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OXIDATION OF AMINO ACIDS BY MANGANOUS IONS AND PYRIDOXAL PHOSPHATE

TERENCE A. SMITH and JACQUELINE H. A. MARSHALL

Research Station, Long Ashton, Bristol, BS18 9AF, U.K.

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; ornithine; pyridoxal phosphate; manganese; oxidative decarboxylation.

Abstract—The oxidation of amino acids by a system consisting of manganese chloride and pyridoxal phosphate was measured by means of an oxygen electrode. The optimum pH for ornithine oxidation was 10.5. At this pH, oxidation of a series of amino acids was in the sequence ornithine > lysine > arginine > citrulline > phenylalanine > methionine > glutamic acid > histidine > tryptophan > proline. The system was greatly stimulated by peroxidase and phenol at pH 7 but no stimulation was found at pH 10.5. In the presence of pyridoxal phosphate, a particulate preparation from an extract of wheat leaves oxidised ornithine, arginine and lysine at pH 9.1 with approximately the same relative activity as the Mn^{2+} system, suggesting that oxidation by the plant extract is Mn^{2+} dependent.

INTRODUCTION

Measurement of $^{14}CO_2$ release from carboxyl-labelled ornithine is frequently used for the estimation of ornithine decarboxylase (ODC; EC 4.1.1.17), an enzyme which forms putrescine, and activity of other amine-forming amino acid decarboxylases is measured by the same principle. The existence of a true ODC in higher plants has now been established [1], and this enzyme has been recently purified to homogeneity from barley seedlings [2]. However, in a study of ODC in many higher plants, Birecka *et al.* [3,4] found that the putrescine which they detected was insufficient to account for the $^{14}CO_2$ released. The ratio of CO_2/amine was often greater than 10 in their experiments. They suggested that this deficit in putrescine could arise by the participation of a mechanism for the decarboxylation of ornithine resulting in products other than putrescine, or by the enzymic oxidation of the putrescine formed by a true ornithine decarboxylase.

A model system consisting of Mn^{2+} and pyridoxal phosphate (PLP) has been shown to cause the oxidative decarboxylation of a wide range of amino acids in the presence of peroxidase and a phenol [5, 6]. The product of methionine oxidation was 3-methylthiopropionamide [7], and tryptophan oxidation yielded indole-3-acetamide [8]. The oxidation of methionine by this system has been used as the principle of a method for estimating Mn^{2+} [9]. An Mn^{2+}/PLP system was also shown to cause the decarboxylation of α-keto acids [10] and the iodination of tyrosine [11]. The products of the oxidative decarboxylation of ornithine by the Mn^{2+}/PLP system have been shown to contain 4-aminobutanamide, but no putrescine could be detected [12]. A similar system, which also converts ornithine to the amide and is dependent on added PLP, is known to occur in extracts of many higher plants [12]. Although this is thermolabile, has multiple pH optima and has a M_r which may be in excess of 4000, circumstantial evidence has indicated that this is also dependent on naturally occurring Mn^{2+}. The present investigation provides additional evidence for the participation of Mn^{2+} in this plant system. Since many assays for amine formation have depended on the release of CO_2 from [1-^{14}C]-labelled amino acids as substrates, it is important that the properties and distribution of the naturally occurring oxidative decarboxylation should be known in order that unambiguous amino acid decarboxylase assays can be developed.

RESULTS AND DISCUSSION

The pH optimum for the oxidation of ornithine by the Mn^{2+}/PLP system was 10.5, with a greater initial lag period at the lower pH ranges (Fig. 1). This contrasts

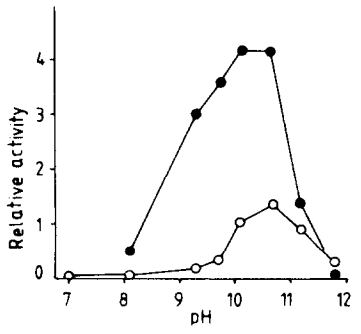

Fig. 1. Relationship between pH and oxygen uptake in a system consisting of $MnCl_2$ (200 μM), pyridoxal phosphate (1 mM) and 0.1 M buffers (sodium phosphate pH 7; borate/HCl pH 8.1–9.3; borate/NaOH pH 9.3–10.7; disodium hydrogen phosphate/NaOH pH 10.8–11.8). The activity was measured 1 min after addition of the amino acid (open symbols) and immediately before total oxygen consumption (closed symbols).

with the pH optimum for the oxidation of methionine by the $MnCl_2$/PLP system [5] conducted in the presence of peroxidase, for which the pH optimum lay between 6.5 and 7.5. The rate of oxidation of ornithine was proportional to Mn^{2+} with a range of concentrations from 10 to 200 μM (Fig. 2). At pH 10.5 ornithine, lysine, arginine and citrulline were oxidised more rapidly than glutamic acid, but at pH 8 oxidation was slower with lysine and citrulline than with glutamic acid, though ornithine was still the best substrate (Fig. 3).

