genotyped (data not shown). Because many of the genotypes were represented by only one sample, analysis of additional samples with these genotypes, as well as prospective analysis of Hb H disease samples, will be needed to verify the robustness of this assay.

This new assay complements an existing deletional multiplex-PCR assay that we developed previously to detect seven common α-thalassemia deletions (6) and is intended to improve the overall mutation detection sensitivity for α-thalassemia, especially for Hb H disease genotyping. Because deletions account for the vast majority of α-thalassemia alleles, samples sent to the molecular diagnostic laboratory for α-thalassemia genotyping should first be screened for common deletional mutations. If no deletions are found or only a single or double α-globin gene deletion is identified in a patient suspected to carry additional mutations, the sample should be further screened for the presence of nondeletional mutations. If the seven-deletion multiplex-PCR assay was used in the deletional analysis (6), the multiplex minisequencing assay can be performed directly on the PCR product of the deletional assay to screen for α-γ-globin gene mutations, with results obtained in less than 3.5 h. If a different deletional assay was performed or if only nondeletional mutations need to be screened, the α-γ-globin gene can be separately amplified for the multiplex minisequencing analysis.

The automated capillary electrophoresis and analysis allow diagnostic laboratories with moderate to high sample volumes to analyze up to 192 DNA samples in a 12-h period, requiring only a 50% effort by one technologist. This includes the α-γ-globin gene PCR (≤3 h), PCR clean-up, multiplex minisequencing and post-minisequencing clean-up (≤3 h), capillary electrophoresis (25 min for 16 samples), and verification of automated genotyping results (5 min for 16 samples). Including all consumables for PCR amplification, multiplex minisequencing, and capillary electrophoresis, but excluding manpower and equipment amortization, this assay costs approximately US $2.90 per sample.

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References

Diagnostic Enzyme Assay That Uses Stable-Isotope-labeled Substrates to Detect l-Arginine/Glycine Amidinotransferase Deficiency, Nanda M. Verhoeven,1‡ Danielle S.M. Schor,1 Birthe Roos,1 Roberta Battini,2 Sylvia Stöckler-Ipsiroglu,3 Gaaja S. Salomons,1 and Cornelis Jakobs1 (1 Medical Unit, Department of Clinical Chemistry, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands; 2 DUNPI Universita di Pisa-IRCCS Fondazione Stella Maris, 56018 Pisa, Italy; 3 Department of Pediatrics, University Hospital and General Hospital of Vienna, A1090 Vienna, Austria; * author for correspondence: fax 31-20-4440305, e-mail N.Verhoeven@vumc.nl)

Arginine-glycine amidinotransferase (AGAT) is the enzyme responsible for the conversion of arginine and glycine into guanidinoacetate (GuAc) and ornithine in creatine biosynthesis. AGAT deficiency was recently described in two patients by Item et al. (1). These two patients [for whom the clinical details were first described by Bianchi et al. (2)] are mentally retarded and have severe creatine deficiency in the brain and decreased urinary GuAc.

Several methods for measuring AGAT activity have been published, but these methods are nonspecific because the measured ornithine formed during the assay can be of different origin (3, 4) or they are impracticable because they use radioactivity (1). We developed a new method in lymphocytes and lymphoblasts that uses stable-isotope-labeled substrates. We used l-[guanido-15N2]arginine and [U-13C,15N]glycine as substrates and analyzed the enzyme product [1,2-13C2,15N2]GuAc (after derivatization) by gas chromatography–mass spectrometry using [1,2-13C2]GuAc as internal standard. We applied this method to measure enzyme activities in lymphocytes and lymphoblasts of controls and in lymphoblasts from a patient affected with AGAT deficiency.

For lymphocyte isolation, whole venous blood from 16 control individuals was drawn into acid-citrate dextrose and stored at room temperature up to 48 h. Written consent was obtained from each participant.

Lymphoblast lines derived from six control individuals were maintained in RPMI-1640 supplemented with 100 mL/L fetal bovine serum and 10 mL/L penicillin/streptomycin. The lymphoblast lines used in this study as controls were originally obtained for carrier screening. No defect was found, and the samples were anonymized. Unless otherwise stated, all chemicals and reagents were purchased from Sigma, Baker, Merck, or Pierce. [U-13C2,15N]Glycine and l-[guanido-15N2]arginine · HCl
were obtained from Cambridge Isotope Laboratories. \([1,2-^{13}C_2]GuAc\) was synthesized as described previously (5).

