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AGE-DEPENDENT CONVERSION OF NITRATE REDUCTASE TO CYTOCHROME *c* REDUCTASE SPECIES IN BARLEY LEAF EXTRACTS

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

Abstract—The stability of nitrate reductase (NR) in extracts from 4-, 5- and 6-day-old primary leaves of barley was examined. The half-time of loss of NR activity was found to be 358, 107 and 70 min, respectively. Bovine serum albumin (BSA) and phenylmethylsulphonylfluoride (PMSF) stabilized NR in extracts from 5- and 6-day-old primary leaves, but BSA was much more effective. The increased instability of NR with age correlated with increased conversion of the MW 203 000 NR complex to smaller NADH cytochrome *c* reductase (CR) species of MW 163 000, 61 000 and 40 000. The MW 163 000 CR species also possessed NR activity. BSA prevented and PMSF retarded the conversion of NR to the smaller CR species. The increased instability of NR in extracts from older tissue may be due to increased conversion of NR to smaller CR species. The ability of PMSF and BSA to stabilize NR and inhibit conversion of NR to the smaller CR species indicates that these phenomena are probably due to proteolytic degradation of NR. This suggestion is supported by the observation that trypsin cleaved NR to 3–4 S CR species and that cleavage was retarded by the presence of BSA. Endogenous proteinase attack at specific sites between domains of the barley NR complex may generate the CR species seen in barley extracts. The MW 40 000 CR species probably carries at least the FAD domain.

INTRODUCTION

In several different developmental systems, e.g. leaf expansion in oat, tobacco and maize [1, 2], maize root elongation [3–5] and cotton cotyledon expansion [6, 7], large amounts of stable NR can be extracted from young tissues. In all these cases, extraction in the presence of BSA (or casein) prevents the decline in amount and stability of extractable enzyme which is characteristic of older tissue.

How BSA brings about these effects is unknown, but it has been suggested that enhancement of NR activity by BSA seen in extracts from older tissue may be due to the ability of BSA to form hydrogen-bond complexes with phenolic compounds, so removing them as inhibitors of NR [6]. The stabilization of NR by BSA (or casein) seen in extracts from older tissue may be due to its ability to prevent the NR molecule from dissociating into subunits [6] (by maintaining a high protein concentration), or alternatively to act as an alternate substrate for a specific NR-inactivating enzyme [5, 8].

We have recently reported that NR from young barley leaves can break down *in vitro* to produce small amounts of a MW 40 000 CR species [9, 10] which may represent either an NR subunit or, more likely, a domain released from the NR complex by endogenous proteinases. In this paper we show that the breakdown of NR to smaller CR species becomes progressively greater with plant age and that breakdown can be prevented if extraction is carried out in the presence of BSA. Possible reasons for these phenomena are discussed.

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RESULTS

Amount and stability of NR extracted from primary leaves of 4-, 5- and 6-day-old barley plants

The amount of NR activity which can be detected by a 10 min assay following tissue extraction and centrifugation of extract for 20 min is shown in Table 1 (column a). Maximum amounts of NR activity were found in extracts from 4-day-old primary leaves and the NR activity decreased with increasing leaf age. A similar age-dependent decrease in NR activity has been reported previously for several other systems (see Introduction).

Table 1. Nitrate reductase activity detected in centrifuged and uncentrifuged extracts of barley primary leaves of different ages

Age of barley primary leaf (day)	NR activity (μmol nitrite produced/ml extract/hr) (a) Centrifuged	(b) Uncentrifuged
4	2.69	2.93
5	1.92	2.70
6	0.90	1.76

Primary leaves were extracted with 50 mM K–Pi buffer containing 1 mM cysteine, 0.1 mM EDTA and 10 μm FAD (pH 7.5) (buffer 1) (1 g tissue/5 ml buffer) and the brei was either centrifuged at 38 000 g for 20 min or filtered through muslin. A 0.1 ml aliquot was then assayed for NR activity for 10 min as described previously [10].

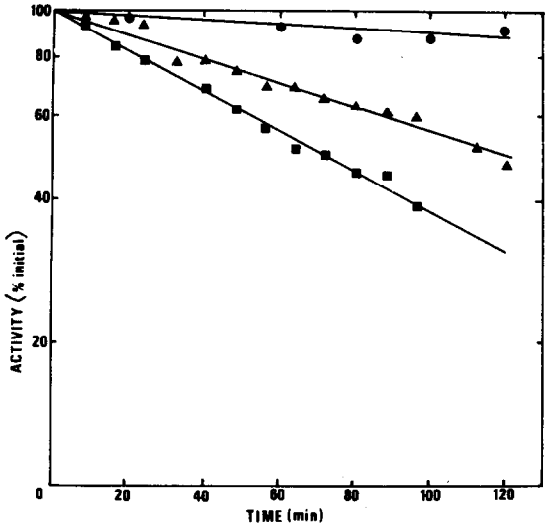

Fig. 1. Stability at 4° of NR present in extracts from 4-day-(●), 5-day-(▲) and 6-day-(■) old primary leaves. Primary leaves were extracted in buffer I and the brei was centrifuged at 38 000 g for 20 min. The extracts were maintained at 4° and aliquots were taken at the times indicated and assayed for NR activity as described previously [10].

However, the results presented in Table 1 (column a) may not reflect the amount of NR present in the tissue at the time of extraction, since 40 min elapsed between the start of the extraction procedure and the end of the assay. The progressive loss of NR with age could be due to the development of some type of age-dependent inactivating mechanism which operates during the 40 min processing period.

The processing period was therefore reduced to 15 min by omitting the centrifugation step. Tissue was extracted, filtered through muslin and assayed for 10 min. The NR activities obtained are shown in Table 1 (column b). Although the NR activity present in extracts from 4-day-old leaves was not different from that obtained if a centrifugation step was employed, the activity found in extracts from 5- and 6-day-old leaves was much higher. This higher activity may be a consequence of the reduced time available for the postulated inactivating mechanism to operate.

