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THE ACTIVITY AND STABILITY OF WHEAT NITRATE REDUCTASE *IN VITRO*

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; nitrate reductase; stability.

Abstract—The activity and decay characteristics of nitrate reductase from wheat (*Triticum aestivum*) were studied in crude, partially-purified and highly-purified preparations. The decay of nitrate reductase activity in crude extracts was due to spontaneous dissociation of the enzyme and to the effects of two decay factors, one present in the 0–30% and the other in the 50–70% saturated $(NH_4)_2SO_4$ fraction of a crude extract. Low rates of factor-mediated NR decay *in vitro* were associated with high levels of NR activity *in vivo*.

INTRODUCTION

Nitrate reductase (NR) (EC 1.6.6.1) is considered to catalyse the rate-limiting step of nitrate assimilation in higher plants [1], and seedling NR activity has been suggested as a possible selection criterion in the breeding of high-protein wheat cultivars [2, 3]. We have reported that a number of ditelosomic lines of wheat cv. Chinese Spring, exhibit NR activities significantly different from that of the euploid [4] but identification of the specific genes responsible necessitates a deeper understanding of the proteins which control NR activity.

In many species, NR is unstable *in vitro* [5, 6] and *in vivo* [7, 8], and causative factors which have been reported include phenolic compounds [9], general proteases [5], NR-specific proteases [10] and NR-binding inactivators [11]. In several species, incorporation of casein [5] or other stabilizing agents [12] has enabled the complete stabilization of NR. A reversible NADH-mediated inactivation of NR has also been reported to operate *in vivo* [13]. We report here on a study of the post-translational control of wheat NR activity *in vitro*.

Abbreviations: NR, nitrate reductase; CR, NADH–cytochrome *c* reductase; MV°, reduced methyl viologen; DTNB, 5,5′-dithiobis (2-nitro-benzoic acid); PMSF, phenylmethylsulphonyl fluoride.

RESULTS AND DISCUSSION

Characteristics of highly-purified wheat NR

The enzyme was purified from a cell-free extract of seedling shoot tissue by precipitation with 30–50% saturated $(NH_4)_2SO_4$ and subsequent elution with 5 μM NADH from Blue Dextran-Sepharose 4B (Table 1). Because of the very low protein concentration in these highly-purified preparations, the homogeneity of the native preparation could not be assessed on polyacrylamide gels, as no protein band could be detected by Coomassie Blue or Amido Black staining, a problem reported by other workers with wheat [14] or barley NR [15]. The specific activity of the highly-purified preparations fell within the range 19.4–23.7 μmol nitrite produced/min/mg protein at 25°, similar to previous reports of highly-purified wheat NR [14], while attempts at further purification by elution from Ultrogel A6 or by sucrose density gradient centrifugation were unsuccessful.

The physical parameters of wheat NR were then determined. A Stokes radius of 6.8 nm was calculated after elution of NR from calibrated Ultrogel AcA22 (Fig. 1) and A6 columns, while a sedimentation coefficient of 7.5 S was determined by centrifugation through a linear 5–20% sucrose density gradient (Fig. 2). From these parameters, a M_r of 202 000 and a frictional ratio (f/f_0) of 1.63 were obtained using the method of ref. [16]. From data

Table 1. Purification of NADH–NR from shoot tissue of wheat (cv. William) seedlings

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (−fold)	Recovery (%)
Crude extract	11.62	1261	0.009	1	100%
30–50% saturated $(NH_4)_2SO_4$	9.53	384	0.025	2.7	82
Blue Dextran-Sepharose 4B	1.63	0.075	21.8	2420	14

One unit of NADH–NR activity produces 1 μmol nitrite per min at 30°.

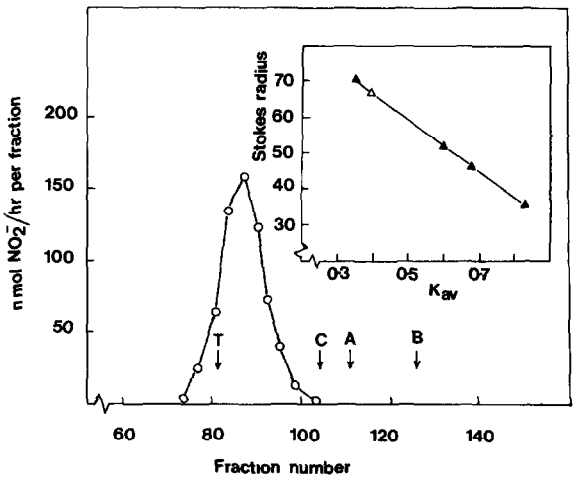

Fig. 1. Ultrogel A6 gel filtration analysis of NADH–NR present in cell-free extracts. Details of sample preparation and elution are described in Experimental. Arrows labelled T, C, A and B refer to the elution volumes of the reference proteins thyroglobulin, catalase, alcohol dehydrogenase and bovine serum albumin, respectively. *Inset*: Relationship between the elution volumes (as K_{av}) of the reference proteins and their Stokes radius values. The open triangle refers to NADH–NR.

