

Characterization of the Porphobilinogen Deaminase Deficiency in Acute Intermittent Porphyria: *IMMUNOLOGIC EVIDENCE FOR HETEROGENEITY OF THE GENETIC DEFECT*

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The molecular pathology of the porphobilinogen (PBG)-deaminase deficiency in heterozygotes for acute intermittent porphyria (AIP) was investigated by means of biochemical and immunologic techniques. The stable enzyme-substrate intermediates (A, B, C, D, and E) of PBG-deaminase were separated by anion-exchange chromatography of erythrocyte lysates from heterozygotes for AIP and normal individuals. In normal lysates, the intermediates eluted in a characteristic pattern with decreasing amounts of activity ($A > B > C > D > E$), the combined A and B intermediates representing >75% of total recovered activity. In contrast, two different profiles were observed in lysates from heterozygotes for AIP. In most heterozygotes, the elution profile was similar to that of normal individuals, but each intermediate was reduced ~50%. A second profile in which the C intermediate had disproportionately higher activity than the A or B intermediates was observed in asymptomatic heterozygotes with high urinary levels of PBG (>5 µg/ml) as well as in heterozygotes during acute attacks. These findings suggested that the C intermediate (the dipyrrole-enzyme intermediate) may be rate limiting in the stepwise conversion of the monopyrrole, PBG, to the linear tetrapyrrole, hydroxymethylbilane. To investigate further the nature of the enzymatic defect in AIP, sensitive immunotitration and immunoelectrophoretic assays were developed with the aid of a rabbit anti-human PBG-deaminase IgG preparation produced against the homogeneous enzyme. Equal amounts [...]

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IMMUNOLOGIC EVIDENCE FOR HETEROGENEITY OF THE GENETIC DEFECT

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ABSTRACT The molecular pathology of the porphobilinogen (PBG)-deaminase deficiency in heterozygotes for acute intermittent porphyria (AIP) was investigated by means of biochemical and immunologic techniques. The stable enzyme-substrate intermediates (A, B, C, D, and E) of PBG-deaminase were separated by anion-exchange chromatography of erythrocyte lysates from heterozygotes for AIP and normal individuals. In normal lysates, the intermediates eluted in a characteristic pattern with decreasing amounts of activity ($A > B > C > D > E$), the combined A and B intermediates representing $>75\%$ of total recovered activity. In contrast, two different profiles were observed in lysates from heterozygotes for AIP. In most heterozygotes, the elution profile was similar to that of normal individuals, but each intermediate was reduced $\sim 50\%$. A second profile in which the C intermediate had disproportionately higher activity than the A or B intermediates was observed in asymptomatic heterozygotes with high urinary levels of PBG ($>5 \mu\text{g/ml}$) as well as in heterozygotes during acute attacks. These findings suggested that the C intermediate (the dipyrrole-enzyme intermediate) may be rate limiting in the stepwise conversion of the monopyrrole, PBG, to the linear tetrapyrrole, hydroxymethylbilane. To investigate further the nature of the enzymatic defect in AIP, sensitive immunotitration and immunoelectrophoretic assays were developed with the aid of a rabbit anti-human PBG-deaminase IgG preparation produced against the homogeneous enzyme. Equal amounts of erythrocyte lysate activity from 32 heterozygotes for AIP from 22 unrelated families and 35 normal individuals

were immunoelectrophoresed. There were no detectable differences in the amounts of cross-reactive immunologic material (CRIM) in lysates from the normal individuals and 25 heterozygotes from 21 of the 22 unrelated families with AIP. In contrast, when equal enzymatic activities were coimmunoelectrophoresed, all seven heterozygotes from one family had ~ 1.6 times the amount of CRIM compared with that detected in normal lysates. Consistent with these findings, immunotitration studies also demonstrated similar quantities of noncatalytic CRIM in lysates from this AIP family. When equal activities of the individual A, B, C, and D enzyme-substrate intermediates from normal and CRIM-positive erythrocytes were immunoelectrophoresed, increased amounts of immunoreactive protein were observed for each intermediate, $B > A \approx C \approx D$, from the CRIM-positive AIP variants. On the basis of these findings, it is hypothesized that the enzymatic defect in the CRIM-positive AIP family resulted from a mutation in the structural gene for PBG-deaminase which altered the catalytic as well as a substrate binding site. These studies of the enzymatic defect provide the first demonstration of genetic heterogeneity in AIP.

INTRODUCTION

Acute intermittent porphyria (AIP),¹ a dominantly inherited inborn error of heme biosynthesis, is characterized by the half-normal activity of porphobilinogen (PBG)-deaminase (EC 4.3.1.8) (1, 2). The enzymatic deficiency has been demonstrated in erythrocytes

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¹Abbreviations used in this paper: AIP, acute intermittent porphyria; CRIM, cross-reactive immunologic material; PBG, porphobilinogen.

(1–6), liver (7, 8), cultured skin fibroblasts (9), amniotic cells (10), and cultured lymphoblasts (11) from heterozygotes for AIP. Comparison of the physical and kinetic properties of the PBG-deaminase activity in erythrocyte lysates from heterozygotes for AIP and normal individuals have shown essentially identical electrophoretic mobilities (3), heat denaturation profiles (12), and apparent Michaelis constant (K_m) values (5, 12). In addition, mitogen-stimulated lymphocytes from heterozygotes for AIP induced only 50% of the activity detected in mitogen-treated lymphocytes from normal individuals (11). Although these studies are consistent with a structural gene mutation (1), it has been suggested that the half-normal levels of PBG-deaminase activity in AIP may be due to a regulatory gene defect (11), but immunologic evidence supporting a structural mutation of the PBG-deaminase gene in AIP, i.e., the demonstration of noncatalytic cross-reactive immunologic material (CRIM), has not been reported to date.

Increasing attention has focused on the characterization of PBG-deaminase from various sources (13–19) and the mechanism by which this monomeric enzyme (~37,000 mol wt) converts the monopyrrole substrate, PBG, to the final tetrapyrrole product, uroporphyrinogen I (20–27). Recently, we reported the first purification of homogeneous PBG-deaminase from human erythrocytes (27). Anion-exchange chromatography of the purified enzyme resolved the activity into five forms, designated A, B, C, D, and E. Interconversion studies and experiments with [^3H]PBG demonstrated that the five forms were stable enzyme-substrate intermediates in the sequential condensation of four PBG molecules into the tetrapyrrole, uroporphyrinogen I (27). The occurrence of these stable PBG-deaminase intermediates was independently confirmed by ^{13}C -nuclear magnetic resonance studies that demonstrated the order of assembly of the four pyrrole rings and established that the end-product of the PBG-deaminase reaction was the linear tetrapyrrole, hydroxymethylbilane, which nonenzymatically cyclized to form the uroporphyrinogen I isomer (22–25). Fig. 1 illustrates the current concept of the stepwise enzymatic conversion of the monopyrrole, PBG, to the tetrapyrrole, hydroxymethylbilane.

