

On the formation of the mixed pyrrole catalysed by porphobilinogen synthase from *Rhodobacter spheroides*

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Abstract

The enzyme porphobilinogen synthase (PBGS) catalyses the formation of porphobilinogen (PBG) from two molecules of 5-aminolevulinic acid (ALA). It has been claimed that the PBGS from *Rhodobacter spheroides* is able to form a mixed pyrrole, from one molecule of 5-aminolevulinic acid and one molecule of levulinic acid. The chemical synthesis of this mixed pyrrole allowed to show, that the compound formed from 5-aminolevulinic acid and levulinic acid with PBGS from *R. spheroides* has not the proposed structure. The putative enzyme catalysed formation of the mixed pyrrole had been used as an argument for the postulated mechanism of PBGS. In view of our results this line of arguments has to be re-evaluated.

Keywords: Porphobilinogen synthase; Porphobilinogen; *Rhodobacter spheroides*; Mixed pyrrole

Porphobilinogen synthase (PBGS = EC 4.2.1.24; earlier name: 5-aminolevulinic acid dehydratase = ALAD) [1,2] catalyses the condensation of two molecules 5-aminolevulinic acid (ALA) to produce porphobilinogen (PBG), a committed intermediate in the biosynthesis of tetrapyrrolic natural products [3] (Fig. 1). PBGS has been purified to homogeneity from different sources [4–7] and the DNA- deduced sequences of several PBGS are known [8–12].

A considerable effort has been devoted to the study of PBGS [2,13–15] since the pioneering contributions from Shemin's laboratories [6,16,17]. Based on the observation, that PBGS from *Rhodobacter spheroides* catalyses the formation of a mixed pyrrole from ALA and levulinic acid [17] (Fig. 2), Shemin and his colleagues [17,18] proposed a mechanism for the enzymatic formation of PBG (Fig. 3). For a long time this mechanism has been widely accepted [19]. However the structure of the mixed pyrrole has never

been proven unequivocally. The structure of the newly formed compound was proposed based on the following observations: paper chromatography, auto radiography and a positive Ehrlich reaction of the newly formed compound [17]. In analogy to the enzyme catalysed PBG formation a pyrrolic structure was assumed for the newly formed compound. Twelve years after the first report, attempts to demonstrate the formation of mixed pyrroles of the type **4** have been singularly disappointing [20].

We report the chemical synthesis of the mixed pyrrole **4**, its unambiguous structure determination as well as the results obtained by repeating Shemin's experiments with PBGS from *Rhodobacter spheroides*. The synthetic mixed pyrrole **4** was used as a reference compound, indicating clearly that the product formed during the enzymatic process has not the structure **4**.

5-Aminolevulinic acid was prepared according to the literature [21]. All other chemicals were either from Fluka (Buchs) or Merck (Darmstadt). Hydroxylapatite Biogel- HTP was purchased from Biorad (Munich). Mixed pyrrole **4** was obtained from the known dimethyl ester [22]. Mixed pyrrole dimethyl ester (245 mg, 1.02 mmol) was hydrolysed in 40 ml 0.5 M NaOH for 3 h at r.t. and under N₂-atmosphere in the dark. The solution was acidified to pH 2 with 4 M HCl, saturated with solid NaCl and extracted 3x with 80 ml ethyl acetate. The combined organic layers were washed with brine (10 ml), dried over

Abbreviations: PBGS, porphobilinogen synthase (5-aminolevulinic acid dehydratase EC 4.2.1.24); ALA, 5-aminolevulinic acid; PBG, porphobilinogen; mixed pyrrole, 3-(2-carboxyethyl)-4-carboxymethyl-5-methyl-1*H* pyrrole; r.t., room temperature; GSH, glutathione red.; A-side, acetic acid side; P-side, propionic acid side

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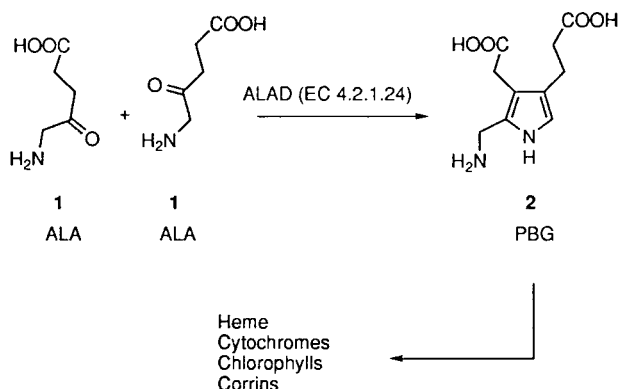