The stability of NR in uncentrifuged leaf extracts was determined by maintaining the extract at 4° and removing aliquots at time intervals for assay (Fig. 1). NR present in extracts from 4-day-old primary leaves was most stable but still lost 10% of the initial activity over the 2 hr period. NR in extracts from 4-day-old primary leaves had a half-life of 358 min, whilst the half-life of NR in extracts from 5- and 6-day-old primary leaves was 107 and 70 min, respectively. The decreased stability of NR in extracts from primary leaves older than 4 days suggests that the postulated inactivating mechanism becomes increasingly effective as the leaf ages.

Effect of the serine proteinase inhibitor, PMSF, and of BSA on the amount and stability of NR extracted from 4-, 5- and 6-day-old primary leaves of barley

Inclusion of the serine proteinase inhibitor PMSF, or BSA, in the extraction buffer had little effect on the NR activity obtained in a 10 min assay following centrifugation of extracts from 4- and 5-day-old primary leaves (Table 2). However, both markedly increased the NR activity in extracts from 6-day-old primary leaves.

The stability of NR in extracts from 4-day-old primary leaves was not markedly affected by the presence of either PMSF or BSA in the extraction buffer, suggesting that PMSF and BSA are not able to protect NR from the small amount of inactivation which occurs at this time. In contrast, the stability of NR in extracts from 5- and 6-day-old primary leaves was increased when PMSF or BSA was present in the extraction buffer. NR in extracts of 5- and 6-day-old primary leaves prepared in the presence of BSA had the same stability as NR in extracts of 4-day-old primary leaves prepared without BSA. Although PMSF stabilized NR to the same extent in extracts from both 5- and 6-day-old primary leaves, it was not as effective as BSA (Table 2).

Table 2. Effect of BSA and PMSF on the amount and stability of nitrate reductase present in centrifuged extracts from barley primary leaves of different ages

Age of barley primary leaf (day)	Treatment	NR activity initially present in the extract (μmol nitrite produced/ml extract/hr)	Activity (% of initial) remaining after 0 hr	2 hr	4 hr
4	Control	2.34	100	89.3	71.8
	+BSA	2.42	100	84.9	71.2
	+PMSF	2.66	100	91.2	80.2
5	Control	2.23	100	50.6	30.0
	+BSA	2.38	100	87.2	76.7
	+PMSF	2.10	100	61.6	38.2
6	Control	0.90	100	32.6	17.8
	+BSA	1.96	100	85.3	75.0
	+PMSF	1.15	100	58.2	43.3

Primary leaves were extracted with buffer I containing either no additions, 3% BSA or 1 mM PMSF and the brei was centrifuged at 38 000 g for 20 min. The extracts were then maintained at 4° and NR activity measured at the times indicated [10].

These results suggest that extracts from 4-, 5- and 6-day-old primary leaves possess an inactivating mechanism which cannot be blocked by either PMSF or BSA, and that extracts from 5- and 6-day-old primary leaves possess an additional inactivating mechanism which can be blocked completely by BSA but only partially by PMSF.

The effect of endogenous protein concentration on stability of NR

It has previously been suggested that stabilization of NR by BSA in extracts from older tissues is due to the higher protein concentration preventing dissociation [6]. However, as may be seen in Table 3, the amount of protein present in extracts of 5- and 6-day-old primary leaves was no lower than that present in extracts from 4-day-old primary leaves.

We also attempted to see if the relatively stable NR present in extracts of 4-day-old primary leaves became more unstable on dilution. However, the stability of NR at 4° actually increased when an extract from 4-day-old primary leaves was diluted (Table 4). We feel that the data in Tables 3 and 4 taken together suggest that the concentration of endogenous protein *per se* does not contribute to NR stability.

Correlation of in vitro *stability of NR with leaf position in field grown barley*

All the results reported above were obtained with barley plants grown in the laboratory. To see whether the age-dependent *in vitro* stability of NR also occurred in field-grown plants, barley was grown during summer 1979 under the conditions described in the Experimental. Eighteen days after sowing, the first, second and third leaf blades were removed and extracted in buffer with or without BSA. After centrifugation, NR activity present in the extract was measured. NR in extracts from the third leaf blade (i.e. the youngest) was most stable and stability did not correlate with the protein content of the extract (Table 5).

Age-dependent conversion of NR to CR species

Although NR from 4-day-old primary leaves is relatively stable *in vitro* (Fig. 1), we recently reported that it is still able to break down to small amounts of a CR species of MW 40000 which we suggested was either a subunit of the NR complex [9] or more likely a domain cleaved from NR by endogenous proteinases [10].

Table 3. Nitrate reductase activity and protein present in centrifuged extracts of barley primary leaves of different ages

Age of barley primary leaf (day)	Protein (mg/ml extract)	NR activity (μmol/ nitrite produced/ml extract/hr)
4	6.74	2.61
5	8.43	2.14
6	6.87	0.59

Primary leaves were extracted in buffer I and the brei was centrifuged at 38000 g for 20 min. NR activity was assayed as previously described [10] and protein was estimated by the method of ref. [43].

The results of the experiments with BSA and PMSF described above support the contention that the increased instability of NR in extracts from leaves older than 4 days is due to increased proteinase attack. We therefore examined the possibility that this increased instability correlated with an increased conversion of NR to the MW 40000 CR species.