In light of the discovery of the stable PBG-deaminase enzyme-substrate intermediates, an investigation was undertaken to characterize the molecular pathology of the enzymatic defect in AIP biochemically and immunologically. We report here (a) the activity profiles of enzyme-substrate intermediates in erythrocyte lysates from normal individuals and hetero-

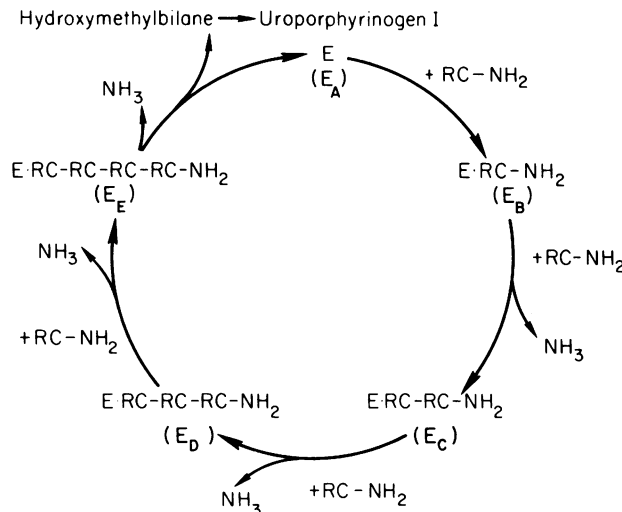


FIGURE 1 Current concept of tetrapyrrole biosynthesis. PBG-deaminase (E) catalyzes the sequential head-to-tail condensation of four molecules of PBG (RC-NH_2) to form the linear tetrapyrrole, hydroxymethylbilane. E_A represents the native enzyme, and E_B , E_C , E_D , and E_E , the mono-, di-, tri-, and tetrapyrrole enzyme-substrate intermediates, respectively.

zygotes for AIP, and (b) the quantification of PBG-deaminase immunoreactive protein in lysates from normal individuals and from AIP heterozygotes in 22 unrelated families. These studies provide the first demonstration of genetic heterogeneity in this dominantly inherited enzymatic deficiency.

METHODS

Materials

PBG was synthesized as described previously (28). Uroporphyrin standards were purchased from Porphyrin Products, Logan, Utah. Diaminobenzidine tetrahydrochloride, δ -aminolevulinic acid hydrochloride, DL-dithioerythritol, DL-dithiothreitol, and human serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo. High purity acetonitrile was purchased from Burdick and Jackson Laboratories, Inc., Muskegon, Mich. Fluorescamine was obtained from Pierce Chemical Company, Rockford, Ill. Enzyme grade ammonium sulfate was a product of Schwartz/Mann, Div. Becton, Dickinson & Co., Orangeburg, N. Y. Female New Zealand rabbits were from the Pocono Rabbit Farm, Canadensis, Pa. Goat anti-rabbit serum and peroxidase-conjugated sheep anti-rabbit IgG were obtained from N. L. Cappel Laboratories, Cochranville, Pa. DEAE-cellulose (DE-52) was a product of H. Reeve Angel & Co., Inc., Clifton, N. J. Seakem agarose was purchased from Marine Colloids, Inc., Div. FCC Corp., Rockland, Maine. High resolution Tris/barbital buffer was obtained from Gelman Instrument Company, Ann Arbor, Mich. The following products were purchased from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.: Sephadex G-100, phenyl-Sepharose CL-4B, octyl-Sepharose CL-4B, Agarose IEF, Pharmalyte (pH 5–8) ampholines, and Gelbond film. All other materials and chemicals were of the highest quality available.

Human subjects and specimen collection

32 heterozygotes for AIP were studied; the diagnosis of each was biochemically documented by demonstration of decreased PBG-deaminase activity in erythrocyte lysates. These subjects were from 22 unrelated families representing at least 10 different ethnic backgrounds or countries of origin. Heparinized blood was collected from each heterozygote and 35 age- and sex-matched normal individuals. Following centrifugation at 2,500 *g*, the erythrocytes were removed, washed twice with 0.9% NaCl, and then either used immediately or stored at -20°C.

Assays

PBG-deaminase assay. Enzymatic activity was determined by the measurement of uroporphyrin fluorescence as previously described (27). One unit of enzymatic activity equals 1 nmol of uroporphyrin produced per hour at 37°C.

Protein assay. Protein concentrations were determined by the fluorescamine procedure as previously described (28).

Separation of PBG-deaminase enzyme-substrate intermediates

Anion-exchange chromatography. Washed, centrifuged erythrocytes (2.0 ml of packed cells) were lysed by the addition of 20 ml of cold distilled water and centrifuged at 35,000 *g* for 20 min. The supernate was dialyzed against 2 liters of 7 mM potassium phosphate buffer, pH 6.8, containing 0.1 mM DL-dithioerythritol.

A 0.9 × 30-cm column of DEAE-cellulose that had previously been acid and base washed was equilibrated with the same buffer used for dialysis (27). Each dialyzed erythrocyte lysate was applied to the column at a flow rate of 1.0 ml/min, and then 60 ml of buffer was passed through the column. PBG-deaminase enzyme-substrate intermediates were eluted as peaks of enzymatic activity using a 400-ml linear NaCl (0 to 0.11 M) gradient. Fractions (2.0 ml) were collected and 0.5 ml was assayed for PBG-deaminase activity at 37°C for 2 h.

Isoelectric focusing. Horizontal agarose slab gels and the Pharmacia system were used for isoelectric focusing of the PBG-deaminase intermediates in accordance with the manufacturer's instructions. Gels were prepared by heating a mixture of 0.3 g Agarose IEF (Pharmacia), 3.6 g sorbitol, and 27 ml distilled water in a boiling water bath. After cooling to 75°C, 1.9 ml of pH 5-8 Pharmalyte ampholines was added. The mixture was then poured into a horizontal casting frame (11.4 × 22.5 cm with Gelbond film backing) that had been preheated to ~60°C using a portable hairdryer. Gels were either allowed to harden at least 1 h at 4°C or stored overnight at 4°C in a moist chamber. Before isoelectric focusing, erythrocyte lysates were heated at 60°C for 1 h, centrifuged at 100,000 *g* for 20 min, and then aliquots of the supernate (50-100 µl) were pipetted onto Whatman no. 17 paper strips (Whatman, Inc., Clifton, N. J.), which were placed 1 cm from the cathode. The cathode contained 1 M NaOH and the anode, 0.05 M H₂SO₄. Focusing was carried out for 2.5 h at 10 W (constant power) at 4°C. The filter paper strips were removed after the hemoglobin had migrated about 1 cm from the origin. Upon completion, a 0.5-cm strip was removed from the gel, cut into 1-cm pieces, soaked in distilled H₂O, and the pH of the leached ampholytes was determined. The gel was overlaid with Whatman no. 3MM filter paper saturated with 0.5 mM PBG, covered with polyvinylchloride film, and incubated at 37°C for 90 min. The

overlay was removed, the gel was exposed to ultraviolet light for 10 min to oxidize the uroporphyrinogen to uroporphyrin, and the red fluorescent bands of PBG-deaminase activity were rapidly photographed under UV light with Polaroid type 58 film (Polaroid Corp., Cambridge, Mass.) with a Wratten no. 4 filter (Eastman Kodak Co., Rochester, N. Y.).

