Structural Defects Underlying Protein Dysfunction in Human Glucose-6-phosphate Dehydrogenase A− Deficiency*

(Received for publication, July 29, 1999, and in revised form, December 23, 1999)

Félix Gómez-Gallego, Amando Garrido-Pertierra, and José M. Bautista‡
From the Departamento de Bioquímica y Biología Molecular IV, Universidad Complutense de Madrid, Ciudad Universitaria, Facultad de Veterinaria, 28040 Madrid, Spain

The enzyme variant glucose-6-phosphate dehydrogenase (G6PD) A−, which gives rise to human glucose-6-phosphate dehydrogenase deficiency, is a protein of markedly reduced structural stability. This variant differs from the normal enzyme, G6PD B, in two amino acid substitutions. A further nondeficient variant, G6PD A, bears only one of these two mutations and is structurally stable. In this study, the synergistic structural defect in recombinant G6PD A− was reflected by reduced unfolding enthalpy due to loss of β-sheet and α-helix interactions where both mutations are found. This was accompanied by changes in inner spatial distances between residues in the coenzyme domain and the partial disruption of tertiary structure with no significant loss of secondary structure. However, the secondary structure of G6PD A− was qualitatively affected by an increase in β-sheets substituting β-turns related to the lower unfolding enthalpy. The structural changes observed did not affect the active site of the mutant proteins, since its spatial position was unmodified. The final result is a loss of folding determinants leading to a protein with decreased intracellular stability. This is suggested as the cause of the enzyme deficiency in the red blood cell, which is unable to perform de novo protein synthesis.

The human gene that codes for glucose-6-phosphate dehydrogenase (G6PD) presents many genetic, pathological, and structural features that make it particularly suitable for the investigation of relationships between mutations and protein dysfunction. The human G6PD gene is located on the X chromosome in a region showing a high degree of genetic variability. More than 100 mutations or combined mutations associated with nearly 200 variants have been detected. Further, it is estimated that over 400 million people in the world are G6PD-deficient, making this the most common human enyzymopathy. G6PD deficiency is associated with acute or chronic hemolytic anemia and neonatal jaundice. Some genetic variants have attained a high incidence in certain parts of the world, since they confer selective advantage against malaria (3, 4).

G6PD catalyzes the oxidation of glucose-6-phosphate to 6-phosphoglucono-δ-lactone with the concomitant reduction of NADP to NADPH. NADPH is the only source of reducing power in red blood cells, where it is required to maintain the redox equilibrium and, in particular, to detoxify hydrogen peroxide and other compounds via glutathione (reviewed in Ref. 5). In African populations or those of African ancestry, the most common polymorphic variant associated with the deficiency is G6PD A−, which accounts for 20–40% of the affected population in western and central Africa (6). This variant differs from the normal G6PD B in that it has two amino acid substitutions, V68M and N126D, which were early identified (7, 8). Furthermore, the most common nondeficient polymorphic variant in Africa, G6PD A, bears a single amino acid replacement, N126D, which is also present in G6PD A−. Nevertheless, the deficient G6PD A− mutation at position 68 alone has not been detected in any variant. This and further haplotyping analyses have led to a suggestion that the nondeficient single mutant G6PD A is more ancient than the deficient double mutant G6PD A− (2). Clinically, the G6PD A enzyme produces no adverse effects in erythrocytes. However, the presence of the second amino acid substitution in G6PD A− leads to a 90% loss of activity with respect to the normal G6PD B (6).

The three-dimensional model proposed for human G6PD (9) shows that the mutations at positions 68 and 126 are only 8 Å apart, suggesting an interactive effect between substitutions as the origin of the deficient G6PD A− phenotype. The positions of the substitutions distant from substrate binding and catalytic centers (9), coupled with the fact that the purified enzyme shows normal specific activity (6) with unchanged Km values for its substrates (10, 11), lends further support to the idea that the two mutations in G6PD A− notably affect the intracellular stability of the enzyme in a synergistic manner (12). It has been previously shown that these two mutations considerably hinder the in vitro refolding capacity of the enzyme molecule to the point that it prevents the formation of the catalytically active dimer (13). In contrast, the presence of the single mutation in G6PD A does not affect refolding capacity or the consequent formation of active molecules (13).

The use of x-ray diffraction to examine discrete dysfunction effects of large mutant proteins is limited by the fact that the lattice energy of crystals is smaller than that stabilizing the protein molecules in solution and therefore not enough to significantly disturb internal structures by single amino acid substitutions (14). In an effort to determine the structural lesions responsible for instability in the deficient G6PD A− molecule leading to low enzyme activity levels in the red blood cell, the aim of this investigation was to perform a complete structural analysis of this protein and to compare it to its ancestral normal phenotypic variants G6PD B and G6PD A.

* This work was supported by Fondo de Investigaciones Sanitarias Grant 92/1179 and by British Council/Acciones Integradas Grant HB1996-0178. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This paper is dedicated to the memory of Prof. Jorge E. Churchich, who enthusiastically proposed the ANS binding experiments.

