PYRROLOOXYGENASES: THE BIOSYNTHESIS OF 2-AMINOACETOPHENONE

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1. Introduction

It has recently been shown [1] that 2-aminoacetophenone (I) can serve, *in vivo* and *in vitro*, as a substrate of a hydroxylating system present in liver microsomes which transformed it into 2-amino-3-hydroxyacetophenone (II), a known metabolic product present as its sulphate in normal and pathological human urines [2]. 2-Aminoacetophenone (I) has been isolated from urine of hens following ingestion of tryptophan [3] but its biosynthetic formation remained unknown and was the subject of much speculation [2, 4]. We describe here the existence of a new enzyme isolated from wheat germ and from rat liver which efficiently oxidizes skatole (IV) and indole-3-acetic acid (V) to give 2-formimidoacetophenone (III), which is subsequently hydrolyzed to (I) by a formylase present in the system. The enzyme belongs to the family of the pyrrolooxygenases, a new type of oxygenases recently described [5].

2. Materials and methods

Skatole, indole-3-acetic acid, NADPH, NADH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, nicotinamide, reduced glutathione, ascorbic acid, cysteine and sodium dithionite were commercial products of analytical grade. 2-Formimidoacetophenone was prepared by synthesis. Wheat germ was a gift of Molinos Rio de la Plata (Buenos Aires). Wistar albino rats weighing 150–200 g were used.

2.1. *Preparation and partial purification of pyrrolooxygenase*

The enzyme was isolated from wheat germ following the general procedure previously described [5]. The enzyme eluted from DEAE-cellulose was used for subsequent work. When rat liver was used as source of the enzyme, 6 to 8 g of wet weight was homogenized with 0.1 M of Tris-HCl buffer (pH 7.6). The homogenate was centrifuged during 20 min at 20,000 *g* and the supernatant solution was fractionated with solid ammonium sulphate. The fraction precipitating at 30–70% saturation (AS 30–70) was dissolved in a small volume of 0.01 M Tris-HCl buffer (pH 7.6) and dialyzed overnight against 4 l of water. The dialyzed fraction (120 mg of protein) was applied to a 1.6 × 15 cm DEAE-cellulose column equilibrated with 0.003 M phosphate buffer (pH 7.4) and most of the activity was eluted with the same buffer. The active fractions were concentrated to one tenth of the original volume with Carbowax and used for subsequent work. Enzymatic activity was very low in the crude extract and in the ammonium sulphate fraction. The DEAE-cellulose fractionation increased strongly the activity. The enzyme could be stored at 0–4° without loss of activity during a month. The wheat germ enzyme had a greater stability and activity than the rat liver enzyme.

A "20,000 *g* supernatant" was prepared by homogenizing 6 to 8 g of rat liver in a buffer containing 0.05 M phosphate, 0.3 M sucrose and 0.15 M potassium chloride (pH 7.4). The homogenate was centrifuged at 5000 *g* during 15 min, followed by a 20,000 *g* centrifugation during 30 min.

This enzyme was also found in rat brain and in spinach chloroplast preparations.

2.2. *Assay of pyrrolooxygenase*

The enzyme, unless otherwise specified, was assayed as follows: 50 nmole of skatole or indole-3-acetic acid, 10 μmole phosphate buffer (pH 7.4), 0.05 μmole of sodium dithionite and enzyme (5 to 20 μg of protein when wheat germ enzyme was assayed and 150–200 μg of protein when rat liver enzyme was assayed) were incubated in a final volume of 100 μl at 37°. Incubation times ranged from 15 to 30 min. Substrate consumption was assayed with Ehrlich's reagent (2% *p*-dimethylaminobenzaldehyde in glacial acetic acid, perchloric acid (84:16 v/v) at 552 nm after previous addition of Hg^{2+}. Two blanks were usually run, omitting either dithionite or enzyme.

3. Results

3.1. *Properties of the enzyme*

The effect of enzyme and substrate concentration can be seen in fig. 1 and fig. 2. The activity increased linearly with time up to 30 min and it had an absolute requirement for a reducing agent and oxygen.