Crossed-immunoelectrophoresis of the separated PBG-deaminase intermediates was performed by removing an unstained lane from the focusing gel and pouring an antibody-containing agarose gel such that the individual intermediates could be electrophoresed directly into it. Rocket immunoelectrophoresis was carried out as described below, except that a larger gel (5.0 × 7.5 cm) containing 8 ml of 1% agarose and 40 µl of antiserum was used.

Immunologic studies

Purification of PBG-deaminase from human erythrocytes. Human PBG-deaminase was purified to homogeneity as previously described by Anderson and Desnick (27). The preparation was routinely stored after the Sephadex G-100 step in the presence of 1.0 mM DL-dithioerythritol. When individual enzyme-substrate intermediates were required, the preparation was subjected to the final DEAE-cellulose chromatographic step (27).

Production of rabbit anti-human PBG-deaminase IgG. New Zealand rabbits were injected intradermally and intramuscularly with 150 µg of homogeneous human PBG-deaminase A in a 1:1 suspension of Freund's complete adjuvant. Booster injections of 150, 100, and 75 µg were given at 1-mo intervals. The titers of rabbit anti-human PBG-deaminase were determined by immunotitration. The IgG fraction from the pooled rabbit sera was partially purified by the method of Harboe and Ingeld (29). The antibody was precipitated by the addition of 4 vol of saturated ammonium sulfate, pH 7.0, to 6 vol of rabbit serum. After stirring 2 h at 4°C, the solution was centrifuged at 2,500 *g* for 20 min and washed twice with 1.5 M ammonium sulfate, pH 7.0. Hemoglobin and albumin were in the supernate and >90% of the IgG was recovered in the pellet. The pellet was resuspended in distilled water and extensively dialyzed against 5 mM potassium phosphate buffer, pH 7.4, containing 0.9% NaCl. The IgG fraction was divided into 1.5-ml aliquots and stored at -20°C.

Immunotitration of anti-human PBG-deaminase. Homogeneous PBG-deaminase A (40 ng of enzyme diluted in bovine serum albumin to 50 µl having a final protein concentration of 40 mg/ml) and 50 µl of rabbit anti-PBG-deaminase IgG (in serial dilutions) were mixed and incubated at 37°C. After 30 min, 50 µl of goat anti-rabbit IgG was added and the mixture was incubated at 37°C for 30 min. To insure quantitative precipitation of the anti-PBG-deaminase antibodies, the samples were left overnight at 4°C. The mixtures were then centrifuged at 4,000 *g* for 15 min. The activity in the supernate was determined by the standard assay described above. For immunotitration of the immunoreactive PBG-deaminase in erythrocyte lysates from normal and AIP heterozygotes, the lysate activities were equalized and then either 50- or 25-µl aliquots were titrated against the antibody as described above.

Quantitation of CRIM in normal controls and individuals with AIP

Sample preparation. Erythrocytes from normal individuals and heterozygotes for AIP were lysed by the addition of 3 vol of 1.0 mM sodium phosphate buffer, pH 7.6,

containing 1 mM DL-dithiothreitol, 1 mM $MgCl_2$, and 0.05% Triton X-100. The lysate was centrifuged at 30,000 g for 20 min and the supernate was removed and used for assays of protein and PBG-deaminase activity. Samples were diluted and then reassayed to ensure that all lysates contained equal enzymatic activity before rocket immunoelectrophoresis.

Rocket immunoelectrophoresis. The rocket immunoelectrophoretic system for human PBG-deaminase was developed as a modification of standard methods (30). A 1% agarose solution was prepared in 0.06 M Tris-barbital buffer, pH 8.8, and 6.0 ml was poured into a 5×7.5 -cm plate in the center of which a $0.4 \times 2.5 \times 7$ -cm double thickness glass microscope slide had been placed. After the agarose had gelled, the slide was removed and 4.0 ml of a 1% agarose solution containing 15 μ l of rabbit anti-human PBG-deaminase was poured into the center area. The agarose had been cooled to 60–65°C before antibody was added.

For routine immunoelectrophoresis of erythrocyte lysates, 11 2-mm wells were cut into the lower portion of the antibody-containing gel and 7- μ l samples were added to each well. Cotton wicks were used to make contact between the gel and the 0.06 M Tris-barbital electrophoresis buffer. The plate was electrophoresed for 4 h at a constant current of 30 mA. Following electrophoresis, the gel was thoroughly washed in 0.9% NaCl for 10–12 h with several changes and then overlaid with 300 μ l of sheep anti-rabbit peroxidase-conjugated IgG that previously had been diluted 1:2 with 0.9% NaCl. The plate was incubated at room temperature in a moist chamber to prevent drying. The gel was again washed with 0.9% NaCl for at least 8 h and then stained for peroxidase. The staining solution was 25 mg diamino-benzidine tetrahydrochloride dissolved in 50 ml 0.1 M Tris/HCl buffer, pH 7.6, containing 0.15 ml of 3% hydrogen peroxide. After the gel and staining solution were allowed to react for 20 min at room temperature, the gel was photographed and stored in 0.9% NaCl. The peroxidase reaction mixture stained the gel brown; rockets appeared white against the brown background. Crossed immunoelectrophoretic gels were stained with Coomassie Brilliant Blue R-250 and destained as previously described (31).

RESULTS

PBG-deaminase enzyme-substrate intermediates in erythrocytes. Fig. 2 shows representative DEAE-cellulose chromatographic elution profiles of the PBG-deaminase enzyme-substrate intermediates in erythrocyte lysates from normal individuals and heterozygotes for AIP. In 12 normal individuals the activity of the PBG-deaminase A and B forms always contained >75% of total recovered activity (Fig. 2A). Occasionally, the C intermediate partially separated into two overlapping peaks. Two different elution patterns were observed in AIP heterozygotes (Fig. 2B and C). Most heterozygotes for AIP ($n = 10$) had profiles similar to normal individuals (compare 2A and B); however the activities of the A, B, and C intermediates were each reduced by ~50%. The low activities in the D and E peaks did not permit comparison. All heterozygotes with this pattern were clinically asymptomatic and had urinary PBG levels <5 μ g/ml. In contrast, eight heterozygotes who were experiencing acute attacks of the disease and/or had elevated urinary

