



รายงานวิจัยฉบับสมบูรณ์

เสถียรภาพของโปรตีนต่อการแสดงออกทางคลินิกของภาวะ
พร่องเอนไซม์ G6PD ชนิดต่าง ๆ

รศ. ดร. อุษา บุญยยืน

พฤษภาคม 2563

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ภาควิชาชีวโมเลกุลและพันธุศาสตร์โรคเขตร้อน
คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล

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Executive Summary

Project title: Contribution of protein stability to clinical manifestations of G6PD variants

Introduction to the research problem and its significance

G6PD deficiency is a hereditary genetic defect which is one of the most prevalent polymorphisms and enzymopathies in human. More than 400 million people worldwide have been affected by G6PD deficiency. Different G6PD variants lead to varying degrees of disease severity and a wide spectrum of clinical manifestations of G6PD deficiency is observed, which includes favism, hemolytic anemia, chronic non-spherocytic hemolytic anemia (CNSHA), spontaneous abortion, and neonatal hyperbilirubinemia resulting in neonatal kernicterus, which can be fatal. Fortunately, most of G6PD deficiencies have no clinical features and, therefore, the conditions remain asymptomatic until the person is exposed to hemolytic triggers. The most common trigger of hemolysis in G6PD deficiencies is fava beans, known as favism. Importantly, the trigger of great public concern is the antimalarials, especially primaquine and tafenoquine which are crucial for malaria elimination and control. Primaquine and tafenoquine are currently available for killing dormant liver stage (hypnozoites) of *Plasmodium vivax* and *P. ovale*. Importantly, primaquine remains the only readily-available drug that actively clears mature gametocytes of *P. falciparum*, a transmission-blocking potential of antimalarial. In addition to primaquine and tafenoquine, a large number of drugs could cause hemolysis in individuals with G6PD deficiency, including sulfanilamide, acetanilide and sulfones. This has raised a significant public concern, regarding the hemolytic toxicity, due to the widespread prevalence of G6PD deficiencies in malaria endemic areas, including Thailand. It is also problematic for the use of antimalarials because the hemolytic anemia observed in individuals with G6PD deficiencies is variable. The clinical hemolytic episode can range from mild and self-limiting to severe and lead to death, depending on the exposure and G6PD genotype.

The wide range of clinical presentations of G6PD deficiency results in diverse understanding of the molecular effects of G6PD variants. In fact, the molecular mechanisms of pathological G6PD variants remain largely unknown. The molecular mechanisms underlying G6PD deficiency were proposed to be either a

decrease in catalytic activity or a reduction in the number of active G6PD molecules. Moreover, it was believed that protein instability in non-nucleated erythrocytes is the principal factor contributing to the clinical manifestations. Biochemical characterization of G6PD variants has revealed that G6PD variants exhibit a range of complex multidimensional effects, including changes in catalytic activity, stability, and protein folding. Though it was proposed that mutations that are located near the structural NADP⁺ or substrate binding sites are associated with severe G6PD deficiency, many Class I G6PD variants are located in other structural regions of G6PD proteins and still cause severe clinical outcomes. The relationship between the structural or biochemical effects of a G6PD variants and their clinical phenotypes remains poorly understood. Moreover, little information is available regarding the effects of additional mutations on the activity and stability of G6PD proteins. This information is also crucial to understand the molecular effects of G6PD variants.

Objectives

The main objective of this study is to provide molecular explanation for a better understanding of mechanisms underlying clinical manifestations of G6PD deficiency. In this study, we want to expand our understanding about molecular mechanisms of G6PD deficiency by investigating the biochemical properties of 11 G6PD variants covering Class I, II and III G6PD variants which include 3 single, 7 double and 1 triple G6PD variants. The study provides information regarding the effects of additional mutations on the biochemical properties of G6PD variants. The ultimate goal is to grant the valuable information regarding the contribution of stability to clinical phenotypes of G6PD variants.

Specific objectives:

1. To construct, express and purify 20 G6PD variants (6 single, 11 double, 2 triple and 1 quadruple)
2. To measure the steady kinetic parameters of G6PD variants

3. To determine the stability of G6PD variants by enzyme activity assay and fluorescence spectrophotometry

Schedule for the entire project

Activities	Month 1-3	Month 4-6	Month 7-9	Month 10-12	Month 13-15	Month 16-18	Month 19-21	Month 22-24
Construction of recombinant DNA of 11 G6PD variants	←→							
Expression and purification of recombinant G6PD variants	←→				→			
Measurement of steady kinetic parameters of G6PD variants	←→					→		
Determination of the stability of G6PD variants		←→					→	
Data analysis and manuscript preparation							←→	→

Output

Title: Functional and structural analysis of double and triple mutants reveals the contribution of protein instability to clinical manifestations of G6PD variants

Journal: International Journal of Biological Macromolecules

Impact factor: 4.784

Budget details

Budget details	1 st year	2 nd year	Total
Honorarium	156,000	156,000	312,000
Reagents and consumables e.g. mutagenesis kit, DNA polymerase, dNTPs, primers, LB broth, LB agar, petri dish, IPTG, antibiotics, G6P, NADP ⁺ , cobalt resin, Tris, Sodium phosphate, imidazole, NaCl, SDS, acrylamide, APS, glycine, tips, tubes	122,675.50	155,742.80	278,418.30
Expenses e.g. sequencing	8,030.00	2,247.00	10,277.00
Total budget for each year	286,705.50	313,989.80	600,695.30

Abstract

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common polymorphism and enzymopathy in humans, affecting approximately 400 million people worldwide. Over 200 point mutations have been identified in *g6pd* and the molecular mechanisms underlying the severity of G6PD variants differ. We report the detailed functional and structural characterization of 11 recombinant human G6PD variants: G6PD Asahi, G6PD A, G6PD Guadalajara, G6PD Acrokorinthos, G6PD Ananindeua, G6PD A⁽²⁰²⁾, G6PD Sierra Leone, G6PD A⁽⁶⁸⁰⁾, G6PD A⁽⁹⁶⁸⁾, G6PD Mount Sinai and G6PD No name. G6PD Guadalajara, G6PD Mount Sinai and G6PD No name are inactive variants and, correlating with the observed clinical manifestations, exhibit complete loss of enzyme activity. Protein structural instability, causing a reduction in catalytic efficiency, contributes to the clinical phenotypes of all variants. In double and triple mutants sharing the G6PD A mutation, we observed cooperative interaction between two and three mutations to cause protein dysfunction. The G6PD A (Asn126Asp) mutation exhibits no effect on protein activity and stability, indicating that the additional mutations in these G6PD variants significantly contribute to enzyme deficiency. We provide insight into the molecular basis of G6PD deficiency, which can explain the severity of clinical manifestations observed in individuals with G6PD deficiency.

Key words: G6PD deficiency, Stability, Catalytic efficiency

บทคัดย่อ

ภาวะพร่องเอนไซม์ G6PD เป็นโรคที่แพร่หลาย โดยพบว่าผู้ป่วยที่มีภาวะพร่องเอนไซม์ G6PD ประมาณ 400 ล้านคนทั่วโลก ในปัจจุบันนี้มีรายงานภาวะพร่องเอนไซม์ G6PD มากกว่า 200 ชนิด โดยภาวะพร่องเอนไซม์ G6PDแต่ละชนิดเกิดจากการกลายพันธุ์บนยีน G6PD ส่งผลให้ภาวะพร่องเอนไซม์ G6PD แต่ละชนิดมีกลไกระดับโมเลกุลที่ส่งผลต่อความรุนแรงของการแสดงออกของโรคแตกต่างกัน ในงานวิจัยนี้ได้ทำการศึกษาผลของการการกลายพันธุ์ต่อการทำงานและโครงสร้างของเอนไซม์ G6PD ทั้งหมด 11 ชนิด ได้แก่ G6PD Asahi, G6PD A, G6PD Guadalajara, G6PD Acrokorinthos, G6PD Ananindeua, G6PD A⁻⁽²⁰²⁾, G6PD Sierra Leone, G6PD A⁻⁽⁶⁸⁰⁾, G6PD A⁻⁽⁹⁶⁸⁾, G6PD Mount Sinai และ G6PD No name โดยจากการศึกษาพบว่า การกลายพันธุ์ 3 แบบ ได้แก่ G6PD Guadalajara, G6PD Mount Sinai และ G6PD No name ส่งผลอย่างรุนแรงต่อการทำงานของเอนไซม์ ทำให้เอนไซม์ไม่สามารถทำงานได้ ซึ่งสอดคล้องกับการแสดงออกของโรคที่พบในผู้ป่วย นอกจากนี้ยังพบว่า การกลายพันธุ์ที่นำมาศึกษาส่งผลต่อเสถียรภาพทางโครงสร้างของเอนไซม์ G6PD ทำให้ประสิทธิภาพในการทำงานของเอนไซม์แย่ง และเป็นกลไกที่ส่งผลต่อการแสดงออกของโรค จากการศึกษากับปฏิสัมพันธ์แบบมีส่วนร่วมของการกลายพันธุ์แบบ 2 และ 3 ตำแหน่ง โดยพบว่า G6PD A ซึ่งเป็นการกลายพันธุ์ที่ไม่ส่งผลต่อการทำงานของเอนไซม์ แต่เมื่อมีการกลายพันธุ์ที่เกิดขึ้นในตำแหน่งที่ 2 หรือ 3 จะส่งผลอย่างมากต่อการทำงานของเอนไซม์ ผลการวิจัยนี้จึงสามารถอธิบายถึงกลไกระดับโมเลกุลที่ส่งผลต่อความรุนแรงต่อการแสดงออกของภาวะพร่องเอนไซม์ G6PD ที่พบในผู้ป่วยได้

คำหลัก: ภาวะพร่องเอนไซม์ G6PD, เสถียรภาพ, ประสิทธิภาพในการเร่งปฏิกิริยา

Introduction

Glucose-6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49) is the first and rate-limiting enzyme in the pentose phosphate pathway, mediating the formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 6-phosphogluconolactone [1]. NADPH plays an essential role in maintaining cellular redox status and reduces oxidative stress via glutathione reduction [2]. G6PD is particularly important in non-nucleated red blood cells (RBCs) where it is the sole source of NADPH production. When NADPH levels cannot be maintained, the amount of reduced glutathione decreases, leading to oxidative damage, which can ultimately lead to hemolysis [3, 4]. The *g6pd* gene is located on the X chromosome (Xq28) and is approximately 18 kb in length, consisting of 13 exons and 12 introns. The *g6pd* gene is highly polymorphic and over 200 mutations are reported [5-8]. Mutation in *g6pd* can cause G6PD deficiency, which affects approximately 400 million people worldwide [6, 9]. Most G6PD variants are single point mutations and double, triple, and quadruple mutations have been reported with much lower frequencies [7]. G6PD deficiency is predominantly found in malaria endemic areas and it is hypothesized that G6PD deficiency confers protection against malarial infection. Patients with G6PD deficiency exhibit a wide spectrum of clinical features, including favism, hemolytic anemia, chronic non-spherocytic hemolytic anemia, and neonatal hyperbilirubinemia [10]. G6PD variants have been classified into 5 classes, based on residual enzyme activity in RBCs and clinical phenotypes [11, 12]. These classes range from Class I G6PD variants, which show less than 5% residual enzyme activity and exhibit the most severe clinical phenotypes, to Class V G6PD variants, which show increased enzyme activity and no clinical manifestations. In individuals with G6PD deficiency, hemolysis can be triggered by infections, stress, food and certain medications, including antimalarials such as primaquine, tafenoquine, chloroquine, dapsone and sulfanilamide [13]. Therefore, hemolytic toxicity of G6PD deficiency has a significant impact on malaria treatment, especially in the radical cure of *Plasmodium vivax* and *P. ovale* using primaquine and tafenoquine [14-18]. The severity of hemolytic anemia varies among individuals with G6PD deficiency, making malaria treatment even more complicated and challenging.

The G6PD protein consists of 514 amino acids (approximately 60 kDa) and is active as a dimer or tetramer. The G6PD three-dimensional (3D) structures (PDB ID: 2BHL, 1QKI and 6E08) show that each subunit binds one molecule of oxidized nicotinamide adenine dinucleotide phosphate (NADP⁺), stabilizing the protein structure [19-21]. The molecular mechanisms underlying the clinical phenotypes of G6PD deficiency depend on the location of the mutation in the 3D structure of the protein. Depending on the mutation location, it can affect protein assembly, dimer formation and stability, binding affinity to substrates and catalytic efficiency [22]. Functional and structural analyses of G6PD variants suggest that mutations at or near the dimer interface and structural NADP⁺ binding site cause protein instability and mostly result in severe deficiency (Class I G6PD variants) [21, 23-27]. Therefore, knowing the effects of mutations on protein function is crucial for understanding the molecular mechanisms underlying the clinical phenotypes observed in individuals with G6PD deficiency. This knowledge can also be beneficial for predicting drug response in malaria treatment.

G6PD A (Asn126Asp), a single mutant commonly found in African populations with a frequency of 20%, is considered a non-deficient variant with no clinical manifestations. [28]. It has been reported that Asn126Asp mutation did not affect the catalytic activity or structural stability of the protein [29]. However, the presence of additional mutation(s) has been found to cause severe enzyme deficiency in people carrying double and triple G6PD mutants sharing the G6PD A mutation [30-39]. In this study, we aim to investigate the cooperative interaction between two and three mutations to cause severe enzyme deficiency in G6PD variants sharing the Asn126Asp mutation. We report detailed biochemical and structural analyses of 11 natural G6PD variants, including Class I (G6PD Guadalajara, G6PD Mount Sinai and, G6PD No name), Class II (G6PD Acrokorinthos and G6PD Ananindeua), and Class III (G6PD A, G6PD Asahi, G6PD A⁻⁽²⁰²⁾, G6PD Sierra Leone, G6PD A⁻⁽⁶⁸⁰⁾ and G6PD A⁻⁽⁹⁶⁸⁾) variants. We also investigated the synergistic interaction between the mutations in the double and triple mutants, G6PD Acrokorinthos (Asn126Asp+His155Asp), G6PD Ananindeua (Asn126Asp+Val291Met), G6PD A⁻⁽²⁰²⁾ (Asn126Asp+Val68Met), G6PD Sierra Leone (Asn126Asp+Arg104His), G6PD A⁻⁽⁶⁸⁰⁾ (Asn126Asp+Arg227Leu), G6PD A⁻⁽⁹⁶⁸⁾ (Asn126Asp+Leu323Pro), G6PD

Mount Sinai (Asn126Asp+ Arg387His) and G6PD No name (Asn126Asp+Val68Met+Leu422Val), which share the Asn126Asp mutation (G6PD A). These results were interpreted in the context of the observed clinical manifestations.

Materials and methods

Site-directed mutagenesis

G6PD variants were created using site-directed mutagenesis. The polymerase chain reaction (PCR) mixture (50 µl) contained 1x KAPA HiFi reaction buffer, 50 ng of template plasmid (pET28a-G6PD wild type (WT)), 100 ng of forward and reverse primers, 0.3 µM of each dNTP, and 1 U of KAPA HiFi DNA polymerase. The primers used for site-directed mutagenesis are listed in **Table 1**. The cycling parameters were as follows: 1 cycle of 95°C for 5 minutes and 16 cycles of 98°C for 20 seconds, 55°C for 15 seconds, and 68°C for 3 minutes and 30 seconds.

Table 1. List of primers used in site-directed mutagenesis

Primer	Sequence
G202A_F	acaccttcatcatgggctatgcc
G202A_R	ggcatagcccatgatgaaggtgt
G311A_F	cttctttgccacaactcctatg
G311A_R	cataggagttgtgggcaaagaag
A376G_F	acagccacatggatgccctccac
A376G_R	gtggaggcatccatgtgctgt
C463G_F	ccaagaacattgacgagtcctgc
C463G_R	gcaggactcgtcaatgttcttg
G680T_F	tctggaacctggacaacatcgc
G680T_R	gcgatgttgcaggtccaga
G871A_F	gaaggtcaagatgttgaatgc
G871A_R	gcatttcaacatcttgaccttc
T968C_F	gggtaccgggacgacc
T968C_R	ggtcgtccgggtaccc
C1159T_F	agcagtgaagtgaacgagctg
C1159T_R	cagctcgttgacttgactgct
C1264G_F	cggagctggacgtgacctacggc
C1264G_R	gccgtaggtcacgtccagctccg

PCR products were digested with *DpnI* to remove the parental plasmid and were then transformed into DH5 α competent cells. The presence of the desired mutations was confirmed by DNA sequencing. Recombinant plasmids harboring desired mutations were transformed into the BL21 (DE3) expression host for protein expression.

Protein expression and purification

Expression and purification of G6PD variants were performed as described previously [24, 25]. Fresh overnight cultures of *E. coli* BL21 (DE3) harboring recombinant plasmid were grown in LB media containing 50 μ g/ml kanamycin at 37°C with 250 rpm shaking. When the OD₆₀₀ reached 1.0, G6PD expression was induced with isopropyl β -D-thiogalactoside at a final concentration of 1 mM. The cultures were further incubated at 20°C with 200 rpm shaking for 20 hours before harvesting by centrifugation at 8,300 \times g for 10 minutes.

