JCI The Journal of Clinical Investigation

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J Clin Invest. 1993;91(4):1436-1444. https://doi.org/10.1172/JCI116348.

Research Article

Variegate porphyria (VP) is characterized by photocutaneous lesions and acute neuropsychiatric attacks. Decreased protoporphyrinogen oxidase activity results in accumulation of protoporphyrin (ogen) IX and coproporphyrin (ogen) III. During acute attacks delta-aminolevulinic acid and porphobilinogen also increase, suggesting that porphobilinogen deaminase (PBG-D) may be rate limiting. We have examined the effects of porphyrinogens accumulating in VP on PBG-D activity in Epstein-Barr virus-transformed lymphoblast sonicates from 12 VP and 12 control subjects. Protoporphyrinogen oxidase activity was decreased and protoporphyrin increased in VP lymphoblasts. PBG-D in control lymphoblasts obeyed Michaelis-Menten kinetics (Vmax 28.7 +/- 1.8 pmol/mg per h, Hill coefficient 0.83 +/- 0.07). VP sonicates yielded sigmoidal substrate-velocity curves that did not obey Michaelis-Menten kinetics. Vmax was decreased (21.2 +/- 2.0 pmol/mg per h) and the Hill coefficient was 1.78 +/- 0.17. Addition of protoporphyrinogen IX and coproporphyrinogen III to control sonicates yielded sigmoidal PBG-D substrate-velocity curves and decreased PBG-D Vmax. Addition of porphyrins or uroporphyrinogen III did not affect PBG-D activity. Removal of endogenous porphyrin (ogens) from VP sonicates restored normal PBG-D kinetics. Purified human erythrocyte PBG-D obeyed Michaelis-Menten kinetics (Vmax 249 +/- 36 nmol/mg per h, Km 8.9 +/- 1.5 microM, Hill coefficient 0.93 +/- 0.14). Addition of protoporphyrinogen yielded a sigmoidal curve with decreased Vmax. The Hill coefficient approached 4. These findings provide a rational explanation for the increased delta-aminolevulinic […]

Allosteric Inhibition of Human Lymphoblast and Purified Porphobilinogen Deaminase by Protoporphyrinogen and Coproporphyrinogen

A Possible Mechanism for the Acute Attack of Variegate Porphyria

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Abstract

Variegate porphyria (VP) is characterized by photocutaneous lesions and acute neuropsychiatric attacks. Decreased protoporphyrinogen oxidase activity results in accumulation of protoporphyrin(ogen) IX and coproporphyrin(ogen) III. During acute attacks delta-aminolevulinic acid and porphobilinogen also increase, suggesting that porphobilinogen deaminase (PBG-D) may be rate limiting. We have examined the effects of porphyrinogens accumulating in VP on PBG-D activity in Epstein–Barr virus–transformed lymphoblast sonicates from 12 VP and 12 control subjects. Protoporphyrinogen oxidase activity was decreased and protoporphyrin increased in VP lymphoblasts. PBG-D in control lymphoblasts obeyed Michaelis–Menten kinetics (V_{max} 28.7±1.8 pmol/mg per h, Hill coefficient 0.83±0.07). VP sonicates yielded sigmoidal substrate-velocity curves that did not obey Michaelis–Menten kinetics. V_{max} was decreased (21.2±2.0 pmol/mg per h) and the Hill coefficient was 1.78±0.17. Addition of protoporphyrinogen IX and coproporphyrinogen III to control sonicates yielded sigmoidal PBG-D substrate-velocity curves and decreased PBG-D V_{max}. Addition of porphyrins or uroporphyrinogen III did not affect PBG-D activity. Removal of endogenous porphyrin(ogens) from VP sonicates restored normal PBG-D kinetics. Purified human erythrocyte PBG-D obeyed Michaelis–Menten kinetics (V_{max} 249±36 nmol/mg per h, K_m 8.9±1.5 μM, Hill coefficient 0.93±0.14). Addition of protoporphyrinogen yielded a sigmoidal curve with decreased V_{max}. The Hill coefficient approached 4. These findings provide a rational explanation for the increased delta-aminolevulinic acid and porphobilinogen during acute attacks of VP. (*J. Clin. Invest.* 1993. 91:1436–1444.) Key words: uroporphyrinogen-I synthase • hydroxymethylbilane synthase • porphyria variegata • acute porphyria

Introduction

Variegate porphyria (VP),[1] an autosomal dominant error of heme metabolism, is characterized by photocutaneous sensitivity and a propensity to develop acute neuropsychiatric attacks with abdominal pain, vomiting, constipation, tachycardia, hypertension, psychiatric symptoms, and, in the worst cases, symmetrical quadriplegia (1–3).

VP is associated with a defect of the penultimate heme synthetic enzyme, protoporphyrinogen oxidase (4–8). This results in accumulation of the distal heme synthetic intermediates protoporphyrin(ogen) IX and coproporphyrin(ogen) III.

During acute attacks porphyrin(ogen) concentrations become even higher and, in addition, delta-aminolevulinic acid (ALA) and porphobilinogen (PBG), proximal intermediates of the heme synthetic pathway, increase (1, 2, 9).

Acute attacks also occur in two other forms of porphyria, acute intermittent porphyria and hereditary coproporphyria (10). Although the site of the inherited enzyme defect varies, PBG deaminase in acute intermittent porphyria (11, 12), coproporphyrinogen oxidase in hereditary coproporphyria (13, 14), and protoporphyrinogen oxidase in VP, neuropsychiatric attacks in all three acute porphyrias are associated with elevated concentrations of ALA and PBG (10).

The elevated concentrations of ALA and PBG are easily explained in acute intermittent porphyria. However, this finding does not tally with the known enzyme abnormality in VP and hereditary coproporphyria. The thesis that ALA and PBG are increased as a result of sequential damming up of products proximal to a block in the distal portion of the heme synthetic pathway is unlikely since increased concentrations of ALA and PBG are not found in porphyria cutanea tarda despite accumulation of large amounts of uroporphyrin(ogen) III, the intermediate immediately distal to PBG (10, 15–17). Similarly it is unlikely that subjects with VP and hereditary coproporphyria have a second inherited defect at the level of PBG deaminase since the heme biosynthetic enzymes appear to be encoded on different chromosomes (9).

In this study we examine the hypothesis that the obligate heme precursors that accumulate in VP (and in hereditary coproporphyria) inhibit PBG deaminase activity and result in rate-limiting effects. We have tested this hypothesis by performing a series of kinetic analyses of PBG deaminase using EBV-transformed lymphocytes from normal and VP subjects. Various porphyrins and porphyrinogens were assessed for their ability to alter kinetic behavior of both lymphoblast PBG deaminase and the purified enzyme.