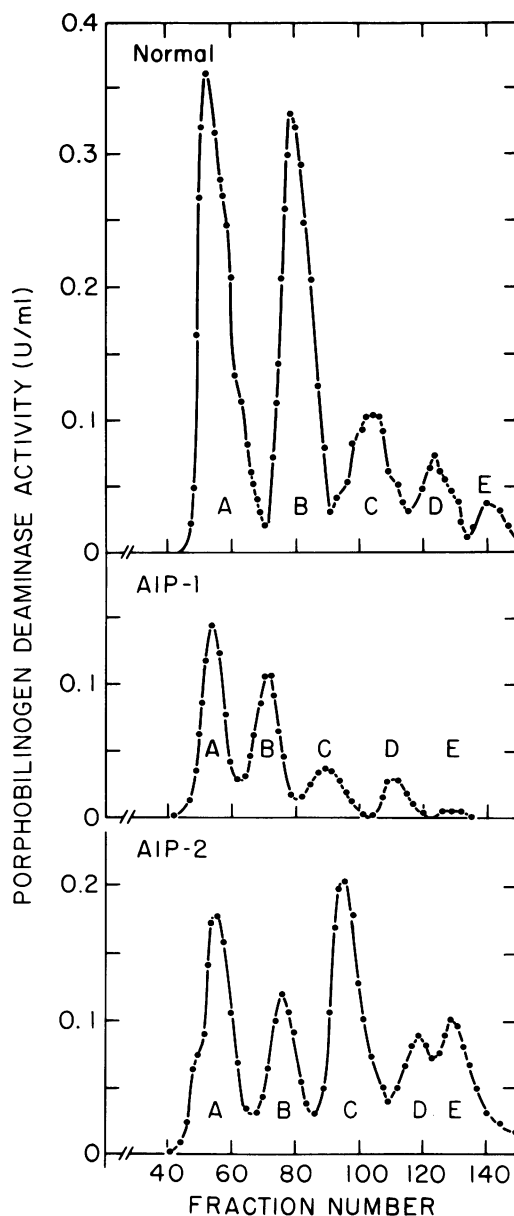


FIGURE 2 DEAE-cellulose chromatographic profiles of PBG-deaminase from normal and AIP erythrocytes. Lysates (2.0 ml) were applied to a 0.9×30 -cm column and PBG-deaminase enzyme-substrate intermediates (A–E) were eluted using a linear 0–0.11 M NaCl gradient as described in Methods. Normal (top graph); AIP type 1 (AIP-1) profile (middle graph), observed in patients in remission when urinary PBG levels were <5 μ g/ml; AIP type 2 (AIP-2) profile (bottom graph), observed during clinical exacerbations and/or when urinary PBG levels were >5 μ g/ml.

PBG levels (>5 μ g/ml) had a different pattern of enzyme-substrate intermediates (Fig. 2C). In these heterozygotes, there was a disproportionate increase in the activity of the C intermediate, particularly when com-

pared with the A and B activities. In two heterozygotes, erythrocyte samples were obtained during an acute attack and subsequently during remission. Both heterozygotes had type 2 profiles (Fig. 2C) during the acute attack and type 1 elution patterns (Fig. 2B) in remission; these profiles also correlated with the level of urinary PBG excretion.

Characterization of anti-PBG-deaminase. Anti-human PBG-deaminase was raised in New Zealand rabbits immunized with the homogeneous A intermediate. The titer of immune sera that resulted in 50% precipitation of purified enzyme was ~1:150. This immune sera was ammonium sulfate precipitated at 40% saturation to remove hemoglobin and albumin (29). After resuspension and dialysis, the anti-PBG-deaminase IgG preparation had a titer of ~1:80. With increasing antibody concentration, PBG-deaminase activity was depleted from the supernate and recovered in the pellet. This antibody preparation was shown to be specific for purified human PBG-deaminase by Ouchterlony double immunodiffusion and competitive immunoprecipitation studies. Immunodiffusion showed a single arc of identity between the homogeneous enzyme and erythrocyte lysates when

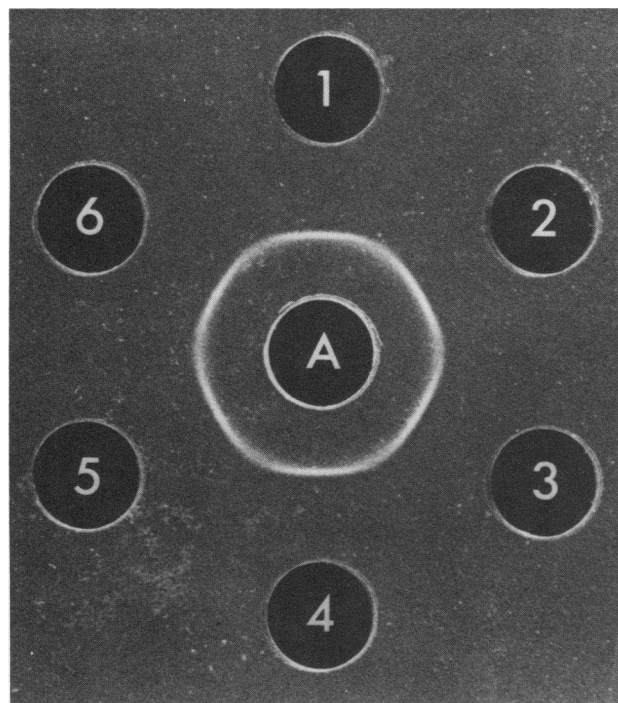


FIGURE 3 Ouchterlony double immunodiffusion demonstrating a precipitin band between rabbit anti-human PBG-deaminase (center well) and purified PBG-deaminase (wells 1, 3, and 5) and erythrocyte lysates from three normal individuals (wells 2, 4, and 6). The precipitin line and PBG-deaminase activity band (not shown) were identical.

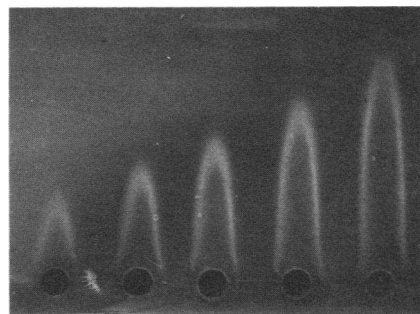


FIGURE 4 Rocket immunoelectrophoresis of PBG-deaminase. Application of homogeneous PBG-deaminase (3–5 ng) in 3-ng increments resulted in progressive increases in rocket peak height. Increasing activity in erythrocyte lysates also gave proportionate increases in peak height (not shown). See Methods for details.

stained for protein and activity using PBG as substrate (Fig. 3).

Evaluation of CRIM in AIP families. A rocket immunoelectrophoretic system was used to quantitate immunoreactive PBG-deaminase protein. As shown in Fig. 4, 3–15 ng of homogeneous enzyme, in 3-ng increments, gave proportionate increases in rocket peak height. Similarly, increasing activity in erythrocyte lysates resulted in proportionate increments. Equal activities of each PBG-deaminase enzyme-substrate

TABLE I
Immunologic Characterization of Defective PBG
Deaminase Activity in AIP Families

Family ancestry*	Number of families	Number of patients	Nonfunctional cross-reacting immunologic material	
			CRIM-positive	CRIM-negative
Basque	1	7	X	
Black, American	3	3		X
English	1	1		X
English/Polish	1	1		X
English/German	1	2		X
German	1	1		X
German/Dutch	1	1		X
Hungarian	1	1		X
Irish	1	1		X
Irish/Scottish	2	3		X
Italian	2	3		X
Norwegian	1	2		X
Polish	3	3		X
Swedish	2	2		X
Swiss	1	1		X
TOTAL	22	32	1	21

* Both parents were from the same ethnic group or country unless otherwise indicated.

intermediate isolated from normal erythrocytes resulted in essentially identical rocket heights. The presence or addition of PBG did not affect peak height.