For protein purification, the pellets were resuspended in lysis buffer (20 mM sodium phosphate buffer pH 7.4, 10 mM imidazole and 300 mM NaCl), disrupted by sonication for 5 min and centrifuged at 36,000 \times g for 1 hour. Protein was purified using immobilized metal affinity chromatography by incubating with TALON cobalt resin for 1 hour and washing the unbound proteins using wash buffer (20 mM sodium phosphate buffer pH 7.4, 20 mM imidazole and 300 mM NaCl). Thereafter, the protein was eluted using imidazole gradient concentrations (40 – 400 mM). The fractions containing the desired protein were pooled. Imidazole was removed by overnight dialysis against 20 mM Tris-HCl pH 7.5 containing 10% glycerol. The purity of the obtained protein was determined by 12% SDS-PAGE staining with Coomassie blue R250. Finally, the protein concentration was measured using the Bradford assay [40].

Determination of steady-state kinetic parameters

G6PD activity was measured spectrophotometrically by monitoring NADP⁺ reduction at 340 nm and 25°C as described previously [24, 25]. Steady-state kinetic properties of recombinant human G6PD WT and G6PD mutants were determined in 1 ml cuvette. The standard reaction mixtures contained 20 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 100 μ M NADP⁺ and 500 μ M glucose-6-phosphate (G6P). The reaction was initiated

with the addition of the enzyme. To determine the K_m for G6P, G6P concentration was varied from 5–1,000 μM and NADP^+ concentration was fixed at 100 μM . The K_m for NADP^+ was also measured by varying NADP^+ concentration from 1–500 μM while fixing G6P concentration at 500 μM . The experiments were performed in triplicate. Steady-state kinetic parameters, K_m , k_{cat} and V_{max} , were obtained by fitting the collected data to the Michaelis-Menten equation using GraphPad Prism software.

Determination of the secondary structure by circular dichroism (CD)

The secondary structure of the G6PD WT and other variants were determined spectroscopically by CD as previously described [24, 25]. Far UV-CD spectra of the G6PD variants at a protein concentration of 0.25 mg/ml were recorded using a Jasco spectrometer, model J-815, equipped with a Peltier temperature control system in a 1 mm path-length quartz cuvette at 25°C. The CD spectra were collected over a wavelength range of 190–260 nm at a scan rate of 50 nm/min. Five scans were averaged for each sample and the buffer scan results were subtracted.

Intrinsic fluorescence and 8-aminonaphthalene-1-sulfonate (ANS)-binding

For intrinsic fluorescence analysis, we used an excitation wavelength of 295 nm, and the emission spectra were recorded in the 300–400 nm range using a Synergy H1 hybrid reader (Biotek) in a 96-well plate at 25°C. The ability of G6PD proteins to bind to ANS was assessed. ANS (100 μM) was used for sample preparation and the G6PD concentration was adjusted to 0.1 mg/ml. G6PD proteins were incubated with ANS at 25°C for 1 hour then the emission spectra at an excitation wavelength of 395 nm were monitored between 400 and 600 nm.

Thermal stability

In a 20 μl reaction mixture, we mixed 5 μg G6PD enzyme, 5x SYPRO Orange reporter dye and various concentrations of NADP^+ (0, 10, and 100 μM). The reaction mixtures were heated at temperatures ranging from 20 to 80°C using a LightCycler 480 real-time PCR machine (Roche, Mannheim, Germany) with excitation and emission of 465 nm and 580 nm, respectively. The experiments were performed in triplicate.

The melting temperature (T_m) was defined as the temperature at which half of the secondary structure unfolded and was calculated for each G6PD variant.

Thermal inactivation

Thermal inactivation analysis was performed as previously described [24, 25]. In brief, 6 μ g of G6PD protein was incubated with varying concentrations of NADP⁺ (0, 10, and 100 μ M) at temperatures ranging from 25 to 65°C for 20 minutes and then cooled to 4°C in a Thermocycler (Eppendorf). Residual enzyme activity was determined and expressed as a percentage of the activity of the same enzyme incubated at 25°C.

Protein stability in the presence of guanidine hydrochloride (Gdn-HCl)

The protein sample (6 μ g) was incubated with varying concentrations of NADP⁺ (0, 10, and 100 μ M) in the presence of different concentrations of Gdn-HCl (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, and 0.5 M) at 37 °C for 2 hours. Thereafter, residual enzyme activity was measured and expressed as a percentage of the activity of the same enzyme incubated at 25°C in the absence of Gdn-HCl.

Susceptibility of G6PD variants to trypsin digestion

The susceptibility of G6PD proteins to trypsin was determined in the presence of different NADP⁺ concentrations (0, 10, and 100 μ M). Recombinant G6PD protein (6 μ g) was incubated with trypsin (final concentration 0.5 mg/ml or 1,250 U/ml) for 5 min at 25°C. Residual enzyme activity was assessed and expressed as a percentage of the enzyme activity in the absence of trypsin.

Size exclusion chromatography of G6PD variants

The oligomeric state of G6PD variants was determined by size exclusion chromatography using AKTA fast protein liquid chromatography (FPLC) equipped with the Superdex 200 Increase 10/300 column (GE healthcare, NJ, USA). Recombinant protein (100 μ g) was loaded onto the pre-equilibrated column. Chromatography was performed at a flow rate of 0.5 ml/min using 50 mM Tris-HCl pH 7.5, and 150 mM NaCl. The column was calibrated with blue dextran (>2000 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and RNase A (13.7 kDa).

Results

Biochemical characteristics of G6PD variants

To understand the molecular mechanisms underlying the clinical manifestations of G6PD deficiency, 11 recombinant human G6PD variants (**Table 2**) were created using site-directed mutagenesis. Recombinant G6PD proteins were expressed in *E. coli* BL21 (DE3), purified to homogeneity using immobilized metal affinity chromatography and visualized using SDS-PAGE analysis (**Figure 1**). Of the 11 variants, G6PD Asahi, G6PD Guadalajara, G6PD Mount Sinai, and G6PD No name severely precipitated during purification, indicating their structural instability. Additionally, G6PD Acrokorinthos did not bind well to the cobalt resin during purification, which may be the result of conformational change caused by the mutations.

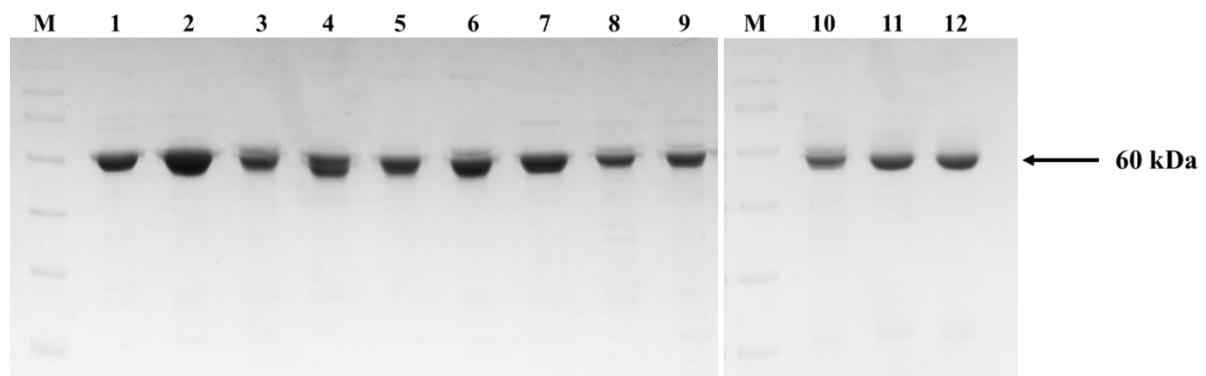


Figure 1. SDS-PAGE analysis of purified recombinant G6PD variants. Lane M, molecular weight marker proteins; lane 1, G6PD WT; lane 2, G6PD Ananindeua; lane 3, G6PD Guadalajara; lane 4, G6PD A⁻⁽⁹⁶⁸⁾; lane 5, G6PD A; lane 6, G6PD Mount Sinai; lane 7, G6PD Acrokorinthos; lane 8, G6PD A⁻⁽²⁰²⁾; lane 9, G6PD A⁻⁽⁶⁸⁰⁾; lane 10, G6PD Sierra Leone; lane 11, G6PD Asahi and lane 12, G6PD No name.

The effects of mutation on the catalytic activity of each G6PD variant were determined (**Table 2 and Figure 2**). The enzyme activity of G6PD Guadalajara, G6PD Mount Sinai and G6PD No name was less than 1%. These three G6PD variants are classified as Class I variants, and individuals with these variants exhibit severe G6PD deficiency with less than 5% residual enzyme activity in RBCs [30-33]. Consistent with previous reports and clinical phenotypes, G6PD A, a Class III variant commonly found in African populations has lower k_{cat} and k_{cat}/K_m values, but slightly higher K_m values, than does of the G6PD WT [29, 41].

Table 2. Kinetic parameters of recombinant G6PD variants.

Construct	Class	Amino acid change	k_{cat} (s ⁻¹)	K_m G6P (μ M)	K_m NADP ⁺ (μ M)	k_{cat}/K_m G6P (μ M ⁻¹ s ⁻¹)	k_{cat}/K_m NADP ⁺ (μ M ⁻¹ s ⁻¹)
WT	-	-	202.4 \pm 11.5	58.6 \pm 8.1	8.7 \pm 1.5	6.2 \pm 1.2	23.3 \pm 5.3
Asahi	III	Val68Met	30.1 \pm 1	48.6 \pm 5.1	8.9 \pm 0.9	0.6 \pm 0.1	3.4 \pm 0.5
A	III	Asn126Asp	131 \pm 11	66 \pm 8	19 \pm 5	1.7 \pm 0.25	6.9 \pm 2.4
A ⁻⁽²⁰²⁾	III	Asn126Asp+Val68Met	11.4 \pm 0.8	43.2 \pm 6.2	12 \pm 3	0.3 \pm 0.1	1 \pm 0.3
Sierra Leone	III	Asn126Asp+Arg104His	14.5 \pm 0.6	81.3 \pm 6.1	14.5 \pm 1.9	0.25 \pm 0.03	1 \pm 0.2
Acokorinthos	II	Asn126Asp+His155Asp	19.3 \pm 0.3	38.1 \pm 3.8	8.4 \pm 0.6	0.5 \pm 0.1	2.3 \pm 0.2
A ⁻⁽⁶⁸⁰⁾	III	Asn126Asp+Arg227Leu	50 \pm 2	54 \pm 6	12 \pm 2	1 \pm 0.1	4 \pm 0.8
Ananindeau	II	Asn126Asp+Val291Met	22.3 \pm 0.7	58.3 \pm 6.9	18 \pm 2.3	0.4 \pm 0.1	1.2 \pm 0.2
A ⁻⁽⁹⁶⁸⁾	III	Asn126Asp+Leu323Pro	37.4 \pm 1.5	37.5 \pm 3.9	18.2 \pm 2.3	1 \pm 0.1	2.1 \pm 0.3
Guadalajara	I	Arg387His	ND	ND	ND	ND	ND
Mount Sinai	I	Asn126Asp+ Arg387His	ND	ND	ND	ND	ND
No name	I	Asn126Asp+Val68Met+Leu422Val	ND	ND	ND	ND	ND

ND: not determined

Class II G6PD variants, G6PD Acrokorinthos and G6PD Ananindeua, have comparable binding affinities toward G6P and NADP⁺ but have a 10-fold reduction in catalytic efficiency compared with the G6PD WT. A significant decrease in catalytic efficiency of G6PD Acrokorinthos and G6PD Ananindeua might be attributable to structural change which will be discussed below. All Class III G6PD variants, G6PD Asahi, G6PD A⁻⁽²⁰²⁾, G6PD Sierra Leone, G6PD A⁻⁽⁶⁸⁰⁾ and G6PD A⁻⁽⁹⁶⁸⁾, show similar K_m values to that of the G6PD WT for G6P and NADP⁺, with the exception of G6PD Sierra Leone, which exhibits a moderately increased K_m . The Val68Met mutation causes a significant decrease (approximately 10-fold) in G6PD Asahi catalytic efficiency compared with that of the native enzyme. A marked decrease in catalytic efficiency (\sim 20-fold) was observed for G6PD A⁻⁽²⁰²⁾ and G6PD Sierra Leone while a moderate reduction in catalytic efficiency (\sim 6-fold) was noted for G6PD A⁻⁽⁶⁸⁰⁾ and G6PD A⁻⁽⁹⁶⁸⁾. A significant decrease in G6PD A⁻⁽²⁰²⁾ catalytic activity might be attributed to the presence of the Val68Met mutation.

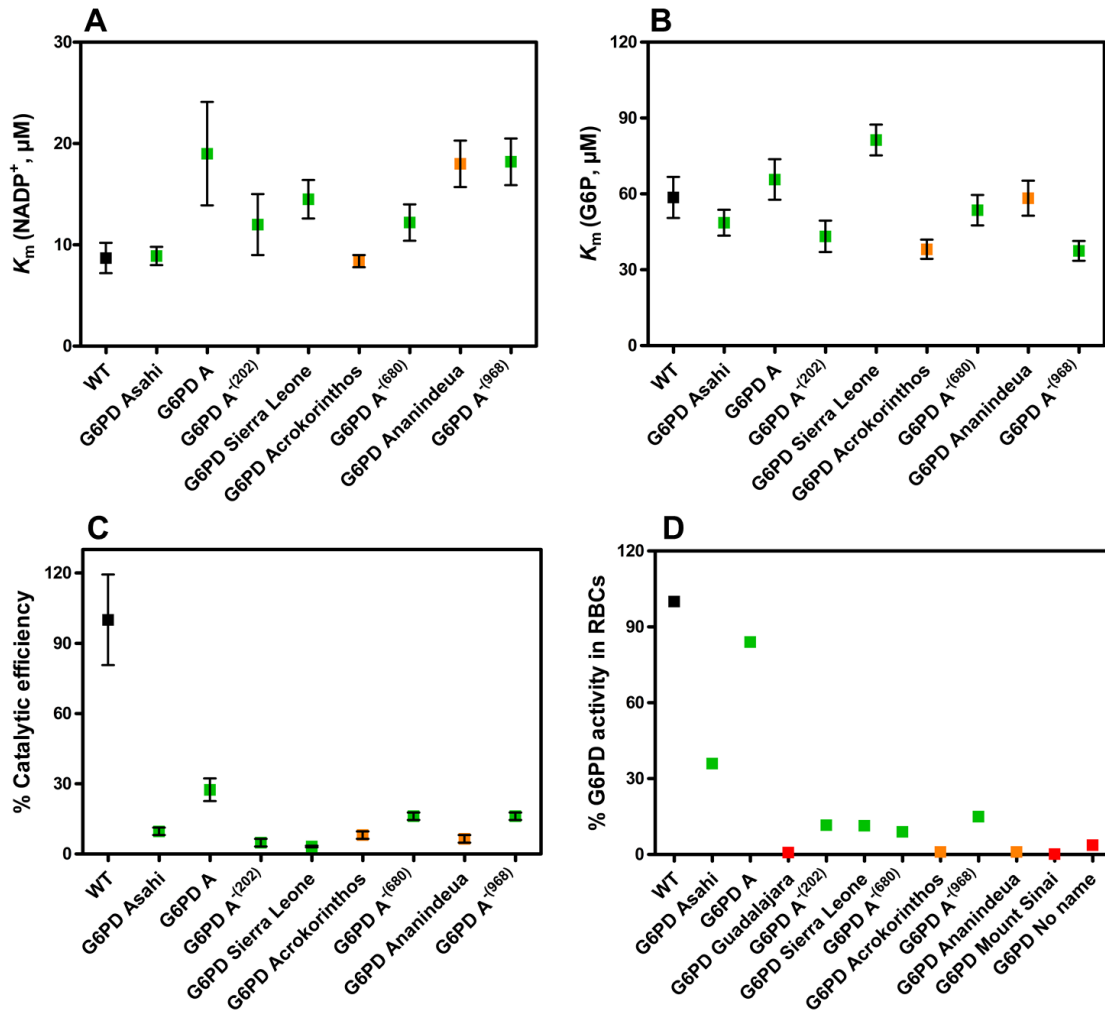


Figure 2. Steady state kinetic parameters and residual enzyme activity of G6PD variants. A) Binding affinity toward NADP⁺ substrate. B) Binding affinity toward G6P substrate. C) The percent of the catalytic efficiency. D) The percent of the residual G6PD activity in RBCs. G6PD WT was present in black. Class I, II and III G6PD variants are present in red, orange and green, respectively.

Structural characteristics of G6PD variants

To assess the effect of mutation on secondary structure, CD spectra of G6PD WT and G6PD variants were recorded in the far-UV region (190–260 nm) (**Figure 3**). All G6PD variants exhibited two negative peaks at 208 and 222 nm, which are characteristic of α -helical proteins, including G6PD [21]. Based on our CD spectra results, the overall G6PD structure was maintained in the analyzed variants. However, when compared with the WT protein, a change in signal intensity was observed for all mutants except G6PD Sierra Leone and G6PD Mount Sinai. A change in signal intensity without changing CD spectral pattern indicates

that the chirality of the chromophores is modified upon mutation and provides information about the stability (flexibility or rigidity) of the secondary structure. The stability of secondary structure of G6PD A, G6PD Ananindeua and G6PD A⁻⁽⁹⁶⁸⁾ was slightly altered and a significant change was observed for G6PD Asahi, G6PD Acrokorinthos, G6PD A⁻⁽⁶⁸⁰⁾ and G6PD Guadalajara. Interestingly, the combination of Val68Met and Asn126Asp mutations resulted in a rigorous change in rigidity of the secondary structure of the G6PD A⁻⁽²⁰²⁾ variant, which may contribute to severe G6PD deficiency and cause the clinical phenotypes observed. A previous study suggested that impaired protein folding, leading to a reduction in the number of active G6PD enzymes, was the cause of deficiency observed in the G6PD A⁻⁽²⁰²⁾ variant [26, 29]. Additionally, secondary structure alteration was described as a cause of G6PD deficiency for other G6PD variants [24, 42].