Fig. 1. Formation of porphobilinogen **2** from two molecules of 5-aminolevulinic acid **1** by the action of porphobilinogen synthase.

magnesium sulfate, filtered and concentrated in vacuum. The crude compound was crystallised from ethyl acetate/hexane yielding analytically pure mixed pyrrole **4** (162 mg, 75%): mp. 146–147° (EtOAc/hexane, dec.). R_f (silica, methanol) 0.69. IR (KBr): 3600–2300m (br.), 3435s, 3010m, 2975m, 2920m, 1695s, 1675s, 1595w, 1525w, 1430m, 1415m, 1405m, 1395m, 1360w, 1320m, 1300m, 1280m, 1240w, 1215m, 1190m, 1160w, 1095w, 1070w, 1020w, 940m (br.), 900w, 830w, 785w, 760w, 730w, 710w, 675w, 660w, 645w, 620w. $^1\text{H-NMR}$ (CD_3OD , TMS): 9.63 (br. *s*, 1H, NH); 6.35 (br. *d*, $^3J = 2.3$, 1H, H-C(2)); 3.34 (*s*, 2H, CH_2COO); 2.68 (*t*, $^3J = 7.8$, 2H, $\text{CH}_2\text{CH}_2\text{COO}$); 2.49 (*t*, $^3J = 7.8$, 2H, $\text{CH}_2\text{CH}_2\text{COO}$); 2.13 (*s*, 3H, $\text{CH}_3\text{-C(5)}$). $^{13}\text{C-NMR}$ (CD_3OD , TMS): 177.6, 176.9 (*s*, 2COO); 126.3 (*s*, C(5)); 122.0 (*s*, C(3)); 113.6 (*d*, C(2)); 111.0 (*s*, C(4)); 36.3 (*t*, $\text{CH}_2\text{CH}_2\text{COO}$); 30.9 (*t*, CH_2COO); 22.0 (*t*, $\text{CH}_2\text{CH}_2\text{COO}$); 11.0 (*q*, $\text{CH}_3\text{-C(5)}$). EI-MS: 212 (7), 211 (73, M^+), 167 (34), 166 (83), 165 (9), 152 (31), 125 (12), 124 (100), 122 (19), 120 (54),

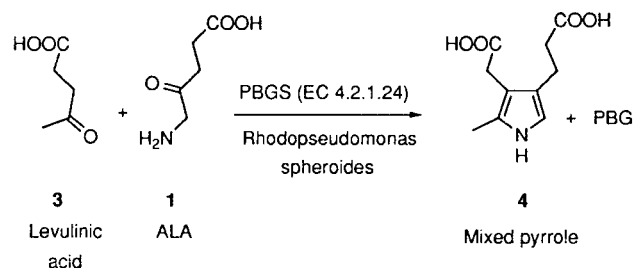


Fig. 2. Formation of a heterologous mixed pyrrole **4** from 5-aminolevulinic acid **1** and from levulinic acid **3** by the action of porphobilinogen synthase from *R. spheroides*.

108 (79), 107 (26), 106 (40), 94 (18), 79 (10), 77 (20), 53 (10), 44 (35), 43 (65), 42 (16), 39 (14), 31 (15). Anal. Calcd. for $\text{C}_{10}\text{H}_{13}\text{NO}_4$ (211): C 56.87, H 6.16, N 6.64; Found: C 56.58, H 6.39, N 6.36.

R. spheroides (ATCC 11167) was grown photosynthetically in 10 l bottles according to Lascelles [23]. PBGS was isolated, purified and assayed as described by Shemin [24], except that the crude low speed centrifuged cell extract was obtained using a French pressure cell (18 000 PSIG) and that the acetone fractionation was replaced by a polymin-P treatment. To the heat treated supernatant was added an aqueous 10% solution of polymin-P (10 $\mu\text{l}/\text{ml}$ extract). After 30 min the precipitate was removed by centrifugation (40 000 $\times g$, 30 min) and the supernatant was used for the fractionation with ammonium sulfate. The following purification procedure was applied: (1) crude extract, (2) heat treatment, (3) fractionation with Polymin-P, (4) fractionation with ammonium sulfate (40%), (5) pH 5 precipitation, (6) fractionation with ammonium sulfate (35%), (7) column chromatography with hydroxylapatite Biogel-HTP (1.6 cm \times 17 cm). The chromatography on hydroxylapatite was performed according to Jordan and

