Enzymatic Dehydration of 3-Hydroxymethyloxindole

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ABSTRACT
Crude and partially purified extracts of wheat (Triticum vulgare, red variety) germ catalyze the dehydration of 3-hydroxymethyloxindole to 3-methyleneoxindole. Examination of the ultraviolet absorption spectrum of a reaction mixture consisting of either the extract or partially purified enzyme and 3-hydroxymethyloxindole, shows that this oxindole has undergone complete dehydration to 3-methyleneoxindole. TPNH-linked 3-methyleneoxindole reductase, also a constituent of the wheat germ extract, can be separated from the dehydrase by passage through an Agarose 15 column. Utilizing these partially purified enzymes, it can be demonstrated that the dehydrase activity found in wheat germ is a discrete enzymatic function.

The pertinence of the oxindole pathway of IAA metabolism to higher plants is suggested by the finding that intact pea seedlings as well as their extracts can oxidize IAA to HMO$^+$ and reduce its dehydration product, MeOx, to 3-methyleneoxindole (7). A highly purified MeOx reductase from peas showed complete substrate specificity for MeOx. Furthermore, MeOx reductase from peas exists in multiple forms, with differential sensitivity to the synthetic auxins, 2, 4-D, and naphthaleneacetic acid (5). Demonstration of the enzymatic oxidation of IAA to HMO and the reduction of MeOx by a highly purified plant MeOx reductase for which MeOx is a specific substrate strongly suggest that the oxindole pathway functions in higher plants.

The nonenzymatic dehydration of HMO can proceed at a measurable rate, especially under conditions of physiological pH and ionic strength (6, 7). An important gap in the evidence for the oxindole pathway of IAA metabolism has been the failure, until now, to demonstrate an enzymatic acceleration of the dehydration of HMO to MeOx. We present evidence that extracts prepared from wheat seedlings and wheat germ can catalyze this dehydration via HMO dehydrase. The current status of the oxindole pathway is described in Figure 1.

MATERIALS AND METHODS

Chemicals. 3-Bromo-oxindole-3-acetic acid was prepared by reaction of IAA with N-bromosuccinimine (2). This compound on solution in water is rapidly converted to MeOx. The latter was purified by chromatography with 5% isopropanol in water (1). HMO was prepared by photooxidation of IAA in the presence of riboflavin (1). Other chemicals were of reagent grade.

Plant Material: Treatment and Fractionation. Wheat seeds (Triticum vulgare, red variety) were rinsed and soaked in distilled water for 16 hr at room temperature. The seeds were covered with moistened vermiculite and incubated at 23 C for 7 days in total darkness. Seedlings (100 g) were rinsed for 1 hr with tap water and homogenized in a chilled blender with 100 ml of 20 mM potassium phosphate, pH 7.0. Subsequent manipulations were carried out at 4 C. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 37,000g for 15 min. The clarified extract was dialyzed overnight against 100 volumes of the same buffer.

Wheat germ, obtained from Nutritional Biochemicals Corporation, was defatted using a previously described method for pea flour (4). One hundred grams of defatted wheat germ were extracted with 350 ml of 20 mM potassium phosphate, pH 7.0, and centrifuged at 37,000g for 15 min. The supernatant was then centrifuged for 90 min at 176,000g. A portion of the clear extract was dialyzed as above, and the remaining portion was made 1% with respect to streptomycin sulfate. The precipitated nucleic acids were removed by centrifugation. The extract was dialyzed as before, and brought to 40% of saturation with ammonium sulfate by addition of solid ammonium sulfate. After 1 hr the precipitate was removed by centrifugation. The ammonium sulfate concentration was then increased to 60% of saturation. After 1 hr, the resultant precipitate was collected by centrifugation, dissolved in a minimal amount of 20 mM potassium phosphate, pH 7.0, and dialyzed against the same buffer for 18 hr. This fraction (500 mg protein) was applied to a column (4.9 cm$^3$ × 83 cm) of Agarose 15 equilibrated with 20 mM potassium phosphate, pH 7.0. The column was eluted with one liter of the same buffer. All the proteins containing HMO dehydrase and MeOx reductase activity were eluted within 750 ml. The fraction size was 10 ml. The levels of the two enzymes in each fraction were determined.

The 100-fold purified MeOx reductase was prepared from pea flour (4).

Analytical Procedures. To determine the concentration and extinction coefficient of HMO, a sample was allowed to undergo complete dehydration to MeOx during storage overnight at room temperature. The concentration of MeOx and therefore that of the original HMO was calculated from absorption at 248 nm using the published extinction coefficient of MeOx (3).

Measurement of MeOx Reductase. MeOx reductase activity of the 176,000g supernatant and the fractions obtained after passage through the Agarose 15 column was measured by a previously described method (4) and expressed as the decrease in absorbance at 340 nm, due to oxidation of TPNH per milligram of protein per min.

