Role of Inosine Monophosphate Oxidoreductase in the Formation of Ureides in Nitrogen-Fixing Nodules of Cowpea (Vigna unguiculata L. Walp.)

BARRY J. SHELP AND CRAIG A. ATKINS
Botany Department, University of Western Australia, Nedlands WA 6009 Australia

ABSTRACT

Cell-free extracts from nodules of cowpea (Vigna unguiculata L. (Walp.) cv Caloona:Rhizobium strain CB756) prepared in the presence of 15% (v/v) glycerol showed high rates (30 to 60 nanomoles NAD reduced per minute per gram fresh weight of nodule) of inosine monophosphate oxidoreductase (EC 1.2.1.14) activity. The enzyme was labile (half-life of activity less than 3 hours) but could be stabilized for up to 18 hours by inclusion of the substrates NAD and inosine monophosphate in the breaking media. Activity showed a broad pH optimum between 8.5 and 9.5, had an apparent K_m (inosine monophosphate) of 4 and 12 micromolar at pH 7.5 and 9.0, respectively, and was largely (96%) associated with the plant cell cytosol fraction of the nodule.

Metabolism of [8-14C]inosine monophosphate and [1-14C]glycine by the cell-free system showed two pathways for purine base production from inosine monophosphate, one via xanthosine monophosphate, xanthosine, and xanthine, the other via inosine and hypoxanthine. The proportion of inosine monophosphate utilized by inosine monophosphate oxidoreductase and the xanthine-based pathway was increased from 30% at 0.5 millimolar to 80% at 0.01 millimolar inosine monophosphate. The data are interpreted to indicate that in vivo inosine monophosphate oxidoreductase activity rather than dephosphorylation is the predominant metabolic route leading to ureide synthesis and that inosine monophosphate provides the link between de novo purine nucleotide synthesis in the plastid and ureide production in the plant cell cytosol.

The ureides, allantoin and allantoic acid, are the major products of nitrogen fixation exported from nodules of many tropical legumes (2, 3, 13, 15). Studies with cell-free extracts of cowpea and soybean have demonstrated that these compounds are formed from the oxidation of purine bases and nucleotides (1, 4, 17, 19, 20) derived by a pathway of de novo purine synthesis which utilizes products of N_2 fixation (5). Although IMP has been detected as the initial nucleotide product of de novo purine synthesis in cowpea nodules (5), it is not clear whether IMP is metabolized principally through inosine and hypoxanthine or through oxidation to XMP^2 (Fig. 1). Allopurinol inhibits both xanthine and hypoxanthine oxidation (Fig. 1) by xanthine oxidoreductase (EC 1.2.1.37) in crude (4) or highly purified preparations (6) of nodules. Application of the inhibitor to intact plants results in xanthine accumulation in the nodules (4, 8, 10) consistent with metabolism through IMP oxidoreductase (EC 1.2.1.14) in vivo. In cell-free extracts, labeled hypoxanthine rather than xanthine accumulates from [8-14C]IMP utilization (1) and, although XMP is readily metabolized to allantoin in preparations from cowpea (1), attempts to assay IMP oxidoreductase in these (1), as well as in extracts from soybean (8, 17), have not been successful. This study reports the presence of IMP oxidoreductase in cell-free extracts of cowpea nodules and provides further information about the nature of intermediate reactions between de novo purine synthesis and ureide formation.

MATERIALS AND METHODS

Cowpea (Vigna unguiculata (L.) Walp. cv Caloona) plants, effectively nodulated with Rhizobium strain CB756, were grown in nitrogen-free sand culture in a naturally lighted glasshouse with a maximum day temperature at 35°C.

Freshly harvested nodules from 4- to 6-week-old plants were washed with deionized H_2O and crushed in a chilled mortar and pestle with 1 to 2 volumes of 50 mM Hepes-KOH buffer (pH 7.5), containing 25 mM KCl, 5 mM MgCl_2, 1% (w/v) PVP, 15% (w/v) glycerol, 10 mM ME, 5 mM DTT, 2 mM GSH, and 0.25 mM sucrose. Following filtration through a 100-μm mesh filter, the homogenate

1 Supported by the provision of a National Science and Engineering Research Council of Canada Post-Doctoral Fellowship to B. J. S. and a grant from the Australian Research Grants Scheme to C. A. A.

