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In Vitro Stability of Nitrate Reductase from Wheat Leaves

III. ISOLATION AND PARTIAL CHARACTERIZATION OF A NITRATE REDUCTASE-INACTIVATING FACTOR[1]

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ABSTRACT

A nitrate reductase (EC 1.6.6.1)-inactivating factor has been isolated from 8-day-old wheat leaves. The purification schedule involved ammonium sulfate precipitation, Sephadex G-100 filtration, DEAE-cellulose chromatography, and Sephadex G-150 filtration. No accurate assessment could be made as to the degree of purification relative to crude extract as the inactivating factor could not be detected in crude extract. However a 2,446-fold purification was achieved from the ammonium sulfate fraction to the pooled enzyme from the Sephadex G-150 step.

The inactivating factor was heat-labile and had a molecular weight of 37,500. The inactivating factor was particularly sensitive to the divalent metal chelators, 1,10-phenanthroline and bathophenanthroline. Evidence indicated that Fe^{2+} may be the functional metal. The trypsin inhibitors *N*-α-*p*-tosyl-L-lysine chloromethyl ketone and α-*N*-benzoyl-L-arginine were inhibitory. However, phenylmethyl sulfonyl fluoride, an inhibitor of serine peptide hydrolases, was not inhibitory. Neither casein nor hemoglobin nor a range of artificial substrates were hydrolyzed by the inactivating factor. Highly purified wheat leaf nitrite reductase (EC 1.7.99.3) and ribulose 1,5-bisphosphate carboxylase:oxygenase (EC 4.1.1.39) were not affected by the nitrate reductase-inactivating factor.

The inactivating factor was more active toward the NADH-nitrate reductase compared to either of the component enzymic activities flavin adenine mononucleotide-nitrate reductase and methyl viologen-nitrate reductase. The NADH-ferricyanide reductase (diaphorase) component was the least sensitive.

In a previous paper (17) we reported on the occurrence in crude extracts from wheat leaves (*Triticum aestivum* L.) of two types of factors which appeared to affect the *in vitro* stability of highly purified nitrate reductase. One of these factors (II) reduced the stability of NR[3] while the other factors (I and III) seemed to confer stability on NR.

In this paper we report on the isolation and partial characterization of factor II. For want of a more specific description we will refer to this as NR-inactivating factor.

MATERIALS AND METHODS

PREPARATION OF HIGHLY PURIFIED NR

Enzyme was prepared from the leaves of 8-day-old wheat seedlings according to the method previously described (16).

ASSAY OF INACTIVATING FACTOR

The procedure employed was based on that described previously (17). Equal volumes, usually 200 μl, of highly purified NR (<1 μg/ml) and inactivating factor were incubated at 10 C. This assay temperature is a compromise, the highly purified NR is very unstable (16), and 10 C was chosen so as to be sufficiently low enough to minimize thermal denaturation of NR but at the same time 10 C is high enough to allow the inactivating factor to affect the NR. After 60 min the residual NADH-NR activity was measured according to the method described by Sherrard and Dalling (16). One unit of inactivating factor activity is equal to 1% loss of NR activity compared to a suitable control, *i.e.*

$$\text{Units per h} = 100 - \left[\frac{\text{NR + factor}}{\text{NR − factor}} \times 100\right]$$

PREPARATION OF OTHER PLANT ENZYME SUBSTRATES

Nitrite reductase was extracted from frozen 8-day-old primary leaves with 25 mM K-phosphate, 5 mM EDTA, 5 mM L-cysteine-HCl adjusted to pH 7.5. The 50 to 65% ammonium sulfate fraction was dissolved in grinding buffer and loaded onto a column (45 × 2.6 cm) of Sephadex G-100 equilibrated with G-100 buffer (20 mM K-phosphate [pH 7.7] containing 0.1 mM DTT). Those fractions with nitrite reductase activity were loaded onto a DEAE-cellulose column (20 × 1.5 cm) equilibrated with the G-100 buffer and the enzyme eluted with a linear gradient of NaCl (0–0.2 M in a volume of 250 ml). Nitrite reductase activity was measured using procedure II as described by Hucklesby *et al.* (3).

Ribulose 1,5-bisphosphate carboxylase:oxygenase was prepared from leaves of 12-day-old primary leaves by double chromatography over Sepharose 6B according to the procedure of Peoples and Dalling (11).