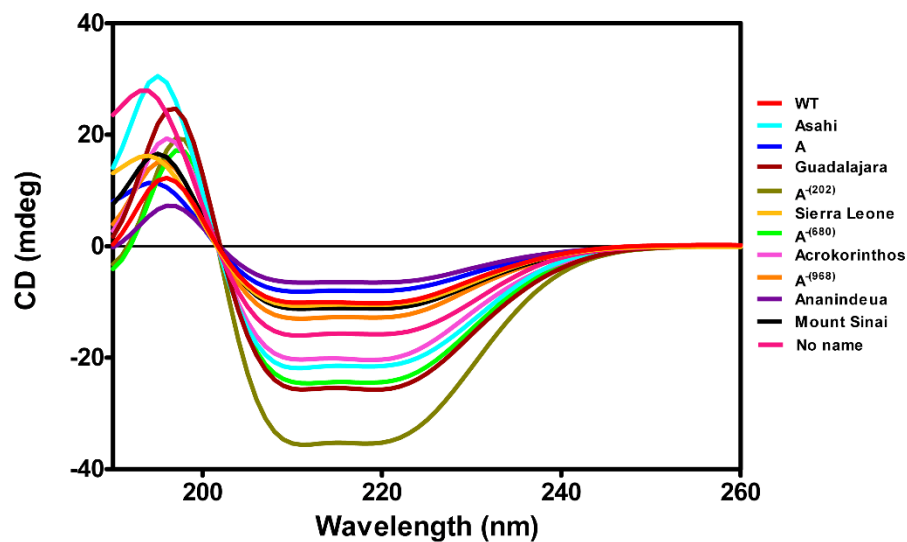


Figure 3. Far-UV CD spectra of recombinant human G6PD variants. The protein concentration was 0.25 mg/ml and CD spectra were collected over a wavelength range of 190–260 nm at a scan rate of 50 nm/min using a Jasco spectrometer, model J-815.

G6PD contains seven tryptophan residues per monomer. Therefore, the effect of mutations on the protein structure can be examined by monitoring tryptophan residue intrinsic fluorescence emission. The fluorescence emission spectra of G6PD variants and G6PD WT were examined (**Figure 4A**). All G6PD variants displayed similar emission spectra to that of the WT enzyme, with emission maxima at 330 nm. However, G6PD Mount Sinai showed greater fluorescence intensity than did the WT, while all other G6PD variants

exhibited less fluorescence intensity than did the WT protein. A decrease in fluorescence intensity could be attributed to electron transfer quenching by the local peptide carbonyl group or by neighboring amino acid side chains as a result of conformational change or interactions with ligands [43]. Hydrophobic interactions play important role in defining protein conformation and interactions in the protein structure. Therefore, ANS, a hydrophobic probe, was used to assess the conformational changes in G6PD variants (Figure 4B). All G6PD variants shared similar ANS emission spectra to that of the WT protein with an emission maxima of 505 nm, except for G6PD Asahi, G6PD A⁽²⁰²⁾, G6PD Guadalajara and G6PD No name, which showed an emission maxima shift to a longer wavelength (515 nm). A change in extrinsic fluorescence intensity demonstrates increasing or decreasing of binding sites for ANS molecules which could be attributed to changing surface hydrophobicity. These results suggest that these mutations cause conformational changes in the protein structure which may partially contribute to the severe clinical manifestations observed in individuals with G6PD deficiency.

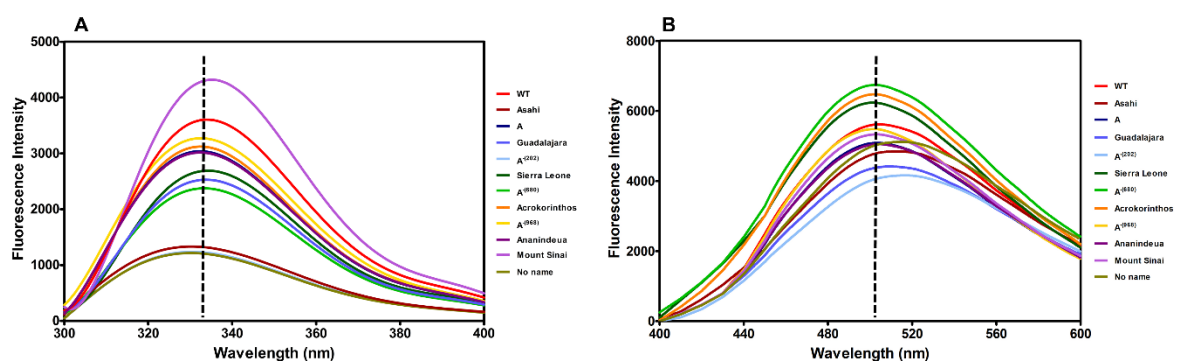


Figure 4. Fluorescence emission spectra of recombinant human G6PD variants. (A) Intrinsic fluorescence spectra. (B) ANS fluorescence spectra. G6PD concentration was 0.1mg/ml. For intrinsic fluorescence spectra, an excitation wavelength of 295 nm was used and the emission spectra were recorded in the 300–400 nm range. For ANS fluorescence spectra, G6PD proteins were incubated with 100 μ M ANS at 25°C for 1 hour and emission spectra between 400 and 600 nm were monitored using an excitation wavelength of 395 nm.

The structural stability of G6PD variants

Reduced structural stability is also responsible for enzyme deficiency in individuals with G6PD deficiency. The effects of mutation on the structural stability of G6PD variants were investigated.

Fluorescence-based thermal shift assay was performed in the absence and presence of different NADP⁺ concentrations (0, 10 and 100 μ M) to assess thermal stability (Figure 5).

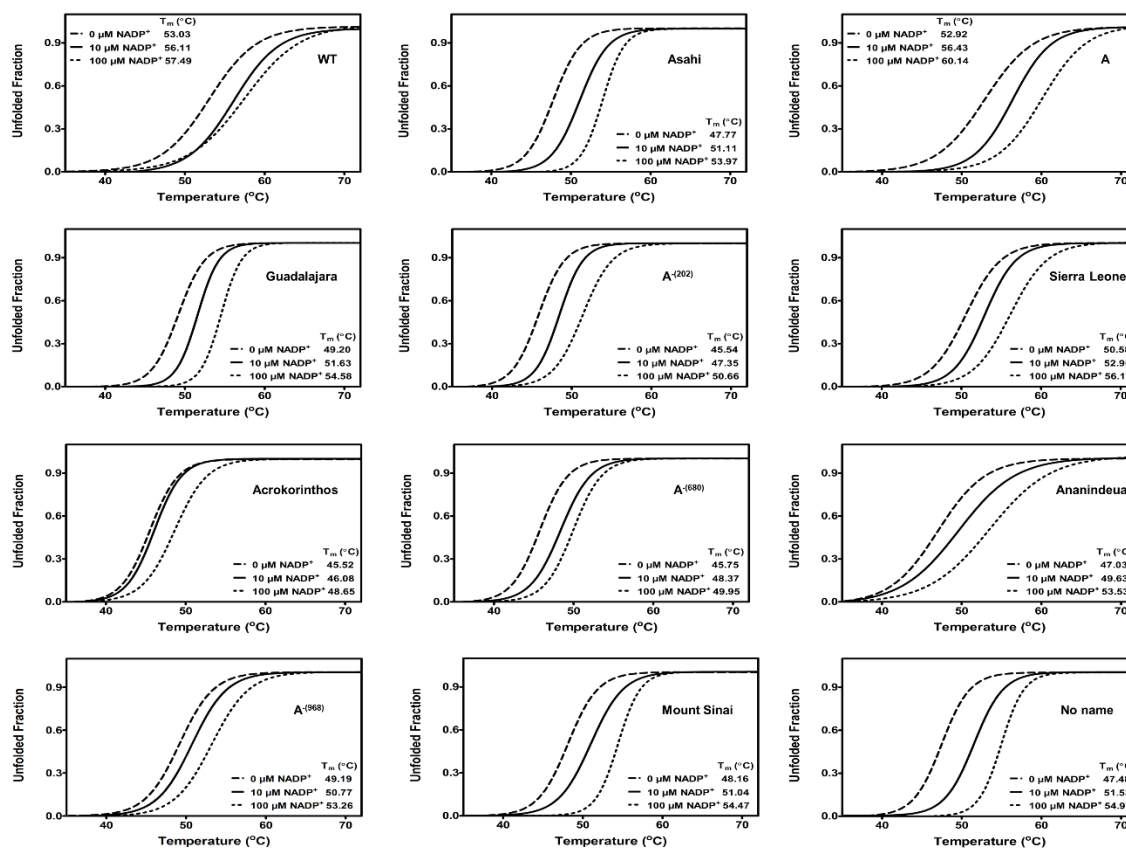


Figure 5. Thermal stability analysis of recombinant human G6PD variants. Proteins were heated in the presence of 5 x SYPRO Orange reporter dye and various NADP⁺ concentrations (0, 10, and 100 μ M). Protein unfolding was monitored by following the emission of fluorescence dye at 580 nm. Error bars represent mean \pm SD of triplicate measurements.

In the absence of NADP⁺, G6PD A showed a T_m value (52.92°C) comparable to that of the G6PD WT (53.03°C) while in the presence of 100 μ M NADP⁺ a higher T_m (60.14°C) than that of the G6PD WT (T_m 57.49°C) was observed, suggesting greater stabilizing effect of 100 μ M NADP⁺ on G6PD A. All other G6PD variants exhibited lower T_m values than did the WT protein, indicating the structural instability of these variants in the absence of NADP⁺. G6PD Acrokorinthos, G6PD A⁽²⁰²⁾ and G6PD A⁽⁶⁸⁰⁾ are the least stable G6PD variants with T_m values of approximately 45°C. G6PD Ananindeua, a Class II G6PD variant, also showed a remarkable reduction in protein structural stability with a T_m of 47.03°C in the absence of NADP⁺, approximately 6°C

lower than that of the WT enzyme. The presence of 100 μM NADP^+ significantly improved the protein stability of G6PD Ananindeua (T_m 53.53°C). The Val68Met mutation moderately affects the structural stability of G6PD Asahi, which had a T_m of 47.77°C in the absence of NADP^+ . G6PD Sierra Leone and G6PD A⁻⁽⁹⁶⁸⁾, the other two G6PD variants belonging to the Class III deficiency category, exhibited only a slight and moderate effect on protein structural stability, respectively. The structural stability of Class I G6PD variants—G6PD Guadalajara, G6PD Mount Sinai and G6PD No name moderately decreased, with a T_m approximately 4–5°C lower than that of the WT enzyme. Consistent with previously reported results, the presence of NADP^+ improved protein stability, and increasing NADP^+ concentration resulted in higher T_m values for all G6PD proteins [24, 25, 42].

Thermal inactivation assays

Thermal inactivation assays were also performed to assess the stability of the active site of G6PD variants upon increasing temperature. Residual enzyme activity was measured in the absence and presence of different NADP^+ concentrations after being incubated at temperature ranging from 25 to 65°C for 20 min (**Figure 6**). Consistent with our thermal stability analysis results, G6PD A had similar $T_{1/2}$ (49.26°C) to the G6PD WT ($T_{1/2}$ 50.06°C) and the activity of all other G6PD variants decreased in the absence of NADP^+ . Compared with the WT protein, a 5–8°C decrease in $T_{1/2}$ was observed for G6PD Asahi, G6PD A⁻⁽²⁰²⁾, G6PD Acrokorinthos, G6PD A⁻⁽⁶⁸⁰⁾ and G6PD A⁻⁽⁹⁶⁸⁾. A 2–3°C decrease in $T_{1/2}$ was observed for G6PD Ananindeua and G6PD Sierra Leone in the absence of NADP^+ . These results show that NADP^+ improves G6PD protein stability and increasing NADP^+ concentration results in higher $T_{1/2}$ values.

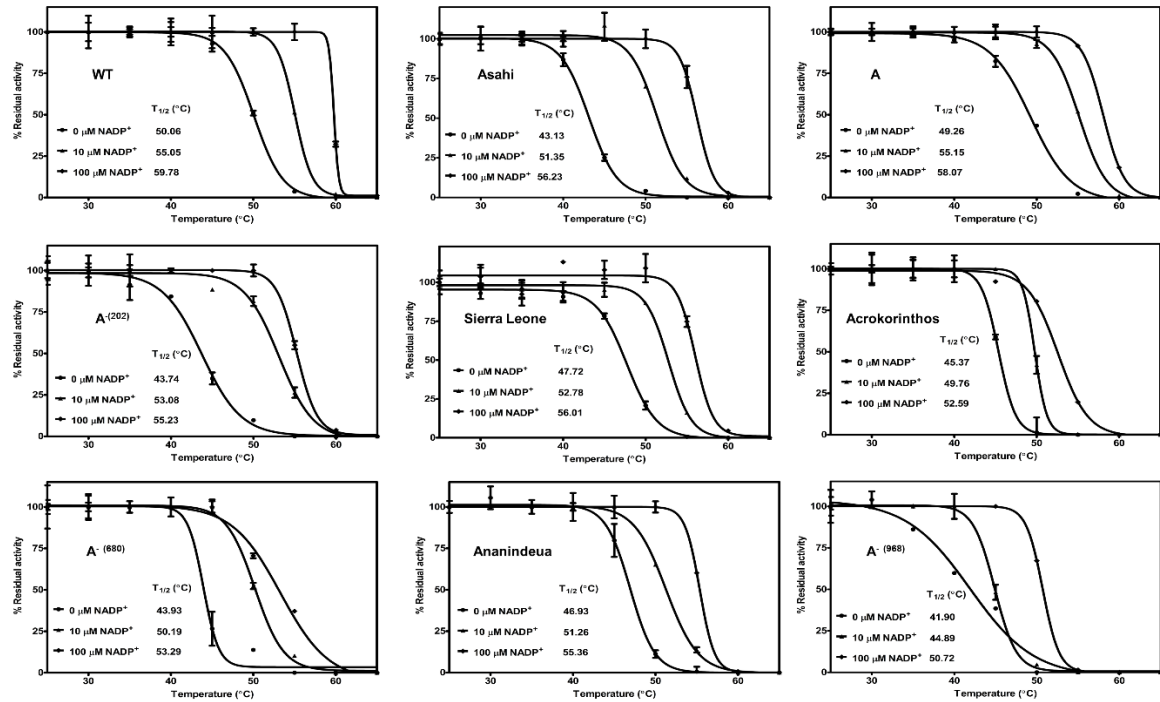


Figure 6. Thermal inactivation analysis of recombinant human G6PD variants. Enzyme activity was measured after the protein was heated in the presence of various NADP⁺ concentrations (0, 10, and 100 μM) for 20 min. $T_{1/2}$ is the temperature at which the enzyme loses 50% of its activity. Error bars represent mean \pm SD of triplicate measurements.

Residual enzyme activity of G6PD variants

Tertiary protein structure unfolding causes a decline in enzyme activity. Therefore, residual enzyme activity in the absence and presence of different NADP⁺ concentrations was determined as a function of Gdn-HCl concentration (**Figure 7**). The loss of G6PD A, G6PD Sierra Leone and G6PD A⁽⁹⁶⁸⁾ tertiary structure occurred similarly to that observed for the WT enzyme. The tertiary structure of G6PD Asahi, G6PD A⁽²⁰²⁾, G6PD A⁽⁶⁸⁰⁾ and G6PD Ananindeua was disrupted, and half of their enzyme activity was lost at 0.09–0.13 M Gdn-HCl. G6PD Acrokorinθος was the variant most susceptible to Gdn-HCl treatment and half of its activity was lost at concentration as low as 0.02 M in the absence of NADP⁺. As NADP⁺ is essential for G6PD protein stability, higher concentrations of NADP⁺ resulted in higher $C_{1/2}$ values for all G6PD variants.

