Decreased Red Cell Uroporphyrinogen I Synthetase Activity in Intermittent Acute Porphyria

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ABSTRACT Intermittent acute porphyria has recently been distinguished biochemically from other genetic hepatic porphyrias by the observation of diminished hepatic uroporphyrinogen I synthetase activity and increased δ-aminolevulinic acid synthetase activity. Since deficient uroporphyrinogen I synthetase may be reflected in nonhepatic tissues, we have assayed this enzyme in red cell hemolysates from nonporphyric subjects and from patients with genetic hepatic porphyria. Only patients with intermittent acute porphyria had decreased erythrocyte uroporphyrinogen I synthetase activity which was approximately 50% of normal. The apparent K_m of partially purified uroporphyrinogen I synthetase was 6×10^{-6} M in both nonporphyrics and patients with intermittent acute porphyria. These data provide further evidence for a primary mutation affecting uroporphyrinogen I synthetase in intermittent acute porphyria. Furthermore, results of assay of red cell uroporphyrinogen I synthetase activity in a large family with intermittent acute porphyria suggest that this test may be a reliable indicator of the heterozygous state.

INTRODUCTION

Intermittent acute porphyria (IAP)[1] is a genetic disorder of heme and porphyrin biosynthesis. It is inherited as an autosomal dominant and is manifested chemically by excessive excretion of the porphyrin precursors, δ-aminolevulinic acid (ALA) and porphobilinogen (PBG). On the basis of clinical features and a unique pattern of excessive excretion of porphyrins and porphyrin precursors, IAP has been distinguished from two related genetic hepatic porphyrias, variegate porphyria and hereditary coproporphyria. In all three of these porphyrias the liver is the primary site of overproduction of porphyrin precursors as a consequence of increased activity of δ-aminolevulinic acid synthetase (ALA-synthetase) (1–8), the first and rate-limiting enzyme in heme biosynthesis (9–11). IAP is also associated with decreased hepatic uroporphyrinogen I synthetase (URO-I-synthetase) activity in contrast to variegate porphyria (7, 12). URO-I-synthetase catalyzes the conversion of PBG to the tetrapyrrole uroporphyrinogen (13). A partial defect in heme synthesis at this site in IAP is therefore entirely consistent with the excessive excretion of ALA and PBG out of proportion to other intermediates in the heme biosynthetic pathway. Moreover, to the extent that heme functions as a repressor ALA-synthetase (7, 10, 14–16) a primary partial defect in heme synthesis could result in a secondary, reciprocal induction of ALA-synthetase.

All mammalian cells may be presumed to have the enzymes necessary for heme biosynthesis. Therefore, if a decrease in URO-I-synthetase characterizes IAP, a relative decrease in this enzyme might be expected in tissues other than liver. Although Heilmeyer and Clotten observed a decreased hepatic production of porphyrins from ALA in a patient with IAP, they did not report such a defect in red cells (17). However, in this report we present data that URO-I-synthetase is diminished in red cells of patients with IAP.

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[1] *Abbreviations used in this paper:* ALA, δ-aminolevulinic acid; IAP, intermittent acute porphyria; PBG, porphobilinogen; URO-I-synthetase, uroporphyrinogen I synthetase.

METHODS

Preparation of red cells for URO-I-synthetase assay. 20 ml of venous blood was collected, anticoagulated with heparin and centrifuged at 1000 $g \times 10$ min at 4°C. Plasma was discarded, and the white cell layer was gently removed with a pasteur pipette. Erythrocytes were washed twice with ice-cold 0.89% NaCl followed by centrifugation at 1000 $g \times 10$ min. The packed cells were then rapidly freeze-thawed three times in acetone-dry ice and diluted 1:20 (v:v) with Tris-HCl 50 mM, pH 8.0. Leukocytes were isolated by the method reported by Rosenberg, Lilleqvist, and Hsai (18).