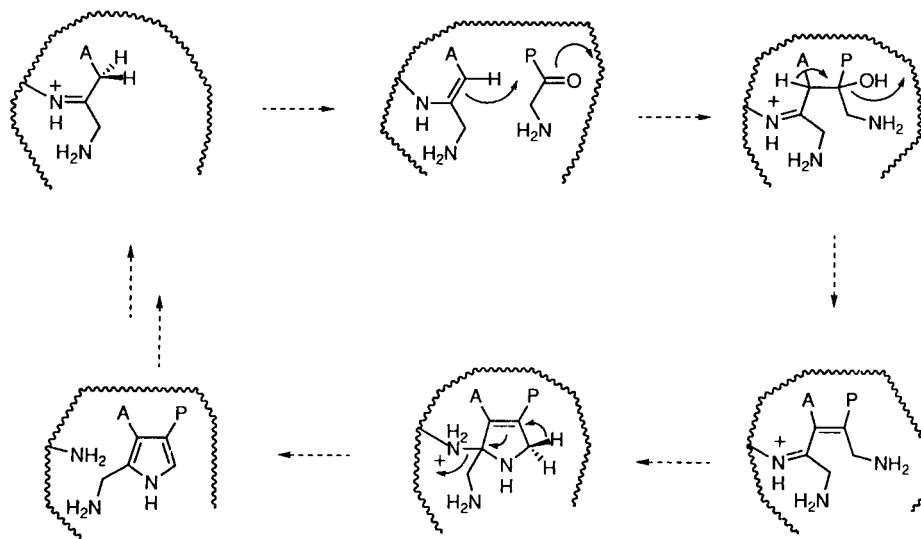


Fig. 3. Proposed mechanism of porphobilinogen formation from 2 molecules of 5-aminolevulinic acid as catalyzed by the enzyme. A side, acetic acid side chain; P side, propionic acid side chain.

Shemin [25]. The partially, 80-times purified PBGS had a specific activity of 26 units. The protein content was estimated by the method of Bradford [26] using the Bio-Rad protein assay. Bovine serum albumin was taken as a standard.

All incubations were performed in centrifugation tubes (16×100 mm) and in a total volume of 3 ml according to Nandi and Shemin [17,24]. Each of the flasks contained 100 μ g of enzyme (specific activity: 15 units) in Tris buffer, 0.1 M, pH 8.6, 50 mM KCl, 5 mM mercaptoethanol and 5 mM GSH. Experiment 1: The incubation mixture contained 10.25 μ mol of ALA and enzyme. Experiment 2: The incubation mixture contained 10.25 μ mol of ALA, 10 μ mol of levulinic acid and enzyme. Experiment 3: The incubation mixture contained 10.25 μ mol of ALA, 50 μ mol of ethyl levulinate and enzyme. Control experiments: (a) the incubation mixture contained 10.25 μ mol of ALA and 10 μ mol of levulinic acid without enzyme; (b) the incubation mixture contained 10 μ mol of levulinic acid and enzyme without the substrate ALA. The tubes containing the reaction mixture were sealed with rubber stoppers and incubated for 4.5 h at 37°C. The tubes were cooled to 0°C and the solutions were acidified to pH 2 with 4 M HCl and lyophilised. The lyophilised probes were diluted with 500 μ l of ethanol and aliquots (20 μ l) were applied to Whatman paper No. 1. Mixed pyrrole (1 mg) was also dissolved in 500 μ l of ethanol and applied on the paper as a reference. Paper chromatography was performed in a solvent system *n*-butanol-water-acetic acid (63:27:10). Pyrroles were located by spraying with Ehrlich's reagent [27].

The development of a new method to synthesise alkyl substituted pyrroles allowed us to prepare and characterize the mixed pyrrole **4** [22]. This compound had been reported to be formed upon incubation of PBGS with ALA **1** and levulinic acid **3** (Fig. 2) [17]. Shemin proposed the following mechanism to explain the formation of the mixed

pyrrole **4** [17,18]. Levulinic acid forms a Schiff base at the A-side, deprotonation gives regioselectively an enamine, which reacts with the carbonyl carbon of ALA bound at the P-side. From this central intermediate a series of steps leads to PBG. Since the enzyme catalysed formation of the mixed pyrrole of structure **4** was one of the relevant factors for Shemin's mechanistic proposal [1], we repeated his experiment in order to prove the structure of the mixed pyrrole by comparison with synthetic material.