Sonicates of lymphoblasts derived from subjects with VP exhibit a significant decrease in PBG deaminase V_{max} activity and display abnormal sigmoidal PBG deaminase substrate-velocity curves with kinetic features suggestive of allosteric inhibition. These changes could be abolished by chromatography aimed at removing porphyrin(ogen)s from the PBG deaminase–containing fraction of VP lymphoblast sonicates and reproduced by addition of protoporphyrinogen IX and coproporphyrinogen III (but not uroporphyrinogen III or the por-

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Received for publication 4 May 1992 and in revised form 19 October 1992.

1. *Abbreviations used in this paper:* ALA, delta-aminolevulinic acid; PBG, porphobilinogen; VP, variegate porphyria.

J. Clin. Invest.

0021-9738/93/04/1436/09 $2.00
Volume 91, April 1993, 1436–1444

phyrins) to sonicates of lymphoblasts from normal subjects. (Unless otherwise stated protoporphyrin[ogen] refers to the series IX isomer, and coproporphyrin[ogen] and uroporphyrin[ogen] refer to the series III isomers.) Finally, kinetic changes similar to those found in VP lymphoblasts were found when protoporphyrinogen was added to purified human PBG deaminase, suggesting that protoporphyrinogen has a direct effect on PBG deaminase activity.

Methods

Subjects. 12 healthy control and 12 quiescent VP subjects were used as lymphocyte donors in this study. All were defined clinically and by quantitative thin-layer chromatographic fluoroscanning of stool, urine, and plasma (18, 19). The VP subjects all had stool protoporphyrin concentrations > 200 nmol/g dry wt and coproporphyrin concentrations > 50 nmol/g dry wt. Control subjects were asymptomatic, had no family history of porphyria, and exhibited normal porphyrin biochemistry. All subjects gave informed consent for the use of their blood, urine, and stool in this study.

EBV-transformed lymphoblast preparation. EBV-transformed lymphoblasts were used in this study, since these cells could be derived from a relatively small amount of whole blood, they were easy to establish in culture, and once established from each original specimen, they readily yielded the large numbers of cells required for kinetic studies (6). Most importantly, studies aimed at validating the lymphoblast system as a "model" of VP tissue allowed us to confidently assume the "VP" nature of these cells (see below).

20 ml of blood from each subject was taken into heparin. Lymphocytes were isolated, cultured, and prepared for assay as previously described (6). Medium containing EBV was prepared from semiconfluent cultures of the EBV-producing marmoset cell line B95/8 (20).

Protoporphyrinogen oxidase activity assay. Lymphoblast protoporphyrinogen oxidase was assayed as previously described (6). Protoporphyrinogen solution (produced by treating protoporphyrin with 4% sodium amalgam) was added at five different concentrations (ranging between 0.75 and 2.5 μM), to a lymphoblast sonicate, and reaction buffer (50 mM Tris-HCl pH 8.5, 1 mM EDTA, 3 mM dithiothreitol, and 1% Brij-35). The reaction was allowed to proceed in the dark for 1 h at 37°C. Protoporphyrin concentrations were measured by direct fluorometry (model 204A fluorescence spectrophotometer; Perkin-Elmer Cetus Instruments, Norwalk, CT). Nonenzymatic production of protoporphyrin was controlled for by measuring spontaneous protoporphyrin production under similar conditions. The rate of nonenzymatic oxidation of protoporphyrinogen was subtracted from the enzymatic rate. Apparent V_{max} and K_m values for protoporphyrinogen oxidase were determined from double-reciprocal Lineweaver–Burk plots. All assays were performed in duplicate.

PBG deaminase activity assay. PBG deaminase activity in lymphoblasts was measured by minimal adaptation of previously described methods for measuring erythrocyte and lymphocyte PBG deaminase activity (21–25).

Lymphoblasts were prepared for assay in reaction buffer (0.1 M Tris-HCl pH 8.2 and 0.1 mM dithiothreitol added fresh) as described above. PBG deaminase was released by sonication. Sonicates were centrifuged at 30,000 *g* for 45 min and the supernatants were used for assay. The reaction mixture contained 100 μl of sample and 600 μl of reaction buffer. PBG concentrations were selected to straddle the reported K_m of PBG deaminase (22, 25–27).

Evaluation of transformed lymphoblasts for assay of protoporphyrinogen oxidase and PBG deaminase. The suitability of lymphoblasts as a model in which to study protoporphyrinogen oxidase and PBG deaminase was assessed as follows. Four normal control lymphoblast lines had protoporphyrinogen oxidase and PBG deaminase assayed at days 30, 40, and 50 posttransformation to assess the variation in activity in culture over a long period. Both enzymes showed remarkably constant activity over this period (protoporphyrinogen oxidase range of activity, 0.91±0.1–0.95±0.1 nmol/mg protein per h; PBG deaminase range of activity, 21.2±1.4–22.4±1.2 pmol/mg protein per h). Reproducibility of the assays was verified by measuring the enzymes in triplicate in the same tissue batch on two separate occasions separated by 2 h. The results differed nonsignificantly by < 14% ($P = 5.853 \times 10^{-1}$).

The effect of transformation on protoporphyrinogen oxidase and PBG deaminase activity. Possible stimulatory or inhibitory effects of the transformation process on protoporphyrinogen oxidase and PBG deaminase were assessed in five control cell lines by comparing activities of the two enzymes 3, 4, 5, 7, 14, 21, and 28 d after initiation of the transformation process.

Both protoporphyrinogen oxidase and PBG deaminase activity reached maximum (fourfold) increases in activity at 7 d. Thereafter, both enzyme activities dropped to a steady level of approximately twice the baseline value. All lymphoblast studies were performed during this steady stage.

Evidence that the VP defect in protoporphyrinogen oxidase activity persists after transformation. We have previously shown that protoporphyrinogen oxidase V_{max} is decreased by 52% in VP-derived lymphoblasts when compared with normal controls ($n = 27$) (6). Additional evidence for persistence of this defect was obtained by assaying protoporphyrinogen oxidase in fresh lymphocytes (500 ml blood) and in lymphoblast cultures prepared from the same normal ($n = 3$) and VP subjects ($n = 3$). Both VP lymphocytes and lymphoblasts showed a similar decrease in protoporphyrinogen oxidase activity (VP lymphocyte V_{max} was 50% diminished, VP lymphoblast V_{max} was 55% diminished). K_m values were unaltered.