MOL WT DETERMINATION

A column (86 × 2.6 cm) of Sephadex G-150 was equilibrated with 50 mM K-phosphate (pH 7.0)-100 mM KCl buffer. The column was calibrated with Cyt *c* (12,500), bovine pancreas chymotrypsinogen A (25,000), ovalbumin (45,000), BSA (68,000) and rabbit muscle aldolase (158,000). Three ml of each standard (2

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[3] Abbreviations: ATEE: *N*-acetyl-L-tyrosine ethyl ester; BAEE: α-*N*-benzoyl-L-arginine ethyl ester; BTEE: *N*-benzoyl-L-tyrosine ethyl ester; $FADH_2$: reduced flavin adenine dinucleotide; $FMNH_2$: reduced flavin mononucleotide; MVH: reduced methyl viologen; NR: nitrate reductase (EC 1.6.6.1); *p*-CMB: *p*-chloromercuribenzoic acid; Phe-β-NA: L-phenylalanine-β-naphthylamide; PMSF: phenylmethyl sulfonyl fluoride; TAME: *p*-tosyl-L-arginine methyl ester; TLCK: *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK: L-1-tosylamide-2-phenylmethyl chloromethyl ketone.

mg/ml) or 3 ml of inactivating factor (approximately 3 μg protein) was loaded and 3.2-ml fractions were collected at a flow rate of 20 ml/h.

RESULTS

ISOLATION OF NR-INACTIVATING FACTOR

As already outlined in the previous paper (17) the presence of the NR-inactivating factor could not be detected in crude extract or when crude extract was fractionated with Sephadex G-100. This was due to the considerable activity of factors I and III. NR-inactivating factor was detected only following ammonium sulfate fractionation of crude extract. Because of this interference the following procedure was adopted.

Ammonium Sulfate Fractionation. Crude enzyme was prepared by grinding 80 g of 8- to 10-day-old primary leaves in 240 ml of an ice-cold buffer which contained 25 mM K-phosphate, 5 mM EDTA, and 5 mM L-cysteine-HCl (pH 7.5). After filtering through Miracloth (Chicopee Mills, Inc., Milltown, N.J.) the extract was centrifuged for 20 min at 35,000*g*. In most experiments the 30 to 45% ammonium sulfate fraction was prepared, and the precipitate dissolved in 32 ml of extraction buffer.

Sephadex G-100 Filtration. The 30 to 45% ammonium sulfate fraction was applied to a Sephadex G-100 column (50 × 7.0 cm) equilibrated with 25 mM K-phosphate (pH 7.5) containing 5 mM EDTA. Ten-ml fractions were collected at a flow rate of 90 ml/h (Fig. 1). Factors I, II, and III are shown.

DEAE-Cellulose Chromatography. Those fractions containing NR-inactivating factor were immediately loaded onto a DEAE-cellulose (Whatman DE32) column (41 × 1.5 cm) equilibrated with 10 mM K-phosphate (pH 7.5). The column was then washed with an equal volume of equilibrating buffer (usually 150 ml) and the NR-inactivating factor was eluted using a linear gradient of NaCl. The gradient was formed by mixing 250 ml equilibration buffer and 250 ml of 0.5 M NaCl in equilibration buffer. The pumping speed was 30 ml/h and 5-ml fractions were collected (Fig. 2).

Sephadex G-150 Chromatography. Those fractions containing NR-inactivating factor were bulked and concentrated by dialysis against PEG 6000. Further purification was achieved by chromatography on Sephadex G-150 (86 × 2.6 cm) equilibrated with 50 mM K-phosphate (pH 7.0). The flow rate was 20 ml/h and 3.2-ml fractions were collected. A summary of the purification procedure is shown in Table I. Yield of NR-inactivating factor (with respect to crude extract) cannot be calculated because of interference from factors I and III. Nevertheless purification is considerable, the specific activity increases by 2,446-fold from the ammonium sulfate fraction to the pooled enzyme from the Sephadex G-150 step.

