We report a new assay of human porphobilinogen deaminase (PBGD). Deficiency in this enzyme activity causes acute intermittent porphyria, the most common disorder of heme biosynthesis. The assay involves incubation of blood erythrocyte lysate with porphobilinogen, the natural PBGD substrate. Two subsequent enzymes in the heme biosynthetic pathway, uroporphyrinogen III synthase and uroporphyrinogen decarboxylase, are deactivated by heating so that their activity does not interfere with the PBGD assay. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) is used to monitor the production of uroporphyrinogen I and thus measure the PBGD activity. A simple and efficient workup using liquid—liquid extraction with >90% product recovery was employed to avoid separation by liquid chromatography. The assays show good reproducibility (±3.3%) and linear dependence of the uroporphyrinogen I formation on incubation time and protein amount. The \( K_m \) of PBGD for porphobilinogen was measured as 11.2 ± 0.5 \( \mu \)M with \( V_{\text{max}} \) of 0.0041 ± 0.0002 \( \mu \)M/(min-mg of hemoglobin). The coefficient of variation of PBGD activity among several unaffected individuals (12%) is significantly lower than the decrease due to acute intermittent porphyria (50%).

Acute intermittent porphyria (AIP), the most common acute porphyria, results from the reduced activity of the enzyme porphobilinogen deaminase (PBGD). PBGD is the third enzyme in heme biosynthesis and catalyzes the stepwise condensation of four molecules of porphobilinogen (PBG) to form hydroxymethylbilane (HMB, Scheme 1). HMB can be converted to uroporphyrinogen III by intramolecular rearrangement and ring closure by the fourth enzyme, uroporphyrinogen III synthase (UROS). An alternative pathway is spontaneous cyclization of HMB to uroporphyrinogen I, which arises by spontaneous, nonenzymatic, cyclization of HMB (Scheme 1). In the absence of UROS, assaying with PBG and monitoring the formation of uroporphyrinogen I can be further converted to other intermediates by uroporphyrinogen decarboxylase (UROD) in the cycle of heme biosynthesis.

AIP has an incidence of 5 in 100 000 in the United States and most other countries but is more common in some European countries, such as Sweden, Britain, and Ireland. An even much higher incidence of 210 in 100 000 was reported in a chronic psychiatric population in the United States. Although half-normal PBGD activity due to AIP is sufficient to maintain functional heme synthesis in most situations, it can become the rate-limiting step for the heme formation when the affected individual is exposed to endocrine factors, steroid hormones, and certain drugs, such as antipyrine, ketoconazole, mephenytoin, etc., to show clinical manifestation.

Several assays have been developed to determine the activity of PBGD that were based on UV—vis spectrophotometry, fluorimetry, or the combination of HPLC and fluorimetry. However, because of the formation of multiple products in the pathway and high background in the spectrophotometric measurements, most of the previous assays could not provide a sensitive and selective method for the diagnosis of AIP. Another recent study used HPLC—mass spectrometry to monitor the level of PBG in patients treated with recombinant PBGD. However, studies which are based on measuring increased substrate levels are not well-suited to distinguish defects of PBGA from those of enzymes downstream in the biosynthetic pathway. Product-based direct assays of PBGD suffer the essential difficulty of having to detect HMB, which is labile and difficult to prepare in a pure state. These properties severely hamper measurements of response and method calibration. The difficulties with HMB can be overcome by inactivating UROS, the next enzyme in the pathway, and monitoring the production of uroporphyrinogen I, which arises by spontaneous, nonenzymatic, cyclization of HMB (Scheme 1). In the absence of UROS, assaying with PBG and monitoring the formation of uroporphyrinogen I can be further converted to other intermediates by uroporphyrinogen decarboxylase (UROD) in the cycle of heme biosynthesis.

We report a simple and efficient workup using liquid—liquid extraction with >90% product recovery to avoid separation by liquid chromatography. The assays show good reproducibility (±3.3%) and linear dependence of the uroporphyrinogen I formation on incubation time and protein amount. The \( K_m \) of PBGD for porphobilinogen was measured as 11.2 ± 0.5 \( \mu \)M with \( V_{\text{max}} \) of 0.0041 ± 0.0002 \( \mu \)M/(min-mg of hemoglobin). The coefficient of variation of PBGD activity among several unaffected individuals (12%) is significantly lower than the decrease due to acute intermittent porphyria (50%).
uroporphyrinogen I provides an indirect but unequivocal measure of PBGD activity. UROS is deactivated by short heating to 56 °C while PBGD is not affected. In general, assaying enzyme activities provides a specific diagnostic tool for inborn errors of metabolism, as reported for several diseases.