Table I summarizes the CRIM status in erythrocyte lysates from 32 AIP heterozygotes from 22 unrelated families representing at least 10 different ethnic backgrounds. When equal amounts of erythrocyte lysate activity from normal individuals and 25 heterozygotes for AIP from 21 unrelated families were immunoelectrophoresed, there were no significant differences in the peak heights of immuno-reactive PBG-deaminase (Fig. 5). For each normal individual and each heterozygote from these 21 AIP families, the amount of activity applied corresponded to the amount of CRIM detected. Thus, the mutations in the AIP heterozygotes from these families were designated as CRIM negative. However, in a family of Basque ancestry, the amount of CRIM was consistently greater in all seven AIP heterozygotes studied (Fig. 6). These findings demonstrated the presence of noncatalytic, immunologically cross-reactive material (CRIM-positive) in this AIP family of Basque ancestry.

Consistent with these results, competitive immunotitration studies (Fig. 7) revealed the presence of noncatalytic PBG-deaminase protein in the AIP heterozygotes from the Basque family (Fig. 7A) and the absence of noncatalytic CRIM in the other AIP families studied (Fig. 7B). When samples containing equal activities of homogeneous PBG-deaminase or PBG-deaminase in erythrocyte lysates from normal individuals or the CRIM-positive family were immunotitrated, the antibody dilutions required to precipitate 50% of the activity in the supernates were ~1:112, 1:112, and 1:68, respectively. A mixture containing equal activities from a CRIM-positive and a normal lysate required on antibody dilution of ~1:86 for 50% immunoprecipitation. On the basis of these findings, the estimated amount of immunoreactive protein in the CRIM-positive variant was 1.65-fold that in normal

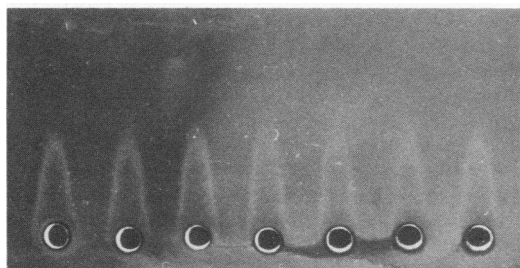


FIGURE 5 Rocket immunoelectrophoresis of PBG-deaminase in erythrocytes from CRIM-negative AIP heterozygotes and normal individuals. Equal enzymatic activities were applied to the gel. No differences in rocket peak heights of immunoreactive enzyme were observed for equal activities of erythrocyte PBG-deaminase from normals (odd channels) and the (CRIM-negative) AIP heterozygotes (even channels).

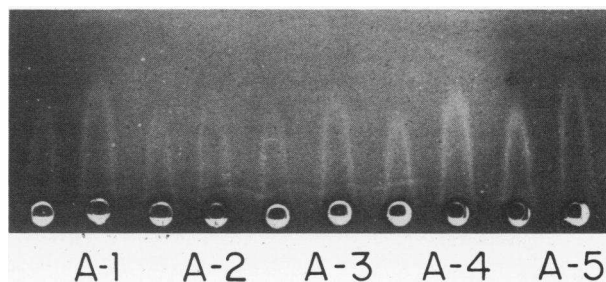


FIGURE 6 Rocket immunoelectrophoresis of PBG-deaminase in erythrocytes from CRIM-positive AIP heterozygotes. Equal enzymatic activity in erythrocyte lysates from heterozygotes of an AIP kindred of Basque ancestry (A1–A5, even lanes) and normal individuals (odd lanes) were applied. The rocket peak heights of immunoreactive enzyme from each AIP heterozygote was increased when compared with those in lysates from normal individuals or unaffected members of this kindred (not shown).

lysates, assuming identical antibody avidities. In contrast, when equal amounts of erythrocyte lysate activity from a CRIM-negative AIP heterozygote and a normal individual as well as purified enzyme were immunotitrated, essentially the same antibody dilution was required to precipitate 50% of activity from each (Fig. 7B), which indicates a direct correspondence between activity and immunotitratable enzyme protein.

Characterization of the noncatalytic PBG-deaminase in the CRIM-positive family. The K_m and stability of the PBG-deaminase activity in erythrocyte lysates from the CRIM-positive AIP heterozygotes were compared with those of CRIM-negative AIP heterozygotes and normal individuals. The K_m values in all three sources were essentially the same (~10 μ M). However, the immunoreactive PBG-deaminase in the CRIM-positive heterozygotes remained stable after heating the lysate for 30 min at 60°C, whereas the immunoreactive enzyme protein in normal and CRIM-negative AIP lysates was in each case decreased ~15% after 30 min of heat treatment. Agarose isoelectric focusing demonstrated no significant differences in the isoelectric points of homogeneous human PBG-deaminase and PBG-deaminase in erythrocyte lysates from normal, CRIM-positive, or CRIM-negative individuals. Routinely, five bands of enzymatic activity were visualized with pI of ~6.4, 6.1, 5.8, 5.5, and 5.3. When samples were subjected to isoelectric focusing in the first dimension and then to crossed-immunoelectrophoresis in the second dimension, no evidence for a mutant protein with different isoelectric properties was obtained; a precipitin line of identity, which corresponded to each of the five PBG-deaminase intermediates, was observed in lysates from normal individuals and CRIM-negative and CRIM-positive AIP heterozygotes (Fig. 8; CRIM-negative AIP lysate not shown). Lysates from the CRIM-positive AIP hetero-

