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DESIGN OF A REUSABLE ENZYMATIC SYSTEM FOR THE PREPARATION OF PORPHYRINS OF BIOLOGICAL INTEREST

Mario D. Gonzalez and A. Ian Scott

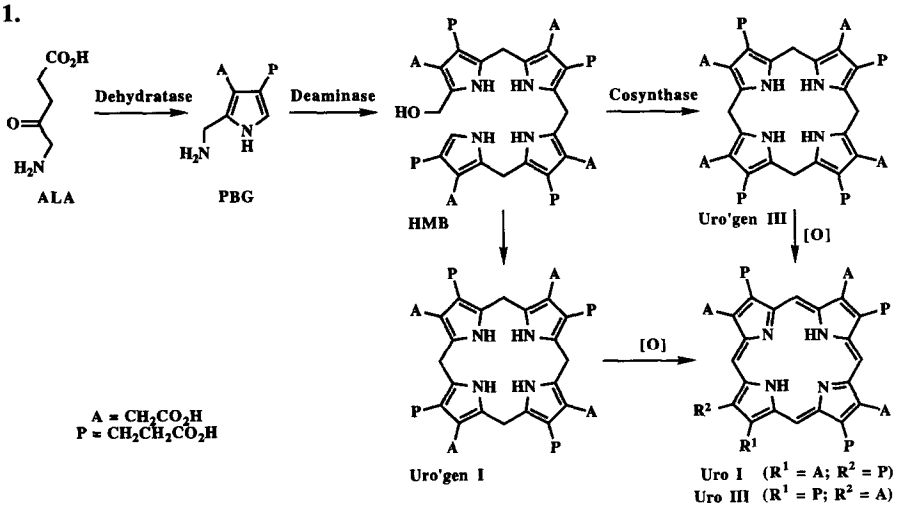
Center for Biological NMR, Department of Chemistry, Texas A & M University, College Station, TX 77843, USA

Abstract. The immobilization of porphobilinogen deaminase, and uroporphyrinogen III synthase was achieved by reaction of each enzyme with BrCN activated Sepharose. The procedure provided stable, reusable systems which, combined with immobilized 5-aminolevulinic acid dehydratase, transformed 5-aminolevulinic acid into uroporphyrinogen III.

Introduction. Aminolevulinic acid dehydratase (Dehydratase; EC 4.2.1.24) is the enzyme responsible for the transformation of 5-aminolevulinic acid (ALA) into porphobilinogen (PBG), which is then tetramerized by PBG deaminase (Deaminase; EC 4.3.1.8) into hydroxymethylbilane (HMB) (Scheme 1). HMB cyclizes chemically into uroporphyrinogen I (Uro'gen I) but, in the presence of uroporphyrinogen III synthase (Cosynthase; EC 4.2.1.75) is rearranged and cyclized into Uro'gen III, the pivotal biosynthetic intermediate of vitamin B_{12}, heme, chlorophylls, and cytochromes.[1] The Uro'gens are conveniently isolated and analyzed as their oxidized forms, uroporphyrins I and III (Uro I and III).

Studies on the later enzymes and intermediates in the biosynthesis of vitamin B_{12} now rely on the availability of advanced precursors, specifically labeled with ^{13}C.[1] The previous lack of rapid and efficient synthetic methodology in this area has now been resolved by overexpression of the genes encoding the early enzymes in the pathway, making the enzymatic preparation of such intermediates the most convenient procedure for their acquisition. In order to scale up the synthetic process, the use of immobilized enzymes[2] provides an attractive method to recover and re-use the biocatalysts.

Scheme 1.