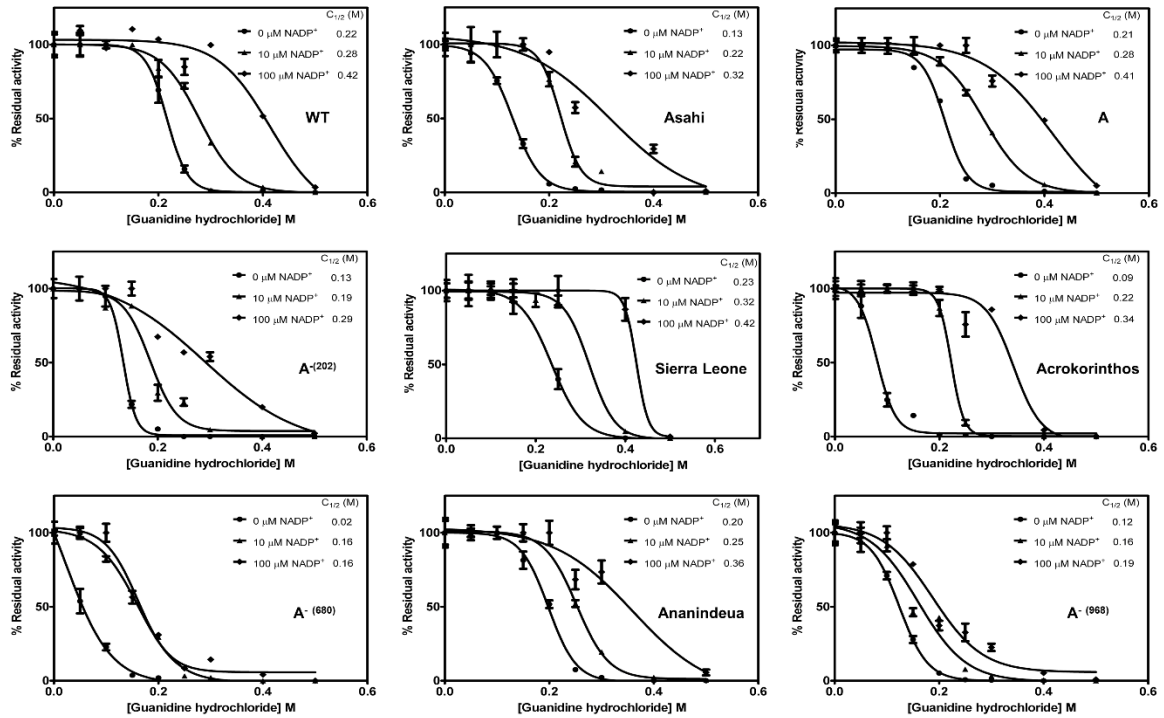


Figure 7. Stability analysis of recombinant human G6PD variants upon Gdn-HCl treatment. Enzymatic activity was measured after incubation with various concentrations of Gdn-HCl and in the presence of various NADP⁺ concentrations (0, 10, and 100 μM) at 37°C for 2 h. Residual enzyme activity is expressed as a percentage of the activity for the same enzyme incubated at 25°C in the absence of Gdn-HCl. $C_{1/2}$ is the Gdn-HCl concentration at which the enzyme loses 50% of its activity. Error bars represent mean \pm SD of triplicate measurements.

The susceptibility of G6PD variants to trypsin digestion

Furthermore, we determined the susceptibility of each G6PD variant to trypsin digestion (**Figure 8**). The WT enzyme lost approximately 60% of its activity during trypsin treatment in the absence of NADP⁺ and the residual enzyme activity was increased to 92% and 100% in the presence of 10 and 100 μM NADP⁺, respectively. G6PD variants G6PD A, G6PD A⁻⁽²⁰²⁾, G6PD Sierra Leone, G6PD A⁻⁽⁶⁸⁰⁾ and G6PD A⁻⁽⁹⁶⁸⁾, were significantly susceptible to trypsin digestion and their residual enzyme activities were between 5–8% in the absence of NADP⁺. The presence of NADP⁺ enhanced structural stability of these G6PD variants. G6PD Asahi, G6PD Acrokorinthos and G6PD Ananindeua were moderately susceptible to trypsin digestion. Nonetheless,

the presence of NADP^+ significantly increased the structural stability of G6PD Asahi, but this was less prominent for G6PD Acrokorinthos and G6PD Ananindeua.

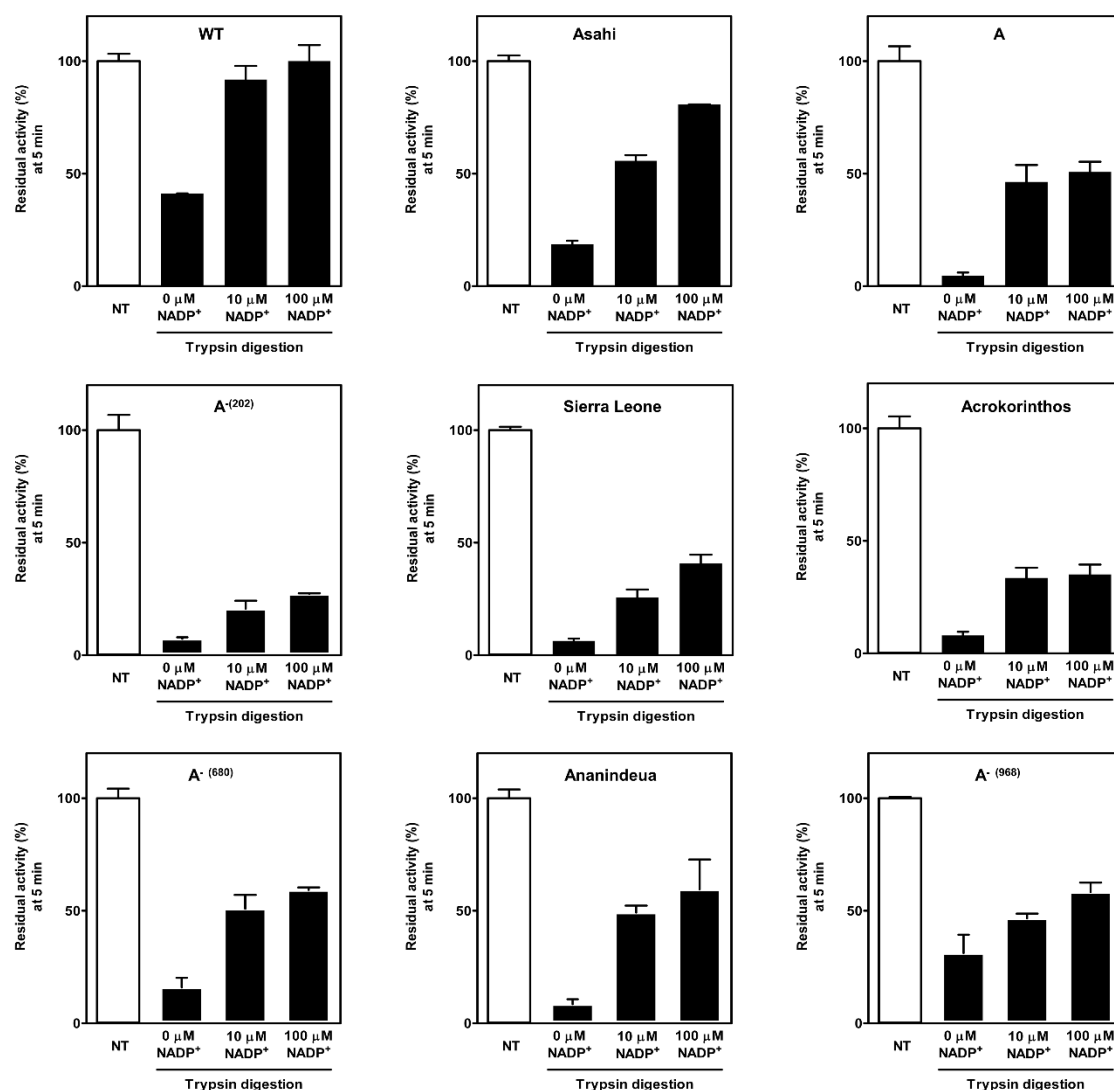


Figure 8. Recombinant human G6PD variant susceptibility to trypsin digestion. Enzymatic activity was measured after incubation with 0.5 mg/ml trypsin (1,250 U/ml) in the presence of various NADP^+ concentrations (0, 10, and 100 μM) at 25°C for 5 min. Residual enzyme activity is expressed as a percentage of the activity for the same enzyme in the absence of trypsin. Error bars represent the mean \pm SD of triplicate measurements. NT: no treatment.

The subunit structure of G6PD variants

Since native human G6PD exists as a dimer or tetramer, size exclusion chromatography was performed to determine the effect of mutations on the subunit structure of G6PD variants (**Figures 9** and

10). G6PD WT, G6PD A, G6PD Sierra Leone, G6PD Ananindeua and G6PD A⁽⁹⁶⁸⁾ primarily existed as dimers. Both dimeric and monomeric forms were observed for G6PD Asahi, G6PD Guadalajara, G6PD A⁽²⁰²⁾, G6PD Acrokorinthos, G6PD A⁽⁶⁸⁰⁾, G6PD Mount Sinai and G6PD No name. These results indicate that the dimeric structure of G6PD Asahi, G6PD Guadalajara, G6PD A⁽²⁰²⁾, G6PD Acrokorinthos, G6PD A⁽⁶⁸⁰⁾, G6PD Mount Sinai and G6PD No name was destabilized, and that the enzyme subunits dissociate into monomers.

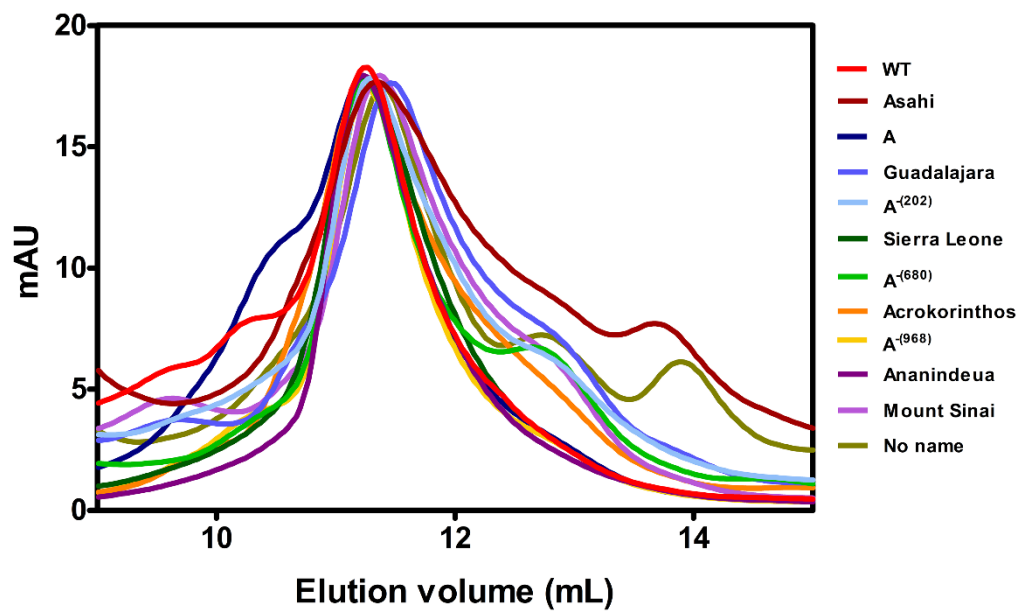


Figure 9. Size exclusion FPLC elution profiles purified recombinant G6PD variants. Proteins were loaded onto Superdex 200 Increase 10/300 equilibrated with 50 mM Tris-HCl and 150 mM NaCl.

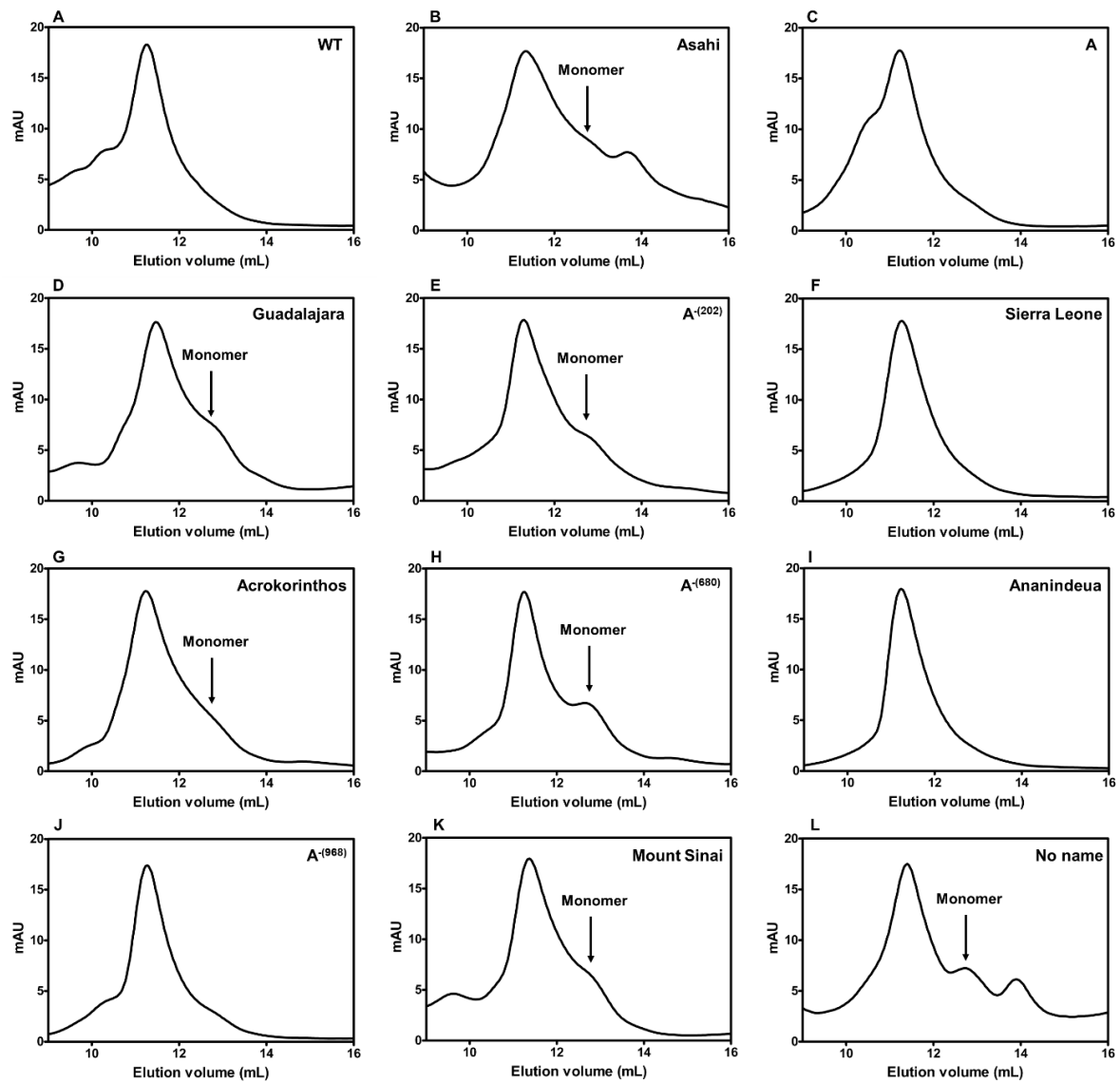


Figure 10. Size exclusion FPLC elution profiles purified recombinant G6PD variants. Proteins were loaded onto Superdex 200 Increase 10/300 equilibrated with 50 mM Tris-HCl and 150 mM NaCl.

Discussion

The molecular mechanisms underlying the clinical manifestations observed in individuals with G6PD deficiency can be attributed to reduced catalytic efficiency and/or decreased structural stability in the G6PD variants. Mutation location in the 3D protein structure is important and can cause decreased catalytic activity or protein stability. G6PD Asahi (Val68Met) was first reported in a 3-year-old Japanese boy who was referred to the hospital with jaundice and anemia, and showed clinical signs of acute hemolysis and only 30% G6PD activity [44]. Sequencing of the all coding exons and flanking regions of adjacent introns

did not reveal additional mutations, suggesting that the Val68Met mutation is entirely responsible for the observed clinical features. Previously, Town *et al.* reported that recombinant G6PD Asahi had specific activity and stability similar to that of WT G6PD and suggested that the Val68Met mutation alone was insufficient to cause G6PD deficiency [29]. However, in our study, we found that the Val68Met mutation causes conformational changes and destabilizes the G6PD protein structure, leading to approximately 10-fold reduction in catalytic efficiency. Our results are consistent with the severe clinical manifestations observed in a boy carrying the G6PD Asahi variant, indicating that a single mutation in G6PD Asahi itself is sufficient to cause severe enzyme deficiency [44].

G6PD A, a Class III variant, is a non-deficient variant with an Asn126Asp mutation, and presents with a frequency of 20% in Africa [28]. The residual enzyme activity in RBCs from patients with G6PD A is in the 70–85% range. Consistent with previously reported results, our results show that the catalytic activity and structural stability of G6PD A is similar to that of the WT enzyme [29]. Taken together, these results indicate that the Asn126Asp mutation does not disrupt G6PD enzyme activity and could explain why G6PD A is an asymptomatic variant and widely distributed in African countries. G6PD Guadalajara (Class I variant) contains a single Arg387His mutation that is wholly accountable for the severe enzyme deficiency (< 5% G6PD activity in RBCs) [30, 31]. Arg387 is located approximately 10°Å from the structural NADP⁺ binding site (**Figure 11**). The Arg387His mutation disrupts NADP⁺ binding, destabilizes the protein structure, and causes the formation of inactive monomers and a complete loss of enzyme activity. Indeed, structural NADP⁺ is known to stabilize the G6PD protein structure [23]. Structural stability is particularly important for enzymes in mature RBCs which are non-nucleated cells.