Sucrose density gradient (SDG) analysis of extracts from 4-day-old primary leaves is shown in Fig. 2a. The major CR species co-sediments with NR activity at 7.7 S [10] whilst the small amount of CR activity in the 3–4 S region represents CR species derived from NR as well as the constitutive MW 27800 CR species [10]. SDG analysis of extracts from 5-day-old leaves showed a different CR profile (Fig. 2b). The CR species sedimenting in the 3–4 S region were more predominant and heterogeneous, whilst the amount of the 7.7 S NR complex present was decreased. There was an additional CR species present which sedimented close to, but more slowly than, the 7.6 S alcohol dehydrogenase marker.

SDG analysis of the extract from 6-day-old primary leaves (Fig. 2c) showed that the major CR species present sediment in the 3–4 S region. Very small amounts of two

Table 4. Effect of dilution on stability at 4° of nitrate reductase in extracts of 4-day-old primary barley leaves

	Stability as % of initial activity			
Time at 4° (min)	Undiluted (1:0)	Diluted (3:1)	Diluted (1:1)	Diluted (1:3)
0	100	100	100	100
20	95.2	96.4	94.4	100.3
40	95.6	100.8	100	107.2
60	94.2	101.9	98.4	107.5
80	87.9	98.7	100.4	101.3
100	89.8	98.6	96.4	104.6
120	86.5	95.3	90.5	106.2

Primary leaves were extracted in buffer I and the brei was centrifuged at 38000 g for 20 min. Buffer I was used to dilute the extract by the appropriate factor and loss of NR activity at 4° was measured [10].

Table 5. Nitrate reductase activity and protein present in extracts from leaves of 18-day-old field-grown barley plants

Leaf	BSA	Protein (mg/ml extract)	NR activity (μmol nitrite produced/ml extract/hr)
First	+	—	0.27
(Oldest)	−	2.95	0.01
Second	+	—	0.36
	−	2.52	0.07
Third	+	—	0.42
(Youngest)	−	2.65	0.34

Leaves were extracted with either buffer I or buffer I supplemented with 3% BSA and the brei was centrifuged at 38000 g for 20 min. NR activity was assayed as previously described [10] and protein was estimated by the method of ref. [43].

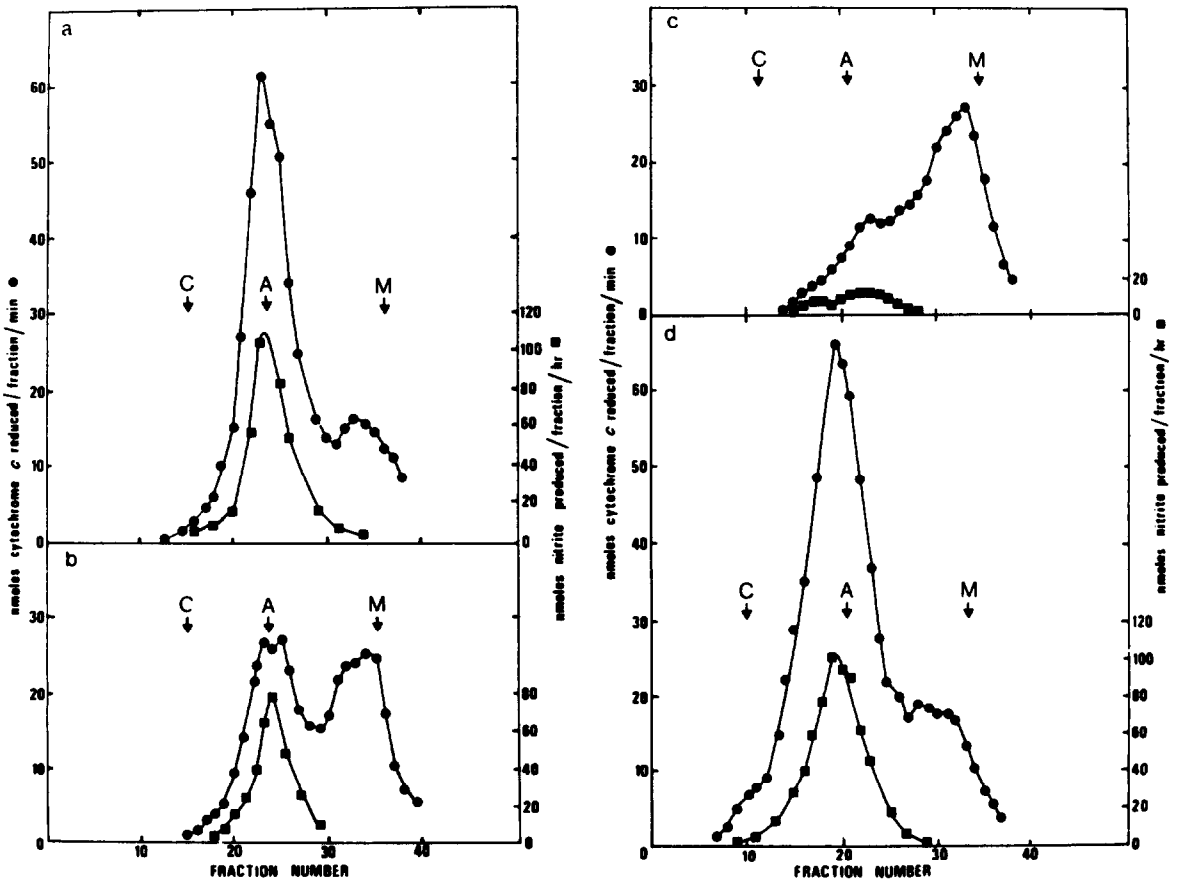

Fig. 2. Distribution of NADH-NR (■) and CR (●) activity after sucrose density gradient analysis of extracts from (a) 4-day-, (b) 5-day-, (c) 6-day-old primary leaves prepared with buffer I and (d) 6-day-old primary leaves prepared with buffer I containing 3% BSA. Conditions of centrifugation and extraction of plant material are described in the Experimental. C, A and M denote the position of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation.