URO-I-synthetase assay. Conversion of PBG to porphyrins was quantitated by porphyrin fluorescence after incubation of homogenate containing 10^{-4} M PBG, Tris-HCl 50 mM, and 1:60 red cell hemolysate in a total volume of 0.15 ml at pH 8 aerobically in the dark for 1 hr at 37°C. The reaction was stopped, and porphyrins were extracted and oxidized with an equal volume of 2 N perchloric acid-ethanol (1:1) in the cold. Samples were centrifuged at 2000 $g \times 10$ min at 4°C. Supernatant fluorescence was compared to a standard coproporphyrin solution at excitation and emission wave lengths of 405 and 595 nm respectively with an Aminco-Bowman spectrophotofluorometer, using a high intensity light source. The fluorescence excitation spectra of the porphyrins formed enzymatically was routinely scanned and found to be characteristic of the extracted porphyrins. Nonenzymatic PBG conversion to porphyrin was determined with hemolysates in which the enzyme had been inactivated by boiling for 20 min. Under the conditions of assay the ratio of enzymatic to nonenzymatic porphyrin formation was greater than 10:1. Recovery of porphyrin, determined fluorometrically by addition of a known amount of uroporphyrin I to duplicate incubations on each subject, was 80–85%. All values were corrected for uroporphyrin recovery and for relative intensity of uroporphyrin fluorescence (19), since more than 95% of the porphyrin formed was uroporphyrin. More than 90% of the hemolysate porphyrin was recovered with the first perchloric acid-ethanol extraction. Product porphyrin was formed linearly with respect to time up to 3 hr with a 1:60 dilution of hemolysate. When the red cells were diluted less than 1:30, heme quenching of porphyrin fluorescence became significant. Porphyrin yield was not increased by anaerobic incubation, the presence of metal chelators such as EDTA or bathophenanthroline, or by addition of glutathione. Substrate inhibition was not produced with 10^{-3} M PBG. 10^{-4} M PBG was at least five-fold the concentration required to produce maximum velocity of the reaction in samples with normal activity. All assays were done in triplicate on at least two different days as well as on repeat blood samples from several subjects. Results were reproducible within 5%. At −45°C the enzyme activity was stable in the freeze-thawed red cells for several days. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (20) using human crystalline albumin as standard.

Chromatography. Thin-layer chromatography of the methyl esters of the enzymatically generated porphyrins was performed by the method of Doss (21, 22). For these experiments a 20-fold increase in the volume of the reactants was used. At the end of the assay the incubation mixture was rapidly frozen and lyophilized to dryness, and the porphyrins were esterified in methanol-sulfuric acid (95:5 v:v) for 12 hr at room temperature, before being extracted with chloroform. After flash evaporation, the porphyrin esters were taken up in a small volume of chloroform and streaked on cleaned silica gel H plates (Riedel-DeHaën AG, Seelze, Hannover, Germany). The plates were first developed in petroleum ether:diethyl ether (4:1) to remove lipids and then in chloroform:benzene:methanol (85:13.5:1.5). Esterified hemin remained at the baseline while the porphyrins with from eight to two methylated carboxyl groups were clearly separated with increasing R_f's as the number of carboxyl groups decreased. Porphyrins were observed under a fluorescent lamp and further identified spectrophotometrically after chloroform elution of the porphyrin bands from silica gel using a millimolar extinction coefficient of 216 at 405.5 nM.

Partial purification of URO-I-synthetase from red cells. 2 ml of freeze-thawed red cells were diluted to 4 ml with cold distilled water and heated for 15 min at 65°C. After centrifugation at 2500 $g \times 10$ min, 0.5 ml of the supernatant was passed through a Sephadex G-25 medium column (0.5 × 5 cm) and the enzyme fraction collected. Hemoglobin was then removed by the method of Hennessey, Waltersdorph, Huennekens, and Gabrio (23, 24). Columns of DEAE-cellulose (Whatman DE-52, 0.5 × 2 cm) were equilibrated with 0.003 M phosphate buffer, pH 7.4 before adding the enzyme solution and were then washed with 15 ml of the same phosphate buffer. Resin was removed from the columns and the enzyme desorbed by repeated washings with 0.134 M phosphate buffer, pH 7.4. The washings were centrifuged at 5000 $g \times 10$ min and the supernatant assayed for URO-I-synthetase activity. These procedures resulted in a 20-fold increase in specific activity and 80–85% recovery of total activity.