2 Abbreviations: XMP, xanthosine 5'-monophosphate; ME, β-mercaptoethanol; FGAR, formylglycinamide ribonucleotide; AICAR, aminimidazole carboxamide ribonucleotide.

Fig. 1. Metabolic pathways for the utilization of the product of de novo purine synthesis, IMP, to form ureides. AP indicates the sites of allopurinol action.

Received for publication February 18, 1983 and in revised form April 24, 1983

Copyright © 1983 American Society of Plant Biologists. All rights reserved.
Table 1. Stability of IMP Oxidoreductase Activity in Extracts of Cowpea Nodules

<table>
<thead>
<tr>
<th>Extraction Medium</th>
<th>Hours after Preparation</th>
<th>nmol NAD reduced/min·g fresh wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0⁺</td>
<td>1.5</td>
</tr>
<tr>
<td>Complete⁺</td>
<td>38.5 (100)³</td>
<td>25.5 (66)</td>
</tr>
<tr>
<td>Complete + 10 mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAD + 8 mM IMP⁺</td>
<td>38.5 (100)</td>
<td>22.4 (58)</td>
</tr>
<tr>
<td>-PVP</td>
<td>36.7 (95)</td>
<td>ND (0)</td>
</tr>
<tr>
<td>-glycerol</td>
<td>19.3 (50)</td>
<td>25.5 (66)</td>
</tr>
<tr>
<td>-ME</td>
<td>38.5 (100)</td>
<td>32.4 (84)</td>
</tr>
<tr>
<td>-DTT</td>
<td>42.4 (110)</td>
<td>33.2 (86)</td>
</tr>
<tr>
<td>-GSH</td>
<td>41.3 (107)</td>
<td></td>
</tr>
</tbody>
</table>

⁺ Zero h after preparation corresponded to approximately 1 h after the initial disruption of the tissue. During this 1 h, the nodules were extracted, a soluble fraction was collected following centrifugation and desalted on a column of G-25 Sephadex equilibrated with the appropriate extraction medium minus PVP.

ª The complete extraction medium contained 50 mM Hepes-KOH (pH 7.5), 25 mM KCl, 5 mM MgCl₂, 2 mM GSH, 5 mM DTT, 10 mM ME, 1% (w/v) PVP, and 15% (v/v) glycerol.

Values in parentheses are per cent of complete at zero h after preparation.

Not detectable; less than 0.1 nmol NAD reduced/min·g fresh nodule tissue.

NAD and IMP were added to the desalted enzyme at zero h after preparation.

![Figure 2](image-url)  
**Fig. 2.** Effect of pH on IMP oxidoreductase activity in cowpea nodule extracts. The buffer used was 50 mM Tris-glycine adjusted to pH 10.2 with NaOH and readjusted to the appropriate pH with HCl. Each data point is the mean of duplicate determinations.

was centrifuged (10,000g, 10 min, 4°C) and the supernatant fraction collected and desalted by passage through a Sephadex G-25 column equilibrated with the extraction buffer mixture minus PVP. The pellet (designated as particulate fraction) remaining was resuspended in extraction buffer minus PVP, twice passed through a French pressure cell and the supernatant collected after centrifugation (10,000g, 20 min, 4°C). For assays of de novo purine synthesis, a PEG precipitate was prepared from the extract of the particulate fraction as described previously (5).

IMP oxidoreductase activity (EC 1.2.1.14) was measured continuously as IMP-dependent NAD or NADP reduction at 340 nm and 30°C. The 1-ml reaction mixture contained 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM MgCl₂, 50 mM KCl, 2 mM NAD (or NADP), 1 mM allopurinol, 1.35 mM IMP, and 0.1 ml of cell-free extract (containing 0.16–0.40 mg protein and equivalent to 40–50 mg fresh weight nodules) prepared as above. Xanthine oxidoreductase (EC 1.2.1.37) activity was measured by replacing IMP with 1 mM xanthine or hypoxanthine and omitting allopurinol

![Figure 3](image-url)  
**Fig. 3.** Lineweaver-Burke plots for IMP oxidoreductase activity as a function of IMP concentration at two pH values in a cell-free system from cowpea nodule extracts. Each data point is the mean of duplicate determinations.

from reaction mixtures.