NR-associated CR species are also present. BSA, which stabilized NR in extracts from 6-day-old primary leaves (Table 2), prevented both the loss of the 7.7 S NR-associated CR species and the appearance of the species sedimenting in the 3–4 S region of the gradient (Fig. 2d). PMSF, which partially stabilized NR in extracts from 6-day-old primary leaves (Table 2) also partially prevented the appearance of the 3–4 S CR species. However, neither PMSF, nor the delayed addition of BSA to the tissue extract was as effective in stabilizing NR and preventing the appearance of the 3–4 S CR species as was the presence of BSA in the extraction buffer (data not shown).

Determination of Stokes radius and sedimentation coefficient of CR species detected in extracts from 5-day-old primary leaves

Extracts from 5-day-old primary leaves were subjected to gel filtration on Sephadex G200 as previously described [10] and the distribution of CR species determined (Fig. 3). This distribution is quite different from that previously determined [10] using extracts from 4-day-old primary leaves. The first eluted CR species (Fig. 3) again elutes just after the void volume. However, in contrast to previous results with 4-day-old primary leaves [10] the next eluted CR species has a Stokes radius of 5.8 nm. An almost identical Stokes radius of 5.7 nm was obtained for the major peak of NR activity (Fig. 3). A heavier NR species, seen as a shoulder on the leading edge of the 5.7 nm NR species, had a Stokes radius of *ca* 6.3 nm. This value is very close to the 6.4 nm we have previously reported for the Stokes radius of the intact NR complex [10].

The last eluted peak of CR activity (Fig. 3) has a Stokes radius of 3.05 nm which corresponds closely to the value previously determined [10] for the major NR-derived CR species. It was not possible, however, to assign sedimentation coefficients to CR species present in fractions derived from the elution profile shown in Fig. 3 since there was not sufficient activity present to allow detection after centrifugation. This problem was circumvented by carrying out gel filtration on Biogel A 1.5 m which separates proteins on the same molecular basis as Sephadex G200. However, due to the faster flow rate it was possible to use a larger column and apply a larger enzyme sample.

The profile of CR species detected after gel filtration through Biogel A 1.5 m of a 0–60 % ammonium sulphate fraction (lacking the MW 27 800 constitutive CR species [11]) from 5-day-old leaves is given in Fig. 4. It is similar to the Sephadex G200 profile in Fig. 3 and reveals the presence of three major size distributions of CR. There is a large peak of CR activity which elutes just after the void volume, an intermediate heterogeneous peak and a third peak of a smaller CR species. This profile is markedly different from the CR profile seen after fractionation on Biogel of an extract from 4-day-old leaves (see Fig. 3 in ref. [10]) since the intermediate peak is heterogeneous and the third eluted peak considerably larger.

Aliquots from three fractions in the Biogel profile in Fig. 4 were subsequently analysed by SDG centrifugation so that sedimentation coefficients of the CR species present could be determined. The first aliquot analysed was from fraction 30 in Fig. 4. This fraction was chosen as likely to be enriched in the NR-associated CR species which sedimented more slowly than the 7.6 S alcohol

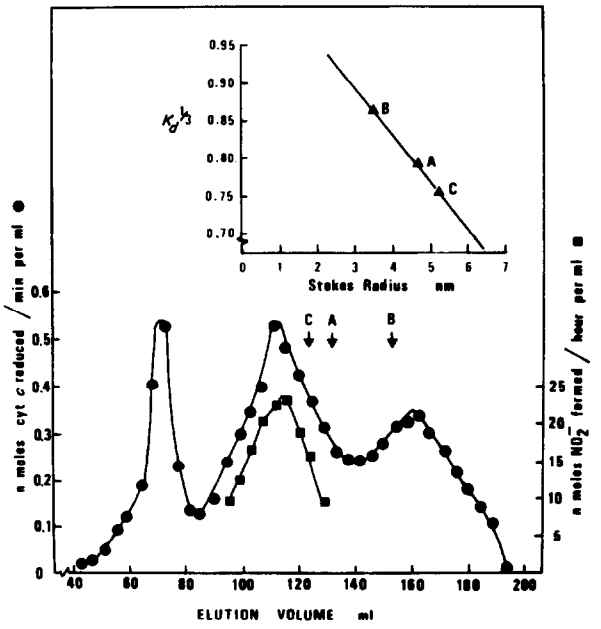

Fig. 3. Distribution of NADH-NR (■) and CR (●) activity after Sephadex G200 gel filtration of an extract from 5-day-old primary leaves. Conditions of gel filtration and extraction of plant material are described in the Experimental. 2 ml fractions were collected. C, A and B denote the positions at which 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 [44].

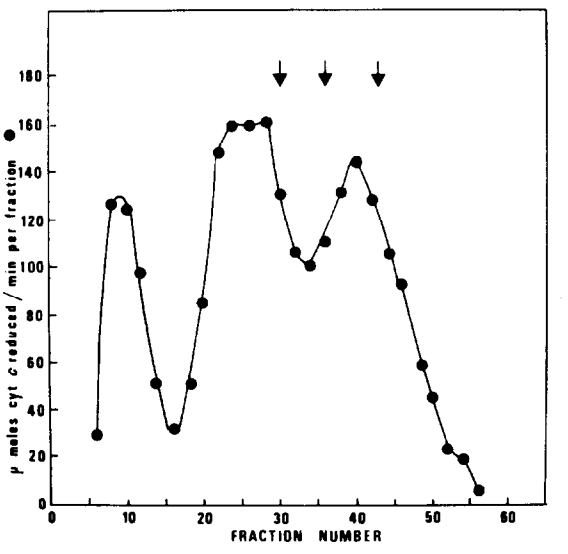

Fig. 4. Distribution of CR activity after Biogel A 1.5 m gel filtration of an extract from 5-day-old primary leaves. Conditions of gel filtration are described in the Experimental. 15 ml fractions were collected. The arrows denote fractions 30, 36 and 43, respectively, which were analysed further by sucrose density gradient centrifugation (Fig. 5).