† To whom correspondence should be addressed: Departamento de Bioquímica y Biología Molecular IV, Universidad Complutense de Madrid, Facultad de Veterinaria, Ciudad Universitaria, 28040 Madrid, Spain. Tel.: 34 91 3943827; Fax: 34 91 3943824; E-mail: bauchem@ eumax.sim.ucm.es.

‡ The abbreviations used are: G6PD, glucose-6-phosphate dehydrogenase; ANS, 8-anilinonaphthalene-1-sulfonic acid; PLP, pyridoxal 5′-phosphate; DSC, differential scanning calorimetry.
**Experimental Procedures**

*Mutagenesis, Protein Purification, and Sample Preparation.—The constructs based on the plasmid pKK232-2 containing full-length human cDNA coding for normal G6PD and for the G6PD A (N126D) and G6PD A (N126D and V68M) variants have been previously described (12, 15). Wild-type G6PD B and its variants were purified to homogeneity by affinity chromatography on 2′,5′-ADP Sepharose 4B as described elsewhere (12, 15). The purity of the enzyme preparations was confirmed by SDS-PAGE. G6PD activity assays were performed at 30 °C by measuring the increase in absorbance at 340 nm (6). Protein samples used for CD, fluorescence, and activity assays were dialyzed and equilibrated at 4 °C against 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.02% 2-mercaptoethanol, 1 mM e-aminocaproic acid, and 1 mM NADP. In the experiments conducted in the presence of urea, the protein samples were preincubated with various denaturant concentrations at 25 °C for 2 h.

*Circular Dichroism and Fluorescence—CD spectra were obtained at 25 °C in the far-UV (200–250 nm) and near-UV (250–300 nm) regions using 0.2-cm path length cuvettes. The protein concentration used to record the spectra was 16.6 μM. Ellipticity, [(θ)], expressed in terms of mean residue ellipticity (deg cm² dmol⁻¹), at 222 and 270 nm, was calculated as follows,

\[
[\theta] = \theta \cdot MRW/10 \cdot l \cdot c
\]

where MRW represents the mean residue weight, l represents the optical path length, and c represents the concentration (g/ml).

The estimation of secondary structural elements was carried out by deconvolution of the far-UV CD curves obtained in 100 mM phosphate buffer (pH 7.0) according to computerized procedures (16). Data were obtained from the eight different CD spectra recorded for each protein preparation (two protein batches per G6PD variant).

Intrinsic protein fluorescence spectra were recorded after excitation at 295 nm, at 25 °C using 1.0-cm path length cuvettes. Samples for 8-anilinonaphthalene-1-sulfonic acid (ANS) binding measurements were prepared by adding 10 mM ANS to the incubation mixtures of protein solutions at various concentrations of urea. ANS fluorescence was monitored by CD spectra in the far and near-UV regions. Samples were prepared by adding aliquots of 5 mM KI to native enzyme solutions. The Stern-Volmer constant (KSV) was defined as follows,

\[
F_0/F = 1 + K_{SV}[Q]
\]

where \( F_0 \) and \( F \) correspond to fluorescence emission in the absence and presence of quencher, respectively, and [Q] is the quencher concentration.

The accessible fraction of fluorophores was calculated from the modified Stern-Volmer equation (17),

\[
F_0/F = 1/f_{\alpha}K_{SV}[Q] + 1/f_{\alpha}
\]

This modified form of this equation permits \( f_{\alpha} \) and \( K_{SV} \) to be determined graphically. A plot of \( F_0/F \) versus \([Q]/Q_0 \) yields \( f_{\alpha} \) as the intercept on the ordinate axis and \( (f_{\alpha}K_{SV})^{-1} \) as the slope. The intercept represents extrapolation to infinite quencher concentration (1/\( Q_0 \) = 0). The value of \( K_{SV} = f_0 - f_{\alpha} \) at this quencher concentration is the reciprocal of the fluorescence quenched. At this concentration, only inaccessible residues fluoresce.

*Pyridoxal 5′-Phosphate (PLP) Experiments—Labeling, Quenching, Polarization, and Fluorescence Energy Transfer—Samples of G6PD (0.4 mg/ml) were dialyzed and equilibrated at 4 °C against 0.1 M Hepes, pH 7.6, 9% glycerol, 0.2% 2-mercaptoethanol, and 1 mM EDTA. The samples were then incubated in the dark at 25 °C with PLP under mild conditions (100-fold molar excess) for 90 min as described elsewhere (18). The Schiff base was reduced by the addition of an equivalent molar amount of NaBH₄ with respect to PLP as reported elsewhere (19). The excess of PLP and NaBH₄ in the samples was removed by dialysis and equilibrated against 50 mM sodium phosphate buffer, pH 7.0, and 1 mM EDTA.

Quenching of the fluorescence of n- α-pyridoxalphenyldiazine by KI was carried out by adding aliquots of 5 mM KI to the samples of labeled enzyme. Readings were obtained at 25 °C using excitation and emission wave lengths of 330 and 390 nm, respectively. The Stern-Volmer constant was calculated as described above. Polarization values of the labeled enzyme were obtained from samples previously incubated at 25 °C with different urea concentrations for 2 h. Readings were recorded using 0.2-cm path length cuvettes at excitation and emission wavelengths of 330 and 390 nm, respectively.