Porphyrins in urine and stool were quantitated fluorometrically after solvent phase partition (19). Urinary porphyrin precursors were determined spectrophotometrically (25).

Statistical methods of Ostle were used (26).

Sources of drugs and chemicals. PBG was synthesized (27) by Dr. G. Kohan and purchased from Protex Research and Development, Registered, Montreal, Canada, and was recrystallized from hydrochloric acid just before use. Purity was verified by UV absorption, reaction with Ehrlich's reagent, and melting point (28). Porphyrins and their methyl esters were purchased from Dr. T. K. With, Copenhagen, Denmark. Other chemicals and reagents were purchased from J. T. Baker Chemical Co., Phillipsburg, N. J., or Sigma Chemical Co., St. Louis, Mo.

RESULTS

Validation of URO-I-synthetase assay. Several studies were done to validate the method of determining URO-I-synthetase activity. To remove small potentially fluorescing molecules, the hemolysates were passed over Sephadex G-25 columns (see Methods). All of the enzyme activity was recovered with the same specific activity and fluorescence excitation spectrum as the crude hemolysate. Isolated leukocytes which had been freeze-thawed three times and diluted 1:60 had no detectable URO-I-synthetase activity. PBG conversion to uroporphyrinogen III is catalyzed by both heat stable URO-I-synthetase and heat labile uroporphyrinogen III cosynthetase (13, 29–31). However, the cosynthetase is normally present in excess so that URO-I-synthetase is rate limiting (30). When the hemolysates were heated at 65°C for

TABLE I

Summary of Clinical and Laboratory Data on Subjects Studied

Subjects	Urine				Stool		Hemoglobin	Reticulocyte	URO-I-synthetase
	ALA	PBG	URO	COPRO	COPRO	PROTO			
	mg/g creatinine		*mg/g creatinine*		*μg/g dry wt*		*g/100 ml*	*%*	*pmoles uroporphyrin/ mg protein per hr*
Nonporphyric controls	0.6–2.1	0.8–1.5	0.01–0.038	0.020–0.115	8–28	2–32	14.0–17.3	0.3–1.4	34 (Range 32–40)
Patients with liver disease in remission	0.4–1.8	0.5–1.2	0.01–0.042	0.023–0.220	7–34	7–45	12.1–16.2	0.7–1.5	36 (Range 30–48)
Intermittent acute porphyria									
1	13.3	79.0	0.433	0.594	67	19	12.5	0.1	20
2	11.0	63.9	0.434	0.495	25	23	13.8	0.7	20
3	19.0	87.0	0.167	0.153	6	37	12.2	1.0	16
4	9.5	52.2	0.206	0.281	62	65	13.7	0.2	17
5	—	24.3	0.720	0.902	46	92	14.0	0.4	12
6	—	10.6	0.880	0.310	40	86	13.0	0.3	14
7	8.2	38.2	0.484	0.360	19	13	12.9	1.4	15
8	6.5	34.0	0.283	0.463	58	54	14.5	0.5	21
9	5.3	13.9	0.015	0.240	18	15	13.7		15
10	5.0	12.2	0.044	0.330	21	24	15.8	0.6	13
Variegate porphyria	3.2	4.8	0.297	0.496	517	539	12.5	0.9	30
Hereditary coproporphyria									
1	—	1.4	0.170	0.703	917	43	15.5	0.2	43
2	—	13.5	0.787	4.500	5370	74	12.0	1.6	44
Range of normal values*	0–4	0–1.8	0.01–0.05	0.02–0.25	0–40	0–100	12–16	0–1.5	

COPRO, coproporphyria; PROTO, protoporphyrin.

* Normal range in our laboratory.

15 min to inactivate the cosynthetase, total enzyme activity was recovered, thus confirming the fact that the cosynthetase was not affecting the velocity of the reaction. Furthermore, these results suggested that enzymatically generated uroporphyrinogen was not being further metabolized by the next enzyme in the pathway, uroporphyrinogen decarboxylase, since this enzyme is also heat labile (32, 33).