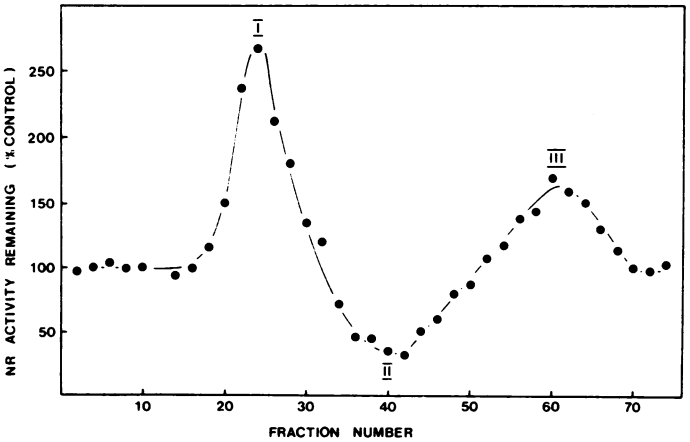

FIG. 1 Separation of factors I, II, and III by Sephadex G-100 chromatography.

Only partial success was achieved using the nonspecific affinity system of hemoglobin coupled to Sepharose 4B according to the method described by Frith *et al.* (2). Although the inactivating factor was completely adsorbed by this system at pH 6.0, recovery of the factor was variable. Elution could only be achieved at pH <3.5 and under these conditions the factor was very unstable. This method was not used routinely.

GENERAL PROPERTIES OF NR-INACTIVATING FACTOR

Several properties of the inactivating factor were difficult to assess because the substrate NR also proved to be very sensitive to most of the variables which were examined. In each of the following experiments, NR-inactivating factor was prepared according to the procedure summarized in Table I.

Effect of Inactivating Factor Concentration. Evaluation of the relation between amount of inactivating factor and activity is confounded by the fact that loss of NR activity is exponential with time (16). However, based upon a standard 60-min incubation at 10 C and a fixed amount of NR (<1 μg) as substrate, a linear relation was observed for loss of NR with increasing amount of inactivating factor up to 0.2 μg (Fig. 3). Because of this effect, the following experiments were run with dilutions of the inactivating factor such that approximately 50% loss of NR occurred in 60 min.

Effect of pH. The effect of pH on activity of the NR-inactivating factor is shown in Figure 4. The optimum pH was found to be 7.0. In this experiment allowance has been made for the instability of NR with change in pH. The inactivating factor was very unstable at low pH with a half-life of less than 5 min at pH 3.2 (data not shown).

Effect of Temperature on Stability. The inactivating factor is heat-labile, losing approximately 50% of its activity when heated for 10 min at 40 C. It is more stable than NR which lost 50% of its activity in 10 min at 31 C (Fig. 5).

Effect of Inhibitors. A wide range of inhibitors were examined for their effect on the NR-inactivating factor (Table II). The alkylating reagent, iodoacetamide, was effective (no effect on NR); the inhibition was not reversed by cysteine. PMSF, which is an inhibitor of serine peptide hydrolases, did not inhibit the inactivating factor; however, the trypsin inhibitor TLCK was inhibitory. TPCK, an inhibitor of chymotrypsin-like enzymes, was examined, but because of difficulties associated with keeping it in solution its effectiveness could not be assessed. The inability of PMSF to inhibit the inactivating factor was not due to the presence of cysteine in the NR preparation as no inhibition was observed when cysteine was omitted. Soybean trypsin inhibitor was not inhibitory. The trypsin inhibitor, α-*N*-benzoyl-L-arginine was inhibitory. However, proflavine hemisulfate, another trypsin inhibitor, could not be assessed as it was a very effective inhibitor of NR (100% at 10 μM).

Several reagents which react with sulfhydryl groups were examined; however, these were particularly difficult to assess because NR showed great sensitivity (50% at 1 μM *p*-CMB). At those concentrations where NR was only partially inhibited neither *p*-CMB nor *N*-ethylmaleimide was inhibitory to the inactivating factor.

The broad spectrum metal chelators EDTA and α,α-dipyridyl were not inhibitory. However, both bathophenanthroline and 1,10-phenanthroline, which are more selective towards Fe^{2+}, were inhibitory (Fig. 6). Bathocuproine was also inhibitory, but only at higher concentrations. When inactivating factor which had previously been inhibited with low concentrations (<20 μM) of bathocuproine was incubated with NR according to the standard assay procedure, the residual NR after 60 min was always greater than that of the control NR incubated under identical conditions; that is, the bathocuproine-treated inactivating factor consistently caused an apparent increase in stability of NR. NR was inhibited by bathocuproine (50% at 6 μM) and bathophenanthroline (50% at