Measurement of HMO Dehydrase. HMO dehydrase was estimated by the increase in absorbance at 248 nm due to the formation of MeOx (6, 7). The optimal pH conditions for this
purified HMO and TPNH was found to greatly accelerate the oxidation of TPNH. Addition of HMO to a 100-fold purified MeOx reductase from peas did not result in any measurable oxidation of TPNH. This finding indicates that the oxidation of TPNH observed after addition of wheat seedling extract is due to a new enzymatic function. Moreover, the HMO used was shown by this assay to be free from contamination with MeOx.

Examination of wheat germ for dehydrase revealed it to be a prolific source of the enzyme. Consequently, all subsequent experiments were performed with wheat germ extracts.

**Dehydration of HMO to MeOx by Wheat Germ Extract.** Examination of the ultraviolet absorption spectrum of HMO in the presence of wheat germ extract reveals that after 45 min the indole has undergone complete dehydration to MeOx (Fig. 2B). The transition of the HMO spectrum to that of MeOx is evident as early as 5 min after incubation. Under the conditions of the assay, however, the two absorption maxima characteristic of MeOx (3) become distinct only after 45 min. The nonenzymatic dehydration of HMO to MeOx is negligible during this period of incubation (Fig. 2A). It normally requires 16 hr to demonstrate a quantitative, nonenzymatic dehydration of HMO to MeOx (7). Addition of 2-mercaptoethanol resulted in the obliteration of the double absorption maxima (Fig. 2B), suggesting the formation of a mercapatan, a reaction characteristic of MeOx (6, 7).

The wheat germ extract used in these experiments also contains a TPNH-linked MeOx reductase, thus permitting the measurement of dehydrase activity, provided that HMO is used as the substrate; HMO is dehydrated to MeOx which is subsequently reduced via the TPNH-linked reductase. A more direct approach for the measurement of the dehydrase activity is the estimation of the increase in absorbancy at 248 nm of reaction mixtures containing HMO and wheat germ extract. The increase in absorbancy is related to the amount of MeOx formed. It may be seen in Table 1 that, the dehydrase activity in extracts of wheat germ can be demonstrated by both of these methods. Heating the extract renders it incapable of catalyzing the dehydration of HMO to MeOx, regardless of whether the activity is assayed indirectly by measuring the oxidation of TPNH at 340 nm, or directly by the formation of MeOx at 248 nm.

**RESULTS**

The presence of HMO dehydrase was first detected in dialyzed extracts of etiolated wheat seedlings. Detection of the dehydrase activity utilized an enzyme coupled reaction which permitted the monitoring at 340 nm of the oxidation of TPNH. This method is feasible due to the fact that under the conditions of the assay, MeOx, the dehydration product of HMO, is an electron acceptor in the oxidation of TPNH. The addition of wheat seedling extract to the reaction mixtures containing

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**Table 1. Heat Inactivation of 3-Hydroxymethyloxindole-Dehydrase Activity of Wheat Germ Extract**

<table>
<thead>
<tr>
<th>Main Constituents of the Reaction Mixture</th>
<th>Activity of HMO-Dehydrase at 340 nm</th>
<th>Activity of HMO-Dehydrase at 248 nm</th>
<th>ΔA/mg protein/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. HMO, pea MeOx-reductase, TPNH</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2. HMO, wheat germ extract, TPNH</td>
<td>0.030</td>
<td>0.030</td>
<td>0.030</td>
</tr>
<tr>
<td>3. HMO, heated wheat germ extract TPNH</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4. Addition of unheated wheat germ extract to No. 3</td>
<td>0.015</td>
<td>0</td>
<td>0.014</td>
</tr>
<tr>
<td>5. HMO, wheat germ extract</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>6. HMO, heated wheat germ extract</td>
<td>0.014</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7. Addition of unheated-wheat germ extract to No. 6</td>
<td>0</td>
<td>0</td>
<td>0.014</td>
</tr>
</tbody>
</table>

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Fig. 1. The oxindole pathway of IAA metabolism in plants.

Fig. 2. Absorption spectra of HMO and reaction of the dehydration product. A: Absorption spectra of HMO at 0 min and after 45 min at room temperature. B: Enzymatic dehydration of HMO. The reaction mixture contained 0.5 mg of protein obtained from a dialyzed extract of wheat germ after centrifugation at 176,000g. Other details are described in the text. Absorption spectrum of HMO was obtained immediately after the addition of extract (o') and after 45 min. Addition of 1.4 mM 2-mercaptoethanol to the enzymatic dehydration product obliterates the two absorption maxima. Using the analytical procedures described in the text, it is found that in the presence of the extract, 35 μM HMO is dehydrated to 30 μM MeOx.