In spite of several different approaches to assaying PBGD, it is advantageous to use methods that are based on a single instrumental platform. To this end, we have been developing tandem mass spectrometric assays of enzymes in biological samples such as dried blood spots and cell lysates for the biochemical analysis of inborn errors of metabolism. Tandem mass spectrometry offers the advantages of analytical sensitivity, selectivity, low background, and speed and is also ideally set up for multiplex analysis, whereby the products of several different enzymes can be quantified during a single infusion into the instrument without time-consuming chromatographic separation. As tandem quadrupole mass spectrometers are becoming standard equipments in clinical laboratories, new assays using electrospray ionization tandem mass spectrometry (ESI-MS/MS) represent a useful and attractive alternative to the existing methods. Our previous communication reported on ESI-MS/MS quantification of enzymes UROD and coproporphyrinogen oxidase in human blood. We envision using ESI-MS/MS to directly quantify all of the enzymes in the heme biosynthetic pathway for the molecular analysis of porphyrias. The method utilizes a designed, often synthetic, substrate for the selected enzyme that is added to a biological sample. After incubation, the amount of enzyme-generated product is quantified, along with a mass-differentiated internal standard, by selective detection with ESI-MS/MS. In this study, we report a direct enzyme assay for PBGD using ESI-MS/MS.

**EXPERIMENTAL SECTION**

**Materials and Methods.** Uroporphyrin I dihydrochloride (cat. no. U830-1) and heptacarboxylporphyrin I dihydrochloride (cat. no. H885-ID) were purchased from Frontier Scientific Inc. (Logan, UT). Porphobilinogen (cat. no. P226) was purchased from Sigma-Aldrich (St. Louis, MO). Anticoagulant tubes with EDTA dipotassium salt (cat. no. 367899), lithium heparin (cat. no. 367886), ACD solution B (trisodium citrate, 13.2 g/L, citric acid, 4.8 g/L, and dextrose 14.7 g/L, 1.0 mL, cat. no. 364816) were obtained from BD (Franklin Lakes, NJ). Hydrochloric acid, 1-butanol, and formic acid were from EMD Chemicals Inc. (San Diego, CA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

**Isolation of Erythrocytes.** Human blood was drawn in a vacuum sealed tube with EDTA (BD Vacutainer, cat. no. 367899). Whole blood (3 mL) was transferred to a 15 mL plastic centrifuge tube. Nine milliliters of NaCl solution (0.9%, w/v) was added, and the sample was centrifuged at 60 g for 10 min at room temper-

---

ature. The supernatant above the erythrocyte pellet was discarded. To the pellet was added 9 mL of NaCl solution, and the cells were resuspended by gently inverting the tube several times followed by centrifugation. This washing step was repeated once more. To the washed erythrocyte pellet was added NaCl solution to bring the volume to 3 mL. After resuspending the cells, the suspension was frozen in a dry ice/acetone bath. The tube was placed in a water bath at room temperature until the solution was fully thawed. This freeze/thaw process was repeated twice. The suspension of lysed cells was centrifuged at 12 000g for 10 min at room temperature. The supernatant was divided into 100 µL aliquots in polypropylene microfuge tubes, which were stored at −80 °C (no loss in PBGD activity was observed when cell lysates were stored frozen up to 4 months). Before use, an aliquot was thawed and diluted 5-fold with distilled water. Sixty microliters (~2 mg) of this diluted stock is used for the PBGD assay (see text).

**Storage of Assay Buffer and Substrate.** Tris–HCl buffer (100 mM, pH 8.2) and PBG (1 mM) were stored into 1.5 mL microfuge tubes at −20 °C.