Since G6PD A is a neutral variant with no effect on enzyme activity and protein stability, we hypothesized that the additional mutation(s) are responsible for the enzyme deficiency and clinical manifestations observed in individuals with G6PD deficiency. To test this hypothesis and examine the cooperation between two and three mutations in G6PD variants, we performed a detailed functional and structural characterization of double and triple mutants carrying the Asn126Asp mutation (G6PD A). G6PD

A⁻⁽²⁰²⁾, a Class III G6PD variant commonly found in Africa, carries Val68Met and Asn126Asp mutations, with 12% residual G6PD activity in RBCs [34, 35]. A previous study suggested that both mutations are required to cause an enzyme deficiency [29]. It was described that both mutations synergistically contribute to protein instability to yield a dramatic effect on enzyme activity. Therefore, it was proposed that Val68Met can only cause enzyme deficiency when it is present in combination with G6PD A. However, our results show that the Val68Met mutation greatly contributes to the enzyme deficiency observed in individuals with the G6PD A⁻⁽²⁰²⁾ variant. By itself, the Val68Met mutation can disrupt protein structure, causing a significant reduction in protein stability and catalytic efficiency (approximately 10-fold). The G6PD No name variant has the same mutations as G6PD A⁻⁽²⁰²⁾ plus an additional mutation at residue 422 to give rise to a Class I variant with the most severe clinical phenotypes [33]. In this study, we have shown that that Leu422 is located approximately 5 Å from the structural NADP⁺ binding site (**Figure 11**). Mutation at G6PD position 422 disrupts the binding of structural NADP⁺, causing protein instability. The Leu422Val mutation has an additive effect on structural stability of G6PD No name, resulting in the complete loss of enzyme activity.

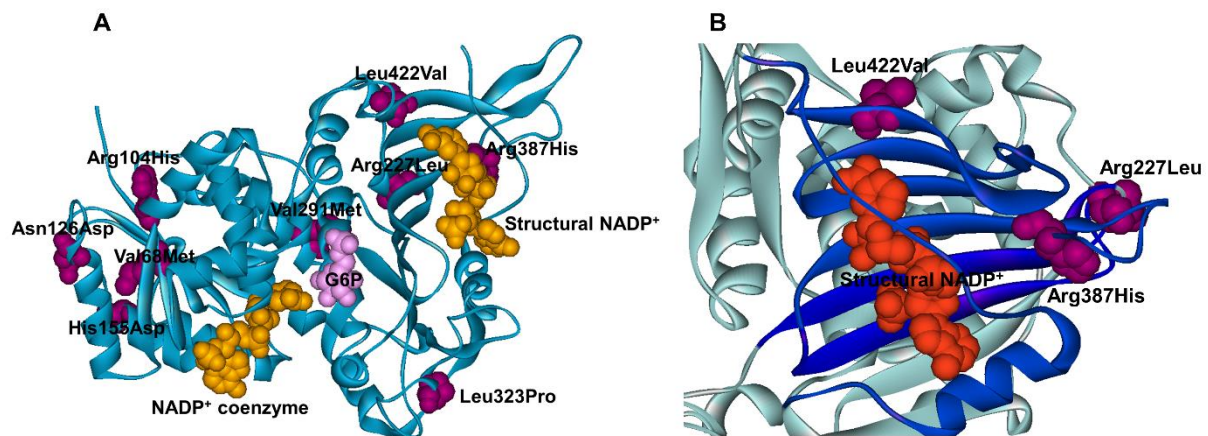


Figure 11. 3D structure of the G6PD protein. A) The monomeric structure of G6PD is shown with structural and coenzyme NADP⁺ binding sites (yellow) and the G6P site (pink). The mutants are shown as CPK representations and labeled. B) The structural NADP⁺ binding site is illustrated (dark blue). Residues Arg227, Arg387, and Leu422 are located within the NADP⁺ binding distance. The graphical illustration was generated using Discovery Studio Visualizer.

A combination of Asn126Asp (G6PD A) and Arg387His (G6PD Guadalajara) mutations causes the severe enzyme deficiency with hemolytic anemia observed in G6PD Mount Sinai [32]. Arg387 is part of the structural NADP⁺ binding domain (**Figure 11**), and the Arg387His mutation significantly interrupts protein stability, causing a marked decrease in enzyme activity. Therefore, we believe that the Arg387His mutation itself is responsible for the severe clinical manifestations observed in G6PD Mount Sinai. The G6PD A⁻⁽⁹⁶⁸⁾ double mutant is caused by the presence of a Leu323Pro mutation in a G6PD A context. The G6PD A⁻⁽⁹⁶⁸⁾ mutant is a Class III variant reported in Mexican populations and has less than 10% residual enzyme activity [35, 36]. Our study shows that the G6PD A⁻⁽⁹⁶⁸⁾ mutant is structurally unstable, resulting in a 5-fold reduction in catalytic activity. Indeed, a single Leu323Pro (G6PD Nefza) mutation was reported in Tunisia where the patient presented with hemolytic anemia triggered by bean [45]. Consistent with our results, it was previously suggested that G6PD Nefza itself was primarily responsible for functional and structural changes observed in the G6PD A⁻⁽⁹⁶⁸⁾ double mutant [46]. Structural instability also accounts for enzyme deficiency in Class II G6PD variant, G6PD Ananindeua. G6PD Ananindeua contains a combination of G6PD A and G6PD Viangchan mutations. The double mutant was reported in Amazonian populations and exhibited extremely low residual enzyme activity in RBCs (< 1%) [37]. G6PD Viangchan, a Class II variant, was previously characterized and was shown to have an extreme effect on protein stability, causing a significant decrease in catalytic efficiency [25]. G6PD Ananindeua is structurally unstable, and has even lower catalytic efficiency than does the G6PD Viangchan mutant. The severe clinical phenotypes observed in G6PD Ananindeua can be explained by G6PD Viangchan making a large contribution to the double mutant enzyme.

Three additional naturally occurring mutants in our study included G6PD Sierra Leone, G6PD Acrokorinthos, and G6PD A⁻⁽⁶⁸⁰⁾. To date, there are no reports of any of these single mutations (Arg104His, His155Asp, and Arg227Leu) occurred separately from the G6PD A variant and we did not create these non-natural single mutations. Biochemical and structural characterization of these double variants indicate that they are structurally unstable. The G6PD Sierra Leone mutant is classified as a Class III G6PD deficiency and is reported in African countries [38]. Similar to the common G6PD A⁻⁽²⁰²⁾ variant, the G6PD Sierra Leone

mutant exhibits low residual enzyme activity in RBCs [38]. However, our results demonstrate an approximate 24-fold reduction in G6PD Sierra Leone catalytic efficiency. In this variant, the presence of Arg104His and Asn126Asp mutants alters binding affinity of both substrates and contributes to trypsin susceptibility. G6PD Acrokorinthos, a Class II variant, was reported in Hellenic populations with severe deficiency and only 1% residual G6PD enzyme activity [39]. The severe deficiency could be explained by a secondary structural change in G6PD Acrokorinthos, leading to 3D conformational change ultimately observed in dimer dissociation and decreased protein stability. Together, this causes a 10-fold reduction in catalytic efficiency. The G6PD A⁽⁶⁸⁰⁾ mutant is found in African populations, causes mild deficiency, and is classified as a Class III variant [35]. A 6-fold decrease in the catalytic efficiency of G6PD A⁽⁶⁸⁰⁾ was observed in this study as a result of structural instability. This variant disrupts the protein secondary structure and causes the dissociation from active dimer to inactive monomer.

Conclusions

We described the functional and structural characteristics of 11 recombinant G6PD variants. We demonstrated that the protein dysfunction observed in G6PD deficient individuals is largely attributable to protein instability, which leads to a reduction in catalytic activity. The location of the mutation in the 3D structure affects the level of enzyme deficiency and leads to the clinical manifestations observed in the patients. The cooperative effect of two and three mutations causing a defect in protein function is evident in the G6PD variants with double and triple mutations. Importantly, when the second and third mutations are present in the context of the non-deficient G6PD A, these additional mutations play essential roles in enzyme deficiency.

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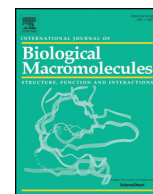
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ภาคผนวก



Functional and structural analysis of double and triple mutants reveals the contribution of protein instability to clinical manifestations of G6PD variants

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ABSTRACT

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common polymorphism and enzymopathy in humans, affecting approximately 400 million people worldwide. Over 200 point mutations have been identified in *g6pd* and the molecular mechanisms underlying the severity of G6PD variants differ. We report the detailed functional and structural characterization of 11 recombinant human G6PD variants: G6PD Asahi, G6PD A, G6PD Guadalajara, G6PD Acrokorinthos, G6PD Ananindeua, G6PD A⁻⁽²⁰²⁾, G6PD Sierra Leone, G6PD A⁻⁽⁶⁸⁰⁾, G6PD A⁻⁽⁹⁶⁸⁾, G6PD Mount Sinai and G6PD No name. G6PD Guadalajara, G6PD Mount Sinai and G6PD No name are inactive variants and, correlating with the observed clinical manifestations, exhibit complete loss of enzyme activity. Protein structural instability, causing a reduction in catalytic efficiency, contributes to the clinical phenotypes of all variants. In double and triple mutants sharing the G6PD A mutation, we observed cooperative interaction between two and three mutations to cause protein dysfunction. The G6PD A (Asn126Asp) mutation exhibits no effect on protein activity and stability, indicating that the additional mutations in these G6PD variants significantly contribute to enzyme deficiency. We provide insight into the molecular basis of G6PD deficiency, which can explain the severity of clinical manifestations observed in individuals with G6PD deficiency.

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1. Introduction

Glucose-6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49) is the first and rate-limiting enzyme in the pentose phosphate pathway, mediating the formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 6-phosphogluconolactone [1]. NADPH plays an essential role in maintaining cellular redox status and reduces oxidative stress via glutathione reduction [2]. G6PD is particularly important in non-nucleated red blood cells (RBCs), where it is the sole source of NADPH production. When NADPH levels cannot be maintained, the amount of reduced glutathione decreases, leading to oxidative damage, which can ultimately lead to hemolysis [3,4]. The *g6pd* gene is located on the X chromosome (Xq28) and is approximately 18 kb in length, consisting of 13 exons and 12 introns. The *g6pd* gene is highly polymorphic and over 200 mutations are reported [5–8]. Mutation in *g6pd* can cause G6PD deficiency, which affects approximately 400 million people worldwide [6,9]. Most G6PD variants are single point mutations and double, triple, and quadruple mutations have been reported with much lower frequencies [7]. G6PD deficiency is predominantly found in malaria-endemic areas and it is hypothesized that G6PD deficiency

confers protection against malarial infection. Patients with G6PD deficiency exhibit a wide spectrum of clinical features, including favism, hemolytic anemia, chronic non-spherocytic hemolytic anemia, and neonatal hyperbilirubinemia [10]. G6PD variants have been classified into five classes, based on residual enzyme activity in RBCs and clinical phenotypes [11,12]. These classes range from Class I G6PD variants, which show <5% residual enzyme activity and exhibit the most severe clinical phenotypes, to Class V G6PD variants, which show increased enzyme activity and no clinical manifestations. In individuals with G6PD deficiency, hemolysis can be triggered by infections, stress, food, and certain medications, including antimalarials such as primaquine, tafenoquine, chloroquine, dapson, and sulfanilamide [13]. Therefore, the hemolytic toxicity of G6PD deficiency has a significant impact on malaria treatment, especially in the radical cure of *Plasmodium vivax* and *P. ovale* using primaquine and tafenoquine [14–18]. The severity of hemolytic anemia varies among individuals with G6PD deficiency, making malaria treatment even more complicated and challenging.

The G6PD protein consists of 514 amino acids (approximately 60 kDa) and is active as a dimer or tetramer. The G6PD three-dimensional (3D) structures (PDB ID: 2BHL, 1QKI and 6E08) show that each subunit binds one molecule of oxidized nicotinamide adenine dinucleotide phosphate (NADP⁺), stabilizing the protein structure [19–21]. The molecular mechanisms underlying the clinical phenotypes

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of G6PD deficiency depend on the location of the mutation in the 3D structure of the protein. Depending on the mutation location, it can affect protein assembly, dimer formation and stability, binding affinity to substrates and catalytic efficiency [22]. Functional and structural analyses of G6PD variants suggest that mutations at or near the dimer interface and structural NADP⁺ binding site cause protein instability and mostly result in severe deficiency (Class I G6PD variants) [21,23–27]. Therefore, knowing the effects of mutations on protein function is crucial for understanding the molecular mechanisms underlying the clinical phenotypes observed in individuals with G6PD deficiency. This knowledge can also be beneficial for predicting drug response in malaria treatment.

G6PD A (Asn126Asp), a single mutant commonly found in African populations with a frequency of 20%, is considered a non-deficient variant with no clinical manifestations. [28]. It has been reported that the Asn126Asp mutation does not affect the catalytic activity or structural stability of G6PD [29]. However, the presence of additional mutation (s) has been found to cause severe enzyme deficiency in people carrying double and triple G6PD mutants sharing the G6PD A mutation [30–39]. This study was designed to investigate the cooperative interactions between two and three mutations that cause severe enzyme deficiency in G6PD variants sharing the Asn126Asp mutation. We report detailed biochemical and structural analyses of 11 natural G6PD variants, including Class I (G6PD Guadalajara, G6PD Mount Sinai and, G6PD No name), Class II (G6PD Acrokorinthos and G6PD Ananindeua), and Class III (G6PD A, G6PD Asahi, G6PD A⁻⁽²⁰²⁾, G6PD Sierra Leone, G6PD A⁻⁽⁶⁸⁰⁾ and G6PD A⁻⁽⁹⁶⁸⁾) variants. We also investigated the synergistic interaction between the mutations in the double and triple mutants, G6PD Acrokorinthos (Asn126Asp + His155Asp), G6PD Ananindeua (Asn126Asp + Val291Met), G6PD A⁻⁽²⁰²⁾ (Asn126Asp + Val68Met), G6PD Sierra Leone (Asn126Asp + Arg104His), G6PD A⁻⁽⁶⁸⁰⁾ (Asn126Asp + Arg227Leu), G6PD A⁻⁽⁹⁶⁸⁾ (Asn126Asp + Leu323Pro), G6PD Mount Sinai (Asn126Asp + Arg387His) and, G6PD No name (Asn126Asp + Val68Met + Leu422Val), which share the Asn126Asp mutation (G6PD A). These results were interpreted in the context of the observed clinical manifestations.

2. Materials and methods

2.1. Site-directed mutagenesis

G6PD variants were created using site-directed mutagenesis. The polymerase chain reaction (PCR) mixture (50 µl) contained 1× KAPA HiFi reaction buffer, 50 ng of template plasmid (pET28a-G6PD wild type (WT)), 100 ng of forward and reverse primers, 0.3 µM of each dNTP, and 1 U of KAPA HiFi DNA polymerase (Kapa Biosystems, MA, USA). The primers used for site-directed mutagenesis are listed in Table S1. The cycling parameters were: 1 cycle of 95 °C for 5 min and 16 cycles of 98 °C for 20 s, 55 °C for 15 s, and 68 °C for 3 min and 30 s.

PCR products were digested with *DpnI* to remove the parental plasmid and were then transformed into DH5α competent cells. The presence of the desired mutations was confirmed by DNA sequencing. Recombinant plasmids harboring desired mutations were transformed into the BL21 (DE3) expression host for protein expression.

2.2. Protein expression and purification

Expression and purification of G6PD variants were performed as described previously [24,25]. Fresh overnight cultures of *E. coli* BL21 (DE3) harboring recombinant plasmid were grown in LB media containing 50 µg/ml kanamycin at 37 °C with 250 rpm shaking. When the OD₆₀₀ reached 1.0, G6PD expression was induced with isopropyl β-D-thiogalactopyranoside at a final concentration of 1 mM. The cultures were further incubated at 20 °C with 200 rpm shaking for 20 h before harvesting by centrifugation at 8300 ×g for 10 min.

For protein purification, the pellets were resuspended in lysis buffer (20 mM sodium phosphate buffer pH 7.4, 10 mM imidazole, and 300 mM NaCl), disrupted by sonication for 5 min, and centrifuged at 36,000 ×g for 1 h. Protein was purified using immobilized metal affinity chromatography by incubating with TALON cobalt resin for 1 h and washing the unbound proteins using wash buffer (20 mM sodium phosphate buffer pH 7.4, 20 mM imidazole, and 300 mM NaCl). Thereafter, the protein was eluted using an imidazole concentration gradient (40–400 mM). The fractions containing the desired protein were pooled. Imidazole was removed by overnight dialysis against 20 mM Tris-HCl pH 7.5 containing 10% glycerol. The purity of the obtained protein was determined by 12% SDS-PAGE staining with Coomassie blue R250. Finally, protein concentration was measured using the Bradford assay [40].

2.3. Determination of steady-state kinetic parameters

G6PD activity was measured spectrophotometrically by monitoring NADP⁺ reduction at 340 nm and 25 °C as described previously [24,25]. The steady-state kinetic properties of the recombinant human G6PD WT and G6PD mutants were determined in 1 ml cuvette. The standard reaction mixtures contained 20 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 100 µM NADP⁺ and 500 µM glucose-6-phosphate (G6P). The reaction was initiated with the addition of the enzyme. To determine the K_m for G6P, the G6P concentration was varied from 5 to 1000 µM and the NADP⁺ concentration was fixed at 100 µM. The K_m for NADP⁺ was also measured by varying the NADP⁺ concentrations from 1 to 500 µM while fixing the G6P concentration at 500 µM. The experiments were performed in triplicate. Steady-state kinetic parameters, K_m , k_{cat} and V_{max} , were obtained by fitting the collected data to the Michaelis-Menten equation using GraphPad Prism software.