Fluorescence energy transfer experiments were conducted at 25 °C using 1.0-cm path length cuvettes. The excitation spectra of the PLP-labeled G6PD variants were recorded at an emission wavelength of 390 nm. The energy transfer efficiency was calculated as follows,

\[
E = (F_{AD} - F_A)/F_{AD}
\]

where \( E_R \) and \( E_{AD} \) are the acceptor relative fluorescence intensities in the absence and presence of the energy donor, respectively.

**Results and Discussion**

*Changes in Secondary and Tertiary Structure—To determine whether some secondary or tertiary structural elements are affected in G6PD A, urea unfolding of the three G6PD variants (B, A, and A−) was monitored by CD spectra in the far and near-UV regions.

Unfolding transitions in the secondary structure of the variants were monitored by the calculation of [θ] at 222 nm, as a function of urea concentration (24). The signal diminished at increasing denaturant concentration (Fig. 1A). At urea concentrations below 2 M, all three variants retained about 90% of their secondary structure. The midpoint (50% of the native CD signal) was achieved at 3.2 ± 0.2 M urea for G6PD B and G6PD A, compared with 2.5 ± 0.1 M for G6PD A−.

The extent of the positive band pattern recorded in the near-UV region as a function of urea concentration permitted observation of unfolding transitions in the tertiary structure (25), which, indeed, was reduced as the denaturant concentrations increased (Fig. 1B). The ellipticity signal, [θ] at 270 nm for the native G6PD A− was 60% lower than for the native G6PD B and G6PD A, indicating reduced tertiary structure in the double mutant. At concentrations of urea under 2 M, there were little differences in the signal of the variants retained: G6PD B, 85 ± 4%; G6PD A, 75 ± 4%; and G6PD A−, 67 ± 4%. Nevertheless, 50% unfolding of all three variants occurred at a very similar urea concentration (2.7 ± 0.2 M for G6PD B and 2.5 ± 0.1 M for G6PD A and G6PD A−).

These results indicate that the loss of tertiary structure in G6PD B and G6PD A occurred at lower urea concentrations than the disruption of secondary structure. However, the presence of two mutations in G6PD A− leads to a simultaneous...
disruption of the secondary and tertiary structure, as exhibited by the identical 50% unfolding values recorded at 222 and 270 nm. This effect is quite unusual, since the tertiary structure of globular proteins generally undergoes disruption before the secondary structural elements (26, 27) and suggests the highly unstable nature of the protein.

In addition, the far-UV CD spectra of the native proteins obtained in 100 mM phosphate buffer (pH 7.0) permitted estimation of the secondary structural weights (16) of each G6PD variant as shown in Table I. These values served to predict specific structural shifts as point mutations were introduced into the enzyme. G6PD A slightly increased its β-sheet and β-turn content at the expense of α-helices. A more dramatic secondary structural shift was observed in G6PD A’, where, in comparison with G6PD A, a 56% increase of β-sheet content with no modification in the amount of α-helix was calculated.

The N126D mutation occurs in an α-helix (αC, Fig. 2) exposed to a polar aqueous environment. The residues asparagine and aspartic acid show a fairly similar low propensity to form α-helices (28, 29), while both have a high conformational preference to form reverse turns (30). When the secondary structural content is predicted by analytic vector decomposition (31, 32) of the 14 amino acids of the αC stretch (AASYQRNLNSHMDAL), and compared with the mutant stretch (AASYQRNLNSHMDAL), both show identical α-helix contents (100%). The solvation of the first four C-O main chain groups in an α-helix can differentially interact with the helix dipole, depending on the nature of the C-cap residue (33, 34). Consequently, the N126D substitution (2 residues away from the C-cap of the αC stretch) could account for a subtle modification in the orientation of the helix and thus increase its natural tendency to form a reverse turn. Indeed, deconvolution of the CD far-UV spectra (Table I) revealed that the N126D mutation in G6PD A resulted in a significant increase in β-turns and β-sheets at the expense of α-helices. This experimental procedure is particularly efficient for calculating the α-helix content of proteins (35), and the present mean value (28.1%) is in good agreement with that calculated for the human G6PD three-dimensional model (27.5%) (9).