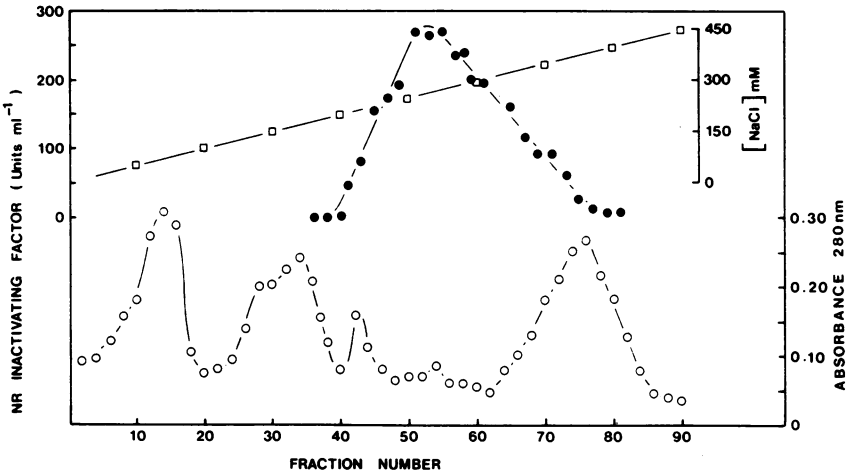

FIG. 2. Elution profile of NR-inactivating factor from DEAE-cellulose. The column was developed with a linear gradient of NaCl as described under "Materials and Methods" (□); NR-inactivating factor (●); $A_{280\ nm}$ (○).

Table I. *Summary of NR-inactivating Factor Purification*

Details of purification are described under "Results." A unit of NR-inactivating factor activity is defined as the per cent loss of highly purified NR when incubated with inactivating factor for 60 min at 10 C.

Purification Step	Volume	Protein	Inactivating Factor Units	
	ml	*mg*	*per ml*	*per mg*
Crude extract	240	1574		
Ammonium sulfate (30–45%)	32	483	175	11.6
Sephadex G-100 (7 × 50 cm)	161	41	239	919
DEAE-cellulose	50	0.5	194	19,400
Sephadex G-150 (2.6 × 86 cm)	45	0.2	126	28,378

65 μM) but not by 1,10-phenanthroline.

Only partial success was achieved in experiments designed to identify the essential metal of the inactivating factor. Treatment of inactivating factor by dialysis against 1 mM 1,10-phenanthroline caused complete inhibition; however, when the inhibitor was removed by prolonged dialysis against 10 mM Tris-HCl (pH 7.0), then 80% of the inactivating factor activity was restored (Table III). Of the divalent metals studied only Fe^{2+} was capable of completely restoring activity to that of the nontreated control. Ca^{2+} and Mg^{2+} both inhibited the inactivating factor. Cu^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} were not assessed because each caused 100% inhibition of NR at the concentrations used.

Effect of Inactivating Enzyme on NR Component Enzymes. Highly purified NR was incubated with inactivating factor as described under "Materials and Methods" and after 60 min the residual activity of the component enzymes $FMNH_2$-NR, MVH-NR, and NADH-ferricyanide reductase was compared to the NADH-NR (Table IV). None of the components was as sensitive to the inactivating factor as the NADH-NR activity. Further purification of the NR eluted from Blue Dextran Sepharose has revealed the presence of an additional NADH-ferricyanide reductase (15). However, this enzyme and the NR component enzyme NADH-ferricyanide reductase were both relatively resistant to the inactivating factor.

Mol Wt. The NR-inactivating enzyme had a mol wt of 37,500 (SE ± 2,000). This was measured from a calibrated Sephadex G-150 column.

Specificity of NR-inactivating Enzyme. Several artificial substrates which are indicative of trypsin-like (TAME, BAEE), chymotrypsin-like (BTEE, ATEE), esterase (ATEE) and aminopeptidase (Phe-β-NA) activity were not hydrolyzed by the inactivating factor. Casein (pH 7.0), with and without mercaptoethanol, and hemoglobin (pH 5.0) were not degraded. The inactivating factor had no apparent effect on highly purified nitrite reductase and ribulose 1,5-bisphosphate carboxylase:oxygenase from wheat leaves.