2.4. Determination of the secondary structure by circular dichroism (CD)

The secondary structure of the G6PD WT and other variants were determined spectroscopically by CD, as previously described [24,25]. Far UV-CD spectra of the G6PD variants at a protein concentration of 0.25 mg/ml were recorded using a Jasco spectrometer, model J-815, equipped with a Peltier temperature control system in a 1 mm path-length quartz cuvette at 25 °C. The CD spectra were collected over a wavelength range of 190–260 nm at a scan rate of 50 nm/min. Five scans were averaged for each sample and the buffer scan results were subtracted.

2.5. Intrinsic fluorescence and 8-anilino-1-naphthalene-sulfonic acid (ANS)-binding

For intrinsic fluorescence analysis, we used an excitation wavelength of 295 nm, and the emission spectra were recorded in the 300–400 nm range using a Synergy H1 hybrid reader (BioTek, VT, USA) in a 96-well plate at 25 °C. The ability of G6PD proteins to bind to ANS was assessed. ANS (100 µM) was used for sample preparation and the G6PD concentration was adjusted to 0.1 mg/ml. G6PD proteins were incubated with ANS at 25 °C for 1 h then the emission spectra at an excitation wavelength of 395 nm were monitored between 400 and 600 nm.

2.6. Thermal stability

In a 20 µl reaction mixture, we mixed 5 µg G6PD enzyme, 5 × SYPRO Orange reporter dye and various concentrations of NADP⁺ (0, 10, and 100 µM). The reaction mixtures were heated at temperatures ranging from 20 to 80 °C using a LightCycler 480 real-time PCR machine (Roche, Mannheim, Germany) with excitation and emission of 465 nm and 580 nm, respectively. The experiments were performed in triplicate. The melting temperature (T_m) was defined as the temperature at

Table 1
Kinetic parameters of recombinant G6PD variants.

Construct	Class	Amino acid change	k_{cat} (s^{-1})	K_m G6P (μM)	K_m NADP ⁺ (μM)	k_{cat}/K_m G6P ($\mu M^{-1} s^{-1}$)	k_{cat}/K_m NADP ⁺ ($\mu M^{-1} s^{-1}$)
WT	–	–	202.4 ± 11.5	58.6 ± 8.1	8.7 ± 1.5	6.2 ± 1.2	23.3 ± 5.3
Asahi	III	Val68Met	30.1 ± 1	48.6 ± 5.1	8.9 ± 0.9	0.6 ± 0.1	3.4 ± 0.5
A	III	Asn126Asp	131 ± 11	66 ± 8	19 ± 5	1.7 ± 0.25	6.9 ± 2.4
A ⁻⁽²⁰²⁾	III	Asn126Asp + Val68Met	11.4 ± 0.8	43.2 ± 6.2	12 ± 3	0.3 ± 0.1	1 ± 0.3
Sierra Leone	III	Asn126Asp + Arg104His	14.5 ± 0.6	81.3 ± 6.1	14.5 ± 1.9	0.25 ± 0.03	1 ± 0.2
Acrokorinthos	II	Asn126Asp + His155Asp	19.3 ± 0.3	38.1 ± 3.8	8.4 ± 0.6	0.5 ± 0.1	2.3 ± 0.2
A ⁻⁽⁶⁸⁰⁾	III	Asn126Asp + Arg227Leu	50 ± 2	54 ± 6	12 ± 2	1 ± 0.1	4 ± 0.8
Ananindeua	II	Asn126Asp + Val291Met	22.3 ± 0.7	58.3 ± 6.9	18 ± 2.3	0.4 ± 0.1	1.2 ± 0.2
A ⁻⁽⁹⁶⁸⁾	III	Asn126Asp + Leu323Pro	37.4 ± 1.5	37.5 ± 3.9	18.2 ± 2.3	1 ± 0.1	2.1 ± 0.3
Guadalajara	I	Arg387His	ND	ND	ND	ND	ND
Mount Sinai	I	Asn126Asp + Arg387His	ND	ND	ND	ND	ND
No name	I	Asn126Asp + Val68Met + Leu422Val	ND	ND	ND	ND	ND

ND: not determined.

which half of the secondary structure unfolded and was calculated for each G6PD variant.

2.7. Thermal inactivation

Thermal inactivation analysis was performed as previously described [24,25]. In brief, 6 μ g of G6PD protein was incubated with varying concentrations of NADP⁺ (0, 10, and 100 μ M) at temperatures ranging from 25 to 65 °C for 20 min and then cooled to 4 °C in a Thermocycler (Eppendorf, Hamburg, Germany). Residual enzyme activity was determined and expressed as a percentage of the activity of the same enzyme incubated at 25 °C.

2.8. Protein stability in the presence of guanidine hydrochloride (Gdn-HCl)

The protein sample (6 μ g) was incubated with varying concentrations of NADP⁺ (0, 10, and 100 μ M) in the presence of different concentrations of Gdn-HCl (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, and 0.5 M) at 37 °C for 2 h. Thereafter, residual enzyme activity was measured and expressed as a percentage of the activity of the same enzyme incubated at 25 °C in the absence of Gdn-HCl.

2.9. Susceptibility of G6PD variants to trypsin digestion

The susceptibility of G6PD proteins to trypsin was determined in the presence of different NADP⁺ concentrations (0, 10, and 100 μ M). Recombinant G6PD protein (6 μ g) was incubated with trypsin (final concentration 0.5 mg/ml or 1250 U/ml) for 5 min at 25 °C. Residual enzyme activity was assessed and expressed as a percentage of the enzyme activity in the absence of trypsin.

2.10. Size exclusion chromatography of G6PD variants

The oligomeric state of the G6PD variants was determined by size exclusion chromatography using AKTA fast protein liquid chromatography (FPLC) equipped with the Superdex 200 Increase 10/300 column (GE Healthcare, NJ, USA). Recombinant protein (100 μ g) was loaded onto the pre-equilibrated column. Chromatography was performed at a flow rate of 0.5 ml/min using 50 mM Tris-HCl pH 7.5, and 150 mM NaCl. The column was calibrated with blue dextran (> 2000 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and RNase A (13.7 kDa).

3. Results

3.1. Biochemical characteristics of G6PD variants

To understand the molecular mechanisms underlying the clinical manifestations of G6PD deficiency, 11 recombinant human G6PD

variants (Table 1) were created using site-directed mutagenesis. Recombinant G6PD proteins were expressed in *E. coli* BL21 (DE3), purified to homogeneity using immobilized metal affinity chromatography and visualized using SDS-PAGE analysis (Fig. S1). Of the 11 variants, G6PD Asahi, G6PD Guadalajara, G6PD Mount Sinai, and G6PD No name precipitated severely during purification, indicating their structural instability. Additionally, G6PD Acrokorinthos did not bind well to the cobalt resin during purification, which may be the result of conformational change caused by the mutations.

The effects of mutation on the catalytic activity of each G6PD variant were determined (Table 1 and Fig. S2). The enzyme activity of G6PD Guadalajara, G6PD Mount Sinai and G6PD No name was <1%. These three G6PD variants are Class I variants, and individuals with these variants exhibit severe G6PD deficiency with <5% residual enzyme activity in RBCs [30–33]. Consistent with previous reports and clinical phenotypes, G6PD A, a Class III variant commonly found in African populations has lower k_{cat} and k_{cat}/K_m values, but slightly higher K_m values, than does G6PD WT [29,41]. Class II G6PD variants, G6PD Acrokorinthos and G6PD Ananindeua, have comparable binding affinities toward G6P and NADP⁺ but have a 10-fold reduction in catalytic efficiency compared with G6PD WT. A significant decrease in the catalytic efficiency of G6PD Acrokorinthos and G6PD Ananindeua might be attributable to structural changes, discussed below. All Class III G6PD variants, G6PD Asahi, G6PD A⁻⁽²⁰²⁾, G6PD Sierra Leone, G6PD A⁻⁽⁶⁸⁰⁾ and G6PD A⁻⁽⁹⁶⁸⁾, show similar K_m values for G6P and NADP⁺ to that of the G6PD WT, with the exception of G6PD Sierra Leone, which exhibits a moderately increased K_m . The Val68Met mutation causes a significant decrease (approximately 10-fold) in G6PD Asahi catalytic efficiency compared with that of the native enzyme. A marked decrease in catalytic efficiency

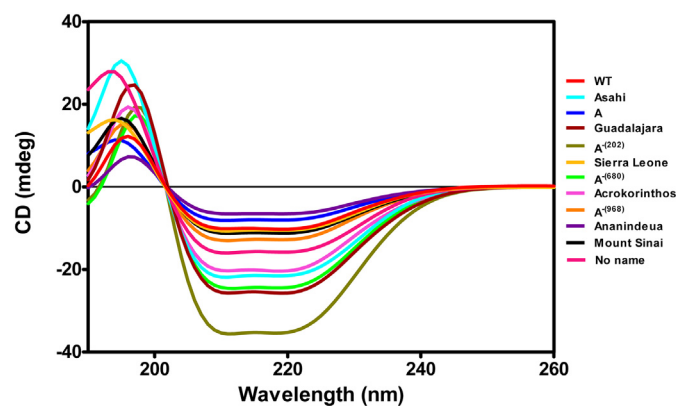


Fig. 1. Far-UV CD spectra of recombinant human G6PD variants. The protein concentration was 0.25 mg/ml and CD spectra were collected over a wavelength range of 190–260 nm at a scan rate of 50 nm/min using a Jasco spectrometer, model J-815.

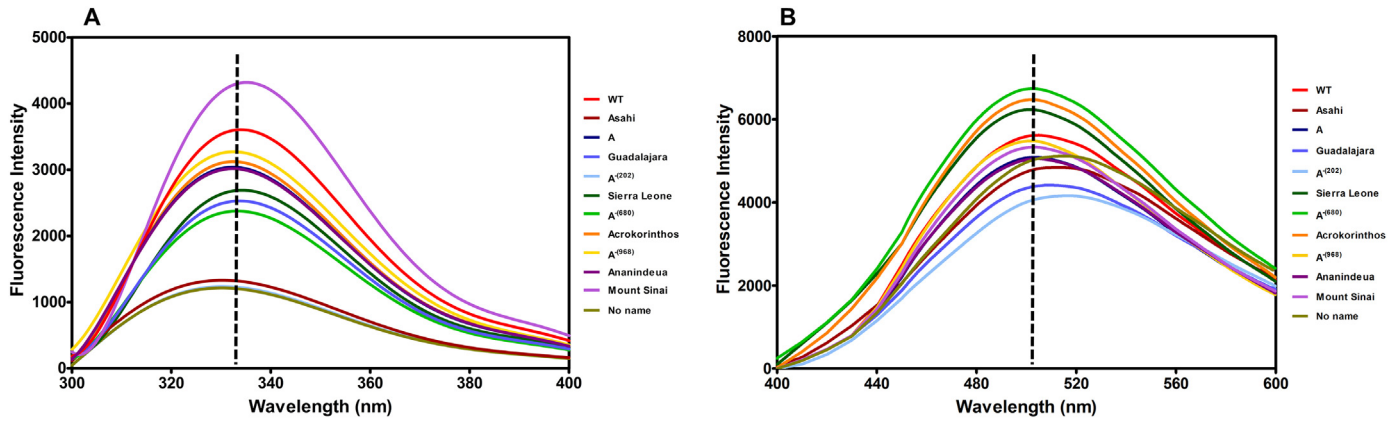


Fig. 2. Fluorescence emission spectra of recombinant human G6PD variants. (A) Intrinsic fluorescence spectra. (B) ANS fluorescence spectra. G6PD concentration was 0.1 mg/ml. For intrinsic fluorescence spectra, an excitation wavelength of 295 nm was used and the emission spectra were recorded in the 300–400 nm range. For ANS fluorescence spectra, G6PD proteins were incubated with 100 μ M ANS at 25 $^{\circ}$ C for 1 h and emission spectra between 400 and 600 nm were monitored using an excitation wavelength of 395 nm.

(~20-fold) was observed for G6PD A⁽²⁰²⁾ and G6PD Sierra Leone while a moderate reduction in catalytic efficiency (~6-fold) was noted for G6PD A⁽⁶⁸⁰⁾ and G6PD A⁽⁹⁶⁸⁾. A significant decrease in G6PD A⁽²⁰²⁾ catalytic activity might be attributed to the presence of the Val68Met mutation.

3.2. Structural characteristics of G6PD variants

To assess the effect of mutation on secondary structure, CD spectra of G6PD WT and G6PD variants were recorded in the far-UV region

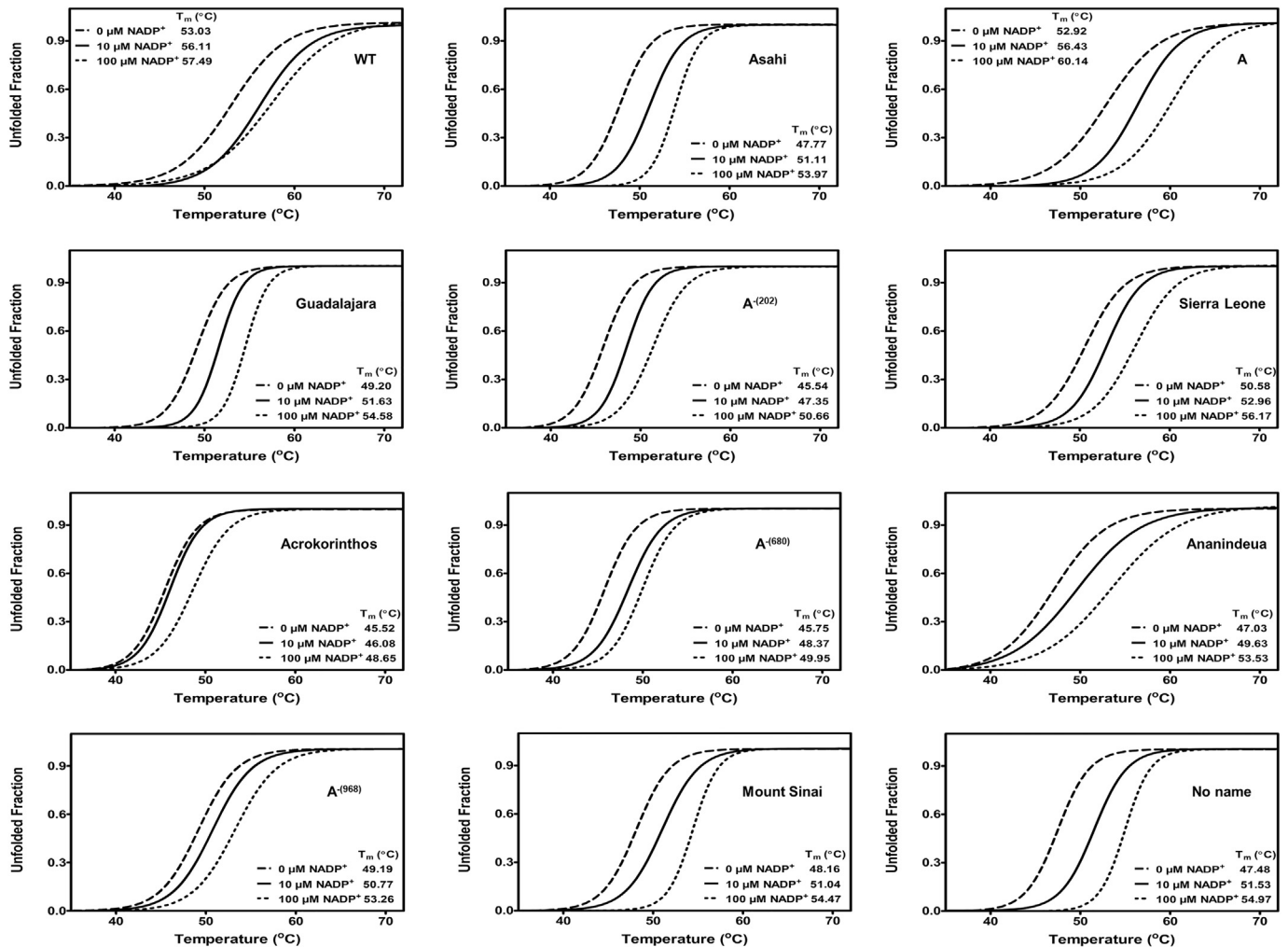


Fig. 3. Thermal stability analysis of recombinant human G6PD variants. Proteins were heated in the presence of 5 x SYPRO Orange reporter dye and various NADP⁺ concentrations (0, 10, and 100 μ M). Protein unfolding was monitored by following the emission of fluorescence dye at 580 nm. Experiments were performed in triplicate.