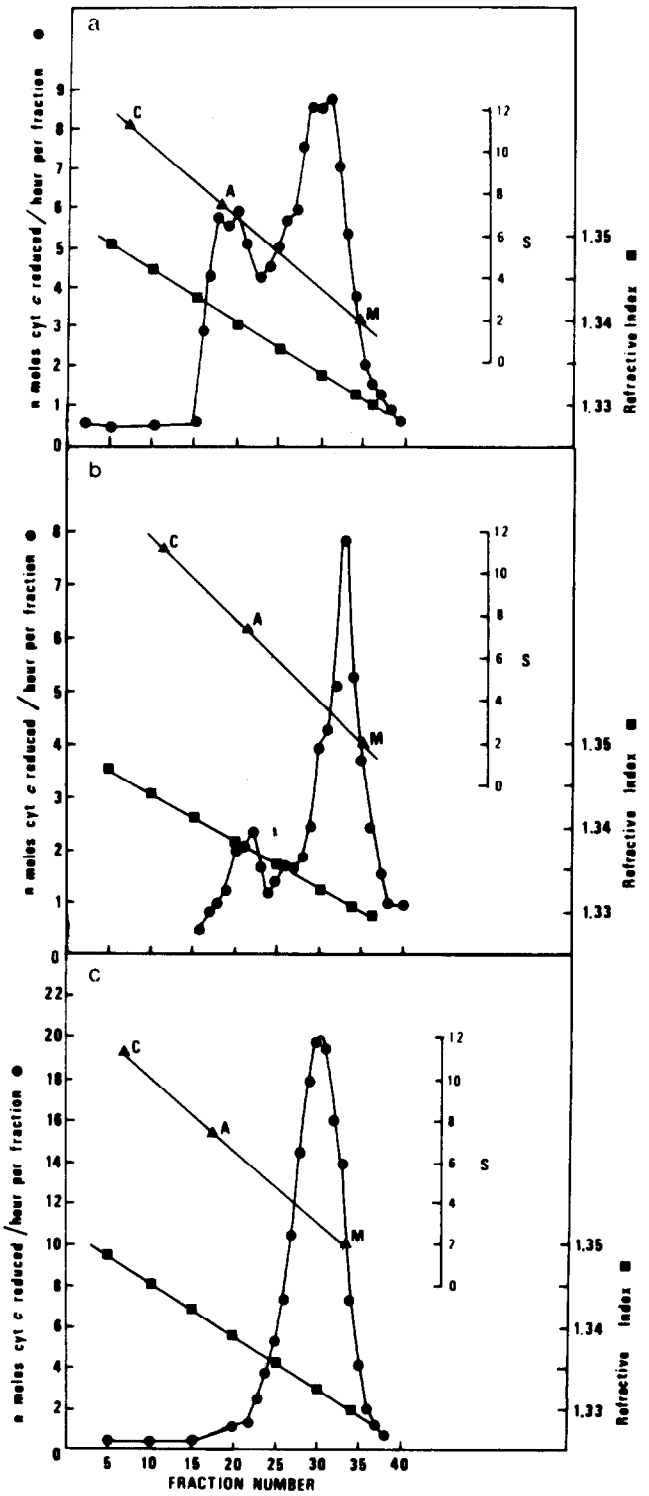

Fig. 5. Distribution of CR activity after sucrose density gradient analysis of aliquots derived from (a) fraction 30 in Fig. 4, (b) fraction 36 in Fig. 4 and (c) fraction 43 in Fig. 4. C, A and M denote the positions of the reference proteins catalase (11.3 S), alcohol dehydrogenase (7.4 S) and myoglobin (2.04 S). Conditions of centrifugation are described in the Experimental.

dehydrogenase marker (seen in Fig. 2b and c) since it is from the trailing edge of the intermediate heterogeneous peak. However, although a 6.8 S CR species was present, together with a 7.7 S CR species (the NR complex) after centrifugation, the predominant CR species sedimented at 3.8 and 3.1 S (Fig. 5a). The predominance of these lighter CR species is surprising in view of the position of fraction 30 in the Biogel elution profile (Fig. 4). We consider that the predominance of the 3.8 S and 3.1 S CR species is due at least in part to the breakdown of the 7.7 S and the 6.8 S CR species. We have previously shown that the 3.8 S and 3.1 S CR species can be derived from the 7.7 S CR species during SDG analysis [10]. In addition to the 6.8 S CR species there is another newly identified CR species which sediments at 5.6 S (Fig. 5a).

SDG analysis of fraction 36 (Fig. 5b) revealed the presence, either as shoulders or distinct peaks, of all the CR species seen in Fig. 5a, although the predominant species present sedimented at 3.1 S. SDG analysis of fraction 43 from the trailing edge of the third peak in Fig. 4 showed the presence of a single CR species sedimenting at 3.1 S (Fig. 5c).

These results confirm the presence of CR species of 7.7, 3.8 and 3.1 S in extracts from barley leaves [10] but in addition provide evidence for the existence of two new species, a 5.6 S species and a 6.8 S NR-associated CR species. This latter CR species has a Stokes radius of 5.8 nm (Fig. 3), a calculated MW of 163 000 [12] and is asymmetrical with an axial ratio of 10:1 and a frictional ratio of 1.61. The Sephadex G200 profile did not reveal the presence of a CR species equivalent to the 5.6 S species and we cannot, therefore, determine its MW.

Tryptic cleavage of nitrate reductase

Incubation of a partially purified NR sample with trypsin led to loss of NR activity (Fig. 6) and the conversion of the 7.7 S NR-associated CR activity to a small CR species which sedimented in the 3–4 S region of the gradient (Fig. 7). BSA retarded tryptic cleavage of NR (Fig. 6).