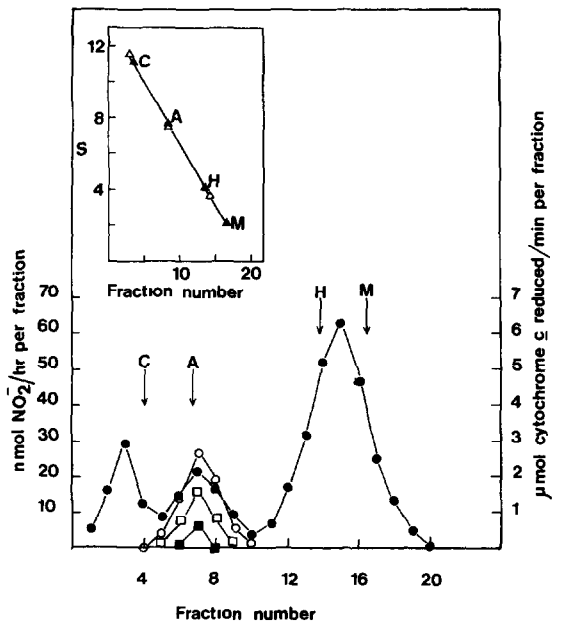

Fig. 2. Sucrose density gradient analysis of NADH–NR (○). $FADH_2$–NR (□), MV°–NR (■) and NADH–CR (●) species present in cell-free extracts. Details of sample preparation and centrifugation are described in Experimental. Arrows labelled C, A, H and M refer to the fractionation volumes of the reference proteins catalase, alcohol dehydrogenase, haemoglobin and myoglobin, respectively. *Inset*: Relationship between the fractionation volumes of the reference proteins and their sedimentation coefficient. The open triangles refer to the three peaks of NADH–CR activity.

presented in ref. [17], an axial ratio of 14:1 was calculated for NR, confirming the cigar shape of the enzyme from other species, as summarised in ref. [18].

The 7.5 S enzyme exhibited several component activities, namely NADH–NR, $FADH_2$–NR, BV°–NR and NADH–CR (Fig. 2). Two additional species of NADH–CR (11.7 S and 3.7 S) were also separated by sucrose density gradient centrifugation of cell-free extracts (Fig. 2). The optimum pH for NADH–NR activity in highly-purified preparations was 7.5, while a second peak at pH 8.8 occurred in cell-free extracts. Activity of highly-purified NADH–NR decreased particularly sharply at lower pH values, due probably to enzyme denaturation. Slight variations in pH optima were observed between the 7.5 S component activities (Table 2). The optimal temperature for activity was 32°. Details of affinity constants for the partial activities are presented in Table 2. Dialysed cell-free extracts exhibited a biphasic kinetic response of NR to nitrate with K_m (nitrate) values of 2.61×10^{-4} M at high concentrations (above 0.2 mM nitrate) and 1.67×10^{-5} M at lower concentrations, agreeing with the bimodal response of NR activity to pH in these preparations.

Maximum activity of highly-purified NR required the presence of 1 mM dithiothreitol, suggesting the existence of active sulphydryl groups. This was confirmed by the inhibition of NADH–NR and NADH–CR activity by iodoacetamide, *p*-chloromercuribenzoate or DTNB at concentrations above 2×10^{-2} mM. The number of sulphydryl groups per molecule of NR was determined by quantification of DTNB-binding, assuming homogeneity of the NR preparation. The stoichiometry of DTNB-binding suggested that there were five or six sulphydryl groups per NR molecule. Pre-incubation of NR with NADH (20 µM) prior to DTNB-treatment reduced the DTNB-binding by 35%, whereas pre-incubation with nitrate (20 mM) had no effect. These data suggest that there are two active sulphydryl groups involved in NADH-binding in NR, i.e. one NADH-binding site per NR subunit.

Decay and inactivation of wheat NR

In all enzyme preparations, NR activity was unstable at temperatures between 2° and 45°, decay being exponential

Table 2. Reaction characteristics of the component activities of the highly-purified 7.5 S complex

Activity	Optimum pH	K_m Donor (M)	K_m Acceptor (M)
NADH–nitrate reductase	7.5	6.0×10^{-5}	3.1×10^{-4}
NADH–cytochrome *c* reductase	7.2	5.8×10^{-5}	8.6×10^{-5}
MV°–nitrate reductase	7.6	4.6×10^{-5}	2.0×10^{-4}
$FADH_2$–nitrate reductase	7.8	6.1×10^{-4}	1.1×10^{-4}

K_m values were determined by regression analyses of (1/V) against (1/S). Similar results were obtained using fresh or stored enzyme preparations, with the exception of NADH–cytochrome *c* reductase, which exhibited a broad pH optimum from 6.6 to 7.3 in stored preparations.

with time. The half-life of NR at 30° in cell-free extracts increased significantly when the enzyme was partially-purified by $(NH_4)_2SO_4$ precipitation (Table 3). Further purification by gel filtration on Ultrogel A6 or by sucrose density gradient centrifugation failed to increase the half-life of NR (Table 4). At very high levels of purification (obtained by affinity chromatography on Blue Dextran-Sepharose 4B), the decay rate of NR increased (Table 3) due probably to the extremely low protein concentration of the preparation. In all preparations examined, maximum NR half-life was obtained at pH 7.5. The most stable preparations were obtained using K–Pi buffers above 120 mM phosphate, the half-life at 30° of partially-purified NR being 17.3 min at 150 mM K–Pi compared to 8.6 min at 20 mM K–Pi.