Nearly all of the enzymatically generated porphyrins cochromatographed as uroporphyrin octamethyl ester (R_f 0.18). A very small amount of heptacarboxyl porphyrin methyl ester was formed (R_f 0.28) as well as a trace of coproporphyrin methyl ester (R_f 0.65). Hemolysate which had been heat treated for 15 min at 65°C generated the same pattern of porphyrin esters on thin-layer chromatography except that no coproporphyrin methyl ester was formed. No porphyrin was present fluorometrically or by thin-layer chromatography in the hemolysates incubated without porphobilinogen. The pattern of porphyrin formation was identical in nonporphyrics and patients with IAP. However, spectrophotometric quantitation of the uroporphyrin octamethyl ester band was much less in IAP patients compared to nonporphyrics, in amounts proportional to the differences found fluorometrically.

URO-I-synthetase assay in controls and patients with different types of porphyria. URO-I-synthetase was assayed from the erythrocytes of 21 nonporphyrics, 10 patients with IAP, one patient with variegate porphyria, and two patients with hereditary coproporphyria (Table I). Of the nonporphyrics, 11 were normal subjects and 10 were patients with hepatic disease in a stable condition. Six had recovered from alcoholic hepatitis, two had recovered from viral hepatitis, and two had active chronic hepatitis. All of the nonporphyrics had hemoglobin, red cell indices, and reticulocyte percentages within the range of normal. All nonporphyrics had urinary porphyrins and precursors as well as fecal porphyrins within the range of normal for our laboratory. Porphyric patients were classified as to type on the basis of clinical manifestations, and, more importantly, patterns of excessive porphyrin and porphyrin precursor excretion characteristic of each disease (34–36). Patients with IAP had excessive urinary excretion of ALA and PBG with slight elevations in urinary uroporphyrin and corproporphyrin and normal fecal coproporphyrin and protoporphyrin. The presence of excessive porphyrin in the urine may reflect the propensity of PBG to polymerize to uroporphyrin nonenzymatically at acid pH, room temperature, and light exposure. However, eluci-

dation of the source of excessive urinary porphyrins in IAP will require further investigation. The patient with variegate porphyria had photosensitive skin lesions as well as increased fecal coproporphyrin and protoporphyrin. Two patients with hereditary coproporphyria had marked increases in urinary and fecal coproporphyrin. All porphyric patients had normal hemograms and normal reticulocyte counts.

Fig. 1 illustrates the mean URO-I-synthetase activity for the patients studied. Patients with IAP had approximately 50% of the activity of nonporphyrics (16±SD 3.3 vs. 35±SD 5.2) which was significant at the 0.001 level. Enzyme activity in patients with variegate and hereditary coproporphyria was within the range observed in nonporphyrics. Enzyme activity on each porphyric patient is listed in Table I.

Kinetic characteristics of partially purified URO-I-synthetase. The apparent K_m of URO-I-synthetase was 6×10^{-6} M in the crude hemolysates from both nonporphyric subjects and patients with IAP, although the specific activity was reduced by about 50% in IAP patients. Similar results were obtained after 20-fold purification of the enzyme. A Lineweaver-Burke plot of one such experiment is depicted in Fig. 2. The apparent K_m was 6×10^{-6} M for both a nonporphyric subject and a patient with IAP. The specific activity was reduced by 50% in the patient with IAP compared to the nonporphyric subject.

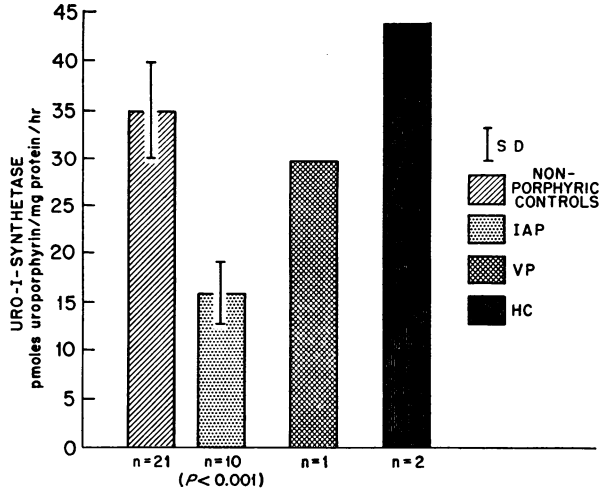

FIGURE 1 Red cell URO-I-synthetase activity in porphyric and nonporphyric subjects. The symbols are: intermittent acute porphyria (IAP), variegate porphyria (VP), and hereditary coproporphyria (HC).