Measurement of lymphoblast porphyrins. Lymphoblasts were centrifuged at 1,000 *g* for 15 min and washed twice in Hanks' Balanced Salt Solution. After addition of 10 ml of 5% sulphuric acid in methanol to the final cellular pellet, the mixture was agitated vigorously and heated in the dark at 55°C for 8 h. This ruptured the cells and esterified any porphyrin present. Next, the mixture was centrifuged (2,000 *g*, 15 min), the supernatant solution was neutralized using a 17% (vol/vol) ammonia solution, and the porphyrin esters extracted and quantitatively analyzed by TLC (Silica gel-60 without fluorescent indicator; Merck, Darmstadt, Germany) and fluoroscanning (TLD-100 scanning densitometer; Vitatron, Dieren, The Netherlands) as previously described (18, 19).

Preparation and stability of porphyrinogens for use in kinetic assays. Protoporphyrinogen, coproporphyrinogen, and uroporphyrinogen (all porphyrins from Porphyrin Products, Inc., Logan, UT) for addition to PBG deaminase assay systems (lymphoblast sonicates or purified PBG deaminase) were prepared by reduction of the corresponding porphyrin using 4% sodium amalgam that was freshly prepared using well-described methods (28, 29).

A potential problem related to the known instability of porphyrinogens: because testing of the hypothesis demanded that porphyrinogens be investigated, it was necessary to examine the stability of various porphyrinogens under assay conditions. The relative stability of proto-, copro-, and uroporphyrinogen under PBG deaminase assay conditions was studied as follows: 5 ml of a 50 μM solution of the appropriate porphyrin was reduced to the porphyrinogen using 4% sodium amalgam. The reduced solution was filtered and the pH was adjusted to 7.5 with glacial acetic acid. 2 ml of this was added to 8 ml of PBG deaminase assay buffer and the solution incubated for 12 h at 37°C in a shaking water bath in the dark for 2 h. A 1-ml aliquot was removed every 15 min and its fluorescence was measured in a fluorometer. After 2 h any porphyrinogen present in the remaining volume was reoxidized by adding 50 μl of a 0.005% (wt/vol) aqueous iodine solution. A small crystal of cysteine was added to decolorize the excess iodine (28). Oxidation of porphyrinogens as judged by reappearance of porphyrin fluorescence was negligible over the first 30 min and very slight over the next 90 min. Protoporphyrinogen was the least stable (14% reoxidation after 120 min), and uroporphyrinogen was the most stable (4% reoxidation after 120 min). It was concluded that the stability of the porphyrinogens under the proposed assay conditions was acceptable.

Addition of porphyrin(ogens) in the kinetic assays. Protopor-

phyrin, coproporphyrin, and uroporphyrin were added to lymphoblast sonicate incubation mixtures 5 min before the assay of PBG deaminase activity. All additions were designed to achieve incubation mixture porphyrin concentrations of 1, 5, and 10 μM. Protoporphyrinogen, coproporphyrinogen, and uroporphyrinogen were added immediately after their preparation by reduction using 4% sodium amalgam.

In a preliminary study we compared two assay methods: direct fluorometry of a 1/10 dilution of reaction mixture yielded a mean PBG deaminase V_{max} of 26.2±4.1 pmol/mg protein per h whereas extraction and quantitative porphyrin TLC gave a mean V_{max} of 28.0±2.1 pmol/mg protein per h. A comparison of these two methods showed a marginally lower V_{max} with a slightly larger scatter using the first method, although the two results were not statistically different ($P = 2.300 \times 10^{-1}$, $n = 6$). Furthermore, thin-layer chromatographic analysis indicated similar quantities of copro- and protoporphyrin both before and after assay, whereas the amount of uroporphyrin increased. It was concluded that the use of fluorescence readings on diluted aliquots of reaction mixture was acceptable for the purposes of this study.

Removal of porphyrin(ogen)s from PBG deaminase in VP lymphoblasts. After sonication of VP lymphoblasts, Sephadex G25 (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) chromatography was used to separate the low molecular weight endogenous porphyrin(ogen)s from fractions containing PBG deaminase activity. Fractions containing PBG deaminase were used for subsequent kinetic studies.

To validate the separation of porphyrins and PBG deaminase, a mixture of 0.5 mM uro-, copro-, and protoporphyrin was added to 10,000 *g* supernatants of control lymphoblast sonicates to ensure visible porphyrin fluorescence in the resulting porphyrin containing fractions eluted from the column. PBG deaminase activity was found to elute in the G25 void whereas porphyrins eluted at 3.2–3.4 times the void volume.

Next, supernatants (10,000 *g*) of sonicated VP lymphoblasts were applied to a 1.5 × 10 cm Sephadex G25 column, equilibrated with 0.1M Tris-HCl buffer (pH 8.2), and eluted, with the same buffer, at a flow rate of 40 ml/h. Fractions containing PBG deaminase activity were pooled, concentrated on Centricon-30 (Amicon, Beverly, MA) columns through a P30 membrane, and assayed for protein content before PBG deaminase assay.

Purification of PBG deaminase. PBG deaminase was purified from human erythrocytes using a procedure adapted from two previously published methods (27, 30). Our purification data have recently been published (31). The clear single protein band obtained with silver staining was estimated to have a molecular mass of 41,200 D (±2,300) and the final specific activity of 241 nmol uroporphyrin formed/mg per h represented ~7,100-fold purification.

Addition of porphyrinogen and porphyrin to purified PBG deaminase. Substrate-velocity data were obtained over a 1.25–20 μM concentration range of PBG at protoporphyrinogen concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 μM. The effect of added protoporphyrin was assessed at 0.1, 1, and 10 μM concentrations. All enzyme assays were performed in duplicate.

Kinetic analysis and statistics. Data obtained from assays of PBG deaminase reaction velocity at different substrate concentrations using lymphoblasts were initially treated by application of Michaelis–Menten equation (32) to obtain VP V_{max} and K_m. Data which did not conform to the Michaelis–Menten rate law were examined using the Hill equation (33). A nonlinear least-squares grid search procedure computed the following parameters from the Hill equation, which were used for comparative purposes: V_{max}, $K_{0.5}$, and the Hill coefficient, n. V_{max} is the maximal catalytic rate (velocity) for PBG deaminase, $K_{0.5}$ is the substrate concentration at which half maximal velocity is achieved. The Hill coefficient describes the extent of cooperativity for ligand-binding processes or the extent to which multiple ligand-binding sites interact. The coefficient n, is always less than the actual number of ligand-binding sites (34). Thus, for single-site substrate-binding processes displaying perfect hyperbolic Michaelis–Menten behavior, the Hill coefficients will approximate to 1. For hyperbolic behavior, K_m as obtained from the Michaelis–Menten equation, is identical to $K_{0.5}$. For processes not following Michaelis–Menten behavior, the K_m per se is meaningless and an equivalent parameter, $K_{0.5}$, is more informative. A Hill plot, $\log v/(V_{max} - v)$ vs $\log [S]$ (where v is the initial velocity at the given substrate concentration and $[S]$ is the substrate concentration) will have a slope approaching n where there is cooperativity. All comparisons were assessed for statistical significance using the Student's t test (35).