The second mutation, V68M, which gives rise to the deficient G6PD A’, did not induce a further loss of α-helix content, as shown by CD spectra deconvolution, while a significant loosening of β-turns shifting to β-sheet was observed, indicating increased rigidity in the β-sheet area of the mutations (Table I). This finding seems to be inconsistent with the comparative prediction of secondary structural content (31, 32) in the 7-residue peptide comprising β-sheet B in the wild type (TFHYGVA; 100% β) and in the mutant (TFIMG YA; 75% β and 25% coil), suggesting the higher β-sheet disruption potential of methio-

![Fig. 1. Plot of [θ] as a function of urea concentration for G6PD B (○), A (△), and A’ (□). CD spectra were recorded at 25 °C in 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.02% 2-mercaptoethanol, 1 μM e-aminocaproic acid, and 1 mM NADP using 0.2-cm path length cuvettes. Values of 50% unfolding are given as the mean ± S.D. of two independent experiments conducted in triplicate. The error bars represent the S.D. range. The absence of error bars indicates an S.D. below 4%. A, changes corresponding to the far ultraviolet region recorded at 222 nm ([θ]222) in the presence of 0–8 M urea. 50% unfolding was achieved at 3.2 ± 0.2 M urea in G6PD B and G6PD A and at 2.5 ± 0.1 M urea in G6PD A’. B, changes corresponding to the near-ultraviolet region recorded at 270 nm ([θ]270) in the presence of 0–8 M urea. 50% unfolding was observed at a very similar urea concentration in all three G6PD variants (2.7 ± 0.2 M in G6PD B and 2.5 ± 0.1 M in G6PD A and G6PD A’).]

![Fig. 2. Model of the human G6PD monomer indicating the position of the substitutions in G6PD A’ in the corresponding secondary structural motifs (V68M in β-sheet B and N126D in α-helix C). Also highlighted are the Lys43 (a) in the active site and all of the protein’s tryptophan (W) residues.]

### Table I

| G6PD variant | α-Helix | β-Sheet | β-Turns | Random coil | Additional chirality contribution |
|--------------|---------|---------|---------|-------------|---------------------------------|
| G6PD B     | 28.1    | 24.4    | 11.9    | 35.6        | 3.2                             |
| G6PD A     | 20.7    | 28.4    | 13.1    | 37.8        | 8.2                             |
| G6PD A’    | 20.0    | 44.4    | 5.7     | 29.9        | 9.5                             |
| G6PD B (3D model) | 27.5 | 17.1    | 8.0–13.0 |             |                                 |

*CD spectra deconvolution values correspond to 100% of the protein structure. The additional chiral contribution is independently given in the last column. Percentages were calculated in 95% of the protein structure, since the 26 N-terminal residues (5% of the protein) were not modeled.

The percentage of secondary structure content of the three G6PD variants was estimated by deconvolution of their respective far-UV CD spectra (16). α-Helix and β-sheet weights were calculated from the three-dimensional (3D) model of G6PD B (9). β-Turn contents were estimated using PROCHECK™ software, which determines this structural element using the three-dimensional model coordinates (M. Adams, personal communication).
fourth amino acid in addition to the close proximity of the α-carbons of the first and fourth residues, which are 0.5–0.6 nm apart (39). In G6PD A and G6PD A”, the loss of β-turn content could be explained in terms of minor changes in the spatial arrangement of some residues induced by the mutations in the molecules. These mutations may favor the stabilization of β-sheet structures in the disturbed area of this enzyme domain.

**Hydrophobic Surface Exposure**—Hydrophobic interactions play a major role in defining conformation and interactions between secondary structural elements. The exposure of the hydrophobic surface of the three variants during urea unfolding was monitored using the hydrophobic probe, ANS. The affinity of ANS for protein molecules significantly increases when the rigidity of the tertiary structure is disrupted while the compactness of the secondary structure is retained (40).

For the native proteins with no urea present, the fluorescence signal obtained at 470 nm for G6PD A” (percentage of $F_0$) = 13.5 ± 1.7% was slightly higher than that corresponding to the nondeficient variants G6PD A (percentage of $F_0$ = 9.8 ± 1.5%) and G6PD B (percentage of $F_0$ = 7.2 ± 0.8%). This is indicative of a greater hydrophobic area accessible to ANS in the deficient variant. Fig. 3 shows the fluorescence emission ratio ($F/F_0$) in the presence of increasing concentrations of urea. During urea unfolding, the three G6PD variants enhanced the fluorescence signal, which reached its maximum at 3 M urea, although each variant showed different maximum peak heights at this urea concentration. With respect to the maximum peak height shown by the native enzyme, 14.0 ± 1.5, 10.4 ± 1.6, and 7.5 ± 1.0-fold increases were recorded for G6PD B, G6PD A, and G6PD A”, respectively, indicating a different degree of hydrophobic surface exposure in each variant. Thus, the presence of the N126D mutation alone in G6PD A leads to reduced ANS binding, which is further reduced when the second mutation V68M in G6PD A” is present, indicating a progressive tendency toward loss in tertiary structure (24) as mutations are introduced into the protein. This is in agreement with the reduced amount of ellipticity signal at 270 nm in the double mutant.