TABLE II

URO-I-Synthetase Activity in a Family with Intermittent Acute Porphyria

Family member	Urinary ALA and PBG	RBC URO-I-synthetase activity
Father	N	15
Mother	N	34
Sibling		
1 F	↑	17
2 F	*	*
3 M	↑	20
4 M	N	33
5 M	N	28
6 M	↑	20
7 M	N	12
8 F	N	34
9 M	N	17
10 M	↑	15
11 F	N	33
12 M	↑	18
13 F	N	*
14 F	N	36

Siblings are listed according to birth order followed by F for female and M for male. Urinary ALA and PBG excretion is listed as N for normal or ↑ for increased. Untested siblings are depicted by an asterisk (*). URO-I-synthetase activity is expressed as picomoles uroporphyrin formed per milligram protein per hour.

URO-I-synthetase activity in a family with IAP. To further evaluate the validity of erythrocyte URO-I-synthetase activity, we studied a large family with IAP. Red cell URO-I-synthetase activity of the members of this family is shown in Table II. Enzymatic activity was determined in both parents and 12 of the 14 siblings. Neither parent had ever had any clinical symptoms suggestive of porphyria. Repeated quantitation of porphyrins and precursors in their excreta was normal. However, the mother had normal URO-I-synthetase activity while the father's was in the range of patients with IAP. Five of the 14 siblings (Nos. 1, 3, 6, 10, and 12) had IAP diagnosed by clinical features plus a typical pattern of excessive excretion of porphyrin precursors. All five had

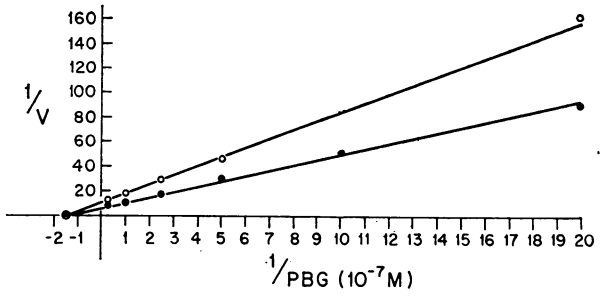

FIGURE 2 Lineweaver-Burke plot of red cell URO-I-synthetase partially purified 20-fold from a patient with intermittent acute porphyria (upper line) and a nonporphyric subject (lower line). The apparent K_m value is 6×10^{-6} M for each plot.

diminished URO-I-synthetase activity. Five siblings tested had normal URO-I-synthetase activity as well as normal porphyrin precursor excretion (Nos. 4, 5, 8, 11, and 14). Two other siblings (Nos. 7 and 9) had diminished URO-I-synthetase activity but no clinical or chemical evidence of IAP.

DISCUSSION

The present data indicate that of all subjects studied, only those with IAP had diminished red cell URO-I-synthetase activity, thus further substantiating similar results obtained in liver and distinguishing IAP from variegate porphyria and hereditary coproporphyria (7, 12). The source of excessive excretion of porphyrin precursors in IAP is primarily hepatic where deficient URO-I-synthetase is associated with a marked increase in activity of the first and rate-controlling enzyme in the heme biosynthetic pathway, ALA-synthetase. Taking into consideration the function of heme as a negative feedback regulator of ALA-synthetase, we have postulated that a primary partial defect in heme synthesis at the level of URO-I-synthetase might result in secondary derepression of hepatic ALA-synthetase and overproduction of the porphyrin precursors ALA and PBG proximal to the site of the partial block. That this mechanism is plausible is supported by the recent demonstration that experimentally produced partial defects in heme synthesis in cultured hepatocytes results in increased ALA-synthetase activity and increased ALA-synthetase induction by 2-allyl-2-isopropylacetamide (37). Although this speculative mechanism is attractive, other possibilities exist (1, 7, 12, 34).