The decay rates of the component activities of the 7.5 S enzyme complex were determined by sucrose density gradient centrifugation of pre-incubated aliquots of NR preparations. This procedure was necessary because of the presence of CR species other than the 7.5 S form in some preparations. It was found that, in all the enzyme preparations tested, NADH–NR was the least stable activity at 30°, while MV°–NR was the most stable (Table 3). NADH–CR decay rates were similar to those of NADH–NR in all preparations studied (Table 3).

These results suggest that two different phenomena are responsible for the decay of NADH–NR activity in crude extracts. One decay mechanism (due probably to a proteolytic enzyme or to an inactivator) could be eliminated by simple methods of enzyme purification, such as $(NH_4)_2SO_4$ fractionation, as demonstrated by the increased stability of NR in the 30–50% saturated $(NH_4)_2SO_4$ fraction relative to the cell-free extract (Table 3). The decay rate of NADH–CR showed a similar response to partial purification to that of NADH–NR, suggesting that this decay factor affected both partial

Table 3. Half-life values of the 7.5 S component activities in crude, partially-purified and highly-purified preparations

Enzyme preparation	Purification (-fold)	Enzyme half-life (minutes) at 30°			
		NADH–NR	NADH–CR	$FADH_2$–NR	MV°–NR
Crude extract	1	15.2	17.0	34.1	71.6
30–50% saturated $(NH_4)_2SO_4$ fraction	2.6	28.4	30.1	42.1	80.1
30–50% fraction eluted from Ultrogel A6	112	29.4	28.5	44.0	83.3
30–50% fraction collected from 5–20% (2/v) sucrose density gradient	98	27.6	31.1	43.5	83.7
30–50% fraction eluted from Blue Dextran-Sepharose 4B	2164	11.1	14.6	21.4	37 6
LSD 5%		3.4	3.1	5.0	3 4

Enzyme preparations were in 150 mM K–Pi buffer, pH 7.5.

Table 4. The effects of buffer supplements on NR activity and half-life in crude and partially-purified preparations

Buffer supplement	NR activity (% of control)		NR half-life (minutes at 30°C)	
	Crude extract	30–50% saturated $(NH_4)_2SO_4$ fraction	Crude extract	30–50% saturated $(NH_4)_2SO_4$ fraction
—	100.0	100.0	16.2	29.8
Dithiothreitol (1 mM)	181.6	183.1	22.1	36.8
L-cysteine (1 mM)	169.2	164.4	21.8	34.7
EDTA (0.5 mM)	108.6	98.1	18.1	28.6
1,10-phenanthroline (1 mM)	106.3	101.4	17.9	28.8
PMSF (0.5 mM)	116.6	92.4	20.6	30.1
Polyclar AT (1 g/5 ml)	96.7	95.3	15.6	27.4
Sodium molybdate (15 mM)	94.8	98.6	16.3	28.2
Casein (3% [w/v])	111.4	104.9	14.6	26.3
Potassium nitrate (20 mM)	102 3	97.8	16.0	30.6
NADH (20 μm)	176.5	43.1	25.6	28.4
FAD (10 μM)	116.8	117.9	19.2	33.9
LSD 5%	4.6	5.1	1.2	2.4

The basic buffer used was 150 mM K–Pi, pH 7.5.

activities (Table 3). After elimination of this decay factor, the partial activities of the 7.5 S complex continued to decay even in highly-purified preparations (Table 3), suggesting that this instability was due to an intrinsic characteristic of the 7.5 S complex.

Maximum NR-stability at 30° was obtained in 30–50% $(NH_4)_2SO_4$ fractions in 150 mM K–Pi buffer, pH 7.5, containing FAD (10 μM) and dithiothreitol (1 mM), where a half-life of 36.8 min at 30° was reported. Other workers succeeded in producing a stable wheat NR preparation at 30° [13], but this method was not successful with wheat cvs. William or Chinese Spring, suggesting that the mechanisms of NR decay may differ between wheat varieties.

Intrinsic in vitro *decay of NR*

As mentioned, half-life estimates for 7.5 S CR were measured after sucrose density gradient centrifugation of the pre-incubated preparations, because of the presence of other CR species. When the 7.5 S CR preparation used was free of other CR species (by elution of a 30–50% saturated $(NH_4)_2SO_4$ precipitate from Ultrogel AcA22), sucrose density gradient centrifugation of the pre-incubated preparation aliquots revealed that whereas 7.5 S CR activity decreased with time (with a half-life at 30° of 33 min), a second CR species was produced during pre-incubation, and the activity of this species increased with time (Fig. 3). The newly-produced CR species had a sedimentation coefficient of 3.7 S, identical to that of the smallest of the three CR forms present in wheat extracts. A mixture of the two 3.7 S species co-eluted from Ultrogel A6 at a volume corresponding to a Stokes radius of 4.1 nm. According to ref. [17], these values correspond to an axial ratio of *ca* 9:1 for both CR species. These data suggest that the intrinsic decay of the 7.5 S complex is due to dissociation into the smaller 3.7 S form found in cell-free extracts, which probably corresponds to the basic subunit of the 7.5 S complex. The dissociation rate of the 7.5 S complex was similar in crude extracts and in enzyme preparations purified more than 120-fold suggesting that this mechanism of NR-decay was spontaneous, rather than factor-mediated.