The change in absorbancy at 248 nm was recorded following the addition of the enzyme at room temperature. The dehydrase activity was also estimated by coupling to MeOx reductase, which was measured by following the oxidation of TPNH at 340 nm.
Failure to detect dehydrase activity in heated wheat germ extract suggests that the dehydration of HMO to MeOx is enzymatic in nature. Addition of unheated extract to such reaction mixtures leads to the formation of MeOx when measured by either method (Table I).

**HMO Dehydrase Activity in Partially Purified Wheat Germ Extracts.** The supernatant obtained after centrifugation of the wheat germ extract at 176,000g was processed as described under "Materials and Methods." The bulk of both HMO dehydrase and MeOx reductase was recovered between 40 and 60% of saturation of ammonium sulfate. Although passage of this fraction through an Agarose 15 column resulted in a slight overlapping of the two enzymatic activities, the eluted fraction containing the maximal reductase activity was well separated from the fraction containing maximal dehydrase activity. Utilizing these two fractions, it was possible to demonstrate that the dehydrase of HMO to MeOx is a discrete enzymatic function of wheat germ extract. It may be seen in Table II that the fraction containing the maximal dehydrase activity can catalyze the dehydration of HMO to MeOx as measured by increase in absorbancy at 248 nm. This fraction is very inefficient in causing the oxidation of TPNH (measured at 340 nm) when MeOx is used as the substrate, indicating that it contains only negligible amounts of MeOx reductase. A similar result was obtained when HMO was used as a substrate, once more indicating that MeOx reductase is the limiting factor. Addition of the reductase fraction to a reaction mixture consisting of HMO, the dehydrase fraction and TPNH, increases the oxidation of TPNH by more than 25-fold (Table II).

Results presented in Table III show that the reductase fraction is almost free of dehydrase activity. In the presence of MeOx, the reductase fraction can cause the oxidation of TPNH. The small amount of MeOx formed when the reductase is incubated with HMO and the increase in absorbancy is measured at 248 nm is probably due to contamination with dehydrase. The reductase fraction can oxidize only a negligible amount of TPNH when HMO is used as the substrate, suggesting that dehydrase in this case is the limiting factor. Addition of the dehydrase fraction accelerates the oxidation of TPNH by 27-fold.

**DISCUSSION**

Crude and partially purified extracts of wheat germ contain a HMO dehydrase activity which catalyzes the dehydration of HMO to MeOx. The extracts also contain a TPNH-linked MeOx reductase which catalyzes the reduction of MeOx to 3-methyloxindole. Although passage of the wheat germ extract through an Agarose 15 column results in only 1.5-fold purification of the dehydrase activity, this treatment makes it possible to demonstrate that the two enzymatic activities are distinct.

So far, attempts to determine Km of the dehydrase have not yielded satisfactory results. This search is complicated by the failure to obtain purified HMO at concentrations greater than 50 μM and due to the tendency of HMO to readily dehydrate to MeOx during the process of purification and under the conditions of the assay. At any rate, the reaction catalyzed by the dehydrase appears to be irreversible.

In contrast to the uncertainty concerning the physical and chemical properties of the dehydrase, there is definite evidence relating to the identity of MeOx, the product of the reaction catalyzed by the dehydrase. Upon addition of wheat germ extract, the ultraviolet absorption spectrum of HMO (Fig. 2) is completely transformed to that characteristic of MeOx (6, 7). Confirmatory tests for MeOx using paper chromatographic procedures are unreliable; the small amounts of HMO used for the dehydrase assay are easily dehydrated to MeOx during chromatography. The results presented in Tables II and III are more reliable. In the presence of TPNH and the dehydrase and reductase fractions HMO is degraded to a compound which serves as substrate for the TPNH-linked reductase. Similar results were obtained by using a 100-fold purified TPNH-linked MeOx-reductase from peas, for which MeOx is a specific substrate (4). These findings strongly suggest that MeOx is the product of the reaction catalyzed by the dehydrase fraction.

The enzymatic oxidation of IAA to HMO and subsequent reduction of its dehydration product, MeOx, to 3-methyloxindole by a highly specific TPNH-linked MeOx reductase has been shown to occur in intact pea seedlings and their extracts (7). Evidence of the enzymatic dehydration of HMO was lacking at that time, which created an important gap in the evidence for the oxindole pathway of IAA metabolism in plants. This gap is bridged by the demonstration of an enzymatic activity in wheat seedlings and wheat germ extracts which catalyzes the dehydration of HMO to MeOx. The relevance of the oxindole pathway of IAA degradation to plant metabolism is strengthened by the detection in plants of all the pertinent enzymes involved in the oxidation of IAA to 3-methyloxindole, the first and last metabolites respectively, of the oxindole pathway (1). Significance of the metabolism of IAA via the oxindole pathway lies in the fact that it generates MeOx, a highly reactive compound capable of producing profound effects on growth and metabolism (7, 8).

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LITERATURE CITED


