Hydroxylamine Reductase Enzymes from Maize Scutellum and Their Relationship to Nitrite Reductase

Received for publication September 26, 1975 and in revised form December 8, 1975

DEREK P. HUCKLESBY AND RICHARD H. HAGEMAN
Department of Agronomy, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Three enzymes contribute to the total hydroxylamine reductase activity of corn (Zea mays L.) scutellum extracts. Two of these resemble enzymes previously prepared from leaves, while the third, which accounts for a major part of the activity, appears to have no counterpart in leaf tissue. One of the hydroxylamine reductases found only in small amounts is associated with nitrite reductase and is induced, together with nitrite reductase, by nitrite. The other two enzymes are noninducible by nitrite and can be totally separated from nitrite reductase, which subsequently remains capable of catalyzing the reduction of nitrite to ammonia. Possible causes of the decline of hydroxylamine reductase activity during the induction of nitrite reductase are discussed.

Nitrite reductase from leaves of vegetable marrow and spinach catalyzes the reduction of nitrite to ammonia stoichiometrically even after most of the hydroxylamine reductase activity is removed (10, 13). The enzyme is a single protein able to transfer all six electrons required for the reduction of nitrite (4, 18). The presence of a separate hydroxylamine reductase is not required for complete reduction of nitrite to ammonia. Several enzymes able to reduce hydroxylamine have been extracted from leaves, but their physiological significance is not understood. In some cases hydroxylamine reductase activity is associated with another enzyme, e.g. with nitrite reductase (9) or with sulfite reductase (2, 19). For contrast, one of the hydroxylamine reductases from vegetable marrow leaves (designated HRs) exists without known relationship to any other enzyme (13).

In the course of studies of nitrite reduction in nongreen tissues, a process which must be presumed to differ fundamentally in nature from that of chlorophyllous tissue (3, 12), it was discovered that corn scutellum extracts possess high activities of hydroxylamine reductase. The present paper describes the enzymes involved and attempts to show their relationship to the leaf enzymes and to the process of nitrite reduction.

MATERIALS AND METHODS

Corn seeds (Zea mays L. var. Ohio 43 × B14) supplied by Crows' Hybrid Corn Co., Milford, Ill., were treated for 45 min in 0.2% (v/v) sodium hypochlorite (Clorox). Seventy disinfected seeds were germinated at 30°C on paper towels in glass trays (34 × 22 × 4 cm). Nutrient solution (100 ml) (12) containing 30 mM potassium nitrate was added with the seed. For experiments involving induction of nitrite reductase in the excised scutella, 5 mM ammonium chloride was substituted for the potassium nitrate. Seedlings were harvested after 4 days, when the shoots were about 4 cm in length. The scutellum was removed from each seed by hand.

Nitrite reductase was induced by immersion of the excised scutella in a solution containing 50 mM potassium phosphate and 1 mM sodium nitrite adjusted to pH 5.5 with KOH. In some experiments the induction medium was 50 mM potassium phosphate, 5 mM ammonium chloride, and 30 mM potassium nitrate at pH 4 (6). The vessels used for induction were as described by Elsner (6) but modified to provide for continuous renewal of the induction medium. The excised scutella (2–10 g per vessel) were circulated in the solution by air pumped through each vessel by means of a Buchler four-channel Polystatic pump at a rate of 120 ml/hr. By this means four vessels could be arranged in parallel.

The assay for nitrite reductase was similar to the method of Miflin described elsewhere (12). Hydroxylamine reductase was assayed by adding enzyme extract (20 μl–0.2 ml) to a mixture of 0.1 μmole of potassium phosphate, pH 7.5, 0.5 μmole of hydroxylamine hydrochloride, and 1.1 μmoles of benzyl viologen. The reaction was started by 0.5 ml of sodium dithionite (7.2 mg/ml in 50 mM potassium phosphate, pH 7.5), giving a final volume of 3 ml. The disappearance of hydroxylamine after 5 to 20 min of incubation at 30°C was measured by assaying 0.25–ml aliquots of the reaction mixture by a method described below.

