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Functional properties of two mutants of human glucose 6-phosphate dehydrogenase, R393G and R393H, corresponding to the clinical variants G6PD Wisconsin and Nashville

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Abstract

Two severe Class I human glucose-6-phosphate dehydrogenase (G6PD, EC1.1.1.49) mutations, $G6PD_{Wisconsin}$ (nt1177 C→G, R393G) and $G6PD_{Nashville}$ (nt1178 G→A, R393H), affect the same codon, altering a residue in the dimer interface close to the "structural" $NADP^+$ site. These mutations are predicted to influence interaction with the bound "structural" $NADP^+$, long supposed to be crucial for enzyme stability. Recombinant proteins corresponding to these mutants have been constructed, expressed and purified to homogeneity. Steady-state kinetic parameters of the mutant enzymes were comparable to those of normal human G6PD, indicating that the mutations do not alter catalytic efficiency drastically. However, investigations of thermostability, urea denaturation, protease digestion, and hydrophobic exposure demonstrated that G6PD R393H is less stable than normal G6PD or R393G, and stability was more $NADP^+$-dependent. Apoenzymes were prepared by removal of "structural" $NADP^+$. Again the $G6PD_{Nashville}$ protein was markedly less stable, and its dissociation constant for "structural" $NADP^+$ is ~500 nM, about 10 times higher than values for R393G (53 nM) and normal G6PD (37 nM). These results, together with structural information, suggest that the instability of the R393H protein, enhanced by the weakened binding of "structural" $NADP^+$, is the likely cause of the severe clinical manifestation observed for $G6PD_{Nashville}$. They do not, however, explain the basis of disease in the case of $G6PD_{Wisconsin}$.

Keywords: Glucose 6-phosphate dehydrogenase deficiency; Site-directed mutagenesis; Steady-state kinetic; Structural $NADP^+$; Dissociation constant; Stability

1. Introduction

Glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) catalyses the first committed step in the pentose phosphate pathway, generating NADPH, which protects the mature erythrocyte cell against oxidative damage by maintaining a high level of reduced glutathione (GSH) and assisting the action of catalase [1–3]. G6PD deficiency accordingly manifests itself through abnormal haematology, and is the most common human enzymopathy, affecting about 400 million people worldwide [4]. Among about 140 different G6PD mutations identified at the DNA level, over 60 are severe Class I mutations which cause chronic non-spherocytic haemolytic anaemia (CNSHA) [5]. The fact that a disease so damaging to blood cells fails to manifest itself in more widespread disruption of metabolism is generally taken as indicating that the problem is one of protein instability rather than low initial activity. The non-nucleate mature erythrocyte has to survive over several months in circulation, but, unlike, say, hepatocytes, is unable to replace enzyme molecules that become denatured over that time period.

It has recently become possible to interpret changes in the DNA and amino acid sequences in terms of the solved three-dimensional structure of the enzyme protein. Interestingly, more than half of the known Class I mutations cluster in exon 10, which encodes residues in the dimer interface and close to

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the "structural" $NADP^+$ binding site [6,7]. Both these structural features are thought to play important roles in the stability and integrity of the active enzyme. Two naturally-occurring Class I mutations, G6PD Wisconsin (nt 1177 C→G, R393G) [8] and G6PD Nashville (nt 1178 G→A, R393H) [9], affect the same codon located in this important region. It is noteworthy that the importance of this position was already noted in advance of a structure solution for human G6PD in a modeling study based on the solved structure of G6PD from *Leuconostoc mesenteroides* [10]. In the bacterial enzyme, possible destabilization of "structural" $NADP^+$ is not an issue, as this feature is missing. However, the residue in question is at the dimer interface in both the bacterial and the human enzyme, and in the modeling study destabilization of the oligomeric structure was therefore postulated as the basis for the clinical defect [10].

So far, G6PD Wisconsin (R393G) has only been reported once, in a 7-year-old white male, without detailed biochemical characterisation owing to insufficient blood sample. In contrast, G6PD Nashville (R393H), first identified in USA, has also been reported in Italy, Portugal, Poland, Mexico etc. Although different names, Nashville, Anaheim, Calgary, and Portici, were initially given to the G6PD variants identified, the same codon change, leading to replacement of Arg-393 by His, has been found at the DNA level [11–15]. Details from the reports suggest that this mutation has recurred in unrelated patients. As a reviewer has pointed out, the greater frequency of the Nashville mutation probably reflects the relative ease of the C→T transition as compared with the G→C transversion required for the Wisconsin mutation. G6PD R393H was reported to show low affinity for G6P and to be extremely unstable both *in vitro* and *in vivo* [9,11]. However, the thermolabile enzyme could be reactivated in the presence of high concentrations of $NADP^+$ [9].