Metabolism of [8-¹⁴C]IMP (0.01–0.5 mM, 0.5 μCi) was determined in the same reaction mixture used for IMP oxidoreductase assay. At varying times following the addition of ¹⁴C-substrate, the reaction was terminated with cold HClO₄ and labeled compounds recovered as described previously (1). As the extracts had been desalted, ¹⁴C recovered in each product of IMP metabolism was equated with the specific activity of [8-¹⁴C]IMP supplied and the amount of product formed calculated as μmol/g fresh weight nodule tissue.

Utilization of [1-¹⁴C]glycine to form products of de novo purine synthesis was assayed in a reaction mixture described previously (5) and containing in addition 2 mM NAD, 1 mM allopurinol, 2 mM GSH, and 5 mM DTT. The source of enzyme used was 0.2 ml
of the PEG precipitate prepared from the particulate fraction (equivalent to 380 mg fresh weight of nodule) together with 0.1 ml of the desalted soluble extract used for IMP oxidoreductase assay (equivalent to 40 mg fresh weight of nodule).

\(^{14}\text{C}\)-Labeled purine nucleotides, nucleosides, bases, and intermediates of the de novo pathway (FGAR, AICAR) were separated and identified using ion-pair and ion-suppression reverse-phase HPLC analysis (5). \(^{14}\text{C}\) Radioactivity, measured by scintillation spectrometry, was determined to a SD of ±5% with quench correction by the channel's ratios method.

**RESULTS**

Detection of IMP Oxidoreductase. Extracts of cowpea nodules prepared in the presence of PVP, glycerol, and a number of reducing agents showed high rates of IMP-dependent NAD reduction in the presence of 1 mM allopurinol (Table I). Under these conditions, assays of xanthine- or hypoxanthine-dependent NAD reduction indicated that xanthine oxidoreductase activity was completely inhibited. In the absence of allopurinol, hypoxanthine-dependent NAD reduction was 100 to 150 nmol/min·g fresh weight nodule. IMP-dependent NAD reduction by the enzyme was relatively slow, being at most 26% of that with NAD.

Enzyme activity was relatively unstable (Table I), and although glycerol markedly increased the level detected compared to extracts prepared in the absence of glycerol, with the complete extraction medium the half-life was less than 3 h. Addition of substrates (NAD, IMP) during storage at 4°C effectively stabilized the enzyme with 80% of initial activity recovered after 18 h (Table I).

Maximum IMP oxidoreductase activities were shown at alkaline pH with a broad optimum between pH 8.5 and 9.5 (Fig. 2). At pH 7.5, the pH used in reaction mixtures to measure \([8-{14}\text{C}]\text{IMP}\) metabolism in this study, around 50% maximum activity was retained. In desalted extracts, the enzyme showed a relatively high affinity for IMP. The apparent \(K_m\), which changed markedly with pH, was around 4 μM IMP at pH 7.5 and around 12 μM at pH 9.0 (Fig. 3). Activity was associated primarily with the soluble fraction of the nodule with relatively little (around 4%) IMP oxidation occurring in the particulate fraction.

Metabolism of \([8-{14}\text{C}]\text{IMP}\). Labeled XMP, xanthosine, inosine, hypoxanthine, and xanthine were recovered following incubation of desalted cell-free extracts with \([8-{14}\text{C}]\text{IMP}\) (Fig. 4) indicating that IMP was utilized by both IMP oxidoreductase and the enzyme or enzymes responsible for dephosphorylation to form inosine. There was however, a marked effect of initial IMP concentration on the extent to which each side of the pathway shown in Figure 1 was utilized. At the highest IMP level, formation of inosine was favored, and although about equal amounts of xanthine and hypoxanthine accumulated, by 60 min 70% of the IMP metabolized was recovered as inosine plus hypoxanthine (Fig. 4A). With the progressive decline in initial IMP level, the proportion of IMP utilized by IMP oxidoreductase increased from 30% at 0.5 mM IMP (Fig. 4A) to 49% at 0.1 mM IMP (Fig. 4B), 68% at 0.025 mM IMP (Fig. 4C), and 80% at the lowest IMP level used, 0.01 mM (Fig. 4D).