Results

Lymphoblast porphyrin measurements. Total porphyrin concentrations of VP lymphoblasts (1.41±0.21 pmol of total porphyrin/10^6 cells, $n = 7$) was significantly more than that of normal control lymphoblast lines (1.10±0.18 pmol of total porphyrin/10^6 cells, $P = 1.935 \times 10^{-3}$). The mean cell volumes of these cells have been previously determined to be $2.05 \pm 0.35 \times 10^{-12}$ liters (36). This therefore represents a porphyrin concentration of ~ 2 μM. Roughly 90% was protoporphyrin and the remainder coproporphyrin.

When heme biosynthesis in lymphoblast cell lines was stimulated by growing in the presence of 0.6 mM ALA for 24 h before harvesting according to previously described methods (21, 22), VP cell lines had significantly higher concentrations of both protoporphyrin (30% increased, $P = 2.163 \times 10^{-4}$) and coproporphyrin (14% increased, $P = 3.254 \times 10^{-3}$) than control cells.

PBG deaminase kinetics in control and VP lymphoblasts. Lymphoblast sonicate PBG deaminase from 12 control subjects gave a mean V_{max} of 25.2±1.7 pmol/mg protein per h and a mean K_m of 8.1±0.7 μM when evaluated by double-reciprocal substrate vs velocity (Lineweaver–Burk) plots. Linearity of the Lineweaver–Burk plot and of the rate/substrate concentration versus rate (Eadie) plot confirmed the adherence of PBG deaminase in lymphoblasts derived from normal subjects (control lymphoblasts) to the Michaelis–Menten rate law (Fig. 1 *A*). Application of the Hill equation to the same data gave mean values for V_{max} of 28.7±1.8 pmol/mg protein per h and $K_{0.5}$ of 8.5±0.8 μM that were not significantly different from those obtained using the Michaelis–Menten equation. The Hill coefficient was 0.83.

Identical experiments in sonicates of lymphoblasts from 12 VP subjects resulted in a 26% decrease in V_{max} (21.2±2.0 vs 28.7±1.8 pmol/mg protein per h; $P = 6.119 \times 10^{-4}$) and yielded sigmoidal substrate-velocity curves (Fig. 1 *B*) with data that did not conform to Michaelis–Menten kinetics. Nonhyperbolic behavior was confirmed by failure of the data to produce a straight line with either the Lineweaver–Burk or Eadie plots. Thus V_{max} and K_m could not be assessed by this method. The Hill equation, however, did allow these determinations and was therefore used.

Addition of porphyrinogens to control lymphoblast sonicates. Addition of protoporphyrinogen at 1, 5, and 10 μM concentrations altered the kinetic behavior of control lymphoblast PBG deaminase (Fig. 2 *A* and Table I). V_{max} activity was reduced and the substrate-velocity plots became sigmoidal, resembling those observed in VP lymphoblasts.

Compared with the values obtained in the absence of added porphyrinogen, V_{max} was decreased by an average of 41% ($P = 7.004 \times 10^{-4}$) whereas the $K_{0.5}$ was unaltered ($P = 3.611 \times 10^{-1}$). V_{max} tended to decrease as the concentration of protoporphyrinogen increased, but the relationship did not achieve

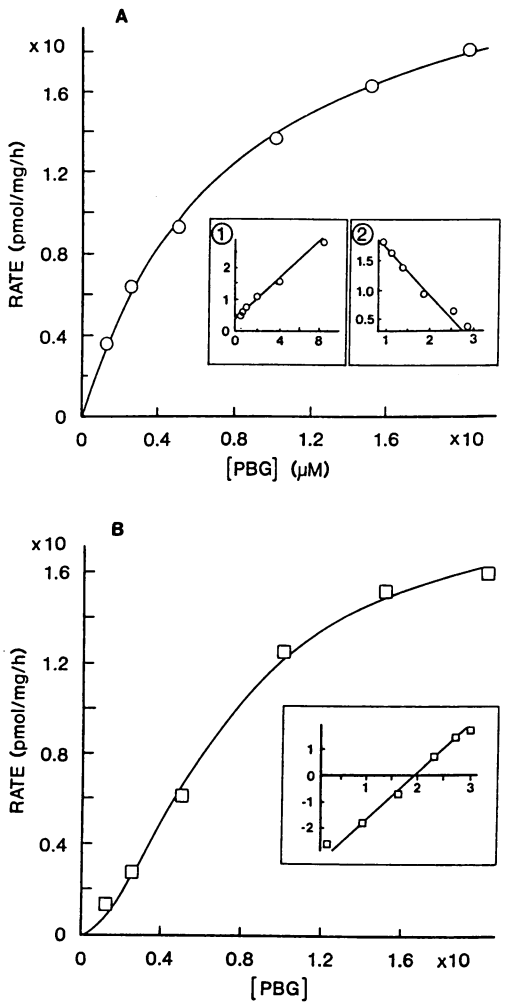

Figure 1. Control and VP lymphoblast PBG deaminase kinetics. (*A*) Control lymphoblast sonicates (n = 12) demonstrated a hyperbolic substrate-velocity plot. Michaelis–Menten kinetics were obeyed as illustrated by the Lineweaver–Burk (inset *1*) and Eadie plots (inset *2*). (*B*) In contrast VP lymphoblast PBG deaminase demonstrated a sigmoidal substrate-velocity plot that could be evaluated using the Hill equation. Inset shows the Hill plot.

statistical significance. The $K_{0.5}$ obtained in the presence of 10 μM protoporphyrinogen was greater than that obtained with 5 μM protoporphyrinogen, which was in turn greater than that in the presence of 1 μM protoporphyrinogen, but none achieved significance.

As the concentration of protoporphyrinogen was increased over a 10-fold range, the Hill coefficient increased to approach a value of 4 in the limit (Table 1). Longer periods of preincubation in the presence of protoporphyrinogen appeared to exert no further effect on the kinetic behavior of control lymphoblast PBG deaminase.

Addition of 1, 5, and 10 μM concentrations of coproporphyrinogen had a similar but less impressive effect on control lymphoblast PBG deaminase. As coproporphyrinogen concentrations were increased over a 10-fold range, V_{max} decreased by an average of 27% ($P = 7.097 \times 10^{-4}$) and the Hill coefficient appeared to approach 3 (in the limit). The mean of the $K_{0.5}$ values between the lymphoblasts with additions and those without were not significantly different ($P = 2.474 \times 10^{-1}$). The distinctive sigmoidal substrate-velocity plot was once again observed, as were the trends in V_{max} and $K_{0.5}$ (Fig. 2 *B* and Table 1). Longer periods of preincubation in the presence of coproporphyrinogen showed no further changes to those measured after 5 min of preincubation.

