mg/ml) in 95 mM $NaHCO_3$ was then added and the reaction was started by gently shaking the tubes to reduce the methyl viologen (tube contents turn blue). After incubation for 1 hr at 25° tube contents were vigorously mixed in a Wirlimixer to reoxidize the methyl viologen and nitrite formation was measured as before [1]. Catalase was assayed by the method of ref. [28] and alcohol dehydrogenase by the method of ref. [29]. Myoglobin was determined by its A at 415 nm and serum albumin by its A at 280 nm.

Determination of MW frictional ratios and axial ratios. The MW and frictional ratio of NR and CR species was determined by the method of ref. [10] from a knowledge of the sedimentation coefficient and Stokes radius. The axial ratio was calculated from the data presented in ref. [11].

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