**Thermodynamics of Unfolding**—Studies conducted on hydrophobic mutants of proteins have shown the part played by hydrophobic residues in protein stability (14). The changes in tertiary structure and surface hydrophobicity observed in G6PD A” prompted us to quantitatively explore the hydrophobic interactions stabilizing the folded conformations of the three enzyme variants. Temperature-induced unfolding was explored by DSC to evaluate the energetic and thermodynamic mechanisms involved in the transition from the folded to the unfolded state. Fig. 4A shows the curves and thermodynamic variables corresponding to the experimental unfolding process. Although the $T_m$ and $\Delta H_{unf}$ values were practically identical for each variant, $\Delta H_{1/2}$ values showed a major difference. The energy released by G6PD A” was half that released by G6PD B and G6PD A, which were in turn very similar. Moreover, although the calculated $\Delta H_{1/2}$ values for the three variants were similar, the $\Delta H_{1/2}/\Delta H_{unf}$ ratio corresponding to G6PD A” was less than 1 (0.48), while those corresponding to G6PD B and G6PD A were close to unity. The van’t Hoff enthalpy value reflects intramolecular interactions within the polypeptide chain (41), while unfolding enthalpy is a more general thermodynamic parameter that also takes into account interactions of the protein with the environment (41). This indicates that in both

![Fig. 3. Hydrophobic surface exposure changes at increasing urea concentrations in G6PD B ( ), A (○), and A” (□). ANS binding was estimated by fluorescence at 470 nm using an excitation wavelength of 350 nm. Determinations were performed at 25 °C in 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.02% 2-mercaptoethanol, 1 μM ε-aminocaproic acid, and 1 nM NADP using 1.0-cm path length cuvettes. F and $F_0$ are the fluorescence signal in the presence and absence of urea, respectively. Maximum peak heights with respect to the native enzyme were obtained in 5 mM urea. Values are expressed as the mean ± S.D. of two independent measurements performed in triplicate. Error bars represent the S.D. range. The absence of error bars indicates an S.D. of less than 5%. Percentage maximum increments with respect to the native state were 14.0 ± 1.5 for G6PD B, 10.4 ± 1.6 for G6PD A, and 7.5 ± 1.0 for G6PD A”.

![Fig. 4. Thermal denaturation of the three G6PD variants. A, DSC plots of the G6PD variants (solid line), A (dotted line), and A” (dashed line) in 50 mM Heps, pH 7.0, 0.02% 2-mercaptoethanol, 1 μM ε-aminocaproic acid, 1 mM NADP, and 0.1 M KCl. Scanning calorimetry was conducted from 35 to 70 °C at a heating rate of 60 °C/min using sample concentrations of 1.50–2.20 mg/ml. Thermogram fitting and data analysis were performed using Origin® software (see “Experimental Procedures”). $T_m$, given in degrees Centigrade, and $\Delta H_{1/2}$ and $\Delta H_{unf}$ enthalpies, expressed in kcal/mol, are the mean ± S.D. of triplicate determinations (see “Experimental Procedures” for details on statistical significance). $\Delta H_{1/2}$ mainly takes into account intramolecular interactions within the polypeptide chain, and $\Delta H_{1/2}$ reflects these intramolecular interactions plus interactions of the polypeptide chain with the environment (41). B, loss of residual enzymatic activity according to temperature in G6PD B ( ), A (○), and A” (□). Several protein samples were heated from 30 to 65 °C in a thermocycler at a rate of 1 °C/min. The protein-containing tubes were removed at a given temperature and chilled on ice. G6PD activity was determined spectrophotometrically at 340 nm as described under “Experimental Procedures.” Results are expressed as the percentage of maximum activity ± S.D. of two independent measurements conducted in triplicate. Error bars represent the S.D. range. The absence of error bars indicates an S.D. of less than 2%. Estimated $T_{1/2}$ values in degrees Centigrade were 57.0 ± 0.3 for G6PD B, 57.0 ± 0.5 for G6PD A, and 55.2 ± 0.2 for G6PD A”.
nondeficient G6PD variants, the transition follows a basic model of unfolding in two states, while the unfolding process in G6PD A reflects cooperativity between the two subunits of the dimer (30). Moreover, the thermal unfolding process was observed to be essentially irreversible in all three variants (data not shown). Since no disruptive effect was observed in G6PD A in comparison with G6PD B, the DSC peaks allowed us to attribute a synergistic destructurization effect to the second mutation. Therefore, the N126D substitution in G6PD A appears to have no effect on the dimeric structure, as shown by the practical absence of modifications in the DSC thermograms and also in the CD spectra discussed above. Hydrophobic interactions are a major stabilizing factor of folded conformations in proteins (42, 43), and correlation between changes in the Gibbs energy and hydrophobic surface exposure upon denaturation has been previously attributed to point mutations in proteins (14, 44). It would therefore seem that the portion of loosened tertiary structure permits the entrance of a certain additional amount of water into the dimer of the double mutant, as detected by the enhanced binding of ANS to the hydrophobic surface. The progressive increase in area of hydrophobicity as each mutation is introduced into the G6PD molecule (see Fig. 3) indicates that the final synergistic structural disruption effect observed by CD and DSC in G6PD A is probably the consequence of several preceding changes in the molecular environment, including hydrophobicity.