Lymphocytes were isolated from whole blood with use of Accuspin® tubes with Histopaque® (Sigma) and were washed twice with Hanks’ balanced salt solution (HBSS; Invitrogen) supplemented with 1 g/L bovine serum albumin and once with HBSS. Lymphoblasts were harvested by centrifugation, and the cells were washed twice with HBSS. Pellets were resuspended in 0.1 mol/L sodium phosphate buffer (pH 7.5) on ice. Cells were lysed by sonic disruption (three times 10 s at standard capacity) using a Soniprep 150 Ultrasonic Desintegrator (MSE). Lysates were centrifuged for 5 min at 4 °C and 8800g. The supernatant was used for the enzyme assay after the protein concentration was measured (bicinchoninic acid protein assay; Sigma).

The incubation was carried out in a capped 1-mL vial with 3000 nmol of \(\mathrm{L-}[\text{guanido}-^{15}\text{N}_2]\)arginine, 4800 nmol of \([\text{U-}^{13}\text{C}_2,^{15}\text{N}_2]\)glycine, and an aliquot of lymphocytes or lymphoblasts lysates corresponding to 50 µg of protein, brought to a final volume of 200 µL with 0.1 mol/L sodium phosphate buffer (pH 7.5). After a period of 4–20 h at 37 °C, the incubation was terminated by addition of 30 µL of 4.2 mol/L perchloric acid. The solution was neutralized by the addition of 20 µL of 6 mol/L potassium hydroxide. After 1.25 nmol of \([1,2-^{13}C_2]\)GuAc was added as internal standard, the samples were prepared for analysis of GuAc by gas chromatography–mass spectrometry as described previously (5). Selected-ion monitoring of the m/z 293 and 290 ions was performed for \([1,2-^{13}C_2,^{15}N_3]\)GuAc and the labeled internal standard \([1,2-^{13}C_2]\)GuAc, respectively.

Enzyme activity was directly related to the formation of \([1,2-^{13}C_2,^{15}N_3]\)GuAc during the incubation with \([\text{U-}^{13}\text{C}_2,^{15}\text{N}_2]\)glycine and \(\mathrm{L-}[\text{guanido}-^{15}\text{N}_2]\)arginine. \([1,2-^{13}C_2,^{15}N_3]\)GuAc formed during the assay was quantified relative to its \(^{13}\text{C}_2\)-labeled internal standard.

We started with the assay conditions described by Ratner and Rochovansky (4), who found a pH optimum of 7.5 for the reaction carried out in 0.1 mol/L phosphate buffer, and used the substrates \(\mathrm{L-}\)arginine and glycine at a ratio of 10:16. The use of a cofactor was not required.

With the assay conditions described, the production of \([1,2-^{13}C_2,^{15}N_3]\)GuAc was linear up to 100 µg of protein and linear in time up to 23 h (not shown). When optimizing the assay for substrate concentrations, we found that full activity was achieved when 15 mmol/L \(\mathrm{L-}[\text{guanido-}^{15}\text{N}_2]\)arginine and 24 mmol/L \([\text{U-}^{13}\text{C}_2,^{15}\text{N}_2]\)glycine were used.

Mean (SD) AGAT activity in eight control lymphoblast lines was 43 (17) pmol·min⁻¹·mg protein⁻¹ (range, 21–70 pmol·min⁻¹·mg protein⁻¹). Reproducibility was tested by assaying one cell line on three different occasions. We obtained values of 25, 23, and 34 pmol·min⁻¹·mg protein⁻¹. The activities in lysates of lymphocytes obtained from 16 control individuals were approximately sevenfold lower than those in lymphoblasts [range, 1.3–8.5 pmol·min⁻¹·mg protein⁻¹; mean (SD), 4.9 (2.1) pmol·min⁻¹·mg protein⁻¹]. In the lymphoblasts from the patient affected with AGAT deficiency, activity was undetectable (<1 pmol·min⁻¹·mg protein⁻¹).

\(\mathrm{L-}\)Ornithine is known to competitively inhibit AGAT in purified form (6) and in crude rat kidney homogenates (7). To investigate the effect of \(\mathrm{L-}\)ornithine in our assay, we performed the assay in the presence of this compound at concentrations of 0.05–30 mmol/L. As shown in Fig. 1, the enzyme in lymphoblasts was almost fully inhibited at a concentration of 30 mmol/L \(\mathrm{L-}\)ornithine.

When we performed the assay in the presence of creatine, no effect of creatine (up to concentrations of 5 mmol/L) was observed (results not shown).

The method reported here is very specific because it measures the formation of \([1,2-^{13}C_2,^{15}N_3]\)GuAc, which can be formed only from both labeled substrates by AGAT. Thus, possible formation of ornithine from arginine by arginase does not interfere. In addition, the method is sensitive and accurate because it uses selected-ion monitoring negative chemical ionization mass spectrometry and the \(^{13}\text{C}_2\)-labeled internal standard \([1,2-^{13}C_2]\)GuAc in the analysis of product formation.