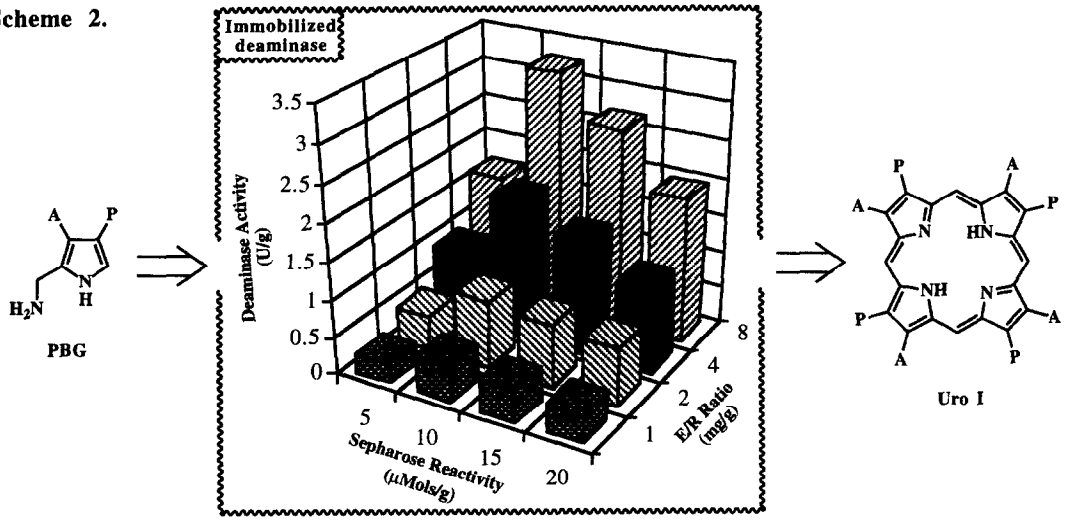


Twenty years ago Gurne and Shemin[3] showed that dehydratase could be immobilized by reaction with BrCN activated Sepharose. The extension of these studies to the next two enzymes, deaminase and cosynthase and the combination of these three immobilized enzymes to provide a very efficient system for the transformation of ALA into Uro'gen III is disclosed in this paper.

Results and discussion. *Dehydratase:*[4,5] The immobilization of dehydratase by the BrCN activated Sepharose method essentially follows the procedure of Gurne and Shemin[3], to produce active resin (13.0 U[5] /g of resin; 55 % of the original activity immobilized), which converted ALA into PBG in 92 % yield.

Deaminase:[5,6] The effect of the density of reactive sites in the resin (μMols/g of Sepharose)[7] and the enzyme to resin ratio (E/R; mg/g) on the amount of deaminase activity immobilized by the above method[3] were analyzed (Scheme 2). The number of reactive sites in the resin is determined by the amount of BrCN utilized during the activation process.[7] Over the E/R ratios tested, resin activated to 10 μMols/g produced the most efficient immobilized product.

Scheme 2.



The fact that resin preparations with greater reactive site density (15-20 μMols/g) exhibited diminishing activity can be rationalized by the increasing possibility of covalent attachment, which could affect the enzyme's conformation and therefore its activity. This rationale is supported by the decrease of the total activity recovered (resin + supernatant and washings) when more reactive resin preparations were utilized (55-75 % activity was recovered using 20 μMols/g Sepharose vs. 70-90 % recovered using 10 μMols/g). This "overimmobilization" effect could not be overcome by increasing the E/R ratio. Finally, when less reactive resin (5 μMols/g) was used, less enzyme activity was immobilized but most of the initial activity (82-90 %) could be recovered, the later results indicating that the decrease in immobilized activity was due to lack of sufficient reactive sites in the resin to retain enzyme, rather than inhibition of the enzyme, which was minimal.

Using 10 μMols/g activated Sepharose the E/R ration was doubled twice (from 1 mg/g) which also doubled the immobilized enzyme activity in each case (Scheme 2). When the ratio was doubled again (to 8 mg/g) a 75 % increase in the immobilized activity was observed. Increasing in the ratio to 12 mg/g (not shown in Scheme 2)

produced a preparation with an activity of 3.75 U/g of resin, which represents only a 15 % enhancement above the previous lead (3.25 U/g), indicating that the "saturation" of the resin capacity to immobilize enzyme occurs at an E/R ratio of ca. 8-12 mg/g. Similar results were observed when Sepharose of different reactivities were used.

Cosynthase:[5,8] The enzyme was immobilized by reaction with activated Sepharose (10 μMols/g resin reactivity, E/R ratio = 4 mg/g), and was mixed with different amounts of immobilized deaminase with the same reactivity and E/R ratio. The resulting two-enzyme system was incubated with a 1 mM solution of PBG (2 g of resin/25 ml solution). When all of the substrate had been consumed, as evidenced by the Ehrlich test,[9] the Uro'gen mixture formed was oxidized and analyzed by HPLC.[10] Combinations which contained between 1 and 25 parts of deaminase per part of cosynthase produced complete transformation of PBG into Uro III, no Uro I being observed in the chromatograms of the different products. When the ratio was increased to 50 parts of deaminase per part of cosynthase, ~2 % of Uro I was observed, reflecting the onset of competition between the enzymatic (Uro'gen III production) and chemical (Uro'gen I production) processes. Finally, when incubation of PBG with a 100:1 ratio of deaminase to cosynthase was tested, the system still was able to produce Uro'gen III with 95 % purity.