A direct comparison of the oxidative ability of the inorganic system with that of the wheat leaf particles was made using 10 μM $MnCl_2$ at pH 9.1 (Fig. 4). The plant extract did not show the lag period which characterises the inorganic system. This may be due to the presence of peroxidase in the plant extracts [6]. However, the specificity of the two systems was very similar, particularly in relation to the oxidation of ornithine and arginine, suggesting that the plant system also depends on Mn^{2+}.

At pH 10.5, 1 mM and 100 μM EDTA inhibited oxidation by the $MnCl_2$/PLP system to 0 and 56% respectively, while at pH 9.3 the activity was reduced to 0 and 70% respectively. In earlier work 100 μM EDTA was shown to inhibit decarboxylation of ornithine by wheat leaf extract at pH 9.3 to 4.5% (particles) and 29% (supernatant).

Although Hill and Mann [6] suggested that illumination accelerates amino acid oxidation at pH 7 in the presence of peroxidase and *p*-cresol, in our work at pH 10.5 the reaction rate was identical in complete darkness and with illumination at 34 W/m^2. All experiments at pH 10.5 were therefore conducted under normal laboratory illumination (*ca* 2 W/m^2).

Dithiothreitol at 1 and 5 mM is rapidly oxidized by the $MnCl_2$/PLP system at pH 10.5. This thiol would therefore tend to deplete the oxygen and hence inhibit the oxidative decarboxylation. Inhibition by thiols was demonstrated in earlier work [12].

Peroxidase and a phenol have been shown to activate the oxidation of amino acids by the Mn^{2+}/PLP system at pH 7 [5, 6]. In the present work the standard mixtures (200 μM $MnCl_2$, 1 mM PLP) and buffers (0.1 M phosphate pH 7, or 0.1 M borate/NaOH pH 10.5) were preincubated with and without 5 μg peroxidase and 5 μM phenol for 2 min prior to adding ornithine (5 mM). Oxygen uptake at pH 7 and 10.5 in the absence of peroxidase/phenol was, respectively, 3.1 and 575 nmol/min, 8 min after the addition of the amino acid. Oxygen uptake at pH 7 and 10.5 in the presence of peroxidase/-phenol was, respectively, 125 and 513 nmol/min. The action of peroxidase/phenol is therefore much more effective at the lower pH.

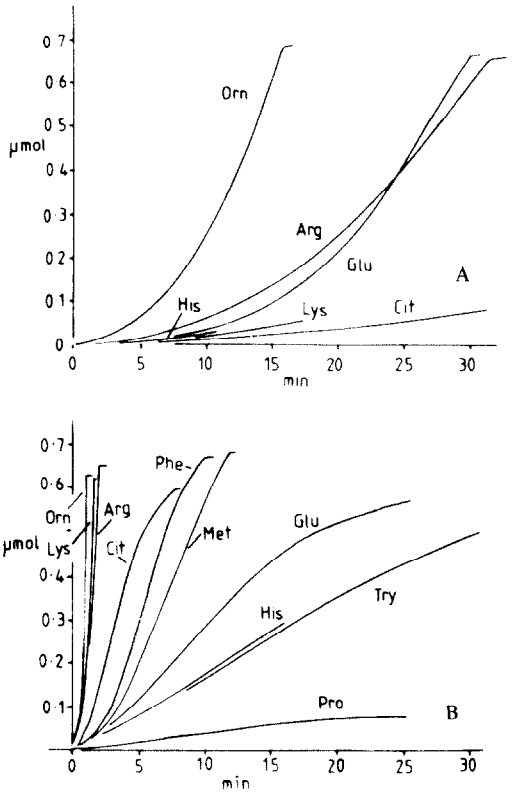

Fig. 3. Oxidation of ornithine (Orn), lysine (Lys), arginine (Arg) citrulline (Cit), phenylalanine (Phe), methionine (Met), glutamic acid (Glu), histidine (His), tryptophan (Try) and proline (Pro) by the manganese/pyridoxal phosphate system at pH 8(A) and pH 10.5(B). No significant oxidation of methionine, phenylalanine, tryptophan or proline could be detected at pH 8.

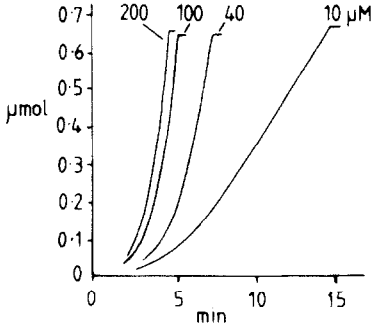

Fig. 2. Effect of $MnCl_2$ concentration (μM) on oxidation of ornithine at pH 10.5 in the presence of pyridoxal phosphate (1 mM).

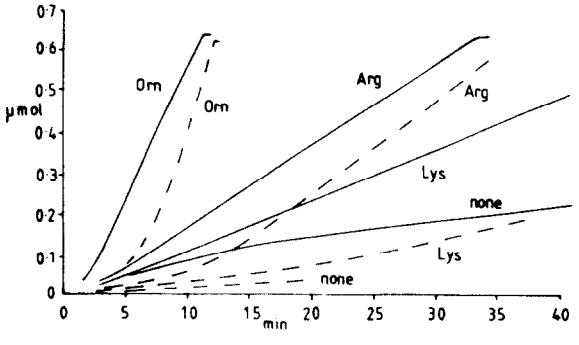

Fig. 4. Oxidation of ornithine (Orn), arginine (Arg) and lysine (Lys) at pH 9.3 by PLP (1 mM) and $MnCl_2$ (10 μM) (dotted line), and by the particulate preparation from wheat leaves in the presence of PLP (1 mM) (continuous line) with no added Mn^{2+}.