(190–260 nm) (Fig. 1). All G6PD variants exhibited two negative peaks, at 208 and 222 nm, which are characteristic of α -helical proteins, including G6PD [21]. Based on our CD spectra results, the overall G6PD structure was maintained in the analyzed variants. However, when compared with the WT protein, a change in signal intensity was observed for all mutants except G6PD Sierra Leone and G6PD Mount Sinai. A change in signal intensity without a change in CD spectral pattern indicates that the chirality of the chromophores is modified upon mutation and provides information about the stability (flexibility or rigidity) of the secondary structure. The stability of G6PD A, G6PD Ananindeua, and G6PD A⁻⁽⁹⁶⁸⁾ secondary structure was slightly altered and a significant change was observed for G6PD Asahi, G6PD Acrokorinthos, G6PD A⁻⁽⁶⁸⁰⁾, and G6PD Guadalajara. Interestingly, the combination of Val68Met and Asn126Asp mutations resulted in a change in the rigidity of the secondary structure of the G6PD A⁻⁽²⁰²⁾ variant, which may contribute to severe G6PD deficiency and cause the clinical phenotypes observed. A previous study suggested that impaired protein folding, leading to a reduction in the number of active G6PD enzymes, was the cause of the deficiency observed in the G6PD A⁻⁽²⁰²⁾ variant [26,29]. Additionally, secondary structure alteration was described as a cause of G6PD deficiency for other G6PD variants [24,42].

G6PD contains seven tryptophan residues per monomer. Therefore, the effect of mutations on protein structure can be examined by monitoring tryptophan residue intrinsic fluorescence emission. The fluorescence emission spectra of G6PD variants and G6PD WT were examined (Fig. 2A). All G6PD variants displayed similar emission spectra to that of the WT enzyme, with emission maxima at 330 nm. However, G6PD Mount Sinai showed greater fluorescence intensity than did the WT, while all other G6PD variants exhibited less fluorescence intensity than did the WT protein. A decrease in fluorescence intensity could be attributed to electron transfer quenching by the local peptide

carbonyl group or by neighboring amino acid side chains as a result of conformational change or interactions with ligands [43]. Hydrophobic interactions play an important role in defining protein conformation and interactions in the protein structure. Therefore, ANS, a hydrophobic probe, was used to assess the conformational changes in G6PD variants (Fig. 2B). All G6PD variants shared similar ANS emission spectra to that of the WT protein with emission maxima of 505 nm, except for G6PD Asahi, G6PD A⁻⁽²⁰²⁾, G6PD Guadalajara and G6PD No name, which showed emission maxima shift to a longer wavelength (515 nm). A change in extrinsic fluorescence intensity indicates an increase or decrease in the number of binding sites for ANS molecules, which could be attributed to changing surface hydrophobicity. These results suggest that these mutations cause conformational changes in the protein structure, which may partially contribute to the severe clinical manifestations observed in individuals with G6PD deficiency.

3.3. Structural stability of G6PD variants

Reduced structural stability is also responsible for enzyme deficiency in individuals with G6PD deficiency. The effects of mutation on the structural stability of G6PD variants were investigated. Fluorescence-based thermal shift assay was performed in the absence and presence of different NADP⁺ concentrations (0, 10 and 100 μ M) to assess thermal stability (Fig. 3 and Table S2). In the absence of NADP⁺, G6PD A showed a T_m value (52.92 $^{\circ}$ C) comparable to that of the G6PD WT (53.03 $^{\circ}$ C) while in the presence of 100 μ M NADP⁺, a higher T_m (60.14 $^{\circ}$ C) than that of the G6PD WT (T_m 57.49 $^{\circ}$ C) was observed, suggesting a greater stabilizing effect of 100 μ M NADP⁺ on G6PD A. All other G6PD variants exhibited lower T_m values than did the WT protein, indicating the structural instability of these variants in the absence of NADP⁺. G6PD Acrokorinthos, G6PD A⁻⁽²⁰²⁾ and G6PD A⁻⁽⁶⁸⁰⁾ are the least stable

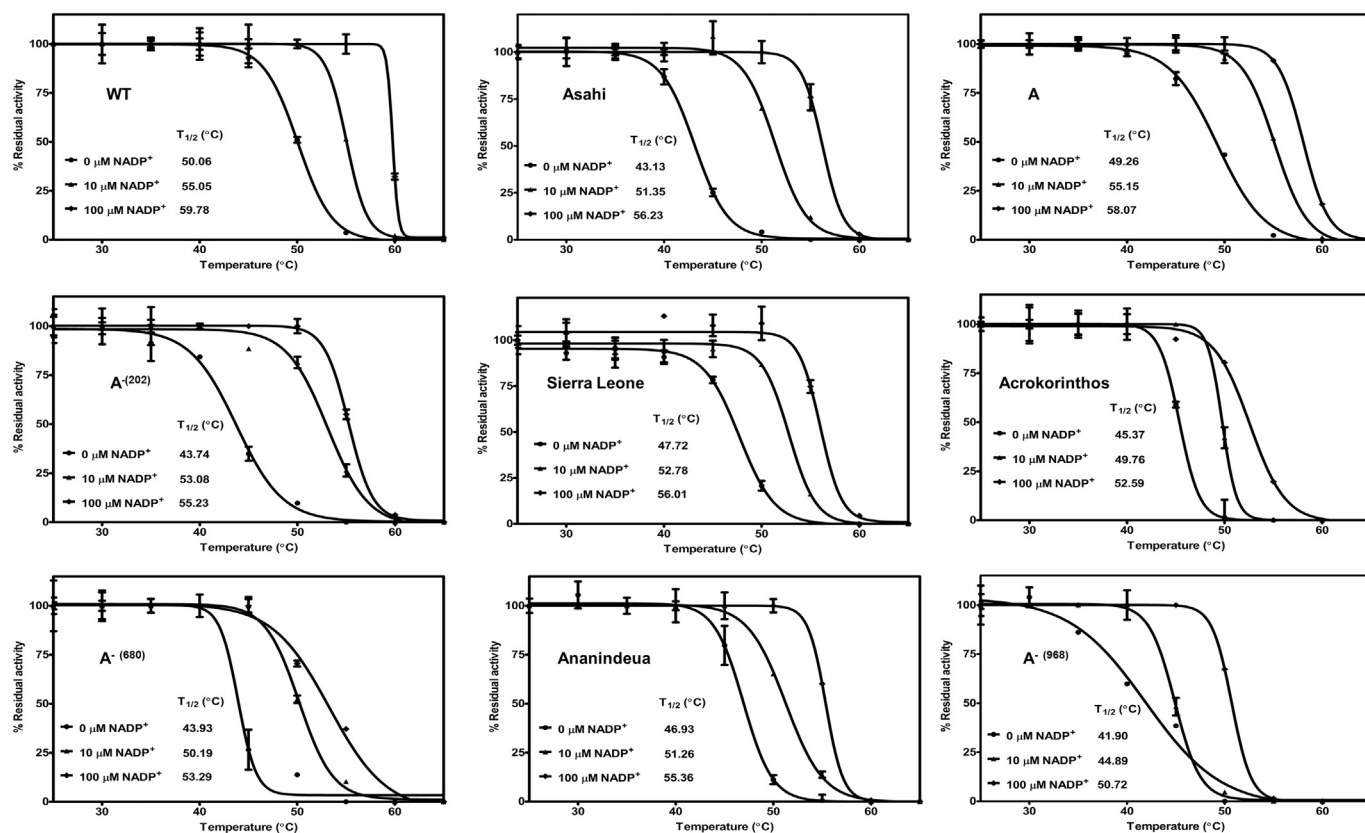


Fig. 4. Thermal inactivation analysis of recombinant human G6PD variants. Enzyme activity was measured after the protein was heated in the presence of various NADP⁺ concentrations (0, 10, and 100 μ M) for 20 min. $T_{1/2}$ is the temperature at which the enzyme loses 50% of its activity. Error bars represent mean \pm SD of triplicate measurements.

G6PD variants with T_m values of approximately 45 °C. G6PD Ananindeua, a Class II G6PD variant, also showed a marked reduction in protein structural stability with a T_m of 47.03 °C in the absence of NADP^+ , approximately 6 °C lower than that of the WT enzyme. The presence of 100 μM NADP^+ improved the protein stability of G6PD Ananindeua (T_m 53.53 °C) significantly. The Val68Met mutation moderately affects the structural stability of G6PD Asahi, which had a T_m of 47.77 °C in the absence of NADP^+ . G6PD Sierra Leone and G6PD A⁻⁽⁹⁶⁸⁾, the other two G6PD variants belonging to the Class III deficiency category, exhibited only a slight and moderate effect on protein structural stability, respectively. The structural stability of Class I G6PD variants—G6PD Guadalajara, G6PD Mount Sinai and G6PD No name decreased moderately, with a T_m approximately 4–5 °C lower than that of the WT enzyme. Consistent with previously reported results, the presence of NADP^+ improved protein stability, and increasing NADP^+ concentration resulted in higher T_m values for all G6PD proteins [24,25,42].

3.4. Thermal inactivation assays

Thermal inactivation assays were also performed to assess the stability of the active site of G6PD variants upon increasing temperature. After incubation at temperatures ranging from 25 to 65 °C for 20 min, residual enzyme activity was measured in the absence and presence of different NADP^+ concentrations (Fig. 4 and Table S3). Consistent with our thermal stability analysis results, G6PD A had similar $T_{1/2}$ (49.26 °C) to the G6PD WT ($T_{1/2}$ 50.06 °C) and the activity of all other G6PD variants decreased in the absence of NADP^+ . Compared with the WT protein, a 5–8 °C decrease in $T_{1/2}$ was observed for G6PD Asahi, G6PD A⁻⁽²⁰²⁾, G6PD Acrokorinthos, G6PD A⁻⁽⁶⁸⁰⁾, and G6PD A⁻⁽⁹⁶⁸⁾. A 2–3 °C decrease

in $T_{1/2}$ was observed for G6PD Ananindeua and G6PD Sierra Leone in the absence of NADP^+ . These results show that NADP^+ improves G6PD protein stability and increasing NADP^+ concentration results in higher $T_{1/2}$ values.

3.5. Residual enzyme activity of G6PD variants upon Gdn-HCl treatment

Tertiary protein structure unfolding causes a decline in enzyme activity. Therefore, residual enzyme activity in the absence and presence of different NADP^+ concentrations was determined as a function of Gdn-HCl concentration (Fig. 5 and Table S4). The loss of G6PD A, G6PD Sierra Leone and G6PD A⁻⁽⁹⁶⁸⁾ tertiary structure occurred similarly to that observed for the WT enzyme. The tertiary structure of G6PD Asahi, G6PD A⁻⁽²⁰²⁾, G6PD A⁻⁽⁶⁸⁰⁾ and G6PD Ananindeua was disrupted, and half of their enzyme activity was lost at 0.09–0.13 M Gdn-HCl. G6PD Acrokorinthos was the variant most susceptible to Gdn-HCl treatment and half of its activity was lost at a concentration as low as 0.02 M in the absence of NADP^+ . As NADP^+ is essential for G6PD protein stability, higher concentrations of NADP^+ resulted in higher $C_{1/2}$ values for all G6PD variants.

3.6. Susceptibility of G6PD variants to trypsin digestion

We determined the susceptibility of each G6PD variant to trypsin digestion (Fig. 6). The WT enzyme lost approximately 60% of its activity during trypsin treatment in the absence of NADP^+ and the residual enzyme activity was increased to 92% and 100% in the presence of 10 and 100 μM NADP^+ , respectively. G6PD variants, G6PD A, G6PD A⁻⁽²⁰²⁾, G6PD Sierra Leone, G6PD A⁻⁽⁶⁸⁰⁾ and G6PD A⁻⁽⁹⁶⁸⁾, were significantly susceptible to trypsin digestion and their residual enzyme activities

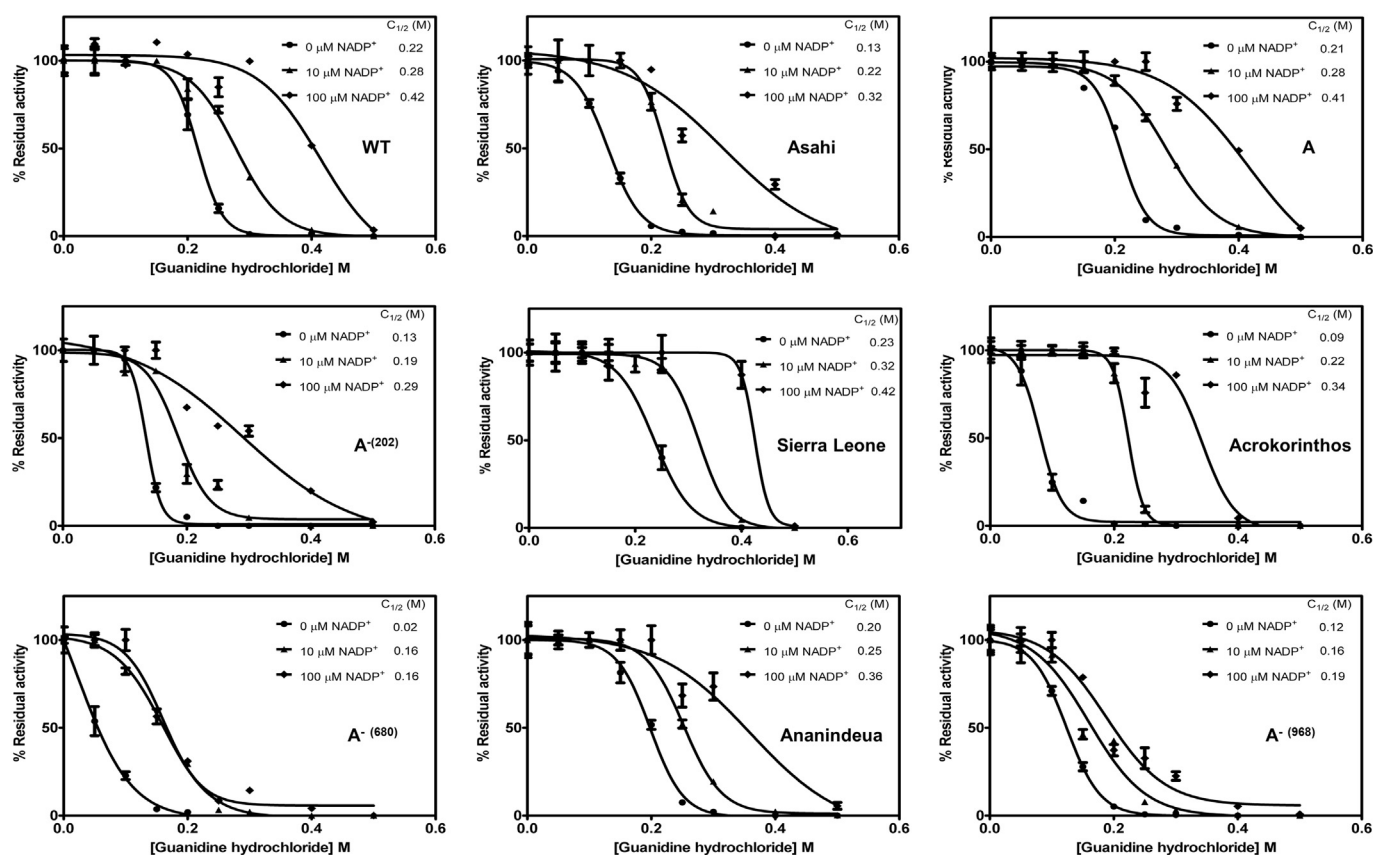


Fig. 5. Stability analysis of recombinant human G6PD variants upon Gdn-HCl treatment. Enzymatic activity was measured after incubation with various concentrations of Gdn-HCl and in the presence of various NADP^+ concentrations (0, 10, and 100 μM) at 37 °C for 2 h. Residual enzyme activity is expressed as a percentage of the activity for the same enzyme incubated at 25 °C in the absence of Gdn-HCl. $C_{1/2}$ is the Gdn-HCl concentration at which the enzyme loses 50% of its activity. Error bars represent mean \pm SD of triplicate measurements.

were between 5 and 8% in the absence of NADP^+ . The presence of NADP^+ enhanced the structural stability of these G6PD variants. G6PD Asahi, G6PD Acrokorinthos and G6PD Ananindeua were moderately susceptible to trypsin digestion. Nonetheless, the presence of NADP^+ increased the structural stability of G6PD Asahi significantly, but this was less prominent for G6PD Acrokorinthos and G6PD Ananindeua.

3.7. Subunit structure of G6PD variants

Since native human G6PD exists as a dimer or tetramer, size exclusion chromatography was performed to determine the effect of mutations on the subunit structure of G6PD variants (Figs. 7 and S3). G6PD WT, G6PD A, G6PD Sierra Leone, G6PD Ananindeua and G6PD $\text{A}^{-(968)}$ primarily existed as dimers. Both dimeric and monomeric forms of G6PD Asahi, G6PD Guadalajara, G6PD $\text{A}^{-(202)}$, G6PD Acrokorinthos, G6PD $\text{A}^{-(680)}$, G6PD Mount Sinai and G6PD No name were observed.

These results indicate that the dimeric structure of G6PD Asahi, G6PD Guadalajara, G6PD $\text{A}^{-(202)}$, G6PD Acrokorinthos, G6PD $\text{A}^{-(680)}$, G6PD Mount Sinai and G6PD No name was destabilized, and that the enzyme subunits dissociate into monomers.