**PBGD Enzyme Assay.** PBGD assay buffer (50 µL; 100 mM Tris–HCl, pH 8.2) was added to a 1.5 mL polypropylene microfuge tube followed by 60 µL (~2 mg of hemoglobin) of diluted red blood cell lysates and 100 µL of distilled water. The tube was capped and incubated in a water bath at 56 °C for 30 min to inactivate enzymes UROS and UROD, and the solution was then cooled in an ice bath. The capped tubes were centrifuged at 1500g for 30 s to spin down the water on the cap back to the aqueous phase. Porphobilinogen (40 µL of a 1 mM solution) was added to the tubes, and they were incubated at 37 °C for 60 min. The water bath chamber was covered with foil to exclude light. After incubation, the reaction was quenched by placing the tubes in an ice bath. Ammonium formate buffer solution (150 mM, pH 3.17) was added to adjust pH to the appropriate range for extraction, followed by 1-butanol (400 µL) and heptacarboxyporphyrin I (15 µL of a 20 µM solution in 1 M HCl) as the internal standard. The mixture was placed on a vortex mixer for 30 s before being centrifuged at 15 000g for 3 min. The top 300 µL of supernatant was transferred using a pipettor with a polypropylene tip to a new 1.5 mL polypropylene microfuge tube containing 150 µL of ammonium formate (20 mM, pH 3.17). After the first centrifugation, proteins were clearly located at the interface of the 1-butanol and water layers. The organic phase should be carefully transferred to a new tube without touching the interface. In order to keep the assay consistent and avoid experimental errors, the volume of the transferred organic layer was kept constant and smaller than the total volume of the 1-butanol phase. The mixture was placed on a vortex mixer for 30 s and centrifuged again for 3 min. The top 150 µL of the butanol layer was transferred to another microfuge tube for ESI-MS/MS analysis.

**Hemoglobin Measurement.** The amount of hemoglobin in red blood cell lysates was measured by using colorimetric determination at 400 nm with a QuantiChrom hemoglobin assay kit (BioAssay Systems, cat. no. DIHB-250, Hayward, CA).

**Mass Spectrometry.** Most ESI-MS and MS/MS experiments were carried out on a Waters Acquity TQD tandem quadrupole instrument operating in positive multiple reaction monitoring (MRM). Ion scanning was carried out with the MassLynx software with the following settings: capillary voltage, 3.5 kV; cone voltage, 120; extractor, 3; rf, 0; source temperature, 80 °C; desolvation temperature, 350 °C; dwell time, 100 ms; desolvation gas, 500 L/h; collision gas, 0.21 L/h; collision energy, 57 eV. The product ion monitored was due to the combined loss of CH$_2$COOH and COOH from the respective precursor MH$^+$ ions (Figure 1). MRM data for product (coproporphyrin) m/z 831 → m/z 727 and internal standard (heptaporphyrin) m/z 787 → m/z 683 were collected. The sample (10 µL of the 150 µL sample in 1-butanol) was infused into the mass spectrometer with a Waters 1525 binary HPLC pump equipped with a sample injection loop. After injection, the solution of 60% methanol (methanol/acetonitrile/formic acid, 90:10:0.1, v/v/v) and 40% water (water/formic acid, 100:0.2 v/v) was infused at 200 µL/min as the flow solvent. The amount of product was calculated from the ratio of integrated ion peak intensities of product to internal standard according to the calibration curve. The ESI-MS/MS responses were calibrated using mixtures of coproporphyrin I and heptaporphyrin I at different molar ratios and a fixed total amount of 300 pmol that were added to the incubation matrix with lysed erythrocytes, followed by standard extraction workup. The calibration curve is given in Figure S4 (Supporting Information).

**RESULTS AND DISCUSSION**

The ESI-MS/MS assay of PBGD relies on the production of uroporphyrinogen I which occurs by spontaneous cyclization of HMB (Scheme 1) after the enzymatic pathway to uroporphyrinogen III has been blocked by deactivating UROS. Spontaneous cyclization of HMB is fast with a half-time of <4 min at 37 °C, which guarantees virtually complete conversion during our incubation time of 60 min. Human UROS has been reported to be unstable to heat, with the half-life of 4 and 1 min at 45 and 60 °C, respectively. Thus, after heating at 56 °C for 30 min UROS is completely deactivated, while the activity of PBGD is not affected. Heat treatment also inactivates the next enzyme in the heme biosynthetic pathway (UROD) and thus prevents depletion of uroporphyrinogen I by subsequent enzymatic decarboxylation.