Speculative interpretations based on molecular modeling [10], though valuable, are no final substitute for direct experimental evidence. The availability of the cloned human G6PD gene and a system for expression and purification [16] offers the possibility of direct comparison of pure mutant and normal enzymes. Accordingly, in order to understand the molecular mechanism underlying G6PD deficiency in these two mutants, and possibly shed further light on the role of "structural" $NADP^+$, recombinant clones corresponding to the clinical mutants, R393G and R393H, were constructed by site-directed mutagenesis, and the expressed proteins were purified to homogeneity for detailed functional studies and comparison with normal, wild-type human G6PD (WT). The results reveal marked and surprising differences in the properties of the two mutant forms of G6PD. The extreme sensitivity to the nature of any substitution at this position was confirmed by less detailed studies of mutants in which R393 was replaced by E, V, L or I.

2. Materials and methods

2.1. Materials

Restriction enzymes, calf intestinal alkaline phosphatase, DNA modifying enzymes, and DNA markers were purchased from NEBiolabs (USA). Agarose was from GIBCO BRL (USA). DNA Miniprep kit and Gel extraction kit were from QIAGEN (USA). All the oligonucleotide primers for both mutagenesis and DNA sequencing were purchased from GIBCO BRL Life Technologies Co. (USA). 2′5′ ADP Sepharose 4B was from Amersham Biosciences (USA). EDTA-free protease inhibitor cocktail was from Roche.

Glucose-6-phosphate, trypsin, chymotrypsin, 8-anilinonaphthalene-1-sulphonic acid (ANS), and $NADP^+$ analogues were obtained from Sigma Chemical Company (UK). Centricon YM-50 was from Millipore Corporation (USA). Roche Diagnostics supplied $NADP^+$ (grade II) and NADPH (grade I). $NADP^+$ concentrations were determined spectrophotometrically at 260 nm ($\varepsilon_{260}=18.0\times10^3\ M^{-1}\ cm^{-1}$), and NADPH at 340 nm ($\varepsilon_{340}=6.22\times10^3\ M^{-1}\ cm^{-1}$).

Spectrophotometric measurements were made throughout with a Cary 300 Bio UV-visible spectrophotometer. Steady-state initial rates and protein fluorescence were measured with a Hitachi F-4500 fluorimeter. All reagents used were analytical grade supplied by Sigma Chemical Co (USA) or BDH (UK).

2.2. Construction of expression plasmid

All DNA manipulations were carried out by standard procedures [17]. The recombinant plasmid pTrc/G6PD, containing the full cDNA sequence of the normal human G6PD gene [16], was used as a template for mutagenesis. The desired mutations in G6PD R393G and R393H, were created by megaprimer-based site-directed mutagenesis [18,19].The two flanking primers (below) were IMPACT NDES, generating an *Nde*I restriction site at the 5′ end of the gene, and G6PD-pQE-*Sal*I-R, creating a *Sal*I restriction site at the 3′ end. The internal mutagenic primers were G6PD-R393G and G6PD-R393H, with the mutagenic base change highlighted in bold in each case.

Primer IMPACT NDES: 5′pCAGGAAACAGCATATGGCAGAGC 3′
Primer G6PD-pQE-*Sal*I-R: 5′pTAGGGCGTCGACTCAGAG-CTTGTGGGGGTTCAC 3′
Mutagenic primer G6PD-R393G: 5′CTGGTGATC**G**GCGTGCAGCCC 3′
Mutagenic primer G6PD-R393H: 5′AGCTGGTGATCC**A**CGTGCAGCC-CAA 3′

In the first-round PCR, the reaction mixture contained 0.5 μl of template (pTrc99A/G6PD, 0.5 μg/μl), 5 μl of Cloned pfu buffer (10×), 2 μl of flanking primer G6PD-pQE-*Sal*I-R (10 pmol/μl), 2 μl of mutagenic primer (10 pmol/μl), 5 μl of 2 mM dNTPs, 35 μl of ddH_2O, and 0.5 μl of Cloned pfu polymerase in a final volume of 50 μl. A 5-min denaturation step at 95 °C was followed by 34 cycles of PCR amplification, each consisting of 36 s at 95 °C, 2 min at 50 °C, 1 min at 72 °C, and followed by a final extension step at 72 °C for 10 min. The amplified PCR products were analysed by agarose gel electrophoresis, and the putative DNA fragments containing desired mutations were purified using the QIA quick gel extraction kit. In the second-round PCR, the product of the first-round PCR, the megaprimer, together with primer IMPACT NDES and the template (pTrc99A/G6PD), were used to synthesize the full-length G6PD cDNA harbouring the mutations. Each reaction mixture contained 0.5 μl of template (pTrc99A/G6PD, 0.5 μg/μl), 5 μl of Cloned pfu buffer (10×), 2 μl of IMPACT NDES primer (10 pmol/μl), 2 μl of megaprimer (first-round PCR product), 5 μl of 2 mM dNTPs, 35 μl of ddH_2O, and 0.5 μl of Cloned pfu polymerase in 50 μl final volume. PCR was run at 95 °C for 5 min, followed by 30 cycles of 36 s at 95 °C, 1 min at 55 °C, 4 min at 72 °C, and a final extension step at 72 °C for 10 min. Amplified products of the second-round PCR were analysed, purified and then digested with *Nde*I and *Sal*I. The resulting fragment was cloned into the expression vector, pET30b, also digested with *Nde*I and *Sal*I.