In order to determine if the destructurization of the molecule occurred at a similar temperature to that corresponding to the global unfolding process, we conducted a series of experiments to estimate the retention of residual enzyme activity in the temperature range of 30–65 °C (Fig. 4B). Again, both G6PD B and G6PD A exhibited very similar behavior as the temperature increased, while the loss of G6PD A activity was apparent even at low temperatures. From 30 to 50 °C, G6PD B and G6PD A retained 100% enzyme activity, while G6PD A exhibited 79.0 ± 1.0% retention. Additionally, in the double mutant, the 50% inactivation temperature (T0.5) was approximately 2 °C lower than that corresponding to G6PD B and G6PD A (55.2 ± 0.2 °C versus 57.0 ± 0.3 and 57.0 ± 0.5 °C, respectively). Thus, the loss in activity of the three variants appears to be concurrent with the unfolding of the protein structure, since both melting temperatures (Tm and Tm*) were practically the same.

Microenvironment—Urea stability assays, in which G6PD activity was monitored in each variant, were conducted (Fig. 5A). Only slight differences were observed in the residual enzyme activity at identical enzyme concentrations (0.2 mg/ml), and up to 2 M urea with 24 ± 3, 29 ± 2, and 38 ± 1% losses in activity was recorded for the B, A, and A variants, respectively. The most dramatic decay in enzyme activity was observed from 2 to 2.5 M urea, with 68.5 ± 0.5, 82.5 ± 2.5, and 86 ± 3% activity losses recorded at 2.5 M urea for G6PD B, A, and A, respectively. G6PD A showed undetectable activity in 3 M urea, while the remaining variants still exhibited some activity at urea concentrations of 3 M (G6PD B, 7.0 ± 2.0%; G6PD A, 1.5 ± 0.3%).

Human G6PD has 7 tryptophan residues/monomer. These are not mutated in either the A or A variant. According to the three-dimensional model proposed for human G6PD, three of these residues (Trp53, Trp54, and Trp165) are found in the coenzyme domain, while the mutations N126D and V68M are located. Fluorescence emission maxima served to monitor modifications in the microenvironment of the tryptophan residues during unfolding. The emission maxima of the native proteins were 344 nm for G6PD B, 346 nm for G6PD A, and 347 nm for G6PD A. In the presence of increasing concentrations of urea, a red shift was produced in the emission spectra, reflecting the gradual exposure of the tryptophan residues to the solvent (Fig. 5B). Shifts of 10, 8, and 7 nm were recorded for the B, A, and A variants, respectively. These displacements of maximum emission toward the red region of the spectrum suggest a slight difference in the microenvironment of some of the tryptophan residues in G6PD B with respect to G6PD A and G6PD A, with the highest degree of tryptophan exposure in the native state exhibited by the double mutant G6PD A. Both A and A showed a linear displacement of emission maxima with rising urea concentration (0–8 M), while in the normal G6PD B this took place in three phases, with an initial stage from 0 to 2 M urea. The second phase, observed in the narrower range of 2–2.5 M urea, involved a 2.5-nm shift in G6PD B. The third linear phase from 2.5 to 8 M urea was of a similar slope in the three species. The shift in the emission maximum from 2 to 2.5 M urea in G6PD B (Fig. 5B) is associated with the dramatic activity loss in the three variants (Fig. 5A), although this shift in fluorescence was inappreciable in the A and A variants. These findings suggest that the microenvironment of some of the tryptophan residues of the A and A variants differs substantially from that of the nondeficient G6PD B in native conditions but does not appear to have a notable effect on catalysis or stability (10, 11).

To further assess the degree of differential exposure of tryptophan residues in the three variants, quenching of intrinsic tryptophan fluorescence was studied, and the accessible fraction f was calculated. The iodide anion is able to quench only the most solvent-exposed residues, whereas acrylamide can penetrate the protein matrix to a certain extent and is therefore capable of additionally quenching tryptophan fluorescence of residues hidden in dynamic structures of high flexibility (45). The Stern-Volmer plots (Fig. 6A) exhibit a downward curve indicating the existence of two populations of tryptophan residues with different degrees of accessibility (17, 45). However, no difference in the degree of quenching was observed between the A and B variants, although variant A did show a less pronounced downward curve in the quenching course, suggesting the greater degree of accessibility of some of the tryptophan residues in this variant. Given the two populations of trypto-
Protein Dysfunction in G6PD Deficiency

FIG. 6. Quenching of tryptophan fluorescence by iodide in G6PD B (●), A (○), and A (□). A, Stern-Volmer plot of tryptophan fluorescence quenching by iodide in native conditions recorded at 25 °C in 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.02% 2-mercaptoethanol, 1 μM e-aminocaproic acid, and 1 mM NADP using 1.0-cm path length cuvettes. Excitation and emission wavelengths were 295 and 340 nm, respectively. Experiments consisted of two independent assays conducted in triplicate. Error bars represent the S.D. range. The absence of error bars indicates an S.D. of less than 4%. B, modified Stern-Volmer plot of fluorescence quenching of the G6PD variants by KI, indicating the 
fA of the tryptophan population. The values of 
fA expressed as the mean ± S.D. of two independent experiments conducted in triplicate were calculated as described under "Experimental Procedures." The experimental 
fA values for each variant were 0.33 ± 0.02 (G6PD B), 0.40 ± 0.02 (G6PD A), and 0.60 ± 0.10 (G6PD A). The respective calculated 
SV values were 3.47 ± 0.10 m−1 (G6PD B), 3.41 ± 0.03 m−1 (G6PD A), and 2.33 ± 0.80 m−1 (G6PD A). Error bars represent the S.D. range. The absence of error bars indicates an S.D. of less than 4%.