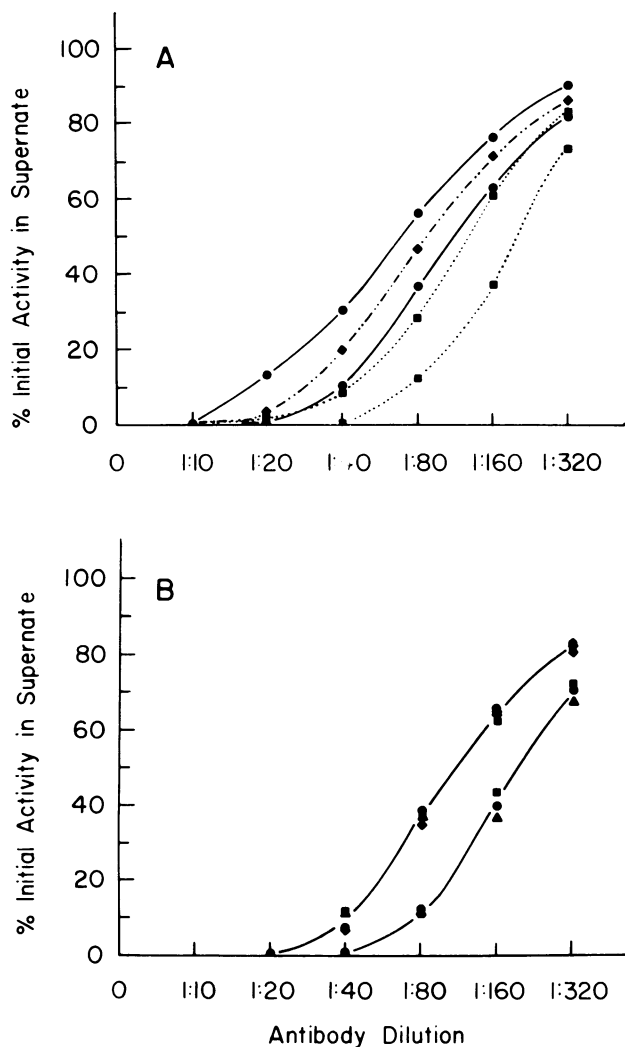


FIGURE 7 Immunotitration of PBG-deaminase with rabbit anti-human PBG-deaminase IgG. Homogeneous normal human PBG-deaminase and enzymatic activity in erythrocyte lysates from a normal individual, a CRIM-positive AIP heterozygote, and a CRIM-negative AIP heterozygote were assayed and adjusted for equivalent enzymatic activity (and protein concentration with bovine serum albumin) as described in Methods. (A) Immunotitration curves are shown for 50- μ l (left) and 25- μ l (right) aliquots of homogeneous normal enzyme (■), a CRIM-positive AIP lysate (●) and a mixture (25 μ l:25 μ l) of homogeneous enzyme and CRIM-positive AIP lysate (◆). Note that antibody dilutions of ~1:68, 1:84, and 1:112 were observed for 50% immunotitration of 50 μ l of the CRIM-positive AIP lysate, the mixture of CRIM-positive lysate and homogeneous enzyme, and 50 μ l of homogeneous enzyme, respectively. (B) Immunotitration curves for 50 μ l (left) and 25 μ l (right) aliquots of homogeneous enzyme (■) and erythrocyte lysates of normal individuals (▲) and CRIM-negative AIP families (●), and a mixture (25 μ l:25 μ l) of homogeneous enzyme and CRIM-negative AIP heterozygote (◆). Note that the curves for 50 μ l of homogeneous enzyme, normal lysate, CRIM-negative AIP lysate, and the mixture of homogeneous enzyme and CRIM-negative AIP lysate were essentially identical.

zygotes, however, appeared to have increased amounts of the immunoreactive PBG-deaminase B intermediate when compared with those observed for an equal amount of normal lysate activity (Fig. 8).

To further estimate the amount of PBG-deaminase protein present in the CRIM-positive heterozygotes, proportionate increments of normal PBG-deaminase activity were compared with a constant amount of activity from a CRIM-positive heterozygote by rocket immunoelectrophoresis. As shown in Fig. 9, the amount of CRIM in this heterozygote corresponded to ~1.6 U of normal immunoreactive protein, which gives a CRIM-activity ratio of 1.6 compared with 1.0 in normal lysates.

To analyze the nature of the enzymatic defect in the CRIM-positive family, the enzyme-substrate intermediates were isolated from erythrocyte lysates of a normal individual and a CRIM-positive heterozygote by DEAE-cellulose chromatography and equal activities of the A, B, C, and D forms were applied to the immunoelectrophoretic gels. As shown in Fig. 10, each of the intermediates from the CRIM-positive heterozygote had elevated levels of immunologically cross-reacting material when compared with an equal amount of activity of the respective normal intermediate, the B form being present in the greatest amount.

DISCUSSION

Recognition that PBG-deaminase catalyzes the formation of uroporphyrinogen I via stable enzyme-substrate intermediates (Fig. 1) (27) suggested that the characterization of these forms might provide insight into the molecular nature of the dominantly inherited enzymatic defect in AIP. Therefore, the PBG-deaminase intermediates in erythrocyte lysates from heterozygotes for AIP and normal individuals were biochemically and immunologically characterized. Following DEAE-cellulose chromatography of erythrocyte lysates, the activity pattern of the enzyme-substrate intermediates for most AIP heterozygotes was similar to that observed in normal individuals. When, however, lysates from the same number of erythrocytes were chromatographed, the activity of each intermediate was approximately half that of the corresponding normal intermediate (type 1 pattern), consistent with expression of only the normal allele. In AIP heterozygotes who had high urinary and circulating concentrations of PBG (e.g., during an acute attack), the dipyrrole intermediate was disproportionately increased (type 2 pattern), which suggests that this intermediate was either the most stable or the rate-limiting step in the conversion of PBG to uroporphyrinogen I, at least in erythrocytes. When the pattern of erythrocyte intermediates was studied before, during, and after an

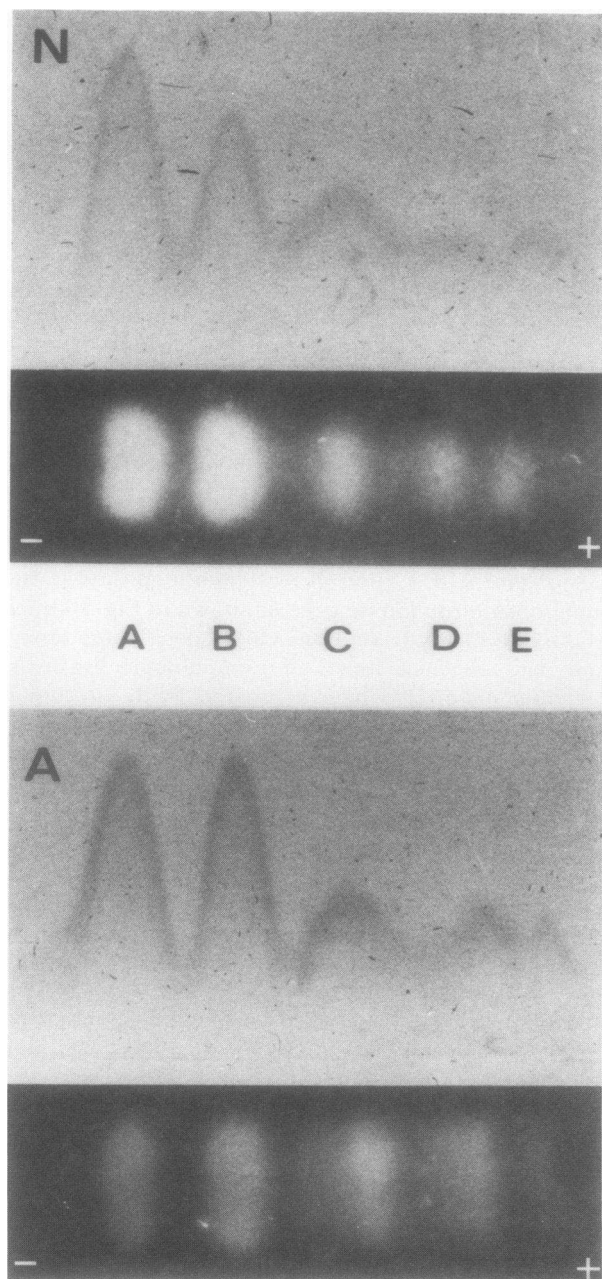


FIGURE 8 Isoelectric focusing and crossed-immunoelectrophoresis of PBG-deaminase in erythrocyte lysates from normal individuals and CRIM-positive AIP heterozygotes. Isoelectric focusing revealed five bands when stained for activity in normal, CRIM-positive, and CRIM-negative (not shown) lysates. No significant differences in the pI values for each band in the different lysates were observed. Following crossed-immunoelectrophoresis of the focused lysate proteins, only immunoreactive proteins corresponding to the activity bands were observed in the normal (N), CRIM-positive (A), or CRIM-negative (not shown) AIP heterozygotes. See Methods for details.

