THE ENZYMATIC OXIDATION OF PORPHOBILINOGEN

Rosalía B. FRYDMAN, María L. TOMARO,
Alfredo WANSCHELBAUM and Benjamin FRYDMAN
Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junin 954, Buenos Aires, Argentina

Received 30 June 1972

1. Introduction

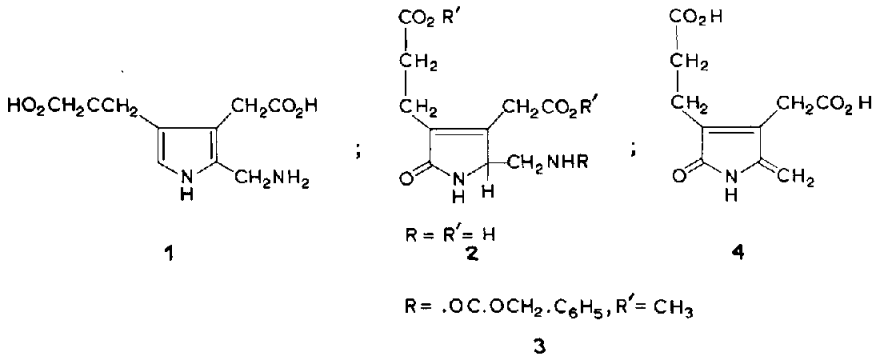
All the natural porphyrins and chlorins originate metabolically in a single monopyrrole – porphobilinogen **1**. Porphobilinogen is thus a crucial intermediate in porphyrin metabolism, and is overproduced and excreted in large amounts during the pathological derangements of this metabolism which result in the metabolic and acquired diseases known as porphyrias [1]. Its only known metabolic pathway was its enzymatic transformation in either uroporphyrinogen III under normal metabolic conditions, or into uroporphyrinogen III and uroporphyrinogen I under pathological conditions, where the overproduction of porphobilinogen leads to the *in vivo* formation of large amounts of porphyrins [1]. In this report we describe the existence of a new enzyme present in plants and animals which efficiently oxidizes porphobilinogen transforming it into 5-oxo-porphobilinogen **2**, a product which is not transformed into porphyrins any longer. The enzyme, for which we propose the tentative name of porphobilinogen oxygenase, belongs to the group of the pyrrolooxygenases, a new type of enzyme recently described [2, 3].

2. Materials and methods

Porphobilinogen was obtained by synthesis [4]. NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, nicotinamide, and sodium dithionite were commercial products of analytical grade. Wheat germ was a gift of Molinos Río de la Plata. Wistar albino rats weighing 150–200 g were used. TLC determinations were run on cellulose coated plates using the upper layer of a butanol:acetic acid:water mixture (4:1:5) as developer. NMR spectra and IR spectra were determined with a Perkin Elmer R 12 and a Perkin Elmer 21 spectrophotometer, respectively.

2.1. *Preparation and purification of porphobilinogen oxygenase*

The enzyme was isolated from wheat germ following



the general procedure described for the isolation of pyrrolooxygenases [2, 3]. The DEAE-cellulose fractionation separated porphobilinogen deaminase from porphobilinogen oxygenase. A more purified enzyme was obtained by filtration of an active DEAE-cellulose fraction (0.5 ml) through Sephadex G-75 (30 cm × 2 cm), equilibrated with 0.05 M phosphate buffer solution (pH 7.4).

The enzyme was also isolated from rat liver. The wet tissue (8 g) was homogenized with 0.1 M Tris-HCl buffer (pH 7.6), and the homogenate centrifuged during 20 min at 20,000 *g*. The supernatant was fractionated by adding solid ammonium sulphate and the fraction precipitating between 30–70% of saturation (AS 30–70) was dissolved in 0.01 M of Tris-HCl buffer (pH 7.6), and used as enzyme source. Activity increased greatly by adsorbing the AS 30–70 fraction on a DEAE-cellulose column and eluting with 0.003 M buffer phosphate (pH 7.4). The enzymatic activity was partially lost after a 12 hr dialysis. Liver microsomes were prepared as described [3]. Porphobilinogen oxygenase was also detected in rat brain and in chloroplasts of spinach and Swiss chard.