FIG. 7. Fluorescence of PLP-labeled lysine 205 in G6PD B (●), A (○), and A (□). The labeling method is described under "Experimental Procedures." A, Stern-Volmer plot of n-ε-pyridoxyllysine fluorescence quenching by iodide in native conditions. Quenching was performed at 25 °C in 50 mM sodium phosphate, pH 7.0, and 1 mM EDTA using excitation and emission wavelengths of 330 and 390 nm, respectively. 
SV values were calculated as described under "Experimental Procedures" and are expressed as means ± S.D. of two independent determinations conducted in triplicate: 10.53 ± 0.29 m−1 for G6PD B, 10.86 ± 0.60 m−1 for G6PD A, and 10.62 ± 0.67 m−1 for G6PD A. Error bars represent the S.D. range. The absence of error bars indicates an S.D. of less than 2%. B, polarization of n-ε-pyridoxyllysine fluorescence according to urea concentration. Readings were obtained at excitation and emission wavelengths of 330 and 390 nm, respectively, using 0.2-cm path length cuvettes. Unfolding transitions were detected at 2.90 ± 0.04 M urea in G6PD B, 2.70 ± 0.03 M in G6PD A, and 2.50 ± 0.04 M in G6PD A. Results are expressed as the mean ± S.D. of two independent assays conducted in triplicate. Error bars represent the S.D. range. The absence of error bars indicates an S.D. of less than 3%.

FIG. 8. Fluorescence energy transfer efficiency from tryptophan residues to PLP-labeled lysine 205 in G6PD B (solid line), A (dotted line), and A (dashed line). Excitation spectra were obtained at an emission wavelength of 390 nm using an excitation wavelength of 260–380 nm. The labeling method, efficiency of energy transfer, and distances calculations are described under "Experimental Procedures." The percentage of energy transfer efficiency permitted calculation of the mean distance (R), in Å, from the Trp53-Trp54 unit to PLP Lys 205 (see "Results and Discussion"). Values are expressed as mean ± S.D. of two independent assays conducted in triplicate.

Tryptophan residues with different degrees of accessibility, we replotted the data according to a modified Stern-Volmer equation that permits the graphical determination of the 
fA of the tryptophan population and the 
SV value of the accessible fraction (Fig. 6B). The values of 
fA and 
SV obtained in the KI quenching of the intrinsic fluorescence of each variant (Fig. 6B) were notably higher than those reported for several other globular proteins (26). As indicated by the 
fA value (17), iodide only had access to less than 3 tryptophan residues out of the 7 in each monomer in the B and A variants. In contrast, the structure of variant A permitted the access to almost 4 tryptophan residues/monomer.

Active Site Geometry—The loss of G6PD A− activity at slightly lower urea concentration and temperature in the denaturation experiments (see Figs. 4B and 5A) prompted us to also analyze the effect of the double mutant on the active site. The lysine residue at position 205 in G6PD has been shown to be essential for the catalysis of glucose 6-phosphate to 6-phosphogluconolactone (46). This residue can be specifically labeled with the fluorescent probe PLP (18). The behavior of this residue within the active site in its interaction with nearby residues was monitored by quenching of pyridoxamine fluorescence and polarization of the pyridoxamine group during unfolding in the three variants.

The Stern-Volmer plots of PLP quenching in the three variants yielded straight lines permitting the calculation of the respective 
SV values: 10.53 ± 0.29 m−1 for G6PD B, 10.86 ± 0.60 m−1 for G6PD A, and 10.62 ± 0.67 m−1 for G6PD A− (Fig. 7A). The virtually identical 
SV constants indicate that the long carbon chain of Lys205 constraining the ε-amino group has a very similar degree of flexibility and shows similar accessibility in the three variants. Moreover, there seemed to be no difference in the local interaction pattern of this residue in the variants, suggesting that the three-dimensional structure shaping the active site was unaffected by the mutations at positions 126 and 68.

Moreover, in the native state, all of the three variants showed a pyridoxalpyruvate 205 polarization of fluorescence value of 0.248 ± 0.002 (Fig. 7B). These findings confirmed those of the quenching experiments and support the idea of an unmod-
disturbances in the tertiary structure of the active site. Greater polarization changes were observed from 2 μm urea onwards (0.084 ± 0.005 for G6PD B, 0.070 ± 0.005 for A, and 0.08 ± 0.007 for G6PD A), suggesting further similar modifications in the secondary structure of the active site.