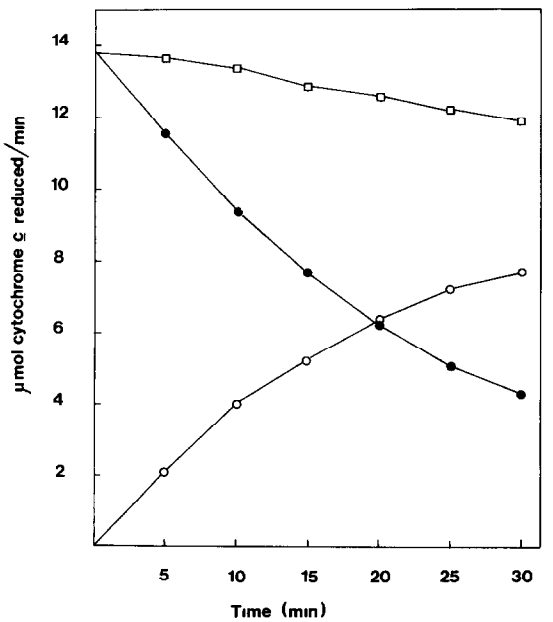

Fig. 3. The rate of change of activity of 7.5 S (●), 3.7 S (○) and total NADH–CR (□) during pre-incubation at 30° of partially-purified 7.5 S CR The 7 5 S enzyme preparation was obtained by elution of a 30–50% saturated $(NH_4)_2SO_4$ fraction from Ultrogel AcA22, using 150 mM K–Pi buffer, pH 7.5. The two species of NADH–CR were separated by centrifugation of the pre-incubated samples for 16 hr at 149 000 *g* through a 5–20% (w/v) linear sucrose gradient.

Dissociation of the 7.5 S complex occurred even at 2°. The inclusion of FAD (25 μm), but not FMN, in buffers reduced the decay rate at temperatures between 2° and 35°, but not by reducing dissociation. NR has a flavin structural component [19], and it has been suggested that FAD may prevent thermal denaturation of NR. Dithiothreitol (1 mM) also reduced the decay rate of partially or highly purified NR by 35–40% by preventing gradual inhibition of NR activity by oxidation effects. No buffer inclusion was shown to reduce the rate of 7.5 S complex dissociation. The discovery of this dissociation phenomenon justifies the use of sucrose density gradient centrifugation to identify the 7.5 S CR during decay studies; without this precaution, there would be a tendency to over-estimate the half-life of 7.5 S CR. This could explain the rather long half-life previously reported for the CR partial activity of wheat NR [14].

In partially purified preparations, the 3.7 S species (obtained either directly, from a cell-free extract, or indirectly, as a dissociation product of 7.5 S) was highly heat-stable, with a half-life at 30° for 261 min. By centrifugation of pre-incubated preparation aliquots through sucrose density gradients, it was shown that, in cell-free extracts the 3.7 S species decayed far more rapidly than in partially-purified preparations, with a half-life at 30° of 56.4 min. Extraction in the presence of PMSF (1 mM) resulted in a partial stabilization of 3.7 S in cell-free extracts (half-life of 96.3 min) although 3% (w/v) casein or BSA proved ineffective, suggesting that a factor containing an active serine group is partly responsible for 3.7 S CR decay in crude extracts.

Factor-mediated decay and inactivation of NR

Characteristics of the decay factor were studied by investigating the stability and activity of NR in the presence (crude extract) and absence (30–50% saturated $(NH_4)_2SO_4$ precipitate) of this factor. The half-life values of the NADH–NR and CR activities of the 7.5 S complex were similar in both preparations (Table 3); knowing that the intrinsic decay mechanism affects both activities equally, this suggests that the decay factor acts on both the 7.5 S NR and the CR activities.

The incubation time course of nitrite production by NADH–NR in cell-free extracts was linear for *ca* 10 min at 30° (Fig. 4). This linear period could not be extended by increasing the nitrate or NADH concentrations of the reaction medium. The linear period of nitrite production could be increased to at least 30 min by $(NH_4)_2SO_4$ precipitation (30–50%) of NR (Fig. 4) or by the use of more highly-purified NR preparations. The time course of nitrite production by $FADH_2$–NR in cell-free extracts revealed that very little nitrite was produced up to