2.2. *Assay of phorphobilinogen oxygenase*

The incubation mixture contained in a final volume of 100 μl: 10 μmoles of phosphate buffer (pH 7.4), 100 nmoles of sodium dithionite, 12 nmoles of porphobilinogen and purified enzyme (5 to 20 μg of protein). Incubations were run at 37° for 15 to 30 min. Two blanks were simultaneously run, omitting either dithionite or enzyme. 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+}. Sodium dithionite consumption was determined by adding an excess of a 1% iodine solution and titrating the excess of iodine with a 0.01 N valorated solution of sodium thiosulfate. Oxygen consumption was measured using an Oxygraph Model K (Gibson Medical Electronics).

3. Results

2.1. *Properties of the enzyme*

The consumption of porphobilinogen by porphobilinogen oxygenase was an enzymatic reaction. The

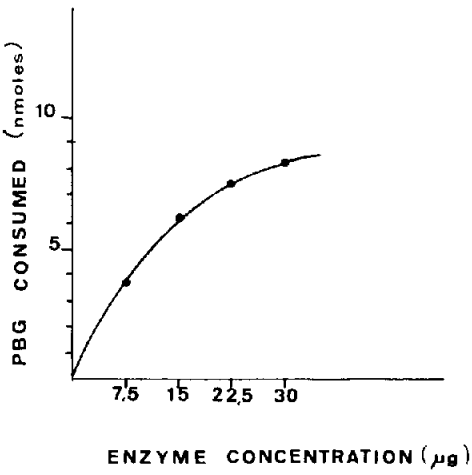


Fig. 1. Time course of the reaction. The Sephadex G-75 purified wheat germ enzyme was used. The incubation was as described in Methods, during the indicated time periods.

time course of the reaction and the effect of enzyme concentration are shown in figs. 2 and 3. The oxygenase activity was entirely destroyed by the action of trypsin on the enzyme during 30 min. The enzyme had an absolute requirement for oxygen and a reducing agent. The best reducing agent was sodium dithionite and to a lesser extent also sodium sulfite and sodium bisulfite. Other reducing agents were ineffective, as

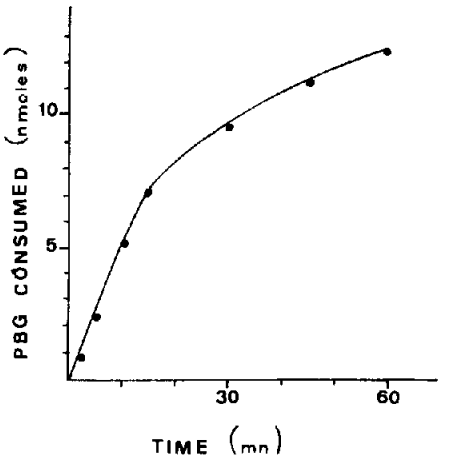


Fig. 2. Effect of enzyme concentration. The Sephadex G-75 purified wheat germ enzyme was used at the indicated concentrations. The incubations were performed as described in Methods for 15 min.

Table 1
Activity of porphobilinogen oxygenase from rat liver.