Uroporphyrinogen I (1) is not detected directly but is converted to the more stable uroporphyrin I (2, Scheme 2) by spontaneous oxidation on exposure to air and light. Product 2 shows favorable properties for electrospray ionization and collision-induced dissociation (CID) which are both necessary for tandem mass spectrometric analysis. Both UROS deactivation and uroporphyrinogen oxidation were studied in detail to ensure robustness and reproducibility of the assay procedure, as described below. UROD deactivation prevents enzymatic formation of heptacarboxyporphyrinogen I. Therefore, heptacarboxyporphyrinogen was chosen as an internal standard because of its similarity to uroporphyrin I.

The ESI-MS/MS assay was based on monitoring the products of ion dissociation of protonated uroporphyrin I, (M + H)$^+$ at m/z 831. On CID, the latter sequentially eliminates COOH and CH$_2$COOH neutral fragments to produce an abundant ion fragment at m/z 727 (Figure 1a) which was used for monitoring. Heptacarboxyporphyrinogen (3) that was used as an internal standard undergoes an analogous dissociation of its (M + H)$^+$ ion (m/z 683).
to form an abundant product ion at m/z 683 (Figure 1b). Although we did not study the mechanism of these ion dissociations, it is of interest to note that the intermediate fragments due to loss of either COOH or CH₂COOH (e.g., m/z 786 and 772, respectively, Figure 1) are very weak and indicate facile dissociation following the loss of the first neutral radical. The fact that the same neutrals are lost from 2 and 3 is advantageous, because it allows one to use MRM based on precursor ion or neutral loss scans. The response in ESI-MS/MS of 3 relative to that of 2 was determined to be 1.47:1, with a 3.1% coefficient of variation.

We note that monitoring the formation of the enzymatic product is superior to monitoring the decrease of the substrate concentration for two reasons. First, product formation is entirely due to enzyme activity and thus product detection can be expected to have a very low blank level, as confirmed by the present data (see below). Second, it is advantageous to maintain a high and
steady substrate concentration to ensure that the measurements are performed in the initial velocity stage of the enzyme reaction (see below).

The effect of blocking UROS prior to the PBGD assay was studied in detail. Four parallel triplicate experiments were carried out in which two assays included heating at 56 °C and two assays were performed without heat deactivation. ESI-MS/MS analysis (Figure 2) indicated that about 40% (±2.2%) of uroporphyrin was lost in the assays performed without heat deactivation. In addition, the assays carried out without heat inactivation showed an increased production of heptacarboxylporphyrin, which is an intermediate product of UROD-catalyzed decarboxylation of uroporphyrinogen I. These results suggested that heat treatment is an important step to deactivate both UROS and UROD to achieve a robust and reproducible assay of PBGD.

Considerable attention was also paid to sample workup following incubation with the goal of optimizing product and internal standard recovery. A standard workup procedure for the isolation of porphyrins is liquid–liquid extraction into ethyl acetate. However, partitioning of 2 between the organic and aqueous phase is largely dependent on pH for different organic solvents. For example, extraction of 2 into ethyl acetate is efficient only in a narrow pH range of 3.0–3.2, whereas 2 is very little soluble in a range of other organic solvents. We found 1-butanol as the optimum choice for the described assays because of its high extraction efficiency (over 90%) over a relatively large pH range of 2.2–3.8. 1-Butanol is sufficiently volatile to be evaporated in the ESI source at a somewhat increased droplet desolvation temperature for mass spectrometric analysis of 2. Ammonium formate buffer, which is volatile and thus compatible with ESI, was used to adjust the pH of the aqueous layer into an optimum range for good recovery yields. These were studied in detail in blank experiments with or without blood and incubation. Figure S1 (Supporting Information) shows that the percent conversion by oxidation to uroporphyrin I in blank assays that contained blood was >90% and was nearly constant in the period of 5–70 min. This showed that the oxidation of uroporphyrinogen I to uroporphyrin I was complete and that the latter was stable in solution under the workup conditions. In contrast, blank assays that were performed without blood showed a much slower oxidation rate (Figure S1) and reached 90% recovery yield only after a period of 60 min. It is possible that the fast oxidation of uroporphyrinogen I to uroporphyrin I is catalyzed by Fe ions from the blood sample.