A simple and sensitive small scale version of the hydroxylamine reductase assay was used for locating the enzyme in column eluates. The reaction mixture comprised 0.1 ml of extract, 10 μmoles of potassium phosphate, pH 7.5, 100 μmoles of hydroxylamine hydrochloride, and 220 μmoles of benzyl viologen in a total volume of 0.3 ml. Sodium dithionite (0.1 ml of the solution described above) was added to start the reaction. After 5 to 20 min, the reaction was stopped by the addition of 5.5 ml of 17 mM potassium phosphate, pH 8.5. The air dissolved in this buffer was sufficient to reoxidize the reduced benzyl viologen. The tubes were agitated for 10 sec by means of a Vortex mixer (Scientific Industries, Springfield, Mass.). Hydroxylamine was determined in this solution by the addition of iodine (1 ml of aqueous solution containing 1.87 g of I2 in 0.56% [w/v]KI), sodium arsenite (1 ml of 0.8% [w/v] solution) followed by 2 ml of a mixture containing sulfanilamide (0.5% [w/v]), 10 mM N-(1-naphthylethylenediamine)hydrochloride, and (1.5 n) HCl. This method is slightly modified from the one described by Hewitt and Nicholas (11). The absorbance at 540 nm was determined after 10 min. Ammonia was estimated by the microdiffusion method of Cedrengalo et al. (5), except that ammonia was expelled from the sample by the addition of 1 ml of saturated potassium carbonate, and the bottles were rocked not rotated. The Russell method (17) for the colorimetric determination of ammonia was used instead of the Nessler method described by Cedrengalo et al. (5). Total protein was assayed by the method of Lowry et al. (15).

To extract the enzymes, scutella were homogenized in 4 volumes of extraction medium in a VirTis homogenizer for 1.5 min at 0.5 line voltage. The extraction medium was 25 mM potassium...
RESULTS

Separation of Nitrite and Hydroxylamine Reductases. Scutella (40 g) were excised from 4-day-old corn seedlings grown with nitrate as nitrogen source. Crude extracts contained nitrite reductase and hydroxylamine reductase in the ratio of approximately 1:5 to 1:10. For separation of the enzymes the extract was centrifuged at 27,000g and then concentrated from 150 ml to 10 ml by dialysis against solid Carbowax 4000. The concentrated was chromatographed on a column of Sephadex G100 (223 ml bed volume, 44 × 2.56 cm) and eluted with 30 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM potassium chloride. A partial resolution of nitrite and hydroxylamine reductases was obtained, with nitrite reductase eluting first.

All fractions containing nitrite or hydroxylamine reductase activity were bulked, and the resulting solution was desalted by dialysis against 20 mM potassium phosphate (pH 7.7). The desalted solution was eluted from a column of DEAE-cellulose using a gradient of chloride concentration. Two nitrite reductase peaks (Fig. 1B) were obtained. These enzymes (NiR₁ and NiR₂) have been described in another paper (12). Two peaks of hydroxylamine reductase activity also eluted from the column (Fig. 1). The second of these (peak B) may be regarded as the principal hydroxylamine reductase of the scutellum in terms of activity, and is benzyl viologen-dependent (Table I). Peak A, which probably represents a mixture of heme proteins, shows hydroxylamine reductase activity in the absence of benzyl viologen, i.e. with dithionite as the sole electron donor. In addition, a small amount of hydroxylamine reductase activity is associated with the nitrite reductase peaks especially NiR₂.

For routine separations of nitrite and hydroxylamine reductases a simplified procedure was devised in which crude extract was adsorbed directly after centrifugation on to a column of DEAE-cellulose and enzymes eluted with a linear chloride gradient, i.e. the method was as described above except that the steps involving concentration and molecular exclusion chromatography were omitted.

Comparison with Leaf Extracts. Maize leaf extracts, fractionated on a column of DEAE-cellulose by the same techniques used for scutellum extracts, show a peak of hydroxylamine reductase activity with chromatographic and spectrophotometric characteristics similar to the dithionite-HR (peak A) from scutellum. A very slight hydroxylamine reductase activity was associated with nitrite reductase in either tissue. The outstanding difference between the two types of tissue was the presence in scutellum extracts of a very active benzyl viologen-hydroxylamine reductase completely separable from nitrite reductase. This enzyme was not detected in leaf extracts.

General Properties of Benzyl Viologen-dependent Hydroxylamine Reductase. This appears to be the principal hydroxylamine reductase of scutellum. It is apparently not represented in leaves and has not been described before. A brief description of some of its characteristics will be given.

Electron Donors. Benzyl viologen reduced by dithionite is an effective electron donor for the enzyme. In contrast to nitrite reductase from the same tissue, ferredoxin does not substitute for benzyl viologen. At high concentrations (0.5 mM) FMN gives slight activity, but this is small by comparison with activities obtained when this cofactor is used with leaf extracts from marrow and spinach (13). FAD (50 mM), 0.15 mM NADH, and 0.13 mM NADPH gave negligible activity.