Enzyme	Reducing agent	Porphobilinogen consumed (nmoles)
Fraction AS 30–70	Dithionite	2.7
Fraction AS 30–70	NADPH[a]	1.3
Microsomes	Dithionite	1.3
Microsomes	NADPH[a]	3.5
Heated microsomes[b]	NADPH[a]	0
DEAE purified enzyme	NADPH[a]	0
DEAE purified enzyme	Dithionite	6.0

The incubation mixture was as described in Methods, a) 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) Heated during 5 min at 80°.

was in the case with other pyrrolooxygenases [2, 3]. When the stoichiometry of the reaction was measured it was found that 6 nmoles of oxygen and 5.4 nmoles of dithionite were used up when 5.6 nmoles of substrate were consumed in 5 min. These results were

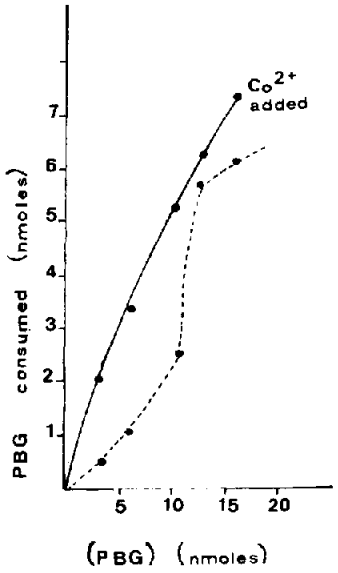


Fig. 3. Effect of substrate concentration. A Sephadex G-75 purified enzyme was used. Conditions were as indicated in assay, except for the different phosphobilinogen (PBG) concentrations. Glycine-sodium hydroxide buffer (pH 8.2) was used (10 μmoles). $CoCl_2$ (25 mM) was used.

consistent with a mixed-function oxidase mechanism.

Porphobilinogen oxygenase activity in crude extracts from rat liver was low due to the presence of a proteic inhibitor, which is partly removed by the DEAE-cellulose purification step (table 1). The enzyme was localized in the microsomes and NADPH replaced sodium dithionite as the reducing agent when the microsomes or the AS 30–70 fraction were used as enzyme source. With the DEAE-purified enzyme NADPH could not replace sodium dithionite, as was the case with the wheat germ enzyme (table 1). Apparently the NADPH reducing specificity was associated to a microsomal electron carrier which could be partially extracted into the AS 30–70 fraction.

The purified oxygenase showed an allosteric behaviour toward substrate (porphobilinogen) concentration, which could be abolished by the addition of Co^{2+}, Ni^{2+} or by freezing (fig. 3). The effect of Co^{2+} is particularly interesting since porphobilinogen oxygenase is a metalloenzyme. In the presence of EDTA (0.5 mM), or α,α'-dipyridyl (5 mM), the Sephadex G-75 purified enzyme was totally inhibited. When the enzyme was dialyzed against EDTA (5 mM), and then filtered through Sephadex G-25 it lost entirely its activity. The same was partially restored by the addition of Fe^{2+} (25 mM) and Mg^{2+} (25 mM), while the addition of Ni^{2+} (25 mM), and Co^{2+} (5 mM) fully restored the enzymatic activity. 2-Mercaptoethanol (5 mM), and dithiothreitol (5 mM) abolished entirely the porphobilinogen consuming activity of the purified enzyme. Sodium cyanide (25 mM) had no effect on the DEAE-cellulose enzyme, but inhibited 50% of the activity of the Sephadex G-75 purified enzyme. The addition of L-tryptophan (2.5 mM) inhibited 70% of the oxygenase activity.

3.2. *Product formed*

To search for formed products, thirty incubation mixtures prepared as described in Methods and incubated during 60 min were pooled, the resulting solution was adjusted to pH 5 with acetic acid, and the mixture was adsorbed on a Dowex-1 X4 column (0.5 cm × 5 cm) in its acetate form. The column was washed with water (25 ml), eluted with 0.8 M acetic acid, and the acidic eluates evaporated to dryness. The obtained product had an R_f of 0.25 (TLC), and was located by spraying with a potassium permangan-