The incubation of PBGS from *R. spheroides* with ALA (Fig. 4, exp. 1) produced an Ehrlich positive compound, which was shown to be PBG. The incubation of PBGS with ALA and levulinic acid gave a second compound besides the dominant formation of PBG (exp. 2). This compound showed a positive Ehrlich reaction and migrated on paper chromatography with an R_f value, which was compatible with the results described by Shemin [17]. The same compound was again observed when PBGS was incubated with ALA and ethyl levulinate. In addition a third Ehrlich positive compound was formed, which was slightly less polar than the substrate ALA in complete agreement with Shemin's report [17]. A close inspection of the paper chromatogram of experiment 1 indicated that this second, Ehrlich positive compound was also present in this experiment but only in trace amounts. In control experiments, incubating PBGS with levulinic acid in the absence of substrate, and incubating levulinic acid and ALA in the absence of PBGS, this second Ehrlich positive spot with a small R_f -value was not observed. This clearly indicates an enzymatic formation of this second Ehrlich positive compound. In contrast the synthetic mixed pyrrole **4** migrates much further than PBG. The additional compound in experiments 2 and 3 to which Shemin had attributed the structure **4**, however, is more polar than PBG. These considerable differences in polarity and chromatographic behaviour unambiguously proves, that the compound detected by Shemin can not have the structure **4**. The struc-

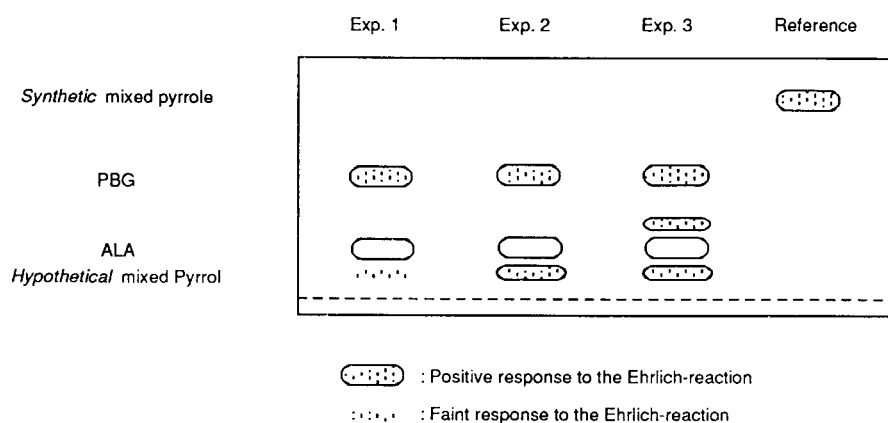


Fig. 4. Paper chromatographical analysis of incubations of porphobilinogen synthase from *R. spheroides* with 5-aminolevulinic **1** acid, levulinic acid **3** and ethyl levulinate. Exp. 1: The incubation mixture contained 10.25 μ mol of ALA and PBGS. Exp. 2: The incubation mixture contained 10.25 μ mol of ALA, 10 μ mol of levulinic acid and PBGS. Exp.3: The incubation mixture contained 10.25 μ mol of ALA, 50 μ mol of ethyl levulinate and PBGS. Reference: synthetic mixed pyrrole **4**.

ture of the enzymatic product from ALA and levulinic acid is therefore still unknown.

Until Jordan's publication of his pulse labelling studies [20], Shemin's proposal was widely accepted. The result of the pulse labelling motivated Jordan to propose an alternative sequence of events for the enzymatic PBG-formation [20]. The pulse labelling experiments do not distinguish between a mechanism where the C-C bond formation is first and a mechanism where the C-N bond formation is first [15]. Even the results of the brilliant NMR studies in Jaffe's group characterising the structure of the Schiff base intermediate in great detail [28–30] do not allow to differentiate between the two mechanistic alternatives.

From a thermodynamic standpoint the formation of the carbon-carbon bond is the central event of the sequence. Once the carbon-carbon bond is formed the rest of the steps leading to the pyrrole ring are occurring in vitro without any outside instruction [22]. This process would therefore follow a pathway of self instruction as described by Eschenmoser [31] (see also [32]). It will be important to determine which one of the two mechanistic alternatives has been chosen during evolution for the enzyme catalysed reaction.

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