acute attack in the same heterozygote, the type 2 pattern (increased C intermediate) occurred during the attack concomitant with high urinary PBG levels. This would suggest that the induction of hepatic δ -aminolevulinic synthase during an acute attack resulted in the increased production of δ -aminolevulinic acid and PBG (32) that gained access to the erythrocyte where the PBG became bound to the PBG-deaminase (33). Thus, the patterns of the PBG-deaminase intermediates in AIP heterozygotes were consistent with the expression of the normal allele and were dependent, in part, on the substrate concentration in erythrocytes.

Immunologic characterization of the enzymatic defect revealed genetic heterogeneity in AIP. Monospecific rabbit anti-human PBG-deaminase was produced for the first time, permitting immunotitration and immunoelectrophoretic studies of the enzyme. Erythrocytes proved to be the enzyme source of choice; the use of cultured lymphocytes or cultured fibroblasts was precluded since 1 ml of packed cultured cells had ~0.5 and 0.18%, respectively, of the total activity in 1 ml of packed normal erythrocytes. When the amount of CRIM was determined by rocket immunoelectrophoresis, erythrocyte lysates from heterozygous members of 21 unrelated AIP families were found to have CRIM-activity ratios of 1.0, i.e., the ratio of immunoreactive protein (estimated by rocket peak height) corresponded to that observed for an equal amount of enzymatic activity from normal lysates (Fig. 5). Similarly, when equal amounts of PBG-deaminase activity in erythrocytes from these AIP heterozygotes and normal individuals were immunotitrated against the monospecific antibody, the 50% immunotitration values were essentially identical, which indicates the absence of noncatalytic immunoreactive protein in the erythrocytes from the heterozygotes in these families (Fig. 7). Thus, the mutation in these 21 unrelated AIP families was designated as CRIM negative.

In contrast, when erythrocyte lysates from all seven heterozygous members (whose clinical manifestations were indistinguishable from the above CRIM-negative heterozygotes) of an AIP kindred of Basque ancestry were subjected to rocket immunoelectrophoresis (Fig. 6) and immunotitration (Fig. 7), noncatalytic immunoreactive protein was observed. The CRIM-activity ratio of PBG-deaminase in the CRIM-positive AIP heterozygotes, as determined by immunoelectrophoresis and immunotitration, were both ~1.6, compared with 1.0 obtained for normal or CRIM-negative AIP heterozygotes. Isoelectric focusing followed by crossed-immunoelectrophoresis of the focused proteins did not reveal differences in the pI values or immunoelectrophoretic profiles of the PBG-deaminase

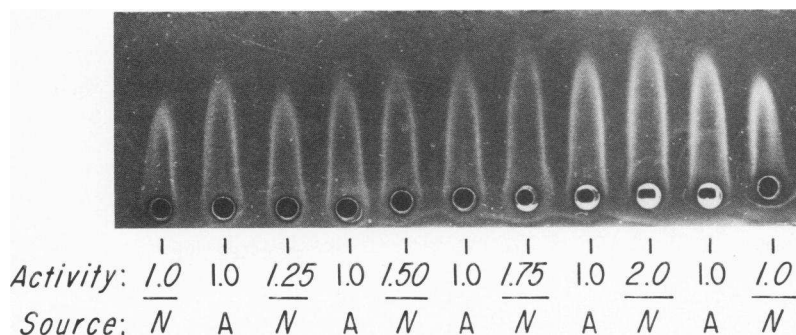


FIGURE 9 Estimation of the CRIM-activity ratio (CRIM/enzymatic activity) in a CRIM-positive AIP heterozygote. Every other channel contained increments of normal (N) lysate activity (1.0, 1.25 . . . and 2.0 U). When compared with a constant amount of activity (1.0 U) from the CRIM-positive AIP heterozygote (A), the amount of immunoreactive PBG-deaminase in the AIP heterozygote was about equal to 1.6 units of normal activity. Thus, the ratio of CRIM to specific activity for the CRIM-positive heterozygote was ~ 1.6 . See Methods for details.

intermediates in lysates from normal individuals or from CRIM-negative or CRIM-positive AIP heterozygotes. Thus, immunoreactive protein was only detected in the regions corresponding to the activity bands for the A, B, C, and D intermediates, which were resolved by isoelectric focusing (Fig. 8). In addition, the immunoreactive PBG-deaminase from the CRIM-positive AIP heterozygotes was thermal stable (60°C for 30 min). These findings suggested that the nonfunctional, immunoreactive protein occurred as stable substrate-bound intermediates that did not have detectably altered isoelectric points. If the nonfunctional immunoreactive protein had an altered charge (e.g., owing to an amino acid substitution), an additional peak(s) of immunoreactive protein should be observed after isoelectric focusing followed by crossed immunoelectrophoresis.

The fact that the noncatalytic, immunoreactive protein focused with the normal catalytic PBG-deaminase intermediates permitted the consideration of several different models for the further characterization of the nature of the enzymatic defect in the CRIM-positive AIP heterozygotes in light of several hypothetical models. Fig. 11 illustrates several different types of mutations that might be revealed by characterizing the CRIM status of the PBG-deaminase intermediates in the CRIM-positive heterozygotes. On the assumption that the normal enzyme monomer (A) has two binding sites for PBG and an active site (34), the stepwise formation of the mono-, di-, tri-, and tetrapyrroles by the enzyme-substrate intermediates, B through E, can be depicted as shown. If substrate binding site-1 was defective, no PBG could bind and only the free enzyme or A intermediate would accumulate. If the second substrate binding was defective, the CRIM-positive B intermediate would accumulate, presumably in an amount greater than the

A form. A defective catalytic site would result in the accumulation of the first three intermediates, presumably the amount of immunoreactive $C > B$, and $B > A$. If a mutation resulted in altered binding and/or catalysis, a kinetic mutation would result (markedly higher K_m), and all of the enzyme-substrate intermediates would accumulate; the amount of immunoreactive B or C intermediate would depend on whether the binding or catalytic site was more defective. Finally, if a mutation altered the enzyme such that the hydroxymethylbilane could not be released, all forms might accumulate, presumably the E intermediate in the greatest amount. To discriminate among these possible mutations, erythrocyte A, B, C, and D intermediates from the CRIM-positive AIP heterozygote were separated and immunologically quantified (Fig. 10). Each of the intermediates from the CRIM-positive AIP heterozygotes had elevated