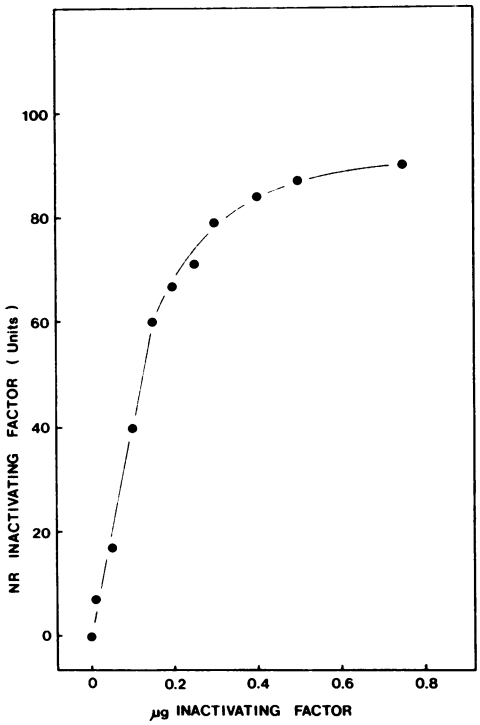

FIG. 3. Effect of varying level of NR-inactivating factor on residual NR activity. Several dilutions of highly purified NR-inactivating factor (1 μg/ml) were prepared and 200 μl of each was incubated with 200 μl NR at 10 C. After 60 min the residual NR activity was measured.

DISCUSSION

There are two possible mechanisms by which the NR-inactivating factor can operate. The first of these is by reversible or nonreversible inhibition and in plants reversible inhibition by high mol wt compounds (probably protein) has been reported for *Lemna* aldolase (14), maize scutellum catalase (18), acid invertase of sweet potato (9) and potato (12), isocitrate lyase from sunflower cotyledons (20), mung bean seed phosphoinositol kinase (8), and soybean leaf NADH-NR (4).

In the case of most of these inhibitors, only partial characterization has been reported, and it is difficult to decide on some

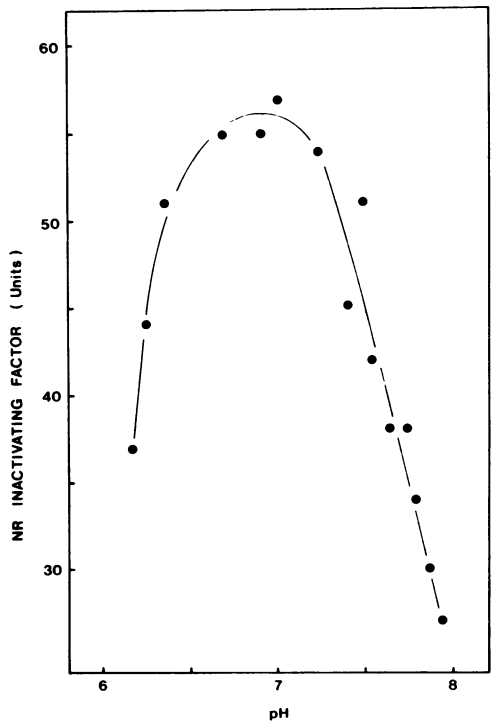

FIG. 4. Effect of pH on activity of NR-inactivating factor. Two parallel experiments were carried out, one to examine the effect of pH on NR (control), and the other to examine the effect of pH on activity of the NR-inactivating factor. The reaction system was 200 μl NR (<1 μg), 200 μl 100 mM K-phosphate buffer. After 60 min at 10 C the residual NR was measured and the units of NR-inactivating factor activity at each pH were calculated as described under "Materials and Methods" after reference to the respective control.

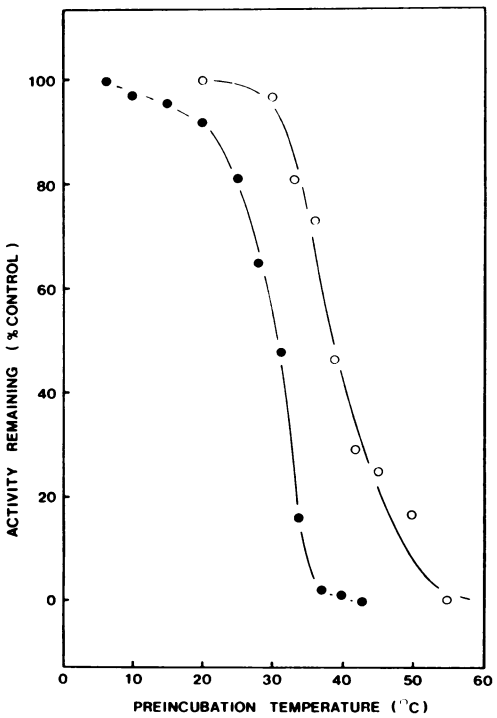

FIG. 5. Effect of temperature of preincubation on stability of NR and NR-inactivating factor. Samples of both were incubated at the temperatures shown for 10 min. Residual activity of NR (●) and NR-inactivating factor (○) were measured according to "Materials and Methods" and the data are plotted relative to a control which was preincubated at 4 C.