Addition of uroporphyrinogen and porphyrins to control lymphoblast sonicates. In contrast to the striking effects noted on the addition of proto- and coproporphyrinogen, uroporphyrinogen failed to influence the kinetic behavior of control lymphoblast PBG deaminase. There were no significant differences in V_{max}, $K_{0.5}$, the Hill coefficient ($P > 0.05$ in all cases), or in the shape of the substrate-velocity plot (Table 1).

Similarly, the addition of 1, 5, or 10 μM proto-, copro-, or uroporphyrin had no effect on the kinetic behavior of PBG deaminase in 12 control lymphoblast sonicates (Table I). Indeed, preincubating lymphoblast preparations with 10 μM proto-, copro-, and uroporphyrin for up to 1 h before assaying PBG deaminase also did not alter PBG deaminase behavior.

Addition of porphyrinogens to VP lymphoblast sonicates. Addition of proto- and coproporphyrinogen to VP lymphoblast sonicates resulted in an exaggeration of the kinetic changes already present. Once again, protoporphyrinogen ap-

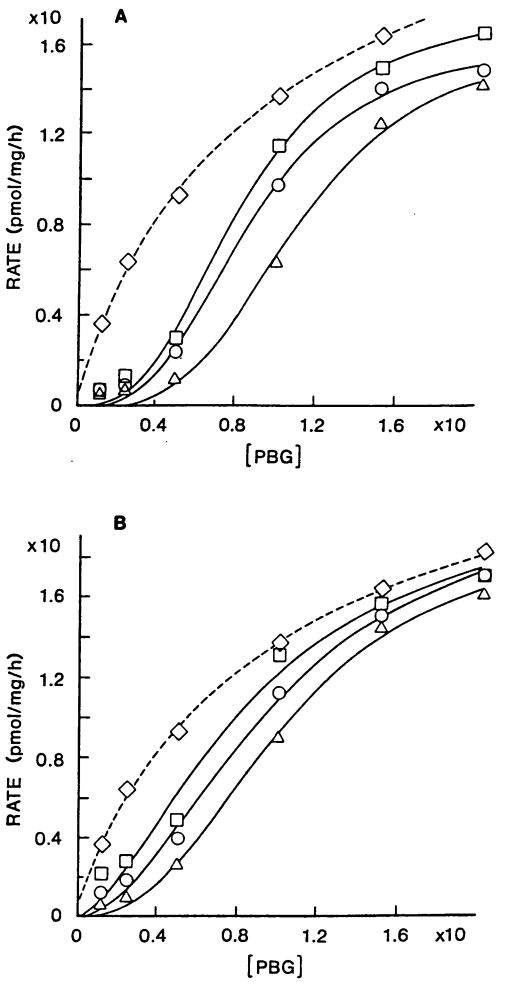

Figure 2. Substrate-velocity plots of PBG deaminase in control lymphoblast sonicates in the absence (*dashed line*) and presence of (*A*) 1 (□), 5 (○), and 10 μM (△) protoporphyrinogen and (*B*) 1, 5, and 10 μM coproporphyrinogen.

Table I. Control PBG Deaminase Kinetic Parameters (as Obtained from the Hill Equation) in the Absence and Presence of 1, 5, and 10 μM Protoporphyrinogen, Coproporphyrinogen, Proto-, Copro-, and Uroporphyrin

Control lymphoblasts	V_{max}	$K_{0.5}$	Hill coefficient
	pmol/mg per h	*μM*	
No additions	28.7±1.8	8.5±0.8	0.83±0.07
+1 μM proto'gen	17.7±1.3	7.7±1.1	2.89±0.43
+5 μM proto'gen	16.4±1.7	8.9±1.1	3.05±0.47
+10 μM proto'gen	16.1±1.6	10.9±2.1	3.61±0.45
+1 μM copro'gen	21.8±1.8	8.5±2.3	1.67±0.51
+5 μM copro'gen	21.5±2.2	9.8±1.3	1.96±0.33
+10 μM copro'gen	19.5±1.9	10.3±1.4	2.51±0.38
+1 μM uro'gen	30.1±1.5	7.8±1.2	0.79±0.26
+5 μM uro'gen	28.7±2.4	8.7±1.0	0.93±0.11
+10 μM uro'gen	29.0±1.6	8.4±1.1	0.89±0.13
+1 μM proto	28.4±2.9	8.4±1.1	0.89±0.10
+5 μM proto	28.4±1.7	8.3±0.9	0.90±0.09
+10 μM proto	29.3±1.6	8.0±1.0	0.87±0.10
+1 μM copro	30.2±2.1	8.8±1.9	0.86±0.10
+5 μM copro	31.2±1.3	8.9±1.2	0.89±0.05
+10 μM copro	30.8±1.6	9.5±0.9	0.88±0.07
+1 μM uro	28.8±1.3	9.0±1.0	0.95±0.11
+5 μM uro	34.5±3.3	8.5±1.3	0.78±0.16
+10 μM uro	30.9±2.0	8.7±1.3	0.82±0.08

Proto'gen, protoporphyrinogen; copro'gen, coproporhyrinogen; uro'gen, uroporphyrinogen; proto, protoporphyrin; copro, coproporphyrin; uro, uroporphyrin.

peared to have a greater effect than coproporphyrinogen. V_{max} did not decrease to levels lower than those observed when 10 μM protoporphyrinogen was added to control lymphoblast sonicates. Similarly, the Hill coefficients never tended to a value > 4.

As in the control lymphoblasts, uroporphyrinogen failed to exert any additional effect on VP PBG deaminase kinetic behavior (Table II). Furthermore, the addition of 1, 5, or 10 μM proto-, copro-, or uroporphyrin had no additional effect on the already anomalous kinetic behavior of PBG deaminase in 12 VP lymphoblast sonicates.

Kinetics of purified PBG deaminase. Substrate-velocity data of pure PBG deaminase yielded a hyperbolic curve (Fig. 3, *dotted line* and Table III) with a V_{max} of 249±36 nmol/mg per h, a $K_{0.5}$ of 8.9±1.5 μM, and a Hill coefficient of 0.93±0.14. Lineweaver–Burk and Eadie plots were linear.

Addition of protoporphyrinogen over a 1,000-fold range of concentrations (0.01–10 μM) transformed the hyperbolic substrate-velocity curve obtained in the absence of protoporphyrinogen to sigmoidal curves similar to those displayed by impure PBG deaminase in the presence of 1, 5, and 10 μM proto-, and coproporphyrinogen (Fig. 3, *solid lines* and Table III). There was an inverse relationship between V_{max} and the protoporphyrinogen concentration. V_{max} in the presence of 10 μM protoporphyrinogen, 169±28 nmol/mg per h, was 32% lower than that in the absence of protoporphyrinogen. The Hill coefficients appeared to approach 4 in the limit, and $K_{0.5}$ was essentially unaltered.