DISCUSSION

The results presented in this paper show clearly that the NR present in extracts of barley primary leaves becomes progressively more unstable as the leaf ages (Fig. 1). A similar age-dependent phenomenon has been previously reported for leaves of oat, tobacco and maize [1, 2], cotyledons of cotton [6, 7] and roots of maize [3–5]. In common with the situation in oat, tobacco and maize leaves [1, 2], the stability and amount of extracted NR was increased if extraction was carried out in the presence of BSA (Table 2). PMSF stabilized NR in extracts of barley primary leaf (Table 2) but was not as efficient as BSA. However, BSA did not appear to stabilize NR through increasing protein concentration *per se* (Table 4).

The ability of PMSF, an inhibitor of serine proteinases to partly stabilize, and of BSA to completely stabilize NR in extracts of 5- and 6-day-old primary leaves (Table 2) suggests that loss of NR activity may be due to proteolytic attack. It is interesting to note that trypsin cleaves NR to 3–4 S CR species (Fig. 7) and that BSA retards this conversion (Fig. 6). We have no direct evidence at present for a proteinase (or proteinases) in barley leaves. They have, however, been reported by other workers [13] and leaf extracts from other plant species have been shown to contain proteinases [14, 15]. Recently a heat-labile factor (II) which has a negative effect on the stability of NR has been identified in wheat leaf extract [16]. The factor had a MW of 37 500, was sensitive to inhibition by Fe^{2+} chelators, but not PMSF, and did not affect nitrite reductase, ribulose-1,5-bisphosphate carboxylase/oxygenase or hydrolyse casein or haemoglobin. The inactivating factor was more active towards NADH-NR activity than either FMNH-NR or MV°-NR, and NADH-ferrocyanide reductase (diaphorase) activity was the least sensitive [16].

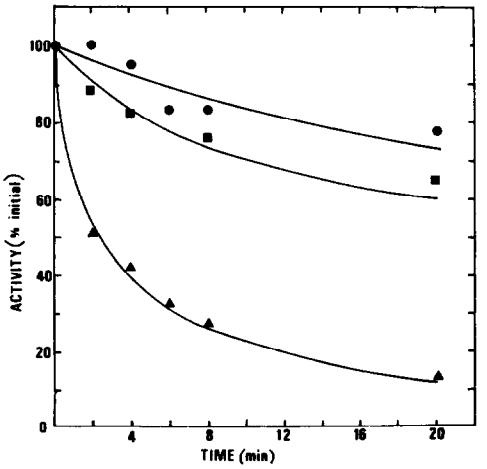

Fig. 6. Stability at 0° of partially purified nitrate reductase in (a) the absence of trypsin (●), (b) the presence of trypsin (0.1 μg/ml) (▲) and (c) the presence of trypsin (0.1 μg/ml) and 3% BSA (■). The stability of NR was determined as described in the Experimental.

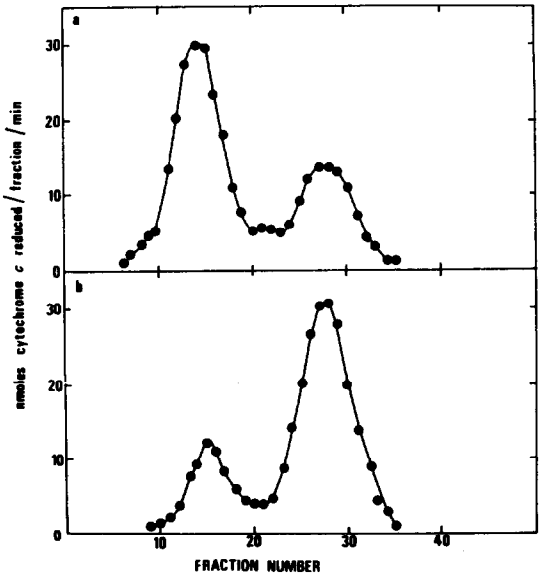

Fig. 7. Distribution of CR activity after sucrose density gradient centrifugation of a partially purified sample of NR either untreated (a) or treated with trypsin (b). Trypsin treatment of NR and conditions of centrifugation are described in the Experimental.

A heat-labile inactivating factor has also been identified in maize roots [4, 5]. Inhibition of the factor by PMSF [5, 8] and casein [5] led to the suggestion that the factor was a serine proteinase. Although originally considered to specifically inactivate NR, later studies showed that it was also able to inactivate tryptophan synthase and was active against the proteinase substrate, azocasein [17]. From a comparison of the rates of loss of NR activity, and the two partial activities CR (diaphorase) and FMNH-NR, Wallace concluded that the maize root proteinase attacked that part of the NR complex which carried CR activity [18].

The available data then suggest that shoot and root extracts of some plant species possess factors, probably proteinases, which can inactivate NR by specific attack at some point on the molecule. The inhibition of the wheat leaf factor by chelating agents [16] does not necessarily conflict with our indirect evidence for a PMSF sensitive proteinase in barley leaf extracts. PMSF only partially stabilized NR in barley leaf extracts (Table 2) and leaf extracts may contain more than one type of proteinase.

We have previously reported that when NR is extracted from barley leaves younger than 4 days old it is able to break down during SDG centrifugation to yield small amounts of CR species of MW 61 000 and 40 000 [10]. We suggested that these CR species were either subunits of the NR complex [9] or domains released from the NR complex by proteinase attack [10]. In the present paper we have shown that NR becomes increasingly unstable in extracts from 5- and 6-day-old primary leaves (Fig. 1). The data in Figs. 1 and 2 show clearly that there is a correlation between the age-dependent loss of NR activity in crude extracts and the conversion of NR to smaller CR species. Further, PMSF and BSA, which were shown to stabilize NR in extracts of 5- and 6-day-old primary leaves were able to prevent (in the case of BSA) or retard (in the case of PMSF) the conversion of NR to the smaller CR species (Fig. 2d). We cannot discount the possibility that other NR-derived CR species, in addition to the MW 163 000, 61 000 and 40 000 species, might exist, but if they do then they are present in relatively smaller amounts and cannot be detected by the techniques used here. An understanding of how the MW 163 000, 61 000 and 40 000 CR species are generated from the NR complex depends on a knowledge of the subunit structure of the enzyme.