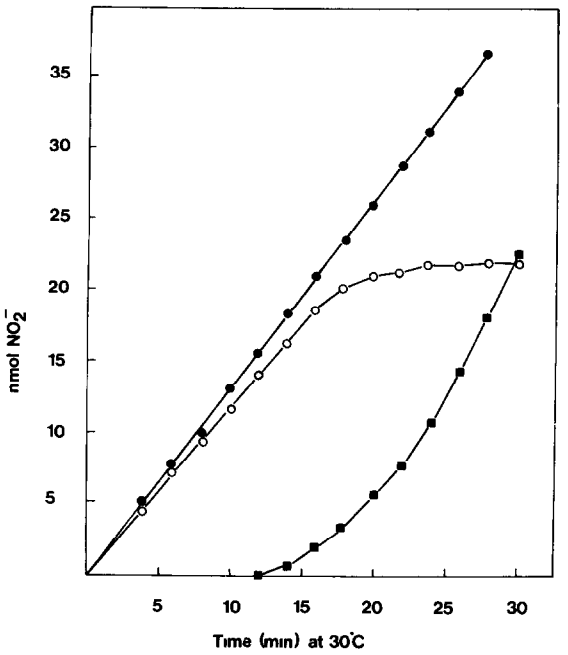

Fig. 4 Time course of nitrite production in cell-free extracts by NADH–NR (○) and $FADH_2$–NR (■), and in desalted 30–50% saturated $(NH_4)_2SO_4$ fractions by NADH–NR (●).

ca 8–10 min at 30°, at which point a rapid increase in nitrate production was observed (Fig. 4). This profile of $FADH_2$–NR activity in cell-free extracts suggests that changes in NR configuration following NADH–NR inactivation allowed $FADH_2$–NR activity to be manifested [20], the inactivating factor being eliminated by $(NH_4)_2SO_4$ preparation of NR. It is not known if this factor is also the decay factor present in crude extracts.

The characteristics of NR-decay in cell-free extracts and 30–50% saturated $(NH_4)_2SO_4$ preparations were then studied. Maximum NR-stability occurred at pH 7.5 in both preparations. The inclusion of sulphydryl reagents (dithiothreitol, L-cysteine) increased the NR half-life in both preparations, by preventing oxidation of active sulphydryl groups (Table 4), while EDTA, 1,10-phenanthroline and PMSF acted to stabilize NR in crude extracts only (Table 4), suggesting that the decay factor may carry metal ions or a serine residue at the active site. The presence of PMSF resulted in a concentration-independent (above 0.5 mM) increase in NR half-life, but this effect was less than that achieved by $(NH_4)_2SO_4$ fractionation of NR (Table 4). This result can be interpreted as being due to the presence of two independent decay factors in the crude extract, the serine factor (inhibited by PMSF) and a second decay factor. A similar result has already been demonstrated here with regard to the decay of 3.7 S CR in crude extracts, suggesting that the two decay factors affect both NADH–NR and 3.7 S CR. Both decay factors were eliminated by $(NH_4)_2SO_4$ fractionation of NR from the cell-free extract, the serine factor being found in the 50–70% saturation $(NH_4)_2SO_4$ fraction while the second decay factor was found in the 0–30% saturation $(NH_4)_2SO_4$ fraction [P. Jones and M. ní Mhuimhneacháin, unpublished results].

Because of the sensitivity of NR to iodoacetamide and related agents, the role of sulphydryl groups in the decay factor could not be ascertained. Polyclar AT and molybdenum had no effect on NR half-life (Table 4).

A major cause of NR decay and subsequently low NR activity in cell-free extracts of many species is general protease action, which can be overcome by inclusion of 1–3% (w/v) casein or BSA, as an alternative protease substrate, in the extraction buffer [5, 6]. In our studies, the presence of 3% (w/v) casein resulted in substantial increases in NR activity in barley cv. Midas (138% increase), maize cv. Earliking (78%) and rye cv. Petkus (126%), the activity exhibiting no significant decay over 30 min at 30°, compared to half-life values of 28.4, 46.1 and 22.1 min, respectively, in the absence of casein. In oat cv. Mostyn and maize cv. Salute, no NR activity could be detected in the absence of casein, whereas the presence of casein resulted in the extraction of high activities of stable NR. The maize data show that the casein effect is variety-dependent [5]. In each case, casein was much less effective if added after extraction. Similar results were obtained using shoot or root tissue. In oat and maize seedlings, casein could be replaced effectively by PMSF (1 mM), demonstrating that NR-decay in cell-free extracts was due to the action of a general serine-protease.

With wheat shoot extracts, however, the presence of 3% (w/v) casein resulted in a small increase in NR activity (Table 4) but a small decrease in NR half-life at 30° suggesting that general protease action was not responsible for NR-decay. At 4°, however, extraction in the presence of 3% (w/v) casein resulted in a NR half-life of 312 min, compared to 221 min in the absence of casein. Casein had no effect on the NR activity or the NR half-life of partially-purified NR preparations (Table 4). In wheat root extracts, however, NR activity could only be detected in the presence of casein (3% [w/v]) or PMSF (1 mM). In contrast to the other species tested, however, wheat root NR activity in the presence of casein was not stable at 30°, exhibiting a half-life of 14.6 min, compared with a value of 13.8 min for the shoot enzyme in the presence of casein. The mechanisms of decay of NR in wheat root extracts are similar to those in shoot extracts, with the additional effect of a general serine protease.