Table 1. Effect of Omission of Constituents of Hydroxylamine Reductase System

<table>
<thead>
<tr>
<th>Activity Hydroxylamine Loss</th>
<th>Enzyme A</th>
<th>Enzyme B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1</td>
<td>Expt 2</td>
<td>Expt 1</td>
</tr>
<tr>
<td>nmole</td>
<td>91</td>
<td>166</td>
</tr>
<tr>
<td>- Benzyl viologen</td>
<td>121</td>
<td>151</td>
</tr>
<tr>
<td>- Dithionite</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>- Enzyme</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Boiled enzyme</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

These data indicate that complete reductase is present in both leaf and scutellum. The reductase from the whole seed contains little or no benzyl viologen reductase activity and is therefore a mixture of both types of enzyme. This is consistent with the results of separations on crude extracts from whole seeds (Table I).


**Substrates.** For the purpose of testing alternative substrates, benzyl viologen was reduced by palladium and hydrogen (8) and dispensed into an evacuated spectrophotometer cell containing enzyme extract. The substrate (500 nmoles) was added from a side-arm and change in absorbance at 715 nm followed by means of a Beckman DB spectrophotometer and recorder. The rate of change in absorbance with hydroxylamine as substrate was 0.088/min. No reaction was observed with acetyl-hydroxamic acid, L-glutamic acid-mono-hydroxamate, or acetone oxime.

Enzyme preparations were also tested for their ability to oxidize ammonia in the presence of O2 alone or with H2O2, NAD, or NADP also present. No ammonia loss was observed and hydroxylamine formation could not be detected.

**Inhibitors.** Cyanide severely inhibited hydroxylamine reduction, whereas EDTA was noninhibitory. Another organic chelate—diethyldithiocarbamate—inhibited the reaction. The enzyme probably contains a rather tightly bound metal. The sulfhydryl inhibitor, \( p \)-chloromercuribenzoate, was also inhibitory.

Partial protection was afforded by glutathione (Table II).

The enzyme was not inhibited by nitrite at concentrations of up to 5 mM, even after contact for 2 hr. This lack of sensitivity differs from the inhibitions observed with hydroxylamine reductases from other higher plant materials (10), and from the dithionite-HR of scutellum. The latter enzyme was inhibited 92 and 61% by 0.2 mM and 0.02 mM nitrite, respectively.

**Molecular Weight.** Preliminary estimates using a calibrated Sephadex G-100 column indicate that the mole wt is approximately 33,000.

**Reaction Product.** Ammonia yield from the reduction of 330 and 305 nmoles of hydroxylamine was 293 and 319 nmoles, respectively, i.e. 89 and 105% recovery of nitrogen as ammonia.

**Stoichiometry of Nitrite Reduction in Absence of Hydroxylamine Reductase.** The separation of two nitrite reductase enzymes from corn scutellum extracts by chromatography on DEAE-cellulose has been described (12). Relatively impure preparations of both enzymes, which may still have possessed hydroxylamine reductase activity, were shown to catalyze the stoichiometric reduction of nitrite to ammonia. In the following purification experiment, the principal objective was to identify the product of nitrite reduction after further removal of hydroxylamine reductase activity.

Scutella (45 g) were removed from 4-day-old seedlings grown with nitrate as nitrogen source. The tissue was homogenized with

Table II. Inhibition of Benzyl Viologen-dependent Hydroxylamine Reductase from Corn Scutellum

<table>
<thead>
<tr>
<th>Inhibitor and Molar Conc of Inhibitor</th>
<th>Activity</th>
<th>Loss NH₂OH</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethyldithiocarbamate (Na) (1 mM)</td>
<td>206</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>333</td>
</tr>
<tr>
<td>EDTA (1 mM)</td>
<td>272</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>252</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyanide (1 mM)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>362</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( p )-Chloromercuribenzoate (1 mM)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>( p )-Chloromercuribenzoate (1 mM) + GSH (5 mM)</td>
<td>238</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>362</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A more definitive study of this type of conversion has been made with nitrite reductase from leaves (10, 13).