The assimilatory NR from eukaryotes is a haemomolybdoflavoprotein [19]. The MW 230 000 *Neurospora crassa* NR consists of two identical subunits of *ca* MW 115 000 each, carrying one FAD and one cytochrome b_{557}, together with a molybdenum-containing component (MCC) of *ca* MW 1000 [20]. The NR from *Rhodotorula glutinis* is similar [21], whilst the larger NR from *Chlorella vulgaris* consists of three identical subunits carrying FAD and cytochrome b_{557} together with one or more MCCs [22, 23]. If barley NR (MW 203 000 [10]) fits this pattern then it would be expected to consist of two MW 100 000 subunits each carrying FAD and cytochrome b_{557} together with an MCC. This proposal is supported by the observation that a 550-fold purified preparation of barley NR produced a major staining band of *ca* MW 100 000 after SDS polyacrylamide gel electrophoresis [11, 24].

All these eukaryotic NRs are also capable of catalysing other reactions such as NAD(P)H–cytochrome *c* reductase, FMNH–nitrate reductase and reduced methyl viologen nitrate reductase activity [19]. These partial activities are considered to be catalysed by specific regions of the NR molecule. Inhibitor studies suggested that the CR activity of barley NR was catalysed by the initial portion of the polypeptide chain [25] and in *N. crassa* NADPH–CR activity is FAD-dependent [26]. It is not clear whether cytochrome b_{557} is obligatorily involved in the expression of CR activity [27], but cytochrome b_{557} is reduced by NADPH only when FAD is also present [26]. Removal of molybdenum [28] or substitution by tungsten [25, 29] abolishes all NR activities but has no effect on the CR activity. Electron flow is generally accepted to be

$$\text{NADH} \rightarrow \text{FAD} \rightarrow \text{Cytochrome } b_{557} \rightarrow \text{Mo} \rightarrow \text{NO}_3.$$

Recent studies on flavocytochrome b_2 from bakers' yeast suggest that the FAD and cytochrome components are organized in the enzyme in domains, that is in independently folded, functionally intact regions of the polypeptide chain, and that these domains are held together by a flexible and loosely structured region which is very sensitive to proteinase attack [30, 31]. Tryptic cleavage of rat liver sulphite oxidase, a haemomolybdoprotein, results in the release of two major functional fragments, one of which contains the cytochrome *b* component, whilst the other contains all the molybdenum. The authors concluded that the molybdenum and cytochrome components of sulphite oxidase were contained in distinct domains which were covalently linked by an exposed hinge region of at least 30 amino acid residues [32, 33].

We consider it highly probable that the FAD and cytochrome b_{557} components of higher plant NR are contained in separate functional domains, whilst an additional domain may be involved in the binding of MCC. The domains are envisaged to be linked by interdomain hinge regions which are hypersensitive to proteinase attack. Evidence which supports the contention that at least FAD is contained in a domain comes from the observation that the CR partial activity of the intact barley NR complex is extremely heat labile and sensitive to *p*CMB whilst the FMNH and reduced methyl viologen NR partial activities are relatively heat stable and insensitive to *p*CMB [25]. This differential sensitivity is what would be expected if the CR activity was expressed by an independently folded, functionally intact region of the NR complex.

Such a model suggests a possible relationship between NR and the smaller CR species released from it. Proteinase attack at a hinge region in one of the MW 100 000 subunits could lead to the release of the MW 40 000 fragment active with respect to CR activity (Figs. 2 and 5). Since FAD is required for CR activity [26], this fragment probably contains at least the FAD domain together with the NADH binding site of the native NR. The resultant MW 163 000 fragment (Figs. 3 and 5) would still be capable of expressing CR and NR activity due to the presence of one intact MW 100 000 subunit and MCC. The two NR species seen after isoelectric focusing of spinach NR [34] may be equivalent to the NR complex and this new MW 163 000 NR fragment.

The MW 61 000 CR species could be generated from the intact MW 100 000 subunit of the MW 163 000 fragment by proteinase attack at a different hinge region

either before or after dissociation of the MW 100000 subunit and the MW *ca* 60000 fragment. The MW 100000 subunit might be equivalent to the 5.6S CR species seen in Fig. 5a and 5b. We are not at present able to detect fragments, derived from the NR complex, which lack CR activity.

We no longer favour our earlier suggestion that these NR-derived CR species represent subunits or subunit associations [35] since (a) the production of these CR species is age-dependent, (b) production of these CR species is inhibited by PMSF and BSA, (c) trypsin cleaves NR to 3–4 S CR species (Fig. 7) and BSA retards this conversion (Fig. 6) and (d) despite exhaustive genetic studies only one structural gene specifying the FAD and cytochrome b_{557}-containing subunit has been identified for the closely related NR from *Aspergillus nidulans* [36] and *Neurospora crassa* [37].

The observed characteristics of the NR seen in the *nit*-3 mutant of *N. crassa* can be explained in terms of our above model. This enzyme is of MW 163000, rather than the MW 235000 of the wild-type enzyme, and lacks NADPH-NR and NADPH-CR activities, although it does express FADH- and reduced methyl viologen NR activities [38]. We propose that this mutant NR lacks the FAD domain from each of its haemoflavoprotien subunits due either to (i) a deletion or nonsense mutation in the gene (exon? [39]) coding for the FAD domain or (ii) cleavage of an abnormal FAD domain from the haemoflavoprotein subunit by proteolytic attack at a postulated hinge region either before or after assembly into the holoenzyme.