Unlike the effect in other species studied, the increase in wheat shoot NR activity in the presence of casein was due not to protection against general protease attack [5], nor was it a consequence of increased NR-extraction in the presence of casein. Extrapolation of the decay data to the time of extraction suggested that *ca* 10–12% less NR activity was extracted in the presence than in the absence of casein. Casein acted to prevent the binding of NR to an inhibitor molecule which was present in cell-free extracts [21]. The inhibitor involved could be eliminated from cell-free extracts by NR-treatments such as $(NH_4)_2SO_4$ precipitation, dialysis or elution from Sephadex G-25, the data suggesting a M_r for the inhibitor of less than 1000. Three of the ditelosomic stocks of *T. aestivum* cv. Chinese Spring which exhibited *in vivo* NR activity significantly greater than that of the euploid (ditelo-$7A^S$, -$7B^S$ and -$2D^L$) [4] did not exhibit the same superiority *in vitro* unless extracted in the presence of casein [4], suggesting that this casein-sensitive NR-inhibitor did not operate *in vivo*.

The effects of nitrate (in the absence of NADH) and NADH (in the absence of nitrate) on NR stability were then investigated in crude and partially-purified preparations, using dialysed preparations of the cell-free extract to eliminate problems of endogenous nitrate or

NADH. Nitrate, in the range 5–60 mM, had no effect on the NR decay rate in either enzyme preparation (Table 4). Pre-incubation in the dark of the dialysed cell-free extract with NADH (20 μM) at 30° resulted in an initial immediate inhibition (60–68%) of NR activity followed by a gradual increase in NRA over a 10 min period to a maximum value *ca* 80% greater than the original control activity. From that point, the half-life of NADH–NR at 30° was 25.6 min compared with 16.2 min for the crude extract enzyme in the absence of NADH (Table 4), while the corresponding values for 7.5 S CR half-life were 29.0 min and 18.6 min, respectively. Pre-incubation of a 30–50% saturated $(NH_4)_2SO_4$ preparation with NADH resulted in the initial inhibition but not the subsequent stimulation, and the decay rate of the residual activity was unchanged (Table 4). As both the NADH–NR and CR activities of the 7.5 S complex appear to be equally affected by the decay factors, it is likely that one of the factors acts *via* the NADH-binding site (shared by both partial activities) of the 7.5 S complex, with NADH stabilizing the enzyme activities by displacing the decay factor. This mechanism of action has been reported for NR-inactivating factors from other species [11, 22].

The inactivation of partially- or highly-purified NR by pre-incubation with NADH could be prevented by the inclusion of nitrate (as low as 50 μM) in the pre-incubation buffer. NADH-inactivated NR could be reactivated by incubation for 5 min with potassium ferricyanide (final concentration of 1 mM) at 20° (Table 5) [13, 23]. The inhibitory effect of NADH was less in the light than in the dark, and illumination of the refrigerated inactivated preparation with fluorescent lighting (*ca* 300 μE/S/m^2) for 5 min resulted in a reactivation of the NR (Table 5). Photographic green or red filters prevented reactivation. Maximal reactivation of NR by direct illumination required the presence of FAD in the buffer (Table 5).

This phenomenon of reversible NADH-mediated inactivation of NR has been shown to play a part in the post-translational regulation of NR activity *in vivo* in several species, with part of the NR existing in an inactive form under certain conditions. Under the standard growth room conditions used here, no inactive NR was extracted (Table 5). Inactive NR could be obtained from plants grown with low levels of nitrate (Table 6), but diurnal fluctuations of *in vivo* NR activity (Fig. 5) were not due to inactivation and reactivation of NR [P. Jones, unpublished results]. Even under those stress conditions where inactive NR was present, the total NR in the tissue (active plus inactive) was still significantly less than that found in the control unstressed plants (Table 6), demonstrating that this mechanism of NR-inactivation is not the only reason for low NR activity in these plants.

Table 5. Inactivation and reactivation of NADH–NR *in vivo* and *in vitro*

Treatment applied to enzyme preparation (30–50% $(NH_4)_2SO_4$ fraction)	NR activity (n moles/minute/mg protein)
—	3.16
NADH (20 μM)	0.62
NADH (20 μM) plus ferricyanide (1 mM)	3.09
NADH (20 μM) plus light (300 μE/S/m^2)	1.68
NADH (20 μM) plus light (300 μE/S/m^2) plus FAD (10 μM)	3.04
NADH (20 μM) plus FAD (10 μM)	0 76
Ferricyanide (1 mM)	3.11
Light (300 μE/S/m^2) plus FAD (10 μM)	3.04
LSD 5%	0.21

Table 6. The effect of nutrient solution nitrate concentration on NR activity and half-life

Nitrate concentration (mM)	NR activity (nmol nitrate/min/mg protein)			*In vitro* NR half-life (min) at 30°	
	In vivo	*In vitro* (−ferricyanide)	*In vitro* (+ferricyanide)	Crude extract	30–50% $(NH_4)_2SO_4$
0	0.38	0.26	0.52	9.1	31.1
1	0.53	0.54	0.84	10.8	28.4
2	0.79	1.21	1.27	13.5	29.8
4	1.23	2.21	2.01	15.9	29.1
7	1.78	2.89	2.98	17.4	31.6
10	2.09	3.31	3.21	16.8	30.4
20	2.20	3.14	3.19	16.2	32.0
40	2.16	3.28	3.24	17.1	30.8
LSD 5%	0.17	0.27		1.2	3.0

Plants were grown on vermiculite at 22° under a 16-hr photoperiod. The ferricyanide treatment consisted of incubation of the enzyme preparation with potassium ferricyanide (1 mM) for 5 min at 20°. Control extracts were incubated in the absence of ferricyanide.