4. Discussion

The molecular mechanisms underlying the clinical manifestations observed in individuals with G6PD deficiency can be attributed to reduced catalytic efficiency and/or decreased structural stability in the G6PD variants. Mutation location in the 3D protein structure is important and can cause decreased catalytic activity or protein stability. G6PD Asahi (Val68Met) was first reported in a 3-year-old Japanese boy who was referred to the hospital with jaundice and anemia, and showed clinical signs of acute hemolysis and only 30% G6PD activity [44]. Sequencing of the coding exons and flanking regions of adjacent

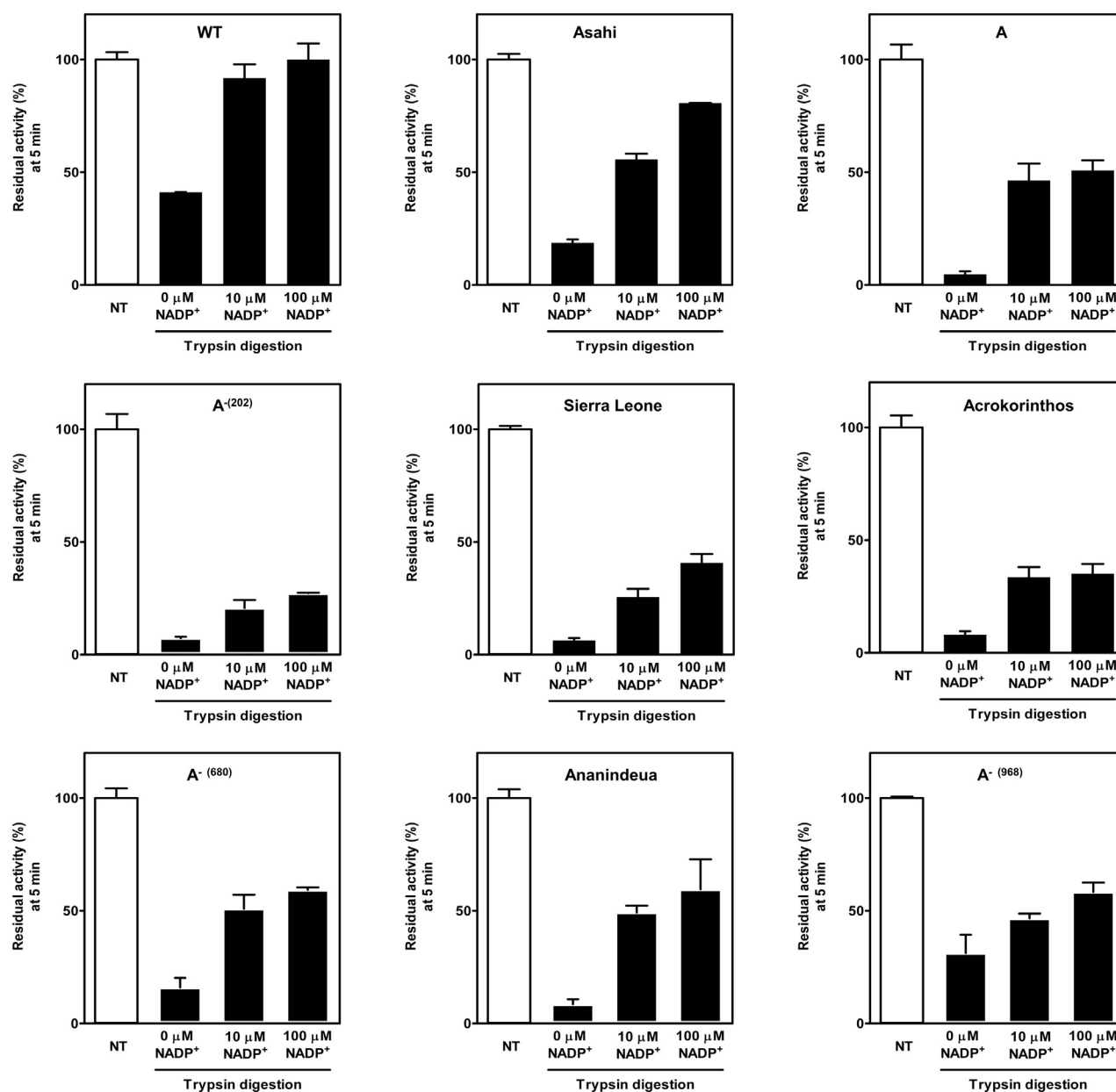


Fig. 6. Recombinant human G6PD variant susceptibility to trypsin digestion. Enzymatic activity was measured after incubation with 0.5 mg/ml trypsin (1250 U/ml) in the presence of various NADP^+ concentrations (0, 10, and 100 μM) at 25 °C for 5 min. Residual enzyme activity is expressed as a percentage of the activity for the same enzyme in the absence of trypsin. Error bars represent the mean ± SD of triplicate measurements. NT: no treatment.

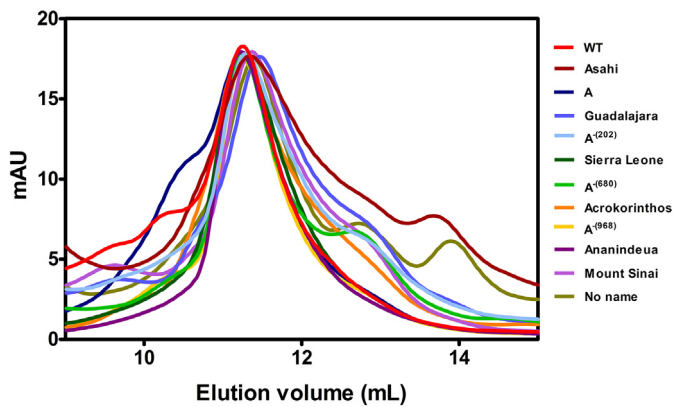


Fig. 7. Size exclusion FPLC elution profiles of purified recombinant G6PD variants. Proteins were loaded onto Superdex 200 Increase 10/300 equilibrated with 50 mM Tris-HCl and 150 mM NaCl.

introns did not reveal additional mutations, suggesting that the Val68Met mutation is entirely responsible for the observed clinical features. Previously, Town *et al.* reported that recombinant G6PD Asahi had specific activity and stability similar to that of WT G6PD and suggested that the Val68Met mutation alone was insufficient to cause G6PD deficiency [29]. However, our study found that the Val68Met mutation causes conformational changes and destabilizes the G6PD protein structure, leading to approximately 10-fold reduction in catalytic efficiency. Our results are consistent with the severe clinical manifestations observed in a boy carrying the G6PD Asahi variant, indicating that a single mutation in G6PD Asahi itself is sufficient to cause severe enzyme deficiency [44].

G6PD A, a Class III variant, is a non-deficient variant with an Asn126Asp mutation, and presents with a frequency of 20% in Africa [28]. The residual enzyme activity in RBCs from patients with G6PD A is in the 70–85% range. Consistent with previously reported results, our results show that the catalytic activity and structural stability of G6PD A is similar to that of the WT enzyme [29]. Taken together, these results indicate that the Asn126Asp mutation does not disrupt G6PD enzyme activity and could explain why G6PD A is an asymptomatic variant and widely distributed in African countries. G6PD Guadalajara (Class I variant) contains a single Arg387His mutation that is wholly accountable for the severe enzyme deficiency (< 5% G6PD activity in RBCs) [30,31]. Arg387 is located approximately 10°A from the structural

NADP⁺ binding site (Fig. 8). The Arg387His mutation disrupts NADP⁺ binding, destabilizes the protein structure, and causes the formation of inactive monomers and a complete loss of enzyme activity. Structural NADP⁺ is known to stabilize the G6PD protein structure [23]; structural stability is particularly important for enzymes in mature RBCs which are non-nucleated cells.

Since G6PD A is a neutral variant with no effect on enzyme activity and protein stability, we hypothesized that the additional mutation (s) are responsible for the enzyme deficiency and clinical manifestations observed in individuals with G6PD deficiency. To test this hypothesis and examine the cooperation between two and three mutations in G6PD variants, we performed a detailed functional and structural characterization of double and triple mutants carrying the Asn126Asp mutation (G6PD A). G6PD A⁻⁽²⁰²⁾, a Class III G6PD variant commonly found in Africa, carries Val68Met and Asn126Asp mutations, with 12% residual G6PD activity in RBCs [34,35]. A previous study suggested that both mutations are required to cause an enzyme deficiency [29]. It was described that both mutations synergistically contribute to protein instability to yield a dramatic effect on enzyme activity. Therefore, it was proposed that Val68Met can only cause enzyme deficiency when it is present in combination with G6PD A. However, our results show that the Val68Met mutation contributes greatly to the enzyme deficiency observed in individuals with the G6PD A⁻⁽²⁰²⁾ variant. By itself, the Val68Met mutation can disrupt protein structure, causing a significant reduction in protein stability and catalytic efficiency (approximately 10-fold). The G6PD No name variant has the same mutations as G6PD A⁻⁽²⁰²⁾ plus an additional mutation at residue 422 to give rise to a Class I variant with the most severe clinical phenotypes [33]. In this study, we have shown that that Leu422 is located approximately 5°A from the structural NADP⁺ binding site (Fig. 8). Mutation at G6PD position 422 disrupts the binding of structural NADP⁺, causing protein instability. The Leu422Val mutation has an additive effect on the structural stability of G6PD No name, resulting in the complete loss of enzyme activity.

A combination of Asn126Asp (G6PD A) and Arg387His (G6PD Guadalajara) mutations causes the severe enzyme deficiency with hemolytic anemia observed in G6PD Mount Sinai [32]. Arg387 is part of the structural NADP⁺ binding domain (Fig. 8), and the Arg387His mutation significantly interrupts protein stability, causing a marked decrease in enzyme activity. Therefore, we believe that the Arg387His mutation itself is responsible for the severe clinical manifestations observed in G6PD Mount Sinai. The G6PD A⁻⁽⁹⁶⁸⁾ double mutant is caused by the presence of a Leu323Pro mutation in a G6PD A context. The G6PD A⁻⁽⁹⁶⁸⁾ mutant is a Class III variant reported in Mexican populations and

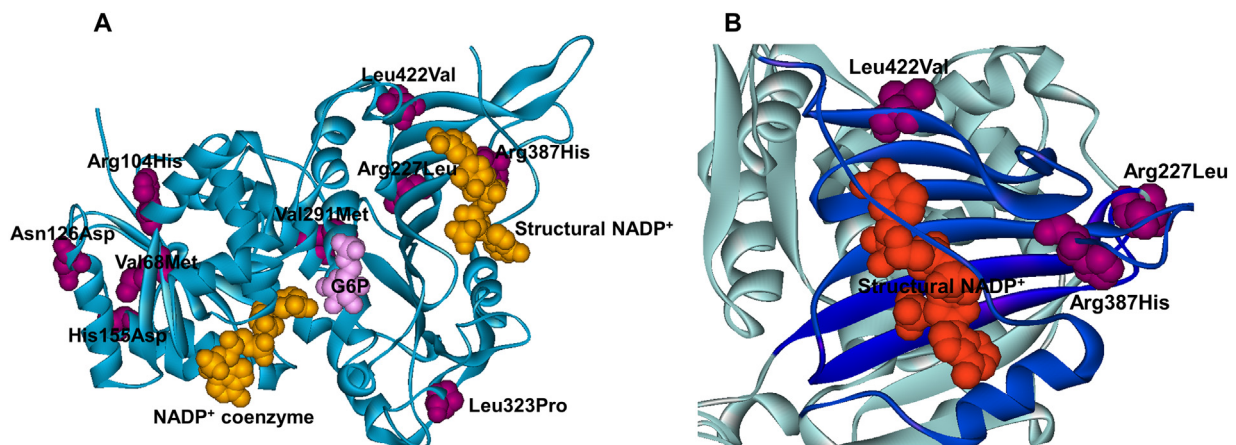


Fig. 8. 3D structure of the G6PD protein. A) The monomeric structure of G6PD is shown with structural and coenzyme NADP⁺ binding sites (yellow) and the G6P site (pink). The mutants are shown as CPK representations and labeled. B) The structural NADP⁺ binding site is illustrated (dark blue). Residues Arg227, Arg387, and Leu422 are located within the NADP⁺ binding distance. The graphical illustration was generated using Discovery Studio Visualizer.

has <10% residual enzyme activity [35,36]. Our study shows that the G6PD A⁻⁽⁹⁶⁸⁾ mutant is structurally unstable, resulting in a 5-fold reduction in catalytic activity. Indeed, a single Leu323Pro (G6PD Nefza) mutation was reported in Tunisia, where the patient presented with hemolytic anemia triggered by bean [45]. Consistent with our results, it was previously suggested that G6PD Nefza itself was primarily responsible for the functional and structural changes observed in the G6PD A⁻⁽⁹⁶⁸⁾ double mutant [46]. Structural instability also accounts for enzyme deficiency in the Class II G6PD variant, G6PD Ananindeua. G6PD Ananindeua contains a combination of G6PD A and G6PD Viangchan mutations. The double mutant was reported in Amazonian populations and exhibited extremely low residual enzyme activity in RBCs (< 1%) [37]. G6PD Viangchan, a Class II variant, was previously characterized and was shown to have an extreme effect on protein stability, causing a significant decrease in catalytic efficiency [25]. G6PD Ananindeua is structurally unstable and has even lower catalytic efficiency than does the G6PD Viangchan mutant. The severe clinical phenotypes observed in G6PD Ananindeua can be explained by G6PD Viangchan making a large contribution to the double mutant enzyme.

Three additional naturally occurring mutants included in our study were G6PD Sierra Leone, G6PD Acrokorinthos, and G6PD A⁻⁽⁶⁸⁰⁾. To date, there are no reports of any of these single mutations (Arg104His, His155Asp, and Arg227Leu) occurring separately from the G6PD A variant and we did not create these non-natural single mutations. Biochemical and structural characterization of these double variants indicate that they are structurally unstable. The G6PD Sierra Leone mutant is classified as a Class III G6PD deficiency and is reported in African countries [38]. Similar to the common G6PD A⁻⁽²⁰²⁾ variant, the G6PD Sierra Leone mutant exhibits low residual enzyme activity in RBCs [38]. However, our results demonstrate an approximate 24-fold reduction in G6PD Sierra Leone catalytic efficiency. In this variant, the presence of Arg104His and Asn126Asp mutations alters the binding affinity of both substrates and contributes to trypsin susceptibility. G6PD Acrokorinthos, a Class II variant, was reported in Hellenic populations with severe deficiency and only 1% residual G6PD enzyme activity [39]. The severe deficiency could be explained by a secondary structural change in G6PD Acrokorinthos, leading to 3D conformational change ultimately caused dimer dissociation, decreased protein stability, and a 10-fold reduction in catalytic efficiency. The G6PD A⁻⁽⁶⁸⁰⁾ mutant is found in African populations, causes mild deficiency, and is classified as a Class III variant [35]. A 6-fold decrease in the catalytic efficiency of G6PD A⁻⁽⁶⁸⁰⁾ was observed in this study as a result of structural instability. This variant disrupts the protein secondary structure and causes the dissociation from active dimer to inactive monomer.

5. Conclusions

We described the functional and structural characteristics of 11 recombinant G6PD variants. We demonstrated that the protein dysfunction observed in G6PD deficient individuals is largely attributable to protein instability, which leads to a reduction in catalytic activity. The location of the mutation in the 3D structure affects the level of enzyme deficiency and leads to the clinical manifestations observed in the patients. The cooperative effect of two and three mutations causing a defect in protein function is evident in the G6PD variants with double and triple mutations. Importantly, when the second and third mutations are present in the context of the non-deficient G6PD A, these additional mutations play essential roles in enzyme deficiency.

Author contributions

Conceived and designed experiments: UB. Performed experiments: AP, TJ and UB. Contributed reagents/materials/analysis tools: UB and MI. Wrote the manuscript: AP and UB. Critical revision of manuscript: MI. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2020.05.026>.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ
Praoparotai A, Junkree T, Imwong M, **Boonyuen U**. Functional and structural analysis of double and triple mutants reveals the contribution of protein instability to clinical manifestations of G6PD variants. Int J Biol Macromol. 2020 May 7;158:884-893. doi:10.1016/j.ijbiomac.2020.05.026 (impact factor = 4.784)
2. การนำผลงานวิจัยไปใช้ประโยชน์
 - เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
 - เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบ ข้อบังคับหรือวิธีทำงาน)
 - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)
 - ☒ เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)องค์ความรู้ใหม่ที่ได้นี้เป็นประโยชน์ทางด้านวิชาการต่อนักวิจัย บุคลากรทางการแพทย์ ที่สนใจทางด้านโรคมาลาเรียและโรคพร่อง G6PD นอกจากนี้องค์ความรู้ที่ได้จากโครงการวิจัยนี้ยังนำไปใช้ในการเรียนการสอนนักศึกษาหลักสูตร Ph.D. (Tropical Medicine) วิชา TMID 608 Blood- and Vector-borne Diseases in the Tropics ในหัวข้อ Resistance mechanism of Plasmodium: a molecular modeling approach ที่คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล
3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)
Praoparotai A and Boonyuen U. Poster presentation: "Contribution of protein stability to clinical manifestations of G6PD variants" at Joint International Tropical Medicine Meeting during 12-14 December 2018, Amari Watergate Bangkok, Thailand.