Biochimica et Biophysica Acta, 659 (1981) 132—140
Elsevier/North-Holland Biomedical Press

BBA 69270

PURIFICATION AND SOME PROPERTIES OF URATE OXIDASE FROM NITROGEN-FIXING NODULES OF COWPEA

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(Received November 5th, 1980)

Key words Urate oxidase, Nitrogen fixation, (Cowpea)

Summary

Urate oxidase (urate oxygen oxidoreductase, EC 1.7.3.3) was purified 166-fold from nitrogen-fixing root nodules of cowpea *Vigna unguiculata* [L.] Walp. The purified enzyme showed a specific activity of 5.7 μmol urate oxidised/min per mg protein, a molecular mass of 100 kdaltons, pH optimum between 9 and 10, isoelectric point at pH 6 8, $K_{m(urate)}$ = 18 μM and $K_{m(oxygen)}$ = 29 μM A number of metal complexing and chelating reagents were inhibitory, as were divalent cations, including Cu^{2+} Iron stimulated the enzyme. Low concentrations of ammonia, glutamine and xanthine were also inhibitory. The regulation of urate oxidase in relation to the assimilation of fixed nitrogen in legume nodules is discussed

Introduction

Many agriculturally important legumes form the ureides, allantoin and allantoic acid, as principal products of nitrogen fixation [1—3] The ureides are exported from the nodules as the main translocated nitrogenous solutes of xylem [1,2,4] and constitute a major source of nitrogen for amino acid and protein synthesis in these plants [4].

Enzymological studies [2,5,6], together with studies of the utilisation of ^{14}C-labelled and unlabelled purine nucleotides and bases by cell-free extracts from cowpea and soybean nodules, indicate that allantoin and allantoic acid are derived from the oxidation of purine bases. While the reactions leading to the bases have not been clearly defined in nodules they are probably formed from de novo synthesis of purine 5′-nucleotides as in other tissues [7].

The formation of allantoin from inosine monophosphate or purine bases in

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a crude cell-free extract of cowpea nodules has been shown to be limited, due to the requirement for a high level of oxygen by urate oxidase (urate· oxygen oxidoreductase, EC 1 7.3.3) [8]. This requirement for high concentrations of oxygen to maximise urate oxidation is significant in view of the low partial pressures of O_2 maintained in legume nodules [9,10], and suggests that in vivo synthesis of ureides could be regulated by the activity of this enzyme.

The present study reports the purification of urate oxidase from cowpea nodules, and examines the kinetic properties of the enzyme in relation to factors likely to modulate its activity in nitrogen fixation.

Materials and Methods

Reagents

In all cases analytical reagent grade chemicals were used without further purification. Sephadex G-200 and Sephacryl G-200 were obtained from Pharmacia, Ultradex gel and Ampholine (pH 6—8) ampholytes from LKB. Apoferritin (horse spleen), myoglobin (horse heart) and catalase (bovine liver) were from Calbiochem, urate oxidase (pig liver) from Boehringer and *o*-phenanthroline, α,α'-dipyridyl, salicylhydroxamate and bovine serum albumin from Sigma.

Plant material

Cowpea *(Vigna unguiculata* [L.] Walp. cv. Caloona) inoculated with *Rhizobium* strain CB756, was grown during spring with nitrogen-free nutrient solution [11] in sand culture in a naturally lighted glasshouse. Nodules were harvested from 40-day-old plants; each plant yielding around 0.5 g fresh weight nodules.

Enzyme assays

The activity of urate oxidase was measured at 30°C (average photo-period temperature during plant growth) by the decrease in absorption at 293 nm due to the disappearance of urate. Reaction mixtures contained 95 μmol Tris-HCl (pH 8.8) and 0.1 μmol freshly prepared sodium urate in a final volume of 1 ml. The reaction was initiated by addition of 5—50 μl enzyme solution. Where the effect of an added compound was measured the addition was made before the reaction was initiated. The pH of solutions used to determine the pH optimum for the enzyme was established using a glass electrode calibrated with standard buffers (BDH Chemicals, Australia). Enzyme activities are expressed as μmol uric acid consumed/min per mg protein.

Catalase (Hydrogen-peroxide· hydrogen-peroxide oxidoreductase, EC 1.11.1.6) was assayed by following O_2 production polarographically [12].