Skatole (IV) and indole-3-acetic acid (V) were both substrates of the pyrrolooxygenase and formed the same products. Skatole was a better substrate, being 52% consumed, while the consumption of indole-3-acetic acid was 33%, at the indicated concentrations (2.2).

Of a series of different reductants: ascorbic acid, cysteine, NADH, reduced glutathione and sodium dithionite, only the last one was capable of carrying out efficiently the enzymatic reaction. NADPH served as a reducing agent when added to the crude rat liver preparation. Although the enzymatic activity was very low (table 1), it paralleled the activity obtained using sodium dithionite. With the DEAE-cellulose purified liver preparations, sodium dithionite was by far the most efficient reducing agent. However, when the "20,000 *g* supernatant" and NADPH were added to the same, the enzymatic activity equalled that obtained by using sodium dithionite (table 1). These results indicated that an inhibitor of the enzyme was present in the crude extracts and in the "20,000 *g* supernatant" and was largely removed during the DEAE-cellulose purification. The natural reducing system, apparently NADPH and a yet unidentified

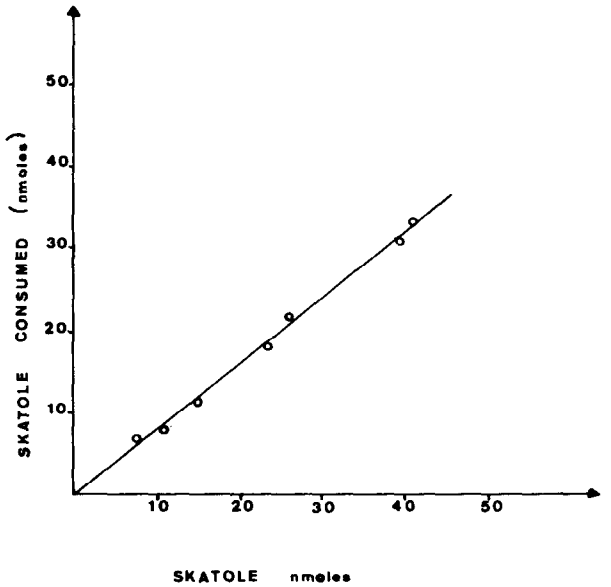


Fig. 1. Effect of skatole concentration: Conditions are as in the standard assay except for the different substrate concentrations. DEAE cellulose enzyme from wheat germ was used (5 μg).

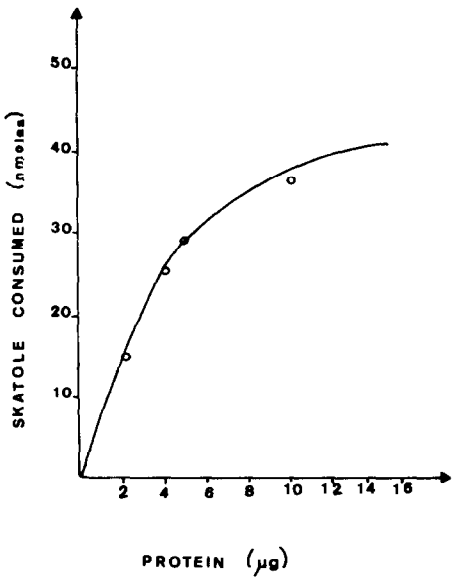


Fig. 2. Effect of enzyme concentration: The DEAE cellulose enzyme from wheat germ was used at the indicated concentrations. The incubations were performed as described in Methods.

Table 1
NADPH as reducing agent of rat liver pyrrolooxygenase.

Enzyme	Addition	Reducing agent	Substrate consumed (nmoles)	Consumption (%)
Fraction AS 30–70%	–	Sodium dithionite	2.4	8
Fraction AS 30–70%	–	NADPH[a]	2.7	9
"20,000 *g* supernatant" (50 μl)	–	Sodium dithionite	1.4	4.6
"20,000 *g* supernatant" (50 μl)	–	NADPH[a]	6.7	22
"20,000 *g* supernatant" (25 μl)	–	NADPH[a]	2.4	8
DEAE concentrated fractions	–	Sodium dithionite	11	36
DEAE concentrated fractions	–	NADPH[a]	2.4	8
DEAE concentrated fractions	"20,000 *g* supernatant"[b]	Sodium dithionite	4	13
DEAE concentrated fractions	"20,000 *g* supernatant"[b]	NADPH[a]	12	40

The incubation mixture was as described in Methods, except for the use of 30 nmoles of skatole.