ase solution or with Ehrlich's reagent followed by heating at 120° (orange colour). The substrate had NMR (D_2O; δ = 0 for NaDSS), δ2.65 (m, 4, CH_2CH_2CO), 3.32 (s, 2, CH_2CO), 3.5 (b, 2, $\underline{CH_2}NH_2$), 4.15 (m, 1, C-2H); IR (KBr), 1690 cm^{-1} ($\underline{CO}$ pyrrolin-2-one), 1720 (CO_2H). By treating the product with benzyloxycarbonyl chloride in the presence of sodium bicarbonate it was transformed into its benzyloxycarbonyl derivative (R_f, 0.9), which by treatment with diazomethane afforded the dimethyl ester **3**. NMR (Cl_3CD; δ = 0, TMS), δ 7.3 (m, 5, C_6H_5), 5.42 (t, 1, NHCO), 5.1 (s, 2, $\underline{CH_2}C_6H_5$), 3.75 (b, 6, CO_2CH_3), 3.6 (s, 2, $\underline{CH_2}CO$), 3.4 (m, 2, $\underline{CH_2}NHCO$), 2.7 (m, 4, $CH_2\underline{CH_2CO}$). By heating the oxidation product in acetic acid (30 min) or in a dilute potassium hydroxide solution (5 min) it was deaminated affording **4**, (R_f 0.80); UV_{max} (ethanol) 265 nm (log ϵ 4.2) (literature [5] gives 265 nm, (log ϵ 4.12) for the 3,4-dimethyl-5-methylen-pyrrolin-2-one chromophore), NMR (D_2O), 5.18 (b, 2, $=CH_2$), 3.40 (s, 2, CH_2CO), 2.70 (m, 4, CH_2CH_2CO). The obtained data secured structure **2** for the oxidation product of porphobilinogen.

4. Discussion

The enzymatic oxidation of porphobilinogen may be of importance for the regulation of porphyrin biosynthesis. It constitutes an alternative pathway for porphibilinogen, diverting it from its exclusive function as porphyrin precursor. It allows an oxygenase to influence directly the porphyrin metabolism. It is interesting to note that phenobarbital (a well known microsomal oxygenase inducer) prevents the induction of chemical porphyria [6]; and allylisopropylacetamide, a powerful inducer of chemical porphyria in rodents, produces a decrease in cytochrome P450 concentration in the same [7]. Cytochrome P450 is closely involved in microsomal oxygenases function.

Tryptophan induced the formation of δ-aminolevulinic acid synthetase [8], and inactivated porphobilinogen oxygenase (see Results), thus allowing for an increased porphobilinogen (and porphyrin) synthesis. The increase in porphobilinogen concentration increased porphobilinogen oxygenase activity (fig. 3), and could prevent further porphyrin synthesis. Tryptophan was also oxidized by tryptophan pyrrolo-oxygenase [3], an enzyme of the same family as porphobilinogen oxygenase.

Acknowledgements

This work was supported by the National Institutes of Health (GM-11973) and the Consejo Nacional de Investigaciones (Argentina). One of us (A.W.) was a recipient of a Fellowship from the Instituto Nacional de Farmacología (Buenos Aires).

References

[1] C. Rimington, Ann. N.Y. Acad. Sciences 104 (1963) 666.
[2] R.B. Frydman, M.L. Tomaro and B. Frydman, FEBS Letters 15 (1971) 305; 17 (1971) 273.
[3] R.B. Frydman, M.L. Tomaro and B. Frydman, Biochim. Biophys. Acta (1972) in press.
[4] B. Frydman, S. Reil, M.E. Despuy and H. Rapoport, J. Am. Chem. Soc. 91 (1969) 2338.
[5] R. Scheffold and P. Dubs, Helv. Chim. Acta 50 (1967) 799.
[6] L. Kaufman, A.L. Swanson and H.S. Marver, Science 170 (1970) 320.
[7] U.A. Meyer and H.S. Marver, Science 171 (1971) 64.
[8] H.S. Marver, D.P. Tschudy, M.G. Perlroth and A. Collins, Science 154 (1966) 501.