Enzyme purification

All operations were carried out at 0—4°C unless stated otherwise. Freshly detached nodules (26 g fresh weight) were homogenised in 2 vol. 0.1 M Tris-HCl (pH 8 8) containing 5 mM dithiothreitol with a mortar and pestle. The macerate was filtered through 100 μm nylon mesh, centrifuged at 37 000 × *g* for 10 min and the supernatant fraction used as the source of enzyme The

purification of urate oxidase from this crude preparation used three steps·

Step 1 Ammonium sulphate fractionation. Finely-powered ammonium sulphate was added with continuous stirring to the crude enzyme solution (0 1 g/1 ml) and after centrifugation at 37 000 × *g* for 10 min the pellet was discarded. Additional ammonium sulphate was added to the supernatant (1.2 g/1 ml), the precipitated enzyme recovered by centrifugation and the pellet dissolved in 0.1 M Tris-HCl (pH 8.8).

Step 2 Gel filtration. Solubilised enzyme obtained from the previous step was loaded onto a 2 × 25 cm column of Sephacryl G-200 equilibrated with 0.1 M Tris-HCl (pH 8.8). The column was developed with the equilibrating buffer at a flow rate of 0.5 ml/min and the eluate collected in 2-ml fractions.

Step 3 Preparative isoelectric focusing The peak fractions of urate oxidase obtained above were combined, concentrated by ammonium sulphate precipitation and dialysed overnight against 2% (w/v) glycine. A flat bed (0.5 × 11 × 24.5 cm) of Ultradex gel containing 2% ampholytes in the pH range 6.0—8.0 (LKB Ampholine) was prepared and the enzyme solution applied by cutting a trough in the gel at right angles to the direction of separation, mixing this removed gel with enzyme and replacing the mixture in the trough. The gel was developed with an initial voltage and current of 3.5 kV and 14 mA, respectively, and reached equilibrium within 17 h. The gel bed was then fractionated into 30 sections using a stainless steel grid and the pH of each section measured by eluting the non-protein containing edges of the gel in distilled water. The remaining, protein-containing gel was eluted with chilled Tris-HCl (pH 8.8) and assayed for urate oxidase activity.

Protein was monitored in column eluates by absorption at 280 nm and measured in enzyme preparations at all stages of purification by the procedure of Lowry et al. [13] using bovine serum albumin as standard.

Supply of oxygen to urate oxidase reaction

Buffers used in routine assays were bubbled with air at 30°C prior to use. Where oxygen concentration was varied, known mixtures of oxygen and nitrogen were generated using a Wostoff gas-mixing pump [14] and buffers equilibrated with the mixture by bubbling for 30 min at 30°C prior to use in the assay. The concentration of dissolved oxygen maintained during assay was checked polarographically.

Results

The step-wise purification of urate oxidase from the soluble proteins of the plant fraction of cowpea nodules yielded a preparation in which there was a 166-fold increase in specific activity of the enzyme although only 12% of the original activity was recovered (Table I). While this purified preparation showed a single protein band associated with urate oxidase on isoelectric focusing the purified enzyme also contained catalase activity.

From measurements of the reaction velocities at a range of urate concentrations the apparent $K_{m(urate)}$ was calculated to be approximately $1.8 \cdot 10^{-5}$ M (Fig. 1) The apparent $K_{m(O_2)}$ of the purified enzyme was determined at saturating urate concentration (Fig. 2) and found to be equivalent to 2.7%

TABLE I

PURIFICATION OF URATE OXIDASE FROM THE SOLUBLE PROTEINS OF THE PLANT FRACTION OF NITROGEN-FIXING NODULES OF COWPEA

	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min per mg protein)	Percent recovery
1 Crude enzyme solution	357 18	12 31	0 034	100
2 Supernatant from ammonium sulphate precipitation 1	316 51	7 84	0 025	64
3 Solubilised pellet from ammonium sulphate precipitation 2	25 43	4 24	0 167	34
4 Gel filtration	5 00	1 08	0 214	9
5 Preparative isoelectric focusing	0 26	1 47	5 654	12

(v/v) O_2 present in the gas phase, or 29 μM dissolved O_2. While the increase in reaction velocity showed a curvilinear response with increasing oxygen the enzyme was not saturated at the concentration in solution in equilibrium with 100% oxygen in the gas phase at the assay temperature used.

The isoelectric point of the purified protein was found to be at pH 6.8 (Fig. 3) and the optimum pH for activity between 9 and 10 (Fig. 4). The enzyme showed significant activity at pH values as high as pH 12 but was inactive at and below neutrality.