---

**Fig. 4.** Distribution of \(^{14}\text{C}\) with time among products of \([8-{14}\text{C}]\text{IMP}\) (10–50 μCi·μmol\(^{-1}\)) metabolism by a cell-free preparation from the cytosol of the central infected tissue of cowpea nodules in the presence of allopurinol. IMP concentration was 0.5 (A), 0.10 (B), 0.025 (C), and 0.01 mM (D). The experiment was carried out three times and the results shown are from a single, typical experiment.
The metabolism of IMP (0.01 mM, 0.5 μCi·nmol⁻¹) by a cell-free preparation from the cytosol of the central infected tissue of cowpea nodules in the presence of allopurinol. The results are from a single, typical experiment.

The relative accumulation of ¹⁴C in these compounds was reversed by the addition of a large excess of unlabeled XMP (1 mM) and xanthosine (1 mM) to the reaction mixture at zero time (Fig. 6) in agreement with a metabolic sequence from IMP to XMP to xanthosine and to xanthine.

A feature of the metabolism of IMP was the extremely small amount of XMP which was recovered (Fig. 4, A–D). Even with 0.01 mM IMP, where the proportional operation of IMP oxidation was greatest, at most 4% of the label was recovered in XMP (Fig. 4D) and at the shortest incubation time used in this experiment (10 min) it seems likely that XMP had reached equilibrium. A more detailed time course during the first 15 min of the metabolism (Fig. 5) showed that, of the compounds formed, the XMP pool was the first to reach equilibrium at between 2 and 4 min and declined after 10 min. The order of labeling indicated that xanthosine and inosine were formed at similar rates but whereas xanthosine accumulated rapidly, even at low xanthosine levels, hypoxanthine formation from inosine was much slower (Fig. 5). Thus, not only did the high affinity of IMP oxidoreductase for substrate favor the formation of xanthine, enzymes responsible for subsequent metabolism of XMP and xanthosine operated more efficiently at lower substrate levels than did those responsible for hypoxanthine synthesis.

Under conditions where the only substrate added was IMP (0.5 mM in Fig. 4A) appreciable ¹⁴C accumulated in xanthine whereas only very small pools of labeled xanthosine and XMP formed. The relative accumulation of ¹⁴C in these compounds was reversed by the addition of a large excess of unlabeled XMP (1 mM) and xanthosine (1 mM) to the reaction mixture at zero time (Fig. 6) in agreement with a metabolic sequence from IMP to XMP to xanthosine and to xanthine.

Purine Synthesis from [1-¹⁴C]Glycine. In the presence of allopurinol and NAD, a reaction mixture containing both the soluble and particulate components from an extract of cowpea nodules
formed xanthine as the principal 14C-labeled purine base from [1-14C]glycine (Fig. 7). By comparison, little labeled inosine or hypoxanthine was recovered indicating that at the purine nucleotide levels generated by the de novo pathway under these conditions oxidation rather than dephosphorylation of IMP was the predominant metabolic route leading to the formation of bases. Recovery of 14C in FGAR, AICAR, and IMP was consistent with the utilization of glycine in the de novo pathway.

DISCUSSION

Metabolism of IMP to form XMP, xanthosine, and xanthine in the presence of allopurinol (Figs. 4–6) accompanied by NAD reduction (Table I) provided convincing evidence for the presence of IMP oxidoreductase in extracts of cowpea nodules. The initial rate of conversion of IMP to XMP (computed as XMP + xanthosine + xanthine) at saturating IMP concentrations (40 and 38 nmol/min·g fresh weight nodule, Fig. 4, A and B, respectively) was stoichiometric with NADH formation (38.5 nmol/min·g fresh weight nodule). Under conditions where the 14C derived from IMP was trapped by the provision of a large pool of unlabelled XMP (Fig. 6), the initial rate of XMP synthesis, though an underestimate due to some 14C xanthosine and xanthine formation, was equivalent to 30 nmol/min·g fresh weight of nodule tissue, again indicating a one to one relationship with NAD reduction and lending further support for the reaction sequence shown in Figure 1.

The apparent lack of IMP oxidation by the cell-free preparations of both cowpea and soybean nodules used in previous studies (1, 17) can be explained by the lability and rapid inactivation of IMP oxidoreductase following isolation (Table I). Activity was extremely labile in the absence of glycerol and inclusion of NAD and IMP in the breaking media used for tissue disruption stabilized the enzyme. Although PVP and sulfhydryl reducing agents were apparently not necessary for the recovering of IMP oxidoreductase (Table I), they have been retained in breaking media to ensure the stability of other enzyme activities associated with purine metabolism and uridine synthesis (1, 4, 5).