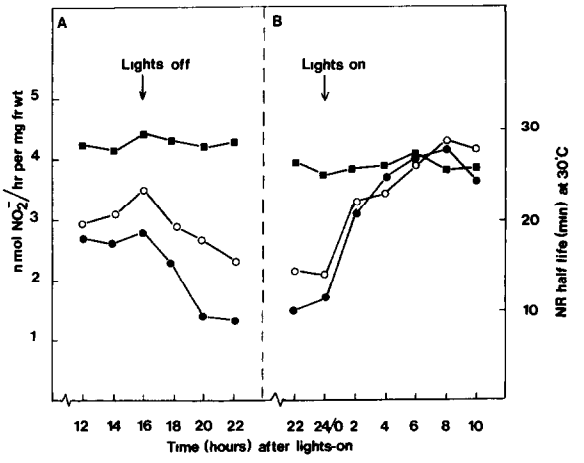

Fig. 5. Diurnal fluctuations of *in vitro* NR half-life at 30° in crude extracts (●) and 30–50% saturated $(NH_4)_2SO_4$ fractions (■), and of NR activity *in vivo* (○). The study was performed as two separate replicated experiments, A (hr 12 to hr 22) and B (hr 22 to hr 10), within a 16-hr photoperiod. Growth room conditions are described in Experimental.

Relationships between in vitro *NR stability and* in vivo *NR activity*

It has been shown in wheat [4] and other species [24] that *in vivo* NR activity exhibits regular diurnal fluctuations. When *in vitro* NR half-life in crude and partially-purified preparations was monitored throughout the light and dark periods of the photoperiod, it was found that whereas *in vitro* NR half-life in crude extract varied in phase with *in vivo* NR activity, little variation in NR decay rates were observed in partially-purified preparations over the study period (Fig. 5). This suggests that variations in the activity of one of the NR-destabilizing factors may be responsible for NR activity fluctuations *in vivo*, although other possible causes have been proposed [24].

The effect of nitrate level in the nutrient medium on *in vivo* NR activity and *in vitro* NR half-life showed that, with increases in nitrate concentration, both activity and half-life of NR increased (Table 6), although the increase in enzyme stability was only observed in crude, rather than partially-purified NR preparations (Table 6). This result supports data from *Neurospora* which demonstrated that the stimulatory effect of nitrate on NR activity may be due to post-translational control (such as reduced activity of the wheat NR-decay factor) as well as transcriptional control [25]. There is no evidence to suggest that variations in NR activity *in vivo* are related to variations in the rate of 7.5 S dissociation.

From these studies, information has been obtained regarding the post-translational control of NR activity and stability *in vitro* in wheat. Several of the mechanisms involved have been identified. Evidence shows that two factors present in wheat shoot cell-free extracts are involved in the decay of NR *in vitro*, and one or both may play a role in determining NR activity *in vivo*. We are currently involved in the isolation and characterisation of these factors.

EXPERIMENTAL

Plant material. The wheat (*Triticum aestivum*) variety used was cv. Chinese Spring, while some experiments were repeated with wheat cv. William. Ditelosomic stocks of Chinese Spring (seed of which was kindly provided by Dr. Colin Law, Plant Breeding Institute, Cambridge, U.K.) were also used in comparative studies with Chinese Spring euploid. Other species studied include maize (*Zea mays*, cvs. Salute and Earliking), barley (*Hordeum vulgare*, cv. Midas), oat (*Avena sativa*, cv. Mostyn) and rye (*Secale cereale*, cv Petkus). Seedlings were grown in sand culture as described previously [26], fed with a nutrient soln containing 10 mM KNO_3 [26] and grown under a 16-hr photoperiod at 20°. For root studies, seedlings were grown under similar conditions in liquid culture [26] For the studies on the effect of nitrate concentration on NR activity and half-life, seedlings were grown in vermiculite.

Extraction. Wheat seedlings were harvested at Seedling Plastochron Index 1.35–1.45 [27], while other species were harvested two days after the emergence of the second leaf. Tissue extraction was carried out according to ref. [4] The basic buffer was 150 mM K–Pi, pH 7.5, and supplements to this buffer are described in the text. All purification procedures were performed at 2°

$(NH_4)_2SO_4$ fractionation The cell-free supernatant was adjusted to 30% satn with $(NH_4)_2SO_4$ by the gradual addition of the appropriate weight of solid $(NH_4)_2SO_4$. After 15 min the precipitated protein was collected by centrifugation at 20000 *g* for 15 min. The supernatant was then adjusted to 50% satn with $(NH_4)_2SO_4$ and the resultant protein pellet, the 30–50% fraction, was resuspended in 1/5 original vol. of extraction buffer. If enzyme activity was to be assayed in the 30–50% fraction, the preparation was desalted prior to assay by elution from a column (3.8 × 26 cm) of Sephadex G-25 (medium), equilibrated with the extraction buffer.