Although the present paper is the first time that the age-dependent production of the 6.8 S and 5.6 S CR species has been reported, closely similar species do appear to be present in spinach extracts under certain circumstances [40]. When spinach plants were grown without molybdenum either with or without tungsten, but with ammonium as an additional nitrogen source to nitrate, CR species of 5.5 and 6.9 S were found after SDG analysis of extracts in addition to the nominal 8.1 S and 3.6 S CR species normally seen in extracts from nitrate- and molybdenum-grown spinach plants. However, only the 8.1 S species possessed NR activity [40]. This suggests to us that the absence of a functional MCC, due either to lack of molybdenum or substitution by tungsten, renders spinach NR more susceptible to proteolytic attack. In this context, one of us has previously reported that the level of CR sedimenting in the 3–4 S region of sucrose gradients is doubled in barley plants treated with tungsten [25]. Perhaps MCC has a role in determining the susceptibility of NR to proteolytic attack as well as a role in the assembly of the haemoflavoprotein subunits.

The ability to reconstitute spinach NR from *ca* 4 S CR species and MCC [41] does not conflict with our suggestion that these CR species are domains (or domain associations) rather than subunits. The 3–4 S region of the gradient would contain the MW 40000 and 61000 CR species together with the proposed molybdenum domain. Cleavage of other enzymes into domains by trypsin or endogenous proteinases leads to the release of functional domains [31–33, 42] which can reassemble to produce a functional enzyme very similar in size to that of the native enzyme [31]. Whether the proposed barley proteinase(s) is involved in *in vivo* turnover of NR and whether *in vivo* turnover involves conversion to the CR species described above remains to be shown.

EXPERIMENTAL

Chemicals. Cysteine-HCl, FAD (Grade III), NAD^+ and NADH (yeast grade III), dithiothreitol, alcohol dehydrogenase (yeast), catalase (bovine liver), myoglobin (whale skeletal muscle, Type II), BSA, cytochrome *c* (horse heart, Type II), PMSF, trypsin (bovine pancreas) and trypsin inhibitor (ovomucoid) were obtained from Sigma. All other chemicals were of the best quality available from the usual commercial sources.

Growth of plant material. With the exception of the data presented in Table 5, barley (*Hordeum vulgare* cv Golden Promise) seeds were sown in trays in vermiculite in the laboratory, watered with tap H_2O and placed in darkness at 28° to germinate. At 24, 48, 72, 96, 120, 144 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°. In the case of field-grown plants (data of Table 5) barley seeds were sown in rows 25 cm apart with Fisons 52 Regular Fertiliser at a rate of 400 kg/ha.

Enzyme extraction. Seedlings were harvested at the times indicated in the table and figure legends and the primary (first emergent) leaf was extracted as described previously [10]. Unless otherwise stated, the homogenates were centrifuged at 38000 *g* for 20 min in a high speed centrifuge at 4° and the supernatants were used as the crude extracts. BSA was either added directly to the extraction buffer or, in the case of delayed addition, a buffer containing twice the desired final concn of BSA was used to dilute the initial 1:1 after centrifugation. PMSF was dissolved in a minimum vol. of propan-2-ol (final concn 5% when PMSF was 1 mM) before addition to the extraction buffer. Extraction with buffer containing 5% propan-2-ol had no significant effect on the activity of NR.

Sucrose density gradient centrifugation, Sephadex G200 and Biogel A 1.5 m gel filtration and enzyme assays. These were performed as described previously [10].

Protein. This was estimated by the method of ref. [43].

Purification of NR for trypsin treatment. Barley shoots (90 hr-old, 250 g) were extracted as previously described [10] and after a streptomycin sulphate treatment, protein precipitating between 0 and 45% satn with respect to $(NH_4)_2SO_4$ was passed through Biogel A 1.5 m [10]. The active fractions from Biogel were pooled and adjusted to 60% satn with $(NH_4)_2SO_4$ and the precipitated protein was dissolved in buffer I. After adjustment to 40% satn with respect to glycerol, the protein was stored at −70° overnight. The protein was again precipitated with $(NH_4)_2SO_4$ at 60% satn to remove glycerol and was dissolved in the minimum vol. of 20 mM K–Pi buffer containing 10 μM FAD, 0.1 mM EDTA, and 1 mM cyteine (pH 7.5) (buffer III). The protein was then applied to a Blue-Dextran Sepharose column (2 cm × 9 cm) equilibrated in buffer III and after washing with buffer III until the E_{280} of the eluate was less than 0.01, NR was eluted with 5 μM NADH in buffer III. The two fractions which contained the greatest amount of NR were pooled and served as the enzyme source for trypsin treatment.

Trypsin treatment. NR (3.6 ml) in buffer III was mixed with 0.4 ml trypsin (1 μg/ml) in buffer III at 0° and 0.1 ml aliquots of the mixture were removed into 0.1 ml of trypsin inhibitor (10 μg/ml) at time intervals before assaying for NR as previously described [10]. For the control (minus trypsin) treatment, the trypsin soln was replaced by an appropriate vol. of buffer III, whilst for the BSA plus trypsin treatment, BSA was added to the enzyme sample to a final concn of 3% (w/v) before trypsin was added.

Sucrose density gradient analysis of the CR species present after trypsin treatment. Aliquots (0.9 ml) of the enzyme were mixed

with either 0.1 ml trypsin (1 μg/ml) in buffer III) or buffer III, and after 10 min at 0° 0.2 ml trypsin inhibitor (200 μg/ml) was added to both treatments. Aliquots (0.4 ml) of the mixture were then analysed on sucrose density gradients as previously described [10].

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