**Induction of Nitrite and Hydroxylamine Reductases by Nitrate or Nitrite.** In the following experiments corn scutella were removed from 4-day-old seedlings, which had been germinated in solutions containing ammonia as nitrogen source, and placed in highly aerated induction media. Enzyme activities were measured at various times during the induction process. Nitrate reductase could be induced by nitrate or by nitrite, and required at least 7 hr induction to reach maximal activity. By contrast, nitrate reductase induced by nitrate reached its maximal activity in about 4 hr (6) with subsequent rapid loss of activity. In the case of nitrite reductase induction in response to nitrate it is not clear whether the inducing ion was nitrate or nitrite (produced by nitrate reductase action) or both ions operating simultaneously. The corn scutellum tissue has an appreciable level of nitrite reductase activity before induction (an observation also made for barley aleurone layers by Ferrari and Varner (7). In 7 hr induction, the activity commonly increased 5- or 6-fold.

Induction of nitrite reductase (Fig. 3) was detectable at a concentration of 0.05 mm nitrite and was optimal at approximately 1 mm nitrite and was optimal at approximately 1 mm. A pH of 5.4 was chosen for inducing the enzyme, although a further small increase in efficiency of induction could be attained by lowering the pH to 4, but the tissue visibly discolored under these latter conditions. Virtually no induction was seen at pH 7.

Scutella from seedlings grown in nutrient solutions containing nitrate or ammonia have high activities of hydroxylamine reductase without recourse to inductive procedures. The ratio of hydroxylamine reductase to nitrite reductase in crude extracts of scutella from ammonia-grown plants is 20:1 to 40:1. During induction with nitrite, the hydroxylamine reductase activity declines (Figs. 2 and 3). In these experiments ammonium sulphate-precipitated extracts were used and the assay did not distinguish between the various hydroxylamine reductases although the benzyl viologen-HR, which is the major (95%) component, was assumed to be the enzyme principally affected by nitrite induction. Fractionation of extracts from induced and noninduced scutella by means of a DEAE-cellulose column confirmed this (Table V) and showed that the decline in HR activity is a complex phenomenon which can be analyzed as follows. (a) The activity of the dithionite-HR is largely unaltered during the induction period. (b) The benzyl viologen-dependent enzyme (peak B) diminishes greatly; most of the decline occurs during the first 1 or 2 hr. (c) That portion of the hydroxylamine reductase activity which is associated with the nitrite reductase peaks is induced by nitrite; presumably this means that the nitrite reductase itself has some capacity for reducing hydroxylamine. Decline in activity of hydroxylamine reductase does not occur when nitrite reductase is induced by nitrate.

**Effect of Cycloheximide on Induction.** This inhibitor (2 μg/ml
of induction media) almost totally suppresses the induction of nitrite reductase. One consequence of this is an accumulation of nitrite in the tissue (Table VI). The decline of hydroxylamine reductase activity during the induction period is hastened by the presence of the cycloheximide (Table VI).

**DISCUSSION**

The hydroxylamine reductase activity of corn scutellum extracts can be fractionated into three parts by chromatography on DEAE-cellulose (Fig. 1). The major fraction (peak B) is apparently not present in leaf extracts, while the two less active components have characteristics in common with hydroxylamine reductases which have previously been isolated from leaves. One of the latter enzymes (peak A), which does not require benzyl viologen for its activity, appears to resemble an enzyme (HR₃) previously prepared from leaf extracts (13, 14). The other enzyme is associated with the main peaks of nitrite reductase, and corresponds in type to hydroxylamine reductase (HR₃) of marrow and spinach leaves (10). In the following discussion, the enzymes are listed in the order of their elution from DEAE-cellulose.

1. **Dithionite-reducible Hydroxylamine Reductase (Peak A).** This enzyme elutes first from a DEAE-cellulose column and has a light absorption maximum at 403 nm. This shifts to 434 nm on reduction as noted for one of the hydroxylamine reductases (HR₃) from the leaves of vegetable marrow (14) with dithionite. Addition of alkaline pyridine gives maxima at 419, 526, 557 nm. Two further similarities have been noted between the enzymes from corn scutellum and marrow leaves (10). Both are inhibited by nitrite and can receive electrons from dithionite in the absence of benzyl viologen. It is possible that this enzyme is not a physiological hydroxylamine reductase, but a heme protein of unknown function which reacts fortuitously with hydroxylamine. Further purification and study are required before its significance can be assessed.

2. **Benzy1 Viologen-dependent Hydroxylamine Reductase (Peak B).** This enzyme, which elutes as the second peak from a DEAE-cellulose column, is responsible for at least 90% of the activity of the crude extract. It has not yet been associated with absorption bands in the visible spectrum and is specific for hydroxylamine among the substrates tested. In assessing its relationship to the nitrite reductase enzymes, the following possibilities were considered.