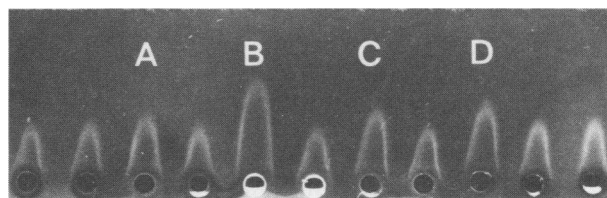


FIGURE 10 Rocket immunoelectrophoresis of PBG-deaminase intermediates. Equal activities of each intermediate, isolated by DEAE-cellulose chromatography from normal and CRIM-positive AIP erythrocytes, were immunoelectrophoresed. From left to right: lanes 1, 2, 4, 6, 8, 10, and 11, normal erythrocyte PBG-deaminase intermediates A, A, B, C, D, A, A, respectively; lanes 3, 5, 7, and 9, PBG-deaminase A, B, C, and D, respectively, from AIP. Note that each intermediate from the CRIM-positive AIP heterozygote had an increased amount of immunoreactive material, the greatest amount in the B or monopyrrole-enzyme intermediate.

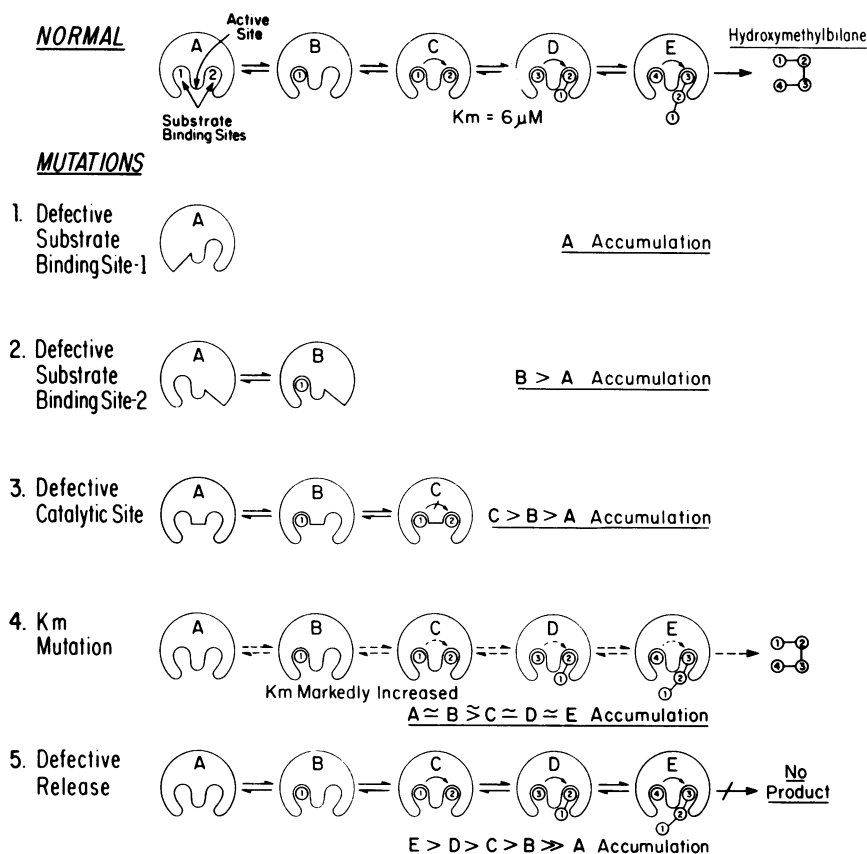


FIGURE 11 Possible alterations in PBG-deaminase resulting from different point mutations in the structural gene. The normal enzyme (1) is assumed to have one active site and two substrate binding sites; the stepwise formation of the mono-, di-, tri-, and tetrapyrrole-enzyme intermediates (B, C, D, and E, respectively) is shown. Different mutations altering substrate binding (1 and 2), the catalytic site (3), the K_m (4) or product release (5), and the expected formation and/or accumulation of the enzyme intermediates for each are depicted. See Discussion for details.

levels of immunologically cross-reacting material when compared with an equal amount of activity for the respective normal intermediate. Notably, the immunoreactive B form was present in the greatest amount. These findings suggested that the structural gene defect in this family best fits the model for a K_m mutation with a markedly altered, but functional, active site and an altered substrate binding site-2.

An unexpected result was the fact that in 21 of the 22 unrelated AIP families, the PBG-deaminase mutation was CRIM negative. Several possible explanations may be advanced to account for these findings. First, the mutation(s) in these families may have altered the enzyme conformation such that its antigenic site or sites was not recognized by our anti-PBG-deaminase. It is, however, unlikely that a rabbit polyvalent antibody preparation (35, 36) would not recognize many, if not most, structurally altered proteins analogous to the experience with other enzyme

deficiency disorders (37). A more likely explanation would be a point mutation that resulted in a markedly unstable PBG-deaminase that would be rapidly degraded. Preliminary attempts to identify a highly unstable enzyme included studies of reticulocyte-enriched preparations and cultured lymphocytes and fibroblasts. Peripheral erythrocytes from two unrelated CRIM-negative AIP heterozygotes and two normal individuals were separated into age cohorts by discontinuous density gradient centrifugation (38). Although the reticulocyte-rich fractions contained increased PBG-deaminase activity when compared with unfractionated circulating erythrocytes, the CRIM-activity ratio for the CRIM-negative AIP and normal reticulocytes were both essentially 1.0. In addition, rocket immunoelectrophoretic studies of cultured lymphocytes and fibroblasts (10^9 cells/sample) from CRIM-negative AIP heterozygotes and normal individuals did not reveal the presence of nonfunc-

tional immunoreactive material, whereas cultured cells from a CRIM-positive AIP heterozygote had non-catalytic immunoreactive enzyme. Further investigation of the molecular nature of the CRIM-negative mutations may require the use of recombinant DNA techniques to obtain appropriate DNA probes to evaluate the possibility that the genetic defects in these AIP kindreds results from chain-terminating defects, mRNA processing defects, or partial or complete gene deletions analogous to those recently identified in the α - and β -thalassemias (39–43).

In summary, these investigations have, for the first time, identified molecular genetic heterogeneity in AIP. The finding of stable enzyme-substrate intermediates for PBG-deaminase is unique among human enzymes, especially for a monomeric protein whose function is necessary for the production of such metabolically essential human molecules as the hemoglobins, cytochromes, and cobalamins. The finding of a genetically defective PBG-deaminase which can be detected immunologically, both as the free protein and as substrate-bound intermediates, provides the first molecular evidence for a structural gene mutation as the primary defect in AIP. Further investigation of the enzymatic defect in other AIP kindreds, using the immunologic techniques reported here, should provide additional insight into the molecular pathology and genetic heterogeneity of this dominantly inherited disorder of human heme biosynthesis.

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