Table II. *Effect of Inhibitors on Activity of NR-inactivating Factor*

Each inhibitor was dissolved in 50 mM K-phosphate (pH 7.0) and 200 μl was mixed with 200 μl inactivating factor (0.1 μg) and after 30-min preincubation at 10 C, 200 μl NR (<1 μg) was added and the incubation continued for a further 60 min. With several inhibitors a small amount of nonaqueous solvent had to be used and the final concentration made up with 50 mM K-phosphate (pH 7.0). In these cases appropriate controls were also included. The data are expressed as per cent inhibition compared to a minus-inhibitor control.

Inhibitor	Concentrations	Inhibition
	mM	%
Iodoacetamide	25.0	91
PMSF	1.0	0
TLCK	2.5	90
α-*N*-Benzoyl-L-arginine	1.0	100
Soybean trypsin inhibitor	0.01	0
N-Ethylmaleimide	0.1	10
p-CMB	0.001	3
EDTA	10.0	0
α,α-Dipyridyl	1.0	0
1,10-Phenanthroline	2.5	93
Bathophenanthroline	1.0	98
Bathocuproine	1.0	85

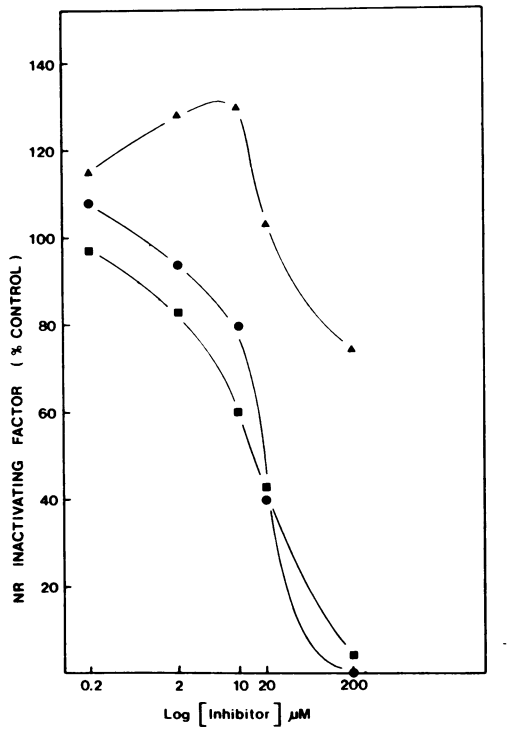

FIG. 6. Effect of varying concentration of 1,10-phenanthroline, bathophenanthroline, and bathocuproine on activity of NR-inactivating factor. Each inhibitor (200 μl) was preincubated with 200 μl inactivating factor (0.1 μg) for 30 min at 10 C; 200 μl of NR (<1 μg) was added and after 60 min at 10 C the residual NR was measured. Data are expressed as NR-inactivating factor activity relative to a minus inhibitor control. In each case the effect of inhibitor on NR was assessed and the calculation of units of NR-inactivation factor activity takes into account any effect on NR. Bathophenanthroline (●), bathocuproine (▲), and 1,10-phenanthroline (■).

unifying feature. However, they are for the most part thermolabile, highly specific and with the exception of the phosphoinositol kinase inhibitor (8), they have mol wt in the range 15,000 to 50,000.

The second mechanism by which NR may be inactivated is

Table III. *Ability of Divalent Metals to Restore Activity of 1,10-Phenanthroline-treated Inactivating Factor*

Inactivating factor (<1 μg/ml) was first dialyzed against 1 mM 1,10-phenanthroline for 24 h at 4 C and then against several changes of 10 mM Tris-HCl (pH 7.0) for 48 h at 4 C. The control was dialyzed against the Tris buffer for 72 h. Inactivating factor (200 μl) was mixed with 200 μl of 10 mM divalent cation and after a preincubation of 60 min at 10 C 200 μl of highly purified NR (<1 μg protein) was added and the incubation continued for a further 60 min. Residual NR was then measured as described under "Materials and Methods." Data show units of inactivating factor activity.