Extracts of wheat leaves showed pH optima of 9 and 10.5 [12] and the peak at the high pH could therefore be attributed to (free) Mn^{2+}. The lower pH optimum (pH 9) and the two pH optima shown by the oat leaf extracts (pH 6.5 and 9) may therefore be due to combined Mn^{2+}, though the form which this takes in wheat (M_r *ca* 4000) [12] is at present unknown. Paper electrophoresis (pH 9.3) of a concentrated preparation of the wheat leaf supernatant gave two bands of activity and separation on G-25 Sephadex showed at least two active peaks, though the pH activity relationship for these has not been studied. The active complex between Mn^{2+} and PLP is assumed to be through the aldehyde and the phosphate residue on the PLP in a 1:1 ratio [13] and the time taken for the formation of this complex probably accounts for the additional lag period for oxygen uptake found on mixing these two components in the oxygen electrode cuvette.

On the basis of the results for the oxygen consumption (Fig. 4) activity of the wheat leaf particulate preparation was *ca* 420 pkat/ml. Oxygen uptake was therefore *ca* 3.5 times the stoichiometric value on the basis of $^{14}CO_2$ release with 4-aminobutanamide formation suggesting that the reaction mechanism is not simple. 4-Aminobutyric acid and pyrroline are additional products which might be expected [12]. It is possible that the PLP is also oxidised [6] though why this should be dependent on the presence of the amino acid is not clear. The conversion of ornithine to pyrroline-2-carboxylic acid is a possible route of oxidation which does not involve decarboxylation and which would, therefore, explain the lack of stoichiometry. The formation of ammonia and the high ratio of O_2 uptake/CO_2 release found by Hill and Mann [6] for the oxidation of ornithine at pH 7 by the Mn^{2+}/PLP system also suggests the operation of this additional pathway.

In view of the commonly used method for determining decarboxylation of amino acids for the formation of amines by measurement of CO_2 release from carboxyl labelled amino acids, it is of interest to compare the levels of Mn^{2+} in biological material, since this may determine the degree of artefactual oxidative decarboxylation in this assay. The Mn^{2+} concentration in wheat and other plants is frequently about 100 μM [14, 15]. The manganese is therefore at a concentration at which significant oxidative decarboxylation could take place. Even in man, values of 7 to 21 μM have been found, which at pH > 8 could give rise to artefactual decarboxylation in the presence of PLP. This Mn^{2+} dependent oxidation can be greatly reduced by adding EDTA and thiols and probably also by lowering the pH [12]. The widespread occurrence of peroxidase and of various phenolics in plants suggests that ammonium sulphate precipitation and/or dialysis should reduce the synergism found at pH 7–8 by these components, though even these procedures may not be effective in eliminating the oxidative decarboxylation [3]. As stated by Birecka *et al.* [3], it is essential to verify the reliability of the standard $^{14}CO_2$ based methods for assessment of amino acid decarboxylases by measurement of the formation of the amine. In the absence of stoichimetry the results obtained by $^{14}CO_2$ release could, at least in part, be a measure of Mn^{2+} concentration, and not enzymic amino acid decarboxylase acitivity.

EXPERIMENTAL

Wheat (*Triticum aestivum* L. cv Timmo) was grown for 8 days in the dark in a nutrient medium [12]. Leaves were extracted in 3 vol. cold H_2O and the crude extract (pH 6) was subjected to ultrasonics (Kerry PUL 125) for 1 hr. The extract was squeezed through muslin and centrifuged at 10000 *g* for 15 min. The supernatant was frozen overnight, thawed, and again centrifuged (10000 *g*, 15 min). The ppt. was suspended in a vol. of pH 9.3 0.1 M borate buffer equivalent to 20% of the original fr. wt. The decarboxylase activity using the $^{14}CO_2$ method of ref. [12] was about 122 pkat/ml.

The Clark electrode [16] was used to measure O_2 consumption at 30°. The electrode was calibrated by measurement of O_2 consumption on adding 250 nmol of ornithine to the $MnCl_2$/PLP system. The oxygen content of the incubate (*ca* 0.65 μmol) corresponds to 260 μM which is within the expected range [17]. In the standard assay the $MnCl_2$/PLP and buffer were pre-incubated in the electrode cuvette for 2 min to stabilize before adding 50 μl of 250 mM amino acid (final concn 5 mM) to start the reaction. Unless otherwise stated, the incubates (total vol. 2.5 ml) consisted of 200 μM $MnCl_2$, 1 mM PLP and 0.1 M buffers as listed in Fig. 1. Starting the reaction with Mn^{2+} or PLP extended the lag period by several min. Oxygen consumption was recorded to the point of exhaustion when practical. The peroxidase was from Sigma (type II).

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