The amount of product in the PBGD assays increased linearly with reaction time from 15 to 150 min (Figure 3a). This indicated that the enzyme reaction was in its initial velocity stage. The PBG substrate conversion was around 11% after 60 min, and this time was chosen for standard assays. We note that PBGD showed a substantially higher stability by comparison with UROD and coproporphyrinogen oxidase, which were studied previously using ESI-MS/MS assays. In addition, the PBG substrate appears to be more stable than porphyrinogen substrates which are generated in situ by chemical reduction. All this resulted in good reproducibility of data points (±3.3% mean SD) and a tight correlation (r² = 0.9996, Figure 3a). The amount of product also showed a linear dependence on the amount of red cell lysate, measured as hemoglobin by colorimetry at 400 nm, which was used for the assay. In the range of 0.08–3.5 mg of hemoglobin from whole blood we obtained a tight linear dependence (r² = 1.000, Figure 3b). From the latter, 2 mg of hemoglobin was chosen for the standard assay to provide an amount of PBGD that could be reliably assayed by ESI-MS/MS with a tandem quadru-

pole mass spectrometer. Comparison of standard and blank assays (no substrate or no hemoglobin) gave the product peak intensity over 150 times of that of the blank. Thus, no blank subtraction was necessary in data evaluation.

Plots of the reaction rate versus the substrate concentration showed the classical hyperbolic behavior for the PBGD assay yielding values of $K_M$ of 11.2 ± 0.5 $\mu$M and $V_{max}$ of 0.0041 ± 0.0002 $\mu$M/(min·mg of hemoglobin), respectively ($r^2 = 0.9968$, Figure 4). This value is close to that from an early fluorimetric determination (13.8 $\mu$M)$^5$ but higher than the value from an HPLC-based assay (6.2 mM)$^8$. The average value for the standard PBGD assay was 1.01 ± 0.12 pmol/(min·mg of hemoglobin) of uroporphyrin I, as determined in blood samples from four healthy individuals (Figure S2, Supporting Information). The coefficient of variation among the healthy individuals from these measurements, CV = 12%, is significantly lower than the expected loss of activity in affected patients (ca. 50%). Thus, by virtue of its robustness and reproducibility, the ESI-MS/MS assay appears to be suitable for clinical diagnostics of patients manifesting AIP.

As a final practical note, we investigated the stability upon storage of blood samples. Blood was collected in standard commercial tubes in the presence of various anticoagulants, e.g., (1) lithium heparin, (2) ACD (trisodium citrate, citric acid, and dextrose), and (3) dipotassium salt of EDTA. All samples were found to give similar PBGD activities without apparent difference after having been stored with the anticoagulant at 4 °C for 2 days (Figure S3, Supporting Information). No loss of activity was observed after storage at −80 °C for 4 months. Thus, PBGD has proven to be a stable enzyme that can be assayed in normal blood samples after weeks of storage under the above-described appropriate conditions.

Figure 4. Lineweaver−Burk plot of reciprocal PBGD velocity in erythrocytes (~2 mg of hemoglobin) over reciprocal concentration of porphobilinogen (PBG).

CONCLUSIONS
Electrospray ionization tandem mass spectrometry has been shown to provide a platform for robust, sensitive, and selective assays of PBGD in human blood samples. Samples can be stored for prolonged time and assayed using a simple procedure that does not require chromatographic separation. Following clinical tests including samples from affected patients, the ESI-MS/MS assay should be suitable for diagnostics of AIP and, in combination with the procedures developed for UROD and coproporphyrinogen III oxidase,$^{15}$ could be employed for the multiplex detection of several porphyrias using a single instrumental platform. We envisage that direct determination of PBGA activity should be preferred over measuring the amount of excreted porphyrins.

ACKNOWLEDGMENT
This work was supported by a Grant from the NDDK—National Institutes of Health (DK67869).

SUPPORTING INFORMATION AVAILABLE
Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review October 30, 2007. Accepted January 10, 2008.
AC702244X