Treatment	Units Inactivating Factor Activity	
	+1,10-Phenanthroline	Control
No additions	37.5	46.8
Ca^{2+}	26.9	24.3
Fe^{2+}	49.6	49.6
Mg^{2+}	10.0	28.6

Table IV. *Susceptibility of NR Component Enzymes to Inactivating Factor*

Highly purified NR was incubated with NR-inactivating factor as described under "Materials and Methods." After 60 min at 10 C the residual activity of NADH-NR and the component enzymes was measured (16). Units of NR-inactivating factor were calculated for each enzyme (substrate) as described for NADH-NR under "Materials and Methods."

Component	Units of Inactivating Factor Activity	
	NR Blue Dextran	NR Sepharose 6B[a]
NADH-NR	75	81
$FMNH_2$-NR	58	62
MVH-NR	49	53
NADH-ferricyanide reductase	19	15

[a] NR (Sepharose 6B) is enzyme which has been further purified beyond elution from Blue Dextran Sepharose by chromatography on Sepharose 6B (15).

proteolysis. Wallace (21) has described in some detail an NR-inactivating enzyme isolated from maize roots. Although this enzyme can hydrolyze casein and is inhibited by PMSF, there is as yet no evidence that the mechanism of NR inactivation is by protein hydrolysis. The inactivating enzyme found by Wallace, although highly specific for NR, is also capable of inactivating yeast tryptophan synthase (24). In this respect, it is similar to inactivators from *Neurospora* (30) and yeast (7) which also inactivate this enzyme. Furthermore, both maize root-inactivating enzyme and the inactivator from yeast, previously designated protease B (13), degraded casein, were inhibited by PMSF and had a neutral to alkaline pH optimum (24). Unlike the heat-inducible NR inhibitor(s) found in the root tips of corn seedlings by Aslam (1), the inactivating enzyme was heat-labile. Its main site of action was thought to be the NAD(P)H Cyt *c* reductase component of NR and the effect on the $FADH_2$-NR component varied between species (23). In a more recent paper, Wallace and Johnson (25) have reported the separation of a number of extramitochondrial types of Cyt *c* reductase from maize, and it appears that more than one is affected by the inactivating enzyme. It is still unclear whether the Cyt *c* reductase enzymes affected by the inactivating enzyme are all related to NR.

Pan and Marsh (10) reported a protein-like macromolecular inhibitor in root extracts of corn seedlings. Although there appeared to be two inhibitor reactions, these could not be separated by chromatography. This inhibitor was relatively specific for NR. A NR inhibitory factor was found in cultured rice cells and a number of other species by Yamaya and Ohira (27, 28). Its characteristics differed from those of the factor reported by Wallace. Although both were inhibited by EDTA and *o*-phenanthroline, the factor from rice cells had no caseolytic activity and was practically unaffected by PMSF. In addition, the rice cell factor alone was susceptible to *p*-CMB. Yamaya and Ohira (29) suggest that the inhibitory factor from rice cells is a protein which binds reversibly to the NR complex by a mechanism similar to the binding of inhibitory proteins to invertase (9, 12). Inactivation by the factor could be reversed by the addition of NADH during incubation, and this protective effect was canceled by addition of nitrate or Cyt *c*. Because these compounds oxidize NR, it appears that the inactivating factor acts only on the oxidized form of the enzyme.

Kadam *et al.* (5, 6) have reported an inhibitor of NR from the roots of rice seedlings. The inhibitor is suggested to be a peroxidase since it has a number of characteristics similar to those of horseradish peroxidase. The effect on NR of the inhibitor and horseradish peroxidase is almost identical. In addition, horseradish peroxidase is known to oxidize several enzymes containing active sulfhydryl groups and NR is sensitive to oxidation in this manner. Both factors inhibit NR from wheat and rice with inhibition being reversed by NADH (6). Inasmuch as peroxidase activity was found to be high in roots compared with leaves, it is possible that this enzyme is responsible for the inhibitor effect observed.