Topology of the Coenzyme Domain —In the active site of G6PD, Lys205 is less than 20 Å away from Trp53 and Trp54 (9). Moreover, these residues are at an equivalent distance from the V68M mutation in the three-dimensional model (see Fig. 2). Specific labeling of Lys205 and the fact that the structure of the active site is not modified in any of the variants permitted the study of the conformational differences that the mutations could cause in the coenzyme domain around tryptophan residues 53 and 54 in the A and A' variants. Indeed, fluorescence energy transfer measurements between tryptophan residues and n-η-pyridoxaloxide have been previously used to calculate inner distances in several proteins (47, 48). We directly measured fluorescence energy transfer in a single excitation spectrum, given the fluorescence properties of the acceptor PLP. The critical distance at which 50% of the excitation energy is transferred to PLP from tryptophan has been calculated as 21 Å for this pair of chromophores (49). Since Trp53 and Trp54 are the only tryptophan residues at least 20 Å from PLP, it is assumed that the possible contribution of the other five tryptophan residues is minimal, since the capacity of excitation energy transfer decreases exponentially with distance (50). Nevertheless, in our system it was not possible to discern the individual energy transfer contribution of Trp53 and Trp54 (see Fig. 2), since these probably behave as a single transfer unit to PLP. The energy transfer efficiency shown by each variant (Fig. 8) decreased as point mutations were incorporated into the native G6PDs, clearly indicating that the distance between the Trp53-Trp54 transfer unit and the PLP-labeled Lys205 is greater in the mutant enzymes (G6PD A’ > G6PD A) than in the normal G6PD B.

The in vitro process of refolding to obtain a catalytically active protein does not entirely correspond to the in vivo folding process. In vitro, folding is less efficient and often requires physicochemical conditions different from those of the intracellular environment. Furthermore, refolding assays involve the whole polypeptide chain, whereas, in vivo, polypeptide folding may start as soon as the N-terminal portion of the nascent chain emerges from the ribosome (51). It has been previously reported that the N126D mutation alone does not affect the in vitro refolding of G6PD A, while the additional presence of the second mutation V68M in G6PD A’ poses a serious constraint to the productive refolding of the protein (13). The onset of in vitro refolding has been associated with the simultaneous collapse of hydrophobic motifs buried within the molecule, the formation of stable secondary structures providing the necessary architecture for the subsequent folding and the formation of covalent bonds (S–S bridges) stabilizing the polypeptide in a given favorable folding conformation (52–55).

In conclusion, it would appear that the combination of changes in hydrophobic surface exposure, shifts in secondary structural elements, and displacements of domains by misinteractions in the deficient G6PD A’ lead to a modified, less stable conformation with altered topology, which shifts the intracellular equilibrium between unfolded/denatured and folded/native states. In the anuclear red blood cell, this results in a 90% reduction of active molecules of G6PD A’.

Acknowledgments—Thanks are due to Susana Perez-Benavente for excellent technical assistance, to Dr. Philip Mason (Imperial College, School of Medicine, London) and Dr. Margaret Adams (University of Oxford, Oxford) for useful discussions on G6PD deficiency mechanisms, to Prof. Carlos Gutierrez-Merino (Universidad de Extremadura, Badajoz) for help and for the provision of the microcolorimeter, to Prof. Francisco Garcia-Blanco and Dr. Susana Corrales (Universidad Complutense de Madrid (UCM) Madrid) for providing the CD spectrometer and for help, and to Dr. Alvaro Martinez del Pozo (UCM) Madrid for use of the deconvolution software.

REFERENCES

1. Vulliamy, T., Luzzatto, L., Hirono, A., and Beutler, E. (1997) Blood Cells Mol. Dis. 23, 302–313
2. Vulliamy, T., Mason, P. J., and Luzzatto, L. (1992) Trends Genet. 8, 138–143
3. Biehnle, U., Ayeni, O., Lucas, A. O., and Luzzatto, L. (1972) Lancet I, 107–110
4. Ruwende, C., Khoo, S. C., Snow, R. W., Yates, S. N., Kwiatkowski, D., Gupta, S., Warn, P., Alspaugh, C. E., Gilbert, S. C., Forscher, N., Newbold, C. I. (1996)
5. Bautista, J. M., Vulliamy, T. J., Luzzatto, L., and Adams, M. J. (1996) Blood 87, 2974–2982
6. Francisc Garcia Blanco and Dr. Susana Corrales (Universidad Complutense de Madrid (UCM) Madrid) for providing the CD spectrometer and for help, and to Dr. Alvaro Martinez del Pozo (UCM) Madrid for use of the deconvolution software.

[The rest of the text is not transcribed due to the nature of the data.]
Structural Defects Underlying Protein Dysfunction in Human Glucose-6-phosphate Dehydrogenase A^- Deficiency
Félix Gómez-Gallego, Amando Garrido-Perttierra and José M. Bautista

J. Biol. Chem. 2000, 275:9256-9262.
doi: 10.1074/jbc.275.13.9256

Access the most updated version of this article at http://www.jbc.org/content/275/13/9256

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 52 references, 11 of which can be accessed free at http://www.jbc.org/content/275/13/9256.full.html#ref-list-1