The requirement of at least one part of immobilized cosynthase for every 25-50 parts of deaminase indicates that a major portion of cosynthase activity was lost during immobilization, since the original specific activity of cosynthase was ca. 1000 times greater than that of deaminase.[6,7]

The coimmobilization of deaminase and cosynthase (50:1 ratio) did not affect the composition of the product, when compared with a mixture of individually immobilized deaminase and cosynthase, as has been found for other enzymatic systems.[11]

Enzyme stability: Immobilized deaminase (E/R = 4 mg/g, 10 μMols/g reactivity) was stirred at 37°C for ca. 2 h with a 1 mM solution of PBG, and the disappearance of PBG was monitored (Ehrlich)[9] in the supernatant. When all of the PBG had been consumed, the suspension was filtered, the resin washed several times with phosphate buffer (pH 8) and stored at 4°C under phosphate buffer containing β-mercaptoethanol (5 mM). This procedure was repeated once a week for 8 weeks. During this period the enzyme activity slowly decreased to 85 % of its original value (from 1.85 U/g to 1.50 U/g). This result indicated not only good storage survival, but also the possibility of reusing the enzyme preparation. In the absence of β-mercaptoethanol, the enzyme activity decreased dramatically after one storage, to less than 20 % of the original value (from 1.82 U/g to 0.34 U/g). Similar results were obtained in the case of dehydratase, which lost its activity after two successive incubations when stored in the absence of β-mercaptoethanol.

A 10:1 mixture of immobilized deaminase and cosynthase (E/R = 2 mg/g) was tested for stability. After consumption of PBG,[9] the supernatant was treated with iodine and the Uro I/Uro III ratio determined by HPLC. After 8 weekly incubations the ratio was still better than 2/98, indicating a very efficient conversion of HMB into Uro'gen III, and hence a very robust immobilized enzyme system.

Three enzyme system: 10 mg of deaminase and 1 mg cosynthase coimmobilized on 3 g of Sepharose were packed into a column (10 mm internal diameter). 6 mg of dehydratase immobilized in 3 g of Sepharose were then packed on top. The resulting column was tested with different concentrations of ALA in phosphate buffer (0.1 M

pH 7.8) at a flow rate of 10 ml/h. The eluates were tested for the presence of residual PBG, Uro composition, and yield of Uro mixture (determined by measuring A_{405} in a 1M HCl dilution).[12]

A 2 mM solution of ALA was converted in Uro III in 83 % yield. No PBG was detected by Ehrlich reagent, nor Uro I by HPLC. When the concentration of ALA was increased to 3, 4 and 5 mM the quality of the porphyrin remained the same (no PBG or Uro I detected) but the yield was decreased (79, 66 and 62 % respectively). Previous reports[13] suggested that dehydratase might be inhibited by 2 mM concentrations of PBG (4 mM ALA), which could explain the decrease in the yield of Uro in spite of complete PBG consumption.

After two days of continuous operation of the column, it was restarted using a 2 mM ALA solution and continued for seven more days. At the end of that period, no reduction of enzymatic activity was observed (83 % yield of Uro III), nor was the quality of the product affected. The immobilized enzymes utilized in this experiment were prepared one month in advance and stored at 4°C, again showing the excellent stability of this system. During these studies ca. 300 mg of Uro III were produced.

Conclusion. The conditions for the immobilization of PBG deaminase and of Uro III synthase were established, very stable enzymatic systems being produced. Such preparations, in combination with immobilized ALA dehydratase were able to transform ALA into Uro III very efficiently (up to 83 % yield) over long periods of time (nine days of continuous operation) , thereby providing a convenient synthesis of the key intermediate for all natural porphyrinoids. In the absence of cosynthase, the enzymatic system forms Uro I, an interesting substrate of S-adenosyl-L-methionine:uro'gen III methyl transferase, which is transformed into the "unnatural" sirohydrochlorin I,[10] as well as factors S_1[11] and S_3.[14]

Immobilization studies are continuing on the succeeding enzymes of the vitamin B12 biosynthetic pathway[1] in order to obtain the rare precorrin intermediates.

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