The IMP oxidoreductase from cowpea nodules resembled the enzyme isolated from pea seeds (18), as well as those from a number of bacteria (9, 14) in having a relatively low apparent Km for IMP; an alkaline pH optimum and greater activity with NAD compared to NADP. However, unlike IMP oxidoreductase from other sources, the enzyme from nodules did not require a specific sulfhydryl reducing agent such as GSH for activity and was extremely labile.

Allopurinol treatment of intact nodulated root systems of both cowpea and soybean led to the accumulation of xanthine rather than hypoxanthine (4, 8, 10) indicating that xanthine was formed in vivo by a mechanism other than that of xanthine oxidoreductase. The presence of IMP oxidoreductase in nodules together with the much higher affinity of this enzyme for substrate compared to those generating hypoxanthine from IMP (Fig. 4, A–D) would appear to provide an explanation for the observed effect of allopurinol in vivo. However, xanthine could also arise from the deamination of guanine formed as a result of GMP synthesis from XMP (2). In support of this, cowpea nodule extracts readily converted [14C]guanine to [14C]urides (19) indicating the presence of guanine aminohydrolase (EC 3.5.4.3). On the other hand, metabolism of GMP was extremely slow compared to uride formation from IMP or XMP (5) and the occurrence of GMP synthetase (EC 6.3.5.2) in plants remains uncertain (1, 2, 12). In the present study, formation of GMP, guanosine, and guanine from IMP could not be detected in the presence of 1 mM NAD, 2 mM glutamine or 10 mM NH4Cl, and 5 mM ATP (data not shown).

IMP oxidoreductase effectively linked de novo purine synthesis and purine oxidation in cell-free extracts comprising both the soluble and particulate components of the nodule. Using the same isolation techniques, the synthesis of IMP has been found associated with plastids in cowpea nodules (16) and recovery of IMP oxidoreductase as a soluble enzyme supports the idea (5, 16) that purine oxidation to form urides is a property of the plant cell cytosol. This suggests that in vivo IMP is effectively transferred across the plastid outer membrane. Although the dephosphorylation of XMP and the formation of xanthine in vitro was apparently more rapid at low nucleotide levels than the comparable metabolism of IMP (Figs. 4 and 5), all purine nucleotides (IMP, XMP, GMP, and AMP) have been shown to be readily metabolized to their respective nucleosides and bases in cell-free extracts of cowpea nodules (1). It is likely that in such extracts nonspecific phosphatases contribute to the nucleotide breakdown while in vivo nucleotide phosphotransferases (EC 2.7.1.77) with varying nucleotide specificity (11) are the functional cytosolic enzymes. Guranowski (12) has purified an inosine nucleosidase (EC 3.2.2.2) from lupin seeds which hydrolyzed other purine nucleosides, albeit at lower rates, in addition to inosine. Hydrolysis of all purine nucleosides to their component bases (1) suggests that in cowpea nodules nucleosidase has broad substrate specificity. The marked difference in rates of xanthine and hypoxanthine formation from similar levels of parent nucleoside in each case (Figs. 4 and 5) may, however, indicate that in nodules xanthosine is more readily utilized at low substrate levels.

The evidence presented supports a role for IMP oxidoreductase in the synthesis of urides from currently fixed nitrogen. While it seems clear that there are at least two possible routes of IMP metabolism more direct proof using 14CO2 and 15N2 or 14N2 labeling of purine pathway intermediates is required to firmly establish the metabolic pathways leading to the formation of xanthine by intact legume nodules.

Acknowledgments—The skilled technical assistance of P. Storer and D. Waldie is gratefully acknowledged.

LITERATURE CITED

5. ATKINS CA, A RITCHIE, PB ROWE, E MCCARROLL, DS SAUER 1982 De novo purine synthesis in nitrogen-fixing nodules of cowpea (Vigna unguiculata L. Walp) and soybean (Glycine max L. Merr). Plant Physiol 70: 55–60
11. GURANOWSKI A 1979 Nucleoside phosphotransferase from yellow lupin seedling cotyledons. Biochim Biophys Acta 569: 13–22
14. MAGASANKI B, HS MOYED, L FERGHIRO 1957 Enzymes essential for the biosynthesis of nucleic acid guanine; inosine 5'-phosphate dehydrogenase of Aero-