A plot of V_{max} and the Hill coefficient versus protoporphyrinogen concentration (Fig. 4) shows a single exponential growth relationship in the case of the Hill coefficient and a single exponential decay for V_{max}. Single exponential analysis reveals that the protoporphyrinogen concentration at which the Hill coefficient is half of its estimated maximum is 0.2 μM, and the concentration at which V_{max} is half its estimated maximum inhibition is 0.19 μM.

Addition of protoporphyrin at 0.1, 1, and 10 μM concentrations had no effect on pure PBG deaminase kinetics.

Sephadex G25 chromatography. PBG deaminase kinetics of control lymphoblast preparations were not changed by Sephadex G25 chromatography. In striking contrast, after Sephadex G25 chromatography, VP lymphoblast PBG deaminase substrate velocity data were identical to those of control lymphoblasts (Fig. 5).

Discussion

Much of the evidence favoring our hypothesis that heme precursors that accumulate in VP inhibit PBG deaminase activity depends on the validity of the EBV-transformed lymphoblast system. Two lines of evidence suggest that the VP defect persists in this system. First, we have confirmed our previously published data that protoporphyrinogen oxidase activity in VP lymphoblasts is reduced by roughly 50% (6). Second, and vital to our hypothesis, there was a significant increase in endogenous protoporphyrin concentration in our VP lymphoblast cultures. Indeed when porphyrin production was stimulated by addition of ALA to the culture medium both protoporphyrin(ogen) and coproporphyrin(ogen) concentrations in VP lymphoblasts exceeded those of similarly treated cells derived from control subjects.

Although diminished protoporphyrinogen oxidase activity has previously been demonstrated in fibroblasts from VP subjects, protoporphyrin(ogen) concentration in that system was not increased even after the addition of ALA (4). The elevated protoporphyrin concentrations found in our study suggest that in transformed lymphoblasts, the flux through the pathway may be high enough for the defect in protoporphyrinogen oxidase to become evident.

Table II. VP PBG Deaminase Kinetic Parameters in the Absence and Presence of 1, 5, and 10 μM Proto-, Copro-, and Uroporphyrinogen

VP lymphoblasts	V_{max}	$K_{0.5}$	Hill coefficient
	pmol/mg per h	*μM*	
No additions	21.2±2.0	7.4±0.7	1.78±0.17
+1 μM proto'gen	17.7±1.8	7.2±1.0	2.25±0.40
+5 μM proto'gen	17.3±1.5	8.5±1.2	2.64±0.49
+10 μM proto'gen	16.6±1.3	8.7±1.1	3.22±0.42
+1 μM copro'gen	19.0±1.5	8.5±1.1	1.88±0.31
+5 μM copro'gen	18.5±2.0	7.9±2.2	2.01±0.33
+10 μM copro'gen	18.3±1.8	8.9±1.4	2.05±0.31
+1 μM uro'gen	20.4±1.4	7.5±1.6	1.75±0.19
+5 μM uro'gen	19.5±1.3	9.4±1.8	1.75±0.18
+10 μM uro'gen	21.3±2.2	7.6±0.9	1.83±0.20

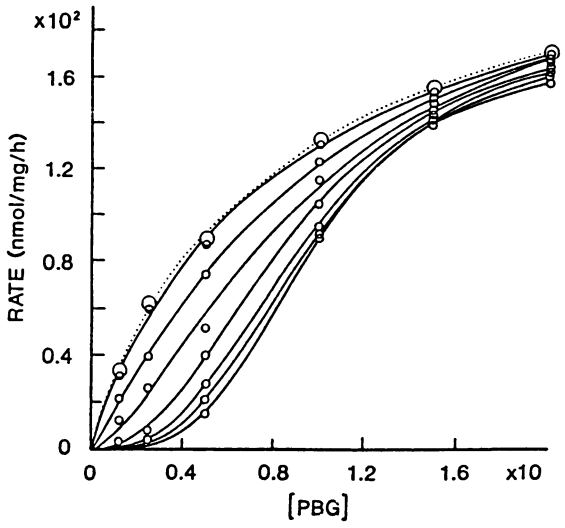

Figure 3. Addition of 0.01–10 μM protoporphyrinogen to pure PBG deaminase resulted in sigmoidal substrate-velocity plots. Dotted line represents substrate-velocity plot without additions.

Substrate-velocity data for PBG deaminase in lymphoblast sonicates were obtained by measuring the rate of formation of uroporphyrinogen at five substrate concentrations. Ideally these concentrations should fall both above and below the K_m for the enzyme (33) so as to cover a wide range around the V_{max}. The literature reports K_m values ranging between 6 and 22 μM (22, 25–27, 30) and our data indicated a K_m (or the equivalent $K_{0.5}$ using Hill analysis) of ~ 8 μM for lymphoblast PBG deaminase originating from healthy control subjects. Thus the choice of PBG concentrations of 1.25, 2.5, 5, 10, and 20 μM is appropriate, since these substrate concentrations cover a range from 13 to 73% of V_{max}.

Analysis of the data obtained in control lymphoblasts by the Michaelis–Menten equation yielded a V_{max} of 25.2±1.7 pmol/mg per h and a K_m of 8.1±0.7 μM. There are only two previous reports of the measurement of PBG deaminase activity in lymphoblasts. One (25) reports an activity of 11 pmol/mg protein per h for human lymphoblast lines, but it is unclear whether these were EBV-transformed lymphoblast lines. The other study (22) found values of 40 and 68 pmol/mg protein per h for PHA- or pokeweed-mitogen–stimulated lymphocytes and EBV-transformed lymphoblasts, respectively. It must be noted that reported erythrocyte PBG deaminase activities show a large range, which underscores the need to establish control values for each individual laboratory and method. Our control lymphoblast results appear to be of the same order as those in other reports and were thus considered valid for the purposes of this study.

Comparison of the indexes of kinetic behavior of VP lymphoblast PBG deaminase with that of control lymphoblasts yielded the unprecedented observation that although control PBG deaminase exhibited a hyperbolic substrate-velocity relationship suggesting enzyme substrate interaction at a single ligand-binding site, VP PBG deaminase did not (Fig. 1).