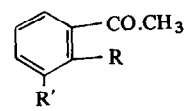
[a] When NADPH was used the system contained NADPH (0.04 μmole), nicotinamide (5 μmole), glucose-6-phosphate (0.5 μmole), glucose-6-phosphate dehydrogenase (5 μl) and $MgCl_2$ (1 μmole).
[b] 25 μl were used.

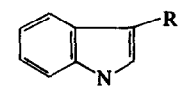
electron carrier system, was also lost in the same purification step but could be reconstructed as described above. NADPH had no effect on the purified wheat germ enzyme.

EDTA (5 mM) inhibited 100% of the skatole consuming activity. α,α′-Dipyridyl (0.5 mM) inhibited 55% of the skatole consuming activity, while all the activity was lost at 1 mM concentration. This indicated that the enzyme probably contains Fe^{2+}. 2-Mercaptoethanol (0.5 mM) inhibited the oxygenase by 25%, while higher concentrations inhibited entirely the activity.

3.2. *Products formed*

The products formed during the incubation of skatole (IV) and indole-3-acetic acid (V) with the enzyme system were extracted with ethyl acetate after adjusting the aqueous solution to pH 5 with citric acid. The extracted products were separated by TLC on silica gel using a benzene-methanol mixture (98.5%–1.5%). The two fluorescent spots (R_f 0.5 and 0.7) were eluted with ethyl acetate and both products identified by spectroscopic analysis.





R: NH_2, *R*′: H (I)
R: NH_2, *R*′: OH (II)
R: NHCHO, *R*′: H (III)

R: CH_3 (IV)
R: CH_2CO_2H (V)

The substance with R_f 0.5 had *m/e* 163 ($C_9H_9NO_2$), 148 ($C_8H_6NO_2$), 135 (C_8H_9ON), 120 (C_7H_6NO), 92 (C_6H_6N), 77 (C_6H_5); IR (KBr), 3330 cm^{-1} (NH), 1695 (CO amide), 1655 (*CO*-CH_3). It was identical with a synthetic sample of 2-formimidoacetophenone (III) when compared by IR, Mp and TLC. The substance with R_f 0.7 had *m/e* 135 (C_8H_9ON), 120 (C_7H_6NO), 92 (C_6H_6N), 77 (C_6H_5), and was identical with an authentic sample of 2-aminoacetophenone (I) when compared by IR and TLC. A short reflux (15 min) of (I) in formic acid transformed it entirely into the formyl derivative (III).

The total yield of (I) and (III) was of 90%, based on consumed skatole. The relative amounts of 2-aminoacetophenone (I) and of its formylated derivative (III) varied with the amount of formylase activity present together with the oxygenase in the different extracts. Formylase activity was evidenced using 2-formimidoacetophenone (III) as a substrate of

the standard incubation mixture (2.2) without addition of sodium dithionite. It was then transformed into 2-aminoacetophenone (I) which was extracted and identified as described above. The formylase was very active in rat liver preparations (DEAE-cellulose enzyme) where (I) comprised 80% of the reaction products, while in wheat germ preparations (DEAE-cellulose enzyme) it comprised only 30% of the formed products.

4. Discussion

The obtained results indicate that 2-aminoacetophenone (I) is a metabolic oxidation product derived from skatole and also from indole-3-acetic acid. In the latter case the product formed is very likely the β-ketoacid, which decarboxylates spontaneously to (III). The pyrrolooxygenase which oxidizes (IV) and (V) is different from the oxygenase which oxidizes tryptophan as such or when it forms part of peptides [5], as will be described in detail elsewhere. The oxygenase is apparently a mixed function oxidase which uses NADPH together with a complex electron-carrier system as the reducing agent, at least in the mammalian enzymes. The enzyme is present normally in the tissues but is inactive due to the presence of a powerful inhibitor.

2-Aminoacetophenone is thus a ubiquitous metabolite of a metabolic pathway the relevance of which must still be assessed.

Acknowledgements

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