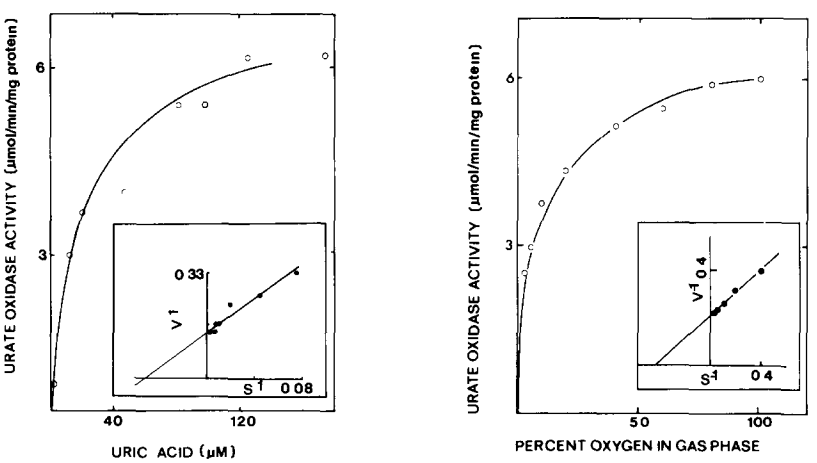

Fig 1 Effect of increasing uric acid concentration on the reaction velocity of purified urate oxidase from cowpea nodules The apparent $K_{m(urate)}$ was found to be 18 μM from the inset Lineweaver-Burk plot

Fig 2 Effect of increasing oxygen concentration on the reaction velocity of purified urate oxidase from cowpea nodules The apparent $K_{m(O_2)}$ was found to be 2 7% (v/v) oxygen (equivalent to 29 μM dissolved O_2) from the inset Lineweaver-Burk plot

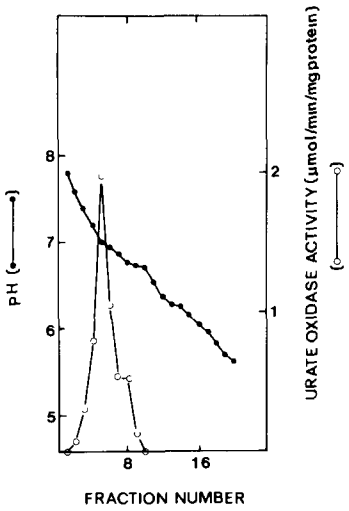

Fig 3 Isoelectric focusing of purified urate oxidase from cowpea nodules on a flat bed gel of Ultradex containing ampholytes in the range pH 6—8

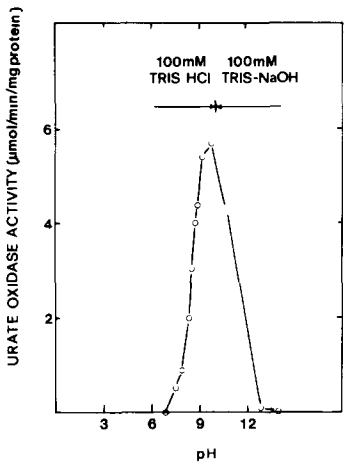

Fig 4 Effect of pH on the reaction velocity of urate oxidase purified from cowpea nodules From pH 6 5—11 the buffer was 0 1 M Tris-HCl and from pH 11—13 0 1 M Tris-NaOH

The molecular mass of the purified enzyme, estimated by gel filtration, was the same as the urate oxidase from pig liver, that is approximately 100 000.

The purified enzyme was relatively stable, retaining complete activity if stored frozen, 60% of initial activity after 7 days at 4°C and 30% after 2 days at 18—20°C.

The enzyme activity was markedly sensitive to low concentrations of a number of metal ions (Table II) and to a range of metal ion complexing and chelating reagents (Table III). As shown in Table II, Cu^{2+}, Mn^{2+} and Co^{2+} were inhibitory, Mg^{2+} had a slightly inhibitory effect but a relatively high concentration and Fe^{3+} stimulated the enzyme. The most effective metal chelating

TABLE II

EFFECT OF METAL IONS ON THE ACTIVITY OF PURIFIED URATE OXIDASE FROM COWPEA NODULES

Fe^{3+} and Mn^{2+} were added as the chloride salts, Cu^{2+}, Co^{2+} and Mg^{2+} as the sulphate salts

Metal	Concentration (M)	Percent inhibition
Fe^{3+}	9 8 10^{-6}	—24 0
	3 9 10^{-5}	—24 0
Cu^{2+}	2 5 10^{-8}	4
	9 8 10^{-8}	72
Mn^{2+}	4 9 10^{-7}	52
	9 8 10^{-7}	100
Co^{2+}	2 5 10^{-7}	88
Mg^{2+}	6 4 10^{-6}	24
	6 6 10^{-5}	48