The peroxidase-like factor from rice seedlings (5, 6) and the factor isolated by Yamaya and Ohira (27–29) are both inhibited by NADH and affect NADH-NR and $FMNH_2$-NR, but neither inhibits the MVH component of NR nor the NADH dehydrogenase activity. However, the possibility of the factor reported by Yamaya and Ohira being a peroxidase is ruled out by the difference in mol wt (200,000 *cf.* about 40,000). In contrast, the mol wt of horseradish peroxidase is similar to that of the factor found by Wallace in the roots of maize (44,000) and in addition both have similar properties in respect to heat lability. However, these two factors differ in response to the presence of nonplant protein. While casein inhibits the action of the inactivating enzyme found by Wallace (22) and was hydrolyzed by the inactivating enzyme, casein had no effect on the peroxidase-like factor isolated by Kadam *et al.* (6).

Recently, Sorger *et al.* (19) and Walls *et al.* (26) reported the isolation and characterization of two NR inactivators from *Neurospora crassa* (inactivator I and inactivator II). These factors differ markedly in pH optimum and sensitivity to EDTA, PMSF, cycloheximide, and heat treatment. Inactivator II appears to be regulated by the nitrogen metabolism of the cell and is thought to be responsible for rapid *in vivo* decay of NR in nitrogen-starved cells. Inactivator II is similar to the inactivating enzyme described by Wallace with respect to PMSF sensitivity and mol wt but differs markedly in pH optimum (5.0 *cf.* 7.0) and EDTA sensitivity.

The difficulty with all of these studies is being able to make an unequivocal choice between proteolysis and inhibition. This is especially so when dealing with NR as the substrate because the amount of protein subject to inactivation is very small (<1 μg) and detection of proteolysis by such conventional techniques as release of trichloroacetic acid-soluble amino-*N* is impossible. However, some distinction can be made from the kinetics of inactivation. Proteolysis is a continuing phenomenon which if given enough time, regardless of the initial NR concentration, will result in irreversible inactivation of all of the NR. In contrast, inhibition will eventually reach an equilibrium level of residual NR which will be dependent on the initial concentrations of NR and inhibitor and the K_i for the inhibitor.

In the case of the NR-inactivating factor from wheat leaves, we have no conclusive evidence that inactivation is due to proteolysis. The inactivating factor has no detectable hydrolytic activity toward casein and hemoglobin, or several artificial substrates that are taken to be indicative of trypsin-like, chymotrypsin-like, ester-

ase or aminopeptidase activity. The inactivating factor is however inhibited by TLCK and α-*N*-benzoyl-L-arginine which are specific inhibitors of trypsin-like enzymes. Proteolysis is further indicated from the kinetics of inactivation in that complete loss of NR will occur if the inactivation reaction is allowed to proceed long enough (17). Finally, inactivation of NR is prevented by either BSA or casein (17).

Apart from a similar mol wt (27,500 *cf.* 44,000) and some sensitivity to TLCK and metal-chelating reagents the maize root NR-inactivating enzyme (21) and the wheat leaf NR-inactivating factor do not have many other properties in common. The wheat leaf-inactivating factor has comparatively little effect on the NADH-ferricyanide reductase (diaphorase) component of NR in marked contrast to the maize root enzyme which has greatest activity toward this component. The NR inhibitor isolated from soybean leaves (4) also exhibits little activity toward the NADH-ferricyanide reductase component of NR. In addition, the inhibitory activity of this factor increases when plants are placed in darkness, a treatment which results in loss of *in vitro* NR activity. Such behavior is very similar to the wheat leaf NR-inactivating factor which apparently increases in activity during the night, a period during which the *in vitro* activity and stability of NR are at a minimum level (17). The wheat leaf NR-inactivating factor also has a similar mol wt to the peroxidase-like factor isolated from rice seedlings (5, 6). However, the rice seedling factor is not inhibited by casein.

Finally, we feel that some general comment should be made about previous studies of the specificity of the various inactivating factors already described. The general consensus seems to be that these NR-inactivating factors are very specific, showing no inhibitory or inactivating activity toward a varied range of other plant enzymes. However, we feel that such studies are of limited value if only partially purified enzyme (substrate) were examined. This conclusion can be attributed to the presence of positive or protection factors in extracts of wheat (17) and soybean leaves (4) which overcome the apparent activity of the NR-inactivating factors isolated from these tissues. As a consequence, a negative result, that is, no apparent inactivating activity toward a particular enzyme, may not be indicative of enzyme specificity but could be due to the presence of contaminating positive or protection factors.

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