Although the mechanism of action of PBG deaminase remains a subject of research activity and debate (37–39), it is generally assumed that in most cases the appearance and rate of production of uroporphyrinogen from PBG by the enzyme follows the Michaelis–Menten rate law. There are, however, reports of nonMichaelian behavior from one group (26, 27, 40). In our study, the impure preparations of PBG deaminase from control lymphoblasts exhibited classic Michaelis–Menten behavior. This was borne out by the linearity of the Lineweaver–Burk plot (1/rate vs 1/[substrate]) and the Eadie plot (rate/[substrate] vs rate) (Fig. 1 *A*).

The Hill equation (33) can also be used for determining kinetic indexes. Under conditions where there is a hyperbolic substrate-velocity relationship, the Hill coefficient approaches 1 and should yield V_{max} and $K_{0.5}$ values equivalent to the Michaelis–Menten V_{max} and K_m. This was the case in control lymphoblast PBG deaminase. The Hill equation is also used to analyze the number of ligand-binding sites and their interdependence in macromolecular species such as proteins. It has been used in this regard to directly demonstrate variable ligand-binding stoichiometries in the interaction of pyridine and *N*-methyl pyridinium cation with ferriprotoporphyrin IX (41). A Hill coefficient close to 1 implies that the enzyme has only one binding site or that multiple sites, if present, are identical and that the binding at any site is independent of the occupation of other sites. It is generally accepted that PBG deaminase exists in monomeric form and that there is only one substrate (ligand)-binding site (22, 30). This is consistent with our value for control lymphoblasts.

The substrate-velocity data for VP lymphoblast PBG deaminase does not fit the Michaelis–Menten equation (Fig. 1 *B*). The most striking difference was a sigmoidal substrate-velocity plot. Fortunately, the data fitted the Hill equation well. Using this equation, the mean V_{max} value of PBG deaminase in VP cells was 21.2±2.0 pmol/mg protein per h, a significant 26% decrease over that of control cells. Comparison of the $K_{0.5}$ values obtained in the VP and control groups showed no significant alterations. This implies that there was no major difference in the thermodynamics of ligand binding by PBG deaminase in the two groups. A sigmoidal substrate-velocity curve implies the presence of more than one cooperative ligand-binding site. The substrate-velocity plot of VP lymphoblast PBG deaminase yielded a Hill coefficient of 1.78±0.17. This suggests that, in addition to the reduction in V_{max} VP lymphoblast, PBG deaminase might exist in a form displaying multiple cooperative binding sites. This might reflect either the unmasking of preexisting additional sites or the formation of a cooperative multimeric structure with decreased catalytic ability.

Table III. Addition of Various Concentrations of Protoporphyrinogen to Pure PBG Deaminase Resulted in Altered Kinetics as Determined by the Hill Equation

Purified control PBG deaminase	V_{max}	$K_{0.5}$	Hill coefficient
	pmol/mg per h	*μM*	
No additions	249±36	8.9±1.5	0.93±0.14
+0.01 μM proto'gen	246±37	8.9±1.0	0.96±0.09
+0.05 μM proto'gen	235±42	9.5±1.5	1.19±0.14
+0.10 μM proto'gen	221±40	9.8±1.1	1.58±0.20
+0.50 μM proto'gen	190±48	9.1±1.9	2.27±0.16
+1.00 μM proto'gen	186±46	9.7±1.6	2.66±0.16
+5.00 μM proto'gen	178±39	9.7±1.4	3.02±0.11
+10.0 μM proto'gen	169±28	9.7±1.6	3.49±0.19

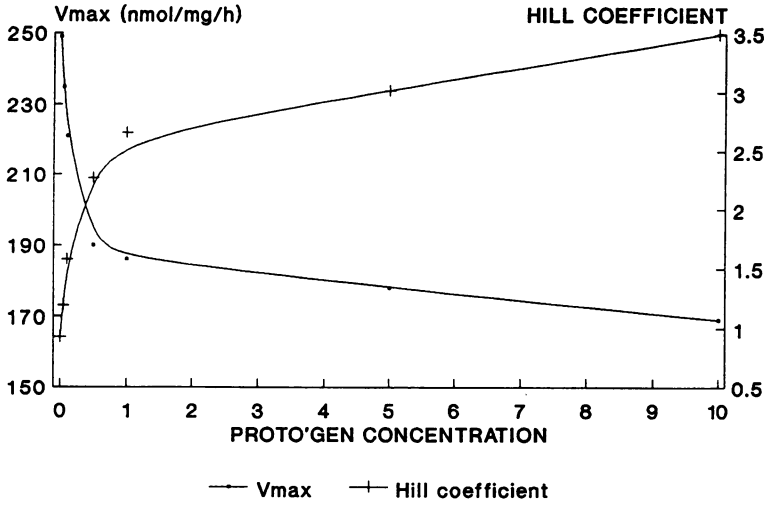

Figure 4. Relationship between added protoporphyrinogen and V_{max} and the Hill coefficient.

Addition of proto- and coproporphyrinogen to control lymphoblast preparations at concentrations bracketing those observed in VP lymphoblasts resulted in PBG deaminase kinetics that closely resembled those seen in VP lymphoblasts. More specifically, addition of protoporphyrinogen (Fig. 2 *A*), decreased V_{max} and changed the substrate-velocity plot to a sigmoidal form. V_{max} decreased most after addition of 10 μM protoporphyrinogen and least after 1 μM protoporphyrinogen (Table I) but this decrease in activity did not appear to be directly proportional to the amount of porphyrinogen added. This might reflect a maximal effect at a concentration of 5 or 10 μM or may result from the use of impure PBG deaminase.

Addition of protoporphyrinogen increased the Hill coefficient to a maximum of 3.61. This would suggest that, in response to addition of protoporphyrinogen, PBG deaminase exhibits up to four ligand-binding sites because of aggregation of enzyme protein or the unmasking of hitherto unsuspected binding sites. Addition of coproporphyrinogen to control lymphoblast preparations produced effects similar to those observed after addition of protoporphyrinogen. The changes in shape of the substrate-velocity curve and the diminution V_{max} activity were less marked than those produced by protoporphyrinogen. For 1 and 5 μM coproporphyrinogen the Hill coefficient approached 2 and for 10 μM coproporphyrinogen approached 3. Thus coproporphyrinogen may be a less "potent" negative effector. Addition of uroporphyrinogen or of porphyrin did not influence control lymphoblast PBG deaminase kinetics.