TABLE III
EFFECT OF METAL COMPLEXING AND CHELATING AGENTS ON THE ACTIVITY OF PURIFIED URATE OXIDASE FROM COWPEA NODULES

Metal chelator or complexing agent	Concentration (M)	Percent inhibition
Sodium fluoride	2.5×10^{-3}	−5
	9.8×10^{-3}	10
α,α′-Dipyridyl	8.5×10^{-5}	53
	2.6×10^{-4}	77
o-Phenanthroline	1.6×10^{-4}	48
	4.9×10^{-4}	88
EDTA	4.9×10^{-3}	11
	2.5×10^{-2}	48
Potassium cyanide	1.2×10^{-3}	85
Salicylhydroxamic acid	7.0×10^{-5}	40

TABLE IV
EFFECT OF NITROGENOUS COMPOUNDS ON THE ACTIVITY OF PURIFIED URATE OXIDASE FROM COWPEA NODULES

Nitrogenous compound	Concentration (M)	Percent inhibition
NO_3^-	2.5×10^{-3}	0
	7.5×10^{-3}	0
NH_4^+	4.9×10^{-7}	15
	6.4×10^{-6}	57
Glutamine	4.9×10^{-7}	5
	6.4×10^{-6}	38
Asparagine	1.5×10^{-4}	−36
	3.0×10^{-4}	17
Glutamic acid	1.0×10^{-4}	−5
	5.0×10^{-4}	14
Aspartic acid	3.0×10^{-4}	8
	9.0×10^{-4}	27

TABLE V
EFFECTS OF INTERMEDIATES OF PURINE NUCLEOTIDE BREAKDOWN ON THE ACTIVITY OF PURIFIED URATE OXIDASE FROM COWPEA NODULES

Compound	Concentration (M)	Percent inhibition
Inosine 5′monophosphate	5.0×10^{-4}	16
	2.5×10^{-3}	46
Guanine	1.0×10^{-4}	7
	3.0×10^{-4}	72
Adenine	1.0×10^{-4}	3
	5.0×10^{-4}	62
Xanthine	1.0×10^{-4}	71
	3.0×10^{-4}	100
Hypoxanthine	1.0×10^{-4}	24
	3.0×10^{-4}	24
Allantoin	1.0×10^{-4}	7
	5.0×10^{-4}	32
Allantoic acid	5.0×10^{-4}	10
	2.5×10^{-3}	10
Glyoxylic acid	5.0×10^{-4}	0
	2.5×10^{-3}	21
Urea	1.0×10^{-4}	7
	5.0×10^{-4}	23

reagents were α,α'-dipyridyl, *o*-phenanthroline and salicylhydroxamate (Table III).

Table IV shows the effect of inorganic and amino nitrogen solutes on the activity of the purified enzyme. Nitrate was without effect whereas ammonia, glutamine and, to a lesser extent, glutamate and aspartate were effective inhibitors. Intermediates of purine nucleotide breakdown also affected enzyme activity (Table V). All were inhibitory, but the purine bases, and especially xanthine were potent inhibitors of urate oxidase at quite low levels.

Discussion

Properties of purified nodule urate oxidase

The markedly alkaline pH optimum (pH 9—10, Fig. 4), slightly acidic isoelectric point (pH 6.8, Fig. 3), molecular mass of 100 000 and relatively high affinity for urate (apparent $K_m = 18$ μM, Fig. 1) are similar to properties shown by urate oxidases purified from a number of animal and microbial sources [15] and by the enzyme partially purified from soybean nodules [6].

Urate oxidase from a number of sources has been reported to be a copper or ion metallo-enzyme, although the functional role of these metals in purified preparations has not been established [15]. The presence of a functional metal for activity of the cowpea nodule enzyme is indicated from the inhibition caused by a range of chelating and metal complexing reagents (Table III). As with the enzyme from soybean nodules [6], the cowpea urate oxidase was particularly sensitive to α,α'-dipyridyl and *o*-phenanthroline, possibly indicating that these enzymes contain iron. This is supported to some extent by the independent stimulation of activity, regardless of concentration, by the addition of Fe^{3+} salts to the purified cowpea nodule enzyme (Table II).