The reference values for AGAT activity that we found in lymphoblasts are 10 times lower than the activities reported by Item et al. (1). It is possible that their filtration
procedure to obtain lymphoblast extracts produced more concentrated enzyme extracts with higher specific activities.

By performing the assay in the presence of various concentrations of ornithine, we confirmed that AGAT is inhibited by ornithine, as reported previously (7). This observation may be of clinical interest in conditions of hyperornithinemia. Decreased brain creatine has been observed in patients affected with gyrate atrophy of the choroid and retina (McKusick 258870). It has been suggested that in this condition the high concentrations of ornithine caused by the inherited deficiency of ornithine-δ-amminotransferase inhibit AGAT and, thus, createine biosynthesis (8). In addition, in hyperornithinemia-hyperammonemia-homocitrullinuria syndrome, creatine excretion has been shown to be low (9).

In our in vitro assay, the amount of ornithine formed posed no problem. Inhibition became significant at an ornithine concentration of ~0.1 mmol/L (93% remaining activity), whereas during incubations over 20 h, at most 3 nmoles of ornithine were formed (0.015 mmol/L).

It is known that creatine regulates AGAT at the pre-translational level. We investigated whether creatine also inhibits AGAT activity directly. We observed no inhibition of AGAT by creatine at concentrations up to 5 mmol/L.

In conclusion, we present a sensitive and specific enzymatic assay for AGAT that enables enzymatic diagnosis of AGAT deficiency. It may help in the diagnosis of more patients with AGAT deficiency. Early recognition and treatment may effectively prevent neurologic damage (1).

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References

Identification and Properties of Glycated Monoclonal IgA That Affect the Fructosamine Assay, Kiyotaka Fujita,1* Linda K. Curtiss,2 Ikunosuke Sakurabayashi,3 Funiko Kameko,3 Nobuo Okumura,3 Funiko Terasawa,3 Minoru Totsuka,4 and Tsutomu Katsuyama4 (1 Department of Immunology, The Scripps Research Institute, 10550 North Torrey Pines Rd., La Jolla, CA 92037; 2 Department of Clinical Laboratories, Omiya Medical Center, Chichi Medical School, Amanuma, Saitama 330-8503, Japan; 3 Department of Biomedical Laboratory Sciences, School of Health Sciences, Shinshu University, Asahi, Matsumoto, Nagano 390-8621, Japan; 4 Department of Laboratory Medicine, Shinshu University School of Medicine, Asahi, Matsumoto, Nagano 390-8621, Japan; * author for correspondence: fax 858-784-9144, e-mail kfyujit@aol.com)

Serum fructosamine is used to monitor short-term (1–3 weeks) average glycemia (1, 2), but controversy remains whether it is influenced by the serum concentrations of albumin (3, 4), IgA (5, 6), or monoclonal IgA (7, 8). We recently demonstrated glycation of monoclonal IgA and the presence of IgA-albumin complexes (9), but the relationship between the glycation of monoclonal IgA and the IgA-albumin complexes was not clear.

In this study, we measured immunoglobulin-albumin complexes, serum fructosamine, and the glycation of immunoglobulin in patients with monoclonal proteinemia and in patients with polyclonal hyper-IgA.

We obtained serum samples from 40 patients with M-proteinemia with no history of diabetes and whose plasma glucose concentrations were <1.1 g/L. The M-protein was IgG in 17 of the patients [mean (SD), 29.2 (21.0) g/L], IgA in 13 [18.5 (16.4) g/L], and IgM in 10 [16.8 (11.3) g/L]. We also analyzed sera from 15 nonglycemic patients with hepatitis who had polyclonal hyper-IgA [5.1 (1.1) g/L] as controls.

Serum fructosamine was measured at 37 °C in an automated analyzer (ICA-RX 10 Clini analyzer; Japan Electron Optics Laboratory) with reagents from Roche Diagnostics Corporation.

Serum protein electrophoresis was performed on agarose gels. Immunoelctrophoresis was based on the method of Scheidegger (10). Fructosamine was detected on agarose gels by incubating the gel for 30 min at 37 °C with a 2× concentration of a fructosamine reagent.

IgA was isolated from serum with use of a jacalin-agarose (Funakoshi Corporation) column (11) and 0.8 mol/L galactose to elute the IgA. The IgA fraction was dialyzed against 0.2 mol/L Tris-HCl buffer (pH 8.0), concentrated by ultrafiltration, loaded on a DEAE-Sephacel (Amersham Pharmacia Biotech) ion-exchange column, and eluted with a linear gradient of 0–0.5 mol/L NaCl in the 0.2 mol/L Tris-HCl buffer.

Sera were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), with or without 2-mercaptoethanol (2-ME), and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (12). Immunostaining was performed with rabbit polyclonal antisera (Dako) as first antibodies and peroxi-