Defective URO-I-synthetase does not necessarily imply impaired hepatic heme synthesis since the K_m of URO-I-synthetase is probably higher than the normal concentration of PBG in the liver. This is supported by the observations that PBG is not detectable in normal liver using a method which can detect about 10^{-6} M PBG and K_m of URO-I-synthetase purified from bovine liver has been reported to be 5×10^{-6} M (38). Thus, induction of ALA-synthetase may sufficiently increase PBG concentration to minimize the enzymatic defect in heme synthesis.

Although patients with IAP have a decreased peripheral red cell volume (39), they do not have an overt defect in heme synthesis in erythroid tissue. Data are not available concerning bone marrow ALA-synthetase and URO-I-synthetase activities in IAP. It is therefore not known if URO-I-synthetase activity is sufficiently depressed to impair heme synthesis and if so, whether this deficiency may be minimized by the high ALA-synthetase activity in bone marrow or by mechanisms which are operative in regulating hepatic heme synthesis. Experiments in mice have shown that URO-I-synthetase activity is very much higher in hematopoietic tissue than in liver (30, 40, 41).

Of particular interest is whether decreased URO-I-synthetase activity in IAP reflects familial or ethnic differences which could modulate excretory patterns resulting from primary overproduction of ALA. Our studies of a large family with IAP do not support this possibility, since one parent and five of the unaffected siblings had URO-I-synthetase activity within the nonporphyric range and family members with overt IAP had diminished enzyme activity.

The observation of diminished enzyme activity in one parent and two siblings who had no clinical symptoms of IAP and who had normal ALA and PBG excretion is of interest. These two siblings (Nos. 7 and 9) and two siblings with normal red cell URO-I-synthetase as well as normal ALA and PBG excretion (Nos. 4 and 5) were studied on a metabolic ward and compared in terms of their in vivo ability to convert porphyrin precursors to porphyrins after a loading dose of ALA. The two siblings with diminished URO-I-synthetase activity resembled patients with IAP in that they had impaired ability to convert ALA to porphyrins in contrast to their two siblings with normal URO-I-synthetase activity, who converted ALA to porphyrins as nonporphyrics (42). These results suggest that the two siblings with diminished URO-I-synthetase are heterozygotes but have as yet not manifested overt clinical or chemical evidence of IAP. Since the disease is transmitted as an autosomal dominant, one of the parents would be expected to have the IAP defect. Consistent with the expectation was the finding of deficient URO-I-synthetase in the father and a normal value in the mother, although neither had ever had clinical or chemical evidence of IAP. The inadequacy of quantitative urinary ALA and PBG determinations in detecting latent IAP in large families has been recognized for many years (34, 43). Our observations raise the possibility that red cell URO-I-synthetase assay may be a more sensitive and specific indicator of the heterozygous state than quantitation of urinary ALA and PBG during the latent stage of IAP. However, sampling of larger populations of porphyrics and nonporphyrics will be required to establish more firmly the level and specificity of deficient URO-I-synthetase in IAP.

The mechanism responsible for decreased URO-I-synthetase activity is not known. It does not appear to result solely from excessive ALA-synthetase activity since URO-I-synthetase activity is not diminished by induction of ALA-synthetase in rats treated with 2-allyl-2-isopropylacetamide or in mice treated with griseofulvin (7, 12). Furthermore, URO-I-synthetase activity is not inhibited by 10^{-3} M PBG in vitro. Mixing experiments with crude and 20-fold partially purified URO-I-synthetase in which equal amounts of IAP and nonporphyric

enzyme were assayed resulted in an additive yield. In addition, the putative inhibitor was not destroyed by heating at 65°C for 15 min. However, these observations do not exclude a tightly bound, heat stable inhibitor nor do they exclude other as yet undefined mechanisms whereby URO-I-synthetase activity could be secondarily diminished.

Alternatively diminished URO-I-synthetase activity with a normal apparent K_m could reflect a primary defect in IAP. This could result from decreased amount of enzyme or a structural alteration resulting in decreased catalytic activity.

The relationship between suggested derangement in steroid metabolism in IAP (44) and decreased URO-I-synthetase activity remains to be investigated as does the relationship of enzymatic aberrations in heme synthesis to the multiple clinical and chemical abnormalities observed in IAP and other hepatic porphyrias (8, 33, 45, 46).

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