Rat liver urate oxidase as well as the enzyme from microorganisms is sensitive to inhibition by low concentrations of Cu^{2+} [15]. This was also the case for the cowpea nodule urate oxidase (Table II) but the effect was not specific for Cu^{2+} and was also caused by higher concentrations of a range of divalent cations.

In cowpea nodules, urate oxidase is largely associated with the cytosol proteins of the bacteroid-containing cells rather than with the bacteroids [2], indicating that the enzyme is of plant rather than bacterial origin. While the cowpea and soybean nodule enzymes are apparently similar in their catalytic properties the only urate oxidase studied from normal plant vegetative tissues (radicle of soybean [6]), shows some quite dissimilar properties This latter enzyme has a neutral pH optimum, apparent $K_{m(urate)}$ of 0.56 μM and requires a low molecular weight (200—300) cofactor for catalytic activity. Thus at least two different urate oxidases may be present in higher plants, the type found in legume nodules being confined to those tissues in which high rates of purine oxidation are associated with major transformations of nitrogen.

Regulation of urate oxidase in nodules

The most significant property of the urate oxidase purified from nodules, and that likely to regulate the activity of the enzyme in vivo, is its relatively low affinity for oxygen ($K_m = 29$ μM dissolved O_2, Fig. 2) Based on measure-

ments of the dissociation constant (O_2) for leghaemoglobin and the content of leghaemoglobin in soybean nodules the concentration of free dissolved oxygen is likely to be 10—200 nM [10]. The high 'oxygen affinity' oxidase of bacteroids functions effectively at 10—100 nM dissolved oxygen [16] and recent measurements of respiration and hydrogen oxidation by bacteroids showed an apparent K_m (O_2) around 10 nm [17]. Clearly, unless the kinetic properties of urate oxidase are quite different in vivo or the enzyme utilises a source of oxygen other than the general tissue supply, the microaerophilic environment maintained in nodules [18] is likley to severely limit oxidation of uric acid. As a consequence ureide formation would be expected to reflect oxygen availability in the tissue.

That this limitation might be a normal feature of nodule functioning in those plants which produce ureides is supported by differential effects of lowered rhizosphere pO_2 on the nature of nitrogenous solutes produced from $^{15}N_2$ by intact soybean plants [19] When pO_2 was changed from 0.2 to 0.1 atm ^{15}N-labelling of allantoin was more severely inhibited than was the formation of $^{15}NH_3$ or the labelling of amino compounds.

A number of nitrogenous compounds likely to be present in nodules as products of nitrogen fixation or intermediates in the oxidation of purine nucleotides were found to modify the activity of purified urate oxidase (Tables IV and V). Ammonia, the initial product of nitrogen fixation secreted into the host cell cytosol from bacteroids, and the initial product of ammonia assimilation, namely glutamine, were the most effective inhibitors of the compounds tested. In each case urate oxidase was inhibited around 50% by 6 μM, a concentration likely to be present in nodules during nitrogen fixation. These observations may indeed explain why exogenously-supplied $^{15}NH_4^+$ and ^{15}N (amide) glutamine were not readily converted to ureides in soybean nodules [20]. By contrast, the other amide found in nodules, asparagine, stimulated the activity of the purified enzyme (Table IV), while the dicarboxylic amino acids only caused significant inhibition at concentrations approaching 1 mM. All intermediates of purine nucleotide breakdown tested were inhibitory although the bases, and especially xanthine, were most effective (Table V). Urate oxidase from soybean nodules as well as from other sources [6,21,22] has also been found to be highly sensitive to inhibition by xanthine.

In most tissues where the intracellular location of urate oxidase has been studied, the enzyme is associated with microbodies [15,23—25] Such a location would result in effective detoxification of hydrogen peroxide produced by urate oxidation releasing oxygen in close proximity to urate oxidase and possibly altering oxygen availability to the enzyme. Catalase added to purified urate oxidase from *Aspergillus flavus* enhanced allantoin formation [26]. However, in the present study the purified enzyme from nodules contained significant catalase activity and additions of purified catalase to reaction mixtures were without effect on the rate of urate oxidation Neither the presence of microbodies nor the intracellular location of urate oxidase in legume nodules has been established. However, in view of the marked sensitivity of urate oxidase to inhibition by the initial products of nitrogen fixation and xanthine, its very alkaline pH optimum and apparently low affinity for oxygen, an organelle location for this enzyme seems likely.

Acknowledgements

This work was supported by funds from the Australian Research Grants Committee (to C.A.A.) and a University of Western Australia Post Graduate Studentship (to R.M.R.).

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