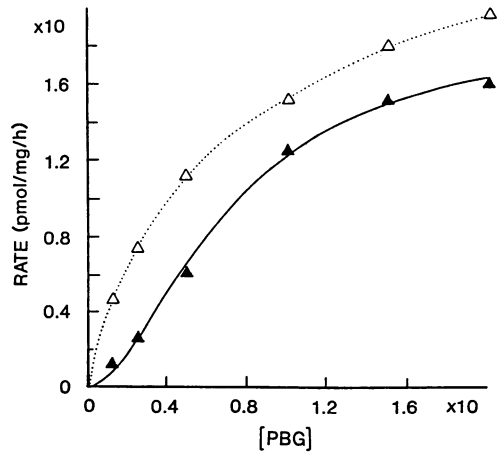

Figure 5. VP lymphoblast PBG deaminase substrate-velocity plots before (▲) and after (△) Sephadex G-25 chromatography. The kinetic parameters were as follows: pre- and (post)-Sephadex G-25 chromatography V_{max}, 19.9±1.9 (30.8±1.6); $K_{0.5}$, 7.9±1.2 (6.7±2.9); Hill coefficient, 1.78±0.21 (0.82±0.33).

The similarity between the behavior of PBG deaminase in VP lymphoblasts and those observed in normal lymphoblasts after addition of protoporphyrinogen and coproporphyrinogen suggest that the sigmoidal curve of VP cell PBG deaminase might be related to intracellular accumulation of these heme intermediates. If this were so, addition of these porphyrinogens to VP lymphoblast PBG deaminase preparations should enhance the changes already present. Our finding that additional protoporphyrinogen and coproporphyrinogen, but not uroporphyrinogen, proto-, copro-, or uroporphyrin, decreased V_{max} and increased the Hill coefficient to levels not dissimilar to those obtained when 10 μM concentrations of proto- and coproporphyrinogen were added to normal lymphoblast sonicates is in keeping with this thesis.

To exclude any confounding effect associated with the use of less pure lymphoblast sonicates, a limited series of experiments were performed using purified human erythrocyte PBG deaminase. Our purification procedure, judged by silver staining of SDS-PAGE, yielded homogeneous PBG deaminase. The final specific activity, 241 nmol uroporphyrin formed/mg per h, was intermediate between the ~ 2300 nmol/mg per h (25) and the 68 nmol/mg per h (27) reported by the two groups on whose techniques our method was based. Our ~ 7,100-fold purification lies between the 3,400-fold (27) and 42,000-fold (25) reported by the two methods. The product ran on SDS-PAGE as a single unit of molecular weight 41,200 (±2,300).

Purified PBG deaminase, in the absence of added porphyrinogen, behaved as a Michaelian enzyme with a single substrate-binding site. Addition of protoporphyrinogen to purified PBG deaminase once again resulted in a decreased V_{max} and a sigmoidal substrate-velocity curve. Once again, the Hill coefficient approached 4 (Fig. 4 and Table III). Both V_{max} and the Hill coefficient were maximally affected at 10 μM protoporphyrinogen and tenuously affected at 0.01 μM. Single exponential analysis of both V_{max} and the Hill coefficient against protoporphyrinogen concentration (Fig. 4) revealed that the concentrations at which the effects were half "maximal" were

almost identical. This strongly suggests that a single mechanism is responsible for affecting both V_{max} and the degree of cooperativity. Addition of protoporphyrin had no effect on the kinetics of pure PBG deaminase. These findings using purified erythrocyte enzyme are so similar to those obtained using our lymphoblast sonicates that they suggest that both erythroid and nonerythroid forms of PBG deaminase are inhibited by protoporphyrinogen.

If the kinetic abnormalities observed in VP lymphoblasts are indeed due to intracellular accumulation of proto- and coproporphyrinogen, removal of these porphyrinogens from VP lymphoblasts should restore normal PBG deaminase kinetic behavior. After establishing that fractions containing PBG deaminase activity and those containing porphyrin(ogen)s could be separated by Sephadex G25 chromatography, this procedure was used to separate endogenous heme intermediates from VP lymphoblast fractions exhibiting reduced PBG deaminase activity. Single passage of VP lymphoblast cytosolic preparations down a Sephadex G25 column resulted in restoration of "normal" kinetic behavior to PBG deaminase. Although it is possible that molecular sieving removed other factor(s) responsible for alteration of PBG deaminase behavior, these results are consistent with our hypothesis.

Finally, if accumulation of proto- and coproporphyrinogen affect PBG deaminase in VP, urinary ALA and PBG concentrations should be marginally elevated, even in quiescent VP. ALA and PBG levels have traditionally been considered to be "normal" in quiescent VP. We have measured urinary concentrations of these precursors in 221 patients with quiescent VP and 93 controls. Urinary ALA and PBG concentrations in quiescent VP subjects were 32.4±26.3 and 15.5±23.6 μmol/10 mmol creatinine, 2.5 and 4.6 times higher, respectively, than those of control subjects (ALA 12.85±5.7, PBG 3.4±2.3) ($P < 0.001$). This increase in urinary ALA and PBG excretion suggests that the decrease in PBG deaminase V_{max} is sufficient to perturb the heme synthetic processes even in quiescent VP.

The mechanism by which protoporphyrinogen and to a lesser degree coproporphyrinogen affect PBG deaminase is not known. Our data suggest that the ability of these intermediates to inhibit PBG deaminase may be related to the number of carboxyl groups on the porphyrinogen. Uroporphyrinogen had no effect, whereas the effect of coproporphyrinogen was less than that of protoporphyrinogen. Thus steric and/or hydrophobic effects may be important. Secondly, the lack of influence of porphyrins on PBG deaminase may be caused by the strongly aromatic nature of these compounds. This is in contrast to the unconjugated macrocyclic ring system of the porphyrinogens. In the porphyrins the conjugated pi-electron systems lie above and below the whole tetrapyrrolic macrocycle and may shield the porphyrin from potential interaction with the enzyme molecule. The porphyrinogens on the other hand are aliphatic and the aromaticity is confined to the individual pyrrolic "corners" (42). This renders the interpyrrolic methylene groups more accessible.

Our data provide several lines of evidence to support our hypothesis that proto- and coproporphyrinogen exert negative allosteric effects on PBG deaminase. This may account for the increases of ALA and PBG seen when heme synthesis is depressed during the acute porphyric attack. Our study offers an explanation for the apparent defect in PBG deaminase in each of the acute porphyrias. In acute intermittent porphyria, PBG deaminase deficiency is inherited. In VP and hereditary coproporphyria, the intermediates accumulating as a result of the inherited enzyme defect may partially inhibit PBG deaminase. The finding that uroporphyrinogen exerted no influence on PBG deaminase is in keeping with our hypothesis, since porphyria cutanea tarda is a nonacute porphyria in which concentrations of ALA and PBG are not elevated despite greatly increased uroporphyrinogen concentrations.

Acknowledgments

We thank R. Schmid, R. Hift, W. Gevers, M. Berman, P. Berman, and M. Moore for advice; E. Sturrock, A. Corrigall, J. Sutherland, B. Davidson, and A. Damon for technical assistance; and I. Batho for manuscript preparation.

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