   (a) This hydroxylamine reductase is an essential intermediary in the conversion of nitrite to ammonia. The enzyme is almost totally separated from both nitrite reductase enzymes by gradient elution from DEAE-cellulose (Fig. 1). Following separation, the nitrite reductases retain their capacity to catalyze the reduction of nitrite to ammonia stoichiometrically (Tables III and IV). Apparently then, this hydroxylamine reductase is not directly concerned with the nitrite assimilation process.

   (b) The enzyme is a degradation product of nitrite reductase. The possibility considered here is that a nitrite reductase which normally mediates a conversion from the +3 state of oxidation (nitrite) to the −3 state (ammonia) might, through damage sustained during extraction or purification, retain only the capacity for catalyzing the conversion from the −1 state (hydroxylamine) to the −3 state (ammonia). The relative inability of the undamaged nitrite reductase to handle hydroxylamine as substrate seems to render this theory improbable. The existence of the hydroxylamine reductase in ammonia-grown tissues prior to the induction of nitrite reductase (Figs. 2 and 3) seems to eliminate the possibility altogether.

   (c) The enzyme is a precursor of the nitrite reductase. The possibility exists that a conversion of hydroxylamine reductase to nitrite reductase occurs during induction of the latter. Support for this idea is provided by the complementary relationship between the two enzymes during the induction period (Fig. 2) and over a range of concentrations (Fig. 3). Experiments with cycloheximide, however, argue against interconversion. The prevention of the induction of nitrite reductase by this inhibitor (Table VI) suggests that nitrite reductase is synthesized de novo. If this is the case, conversion of hydroxylamine reductase (mol wt = 33,000) to nitrite reductase (mole wt = 63,000) must be assumed to occur by the de novo synthesis of a segment of protein and its subsequent addition to the hydroxylamine reductase. If nitrite reductase synthesis depended upon interconversion, suppression of the induction of this enzyme by cycloheximide should be accompanied by a concomitant arrest of the decline in hydroxylamine reductase activity. In fact, the decline in activity is accelerated (Table VI).

   (d) The nitrite reductase and hydroxylamine reductase are not related in any direct way.

   Logically, the nitrite reductase enzymes described in this paper, because of their inducibility by nitrate or nitrite, must be regarded as genuine components of the nitrate assimilatory system, whereas the hydroxylamine reductase, which is present in plants grown with ammonia as nitrogen source, could conceivably be involved in some other process. Possibly hydroxylamine is not its physiological substrate. However, the enzyme is not a sulphite reductase, and did not reduce several other compounds containing -NO₂ or -NO₂H groups.

   The decline of hydroxylamine reductase activity during induction of nitrite reductase would be accelerated if hydroxylamine reductase were inhibited irreversibly by the nitrite ion or by free nitrous acid, since nitrite concentration rises temporarily during induction (Table VI). The accelerated decline of hydroxylamine reductase activity when cycloheximide is present during the induction period (Table VI) would be very satisfactorily explained in the same way, since the presence of this inhibitor
causes a large increase in nitrite concentration in the tissue due to its suppression of nitrite reductase synthesis. Although other higher plant hydroxylamine reductases are sensitive in vitro to nitrite inhibition, this particular enzyme is not appreciably inhibited by concentrations of nitrite as high as 5 mM at pH values as low as 5.5. This concentration of nitrite is in 10-fold excess of the overall concentration observed in the scutellum tissue during induction. Nitrite inhibition of HR activity is unlikely, therefore, to be the cause of decline of this enzyme during nitrite reductase induction. A further possible cause is interference by nitrite with synthesis of hydroxylamine reductase; decline in level of the enzyme in the presence of cycloheximide suggests that it is undergoing rapid synthesis and breakdown. It is necessary to assume, in this case, that the half-life of the enzyme is almost as short as that reported for ornithine carbamoylase (16).

3. Hydroxylamine Reductase Associated with Nitrite Reductase Peaks. The two nitrite reductase peaks, especially the second one to elute from a DEAE-cellulose column, are both associated with a small amount of hydroxylamine reductase activity (Fig. 1). This appears when the nitrite reductase is induced by nitrite (Table V), suggesting that the nitrite reductase is not totally specific for nitrite but has a small capacity for reducing hydroxylamine. The ratio of activities (HR-NiR) is very much less for corn scutellum enzymes than for those extracted from the leaves of marrow and spinach (10) where ratios approaching unity were found.

LITERATURE CITED