NR-purification. 140 g fresh weight tissue were extracted and fractionated to obtain the 30–50% saturated $(NH_4)_2SO_4$ preparation. This sample (120 ml) was run through a column (4 × 10 cm) of Blue Dextran-Sepharose 4B prepared according to ref. [14]. The preparation buffer and the column equilibration buffer were 10 mM K–Pi buffer, pH 7.5, containing dithiothreitol (1 mM) and FAD (10 μM). Loading, washing and elution were carried out at a rate of 35 ml/hr. NR was eluted by the application to the column of equilibration buffer supplemented with NADH (5 μM).

Ultrogel A6 gel filtration. A 10 ml sample of enzyme preparation was applied to a column (2.8 × 86 cm) of Ultrogel A6 equilibrated with 50 mM K–Pi buffer, pH 7 5, containing dithiothreitol (1 mM) unless CR activity was to be assayed in the fractions. An elution rate of 20 ml/hr was used. The column was calibrated using the elution vols of standard proteins of known Stokes radius namely thyroglobulin (porcine, 7.0 nm), catalase (bovine liver, 5.2 nm), alcohol dehydrogenase (yeast, 4.6 nm) and albumin (bovine serum, 3.5 nm). Blue Dextran and chymotrypsinogen were used to determine V_0 and V_t values, respectively, and the elution data were plotted as K_{av} against Stokes radius.

Ultrogel AcA22 gel filtration. A 5 ml sample was applied to a column (2.5 × 33 cm) of Ultrogel AcA22. The equilibration buffer and the calibration standards were as for Ultrogel A6 (with the omission of BSA), an elution rate of 12 ml/hr was used, and 1 ml fractions were collected.

Sucrose density gradient centrifugation. The enzyme sample (0.2 ml) was layered onto the 4.4 ml linear 5–20% (w/v) sucrose gradient, over a 0.5 ml 50% (w/v) sucrose cushion. The sucrose solns were prepared in 50 mM K–Pi buffer pH 7.5 containing dithiothreitol (1 mM), unless CR activity was to be assayed, and FAD (10 μM). After centrifugation at 149000 *g* for 16 hr at 2°, the gradients were fractionated from the bottom of the tube into 20 fractions. The gradients were calibrated by the use of standard proteins of known sedimentation coefficient, namely catalase (bovine liver, 11.3 S), alcohol dehydrogenase (yeast, 7.4 S), haemoglobin (bovine blood, 4.11 S) and myoglobin (bovine skeletal muscle, 2.04 S). Fractionation data were plotted as sedimentation coefficient against fraction number.

Disc gel electrophoresis. Electrophoresis was carried out at 4° according to the method of ref. [28], using 7.5% running gels and a running buffer pH of 8.5. The gels were stained for protein using Coomassie Blue or Amido Black.

Enzyme assays. In vivo NADH–NR activity was assayed by the method of ref. [26], while the activity *in vitro* was assayed using the method of ref. [4]. When *in vitro* NADH–NR was to be assayed in fractions from Ultrogel columns or sucrose density gradients where the buffer lacked dithiothreitol, dithiothreitol was added to the incubation medium to a final concn of 1 mM. In those experiments where the effects of different concns of NADH were compared, e.g. Km (NADH) determination and NR-inactivation by NADH, excess NADH was eliminated by the addition of 10 mg activated charcoal after the enzyme reaction had been terminated. $FADH_2$–NR and MV°–NR were assayed by the method of ref. [29]. NADH–CR was assayed by incubating 2 4 ml 200 mM K–Pi buffer, pH 7.5, 0 2 ml 100 μM cytochrome *c*, 0.2 ml enzyme preparation and 0.2 ml 2 mM NADH in a cuvette at 25°, and measuring the rate of change of *A* at 550 nm

Protein determination. The method used depended on the protein concentration of the preparation. In high-protein preparations, e.g. cell-free extracts, 30–50% satd $(NH_4)_2SO_4$ preparations, the method of ref. [30] was used, while at low levels of protein, such as those found in fractions eluted from Ultrogel or Blue Dextran-Sepharose 4B columns, the procedure described in ref. [31] was used.

Enzyme half-life ($t_{1/2}$) *values.* Aliquots of the enzyme preparation were pre-incubated at 30° for from 0 to 40 min, after which time the residual activity remaining in the sample was assayed. In the case of the partial activities of the 7.5 S complex, the aliquots were subjected to sucrose density gradient centrifugation after pre-incubation and the residual 7.5 S activities remaining in the gradient were measured for reasons described in the text. In all cases, the $t_{1/2}$ value was calculated from the regression of logarithm [% residual activity] against time.

DTNB-binding. Studies on the binding of DTNB to NR were performed by the method of ref. [32]. A sample (0.5 μg protein) of highly-purified NR was incubated, in the absence of dithiothreitol, with 1 μmol DTNB in 20 mM K–Pi buffer, pH 7.5, under reducing conditions. The stoichiometry of the steady-state change in A at 412 nm was calculated using a millimolar extinction coefficient for 2-nitro-thiobenzoic acid of 13.6 [32].

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