In an exactly similar fashion, four new mutations that have not so far been clinically reported were also made at the same position to replace Arg 393 with Glu, Leu, Val and Ile. In all cases DNA constructs were sequenced both to confirm the correct incorporation of the mutation and to check for any unintended mutations elsewhere in the gene.

2.3. Expression and purification of recombinant G6PD

Recombinant constructs with the desired mutation(s) were transformed into competent *E. coli* BL21(DE3) cells, which were grown at 37 °C overnight on a

Luria–Bertani (LB) plate containing 50 μg/ml kanamycin. Single recombinant clones containing either the entire wild-type gene or the variant cDNA insert were separately inoculated into 5 ml LB medium with kanamycin and grown overnight. 2 ml overnight culture was inoculated into 200 ml LB medium with kanamycin and grown at 37 °C with shaking. When A_{600} of the culture reached ~0.8, IPTG was added to a final concentration of 0.5 mM. The culture was allowed to grow for another 5 h at 30 °C before harvesting by centrifugation. The wild-type and mutant G6PDs were successfully purified by affinity chromatography on 2′,5′-ADP-Sepharose 4B as described previously [20].

2.4. Initial rate measurements

Steady-state kinetic studies were carried out fluorimetrically at 25 °C [16]. The reaction buffer was 0.1 M Tris/HCl, pH 8.0, containing 0.01 M $MgCl_2$. $NADP^+$ concentrations ranged from 2 to 50 μM and G6P concentrations from 40 to 1000 μM.

2.5. Stability in the presence or absence of urea

In order to study susceptibility to denaturation, the purified enzyme was first diluted extensively so that the final concentration of free $NADP^+$ was ~1 μM (75 μM $NADP^+$ is used to elute the enzyme from the column during purification and to stabilize during storage). Different concentrations of urea were added to separate samples, which were then incubated at room temperature. Residual enzyme activity was measured at intervals over a period of up to 3 h.

2.6. Protease digestion

Trypsin and chymotrypsin were used to probe proteolytic sensitivity of G6PD WT, R393G, and R393H. G6PD samples were diluted to a final protein concentration of 0.01 mg/ml. Trypsin (0.5 mg/ml) or chymotrypsin (0.05 mg/ml) and different amounts of $NADP^+$ from 1 μM to 100 μM were added to individual samples. The mixtures were kept at room temperature and the residual G6PD activity was measured at intervals. Control samples without protease kept over 90% of their activity throughout the incubation.

2.7. Hydrophobic surface exposure experiment

The exposure of hydrophobic areas, a feature of a putative 'molten-globule' state [21], was assessed by fluorimetric assay of the binding of 8-anilinonaphthalene-1-sulphonic acid (ANS). The protein (10 μg/ml), with various concentrations of urea, was incubated with 20 μM ANS. A Hitachi F-4500 spectrofluorimeter was used at a working power of 700 w. Excitation and emission wavelengths were 350 nm and 470 nm, respectively, with a slit of 10 nm in each case.

2.8. Determination of dissociation constant of "structural" $NADP^+$

"Structural" $NADP^+$ was quantitatively stripped from the holoenzymes as described [22]. In brief, "structural" $NADP^+$ could be converted to NADPH by adding G6P to the holoenzyme, followed by gel filtration to remove NADPH and the remaining G6P. The resulting apoenzyme was titrated with $NADP^+$ using a Hamilton microsyringe, and the fluorescence quench was monitored as described in the same report.

2.9. Apoenzyme stability test

The apoenzymes were incubated at 37 °C, with and without 10 μM $NADP^+$, at a protein concentration of 10 μg/ml. Enzyme activity was followed during the incubation by removing samples for assay.

3. Results

3.1. Enzyme preparation

Under the conditions described, 1300 IU, 1200 IU, and 1000 IU G6PD activity per litre culture were obtained for G6PD WT, R393G, and R393H in the initial crude extract, and quite similar figures were also obtained for the E, V, L and I mutants (Table 1). This suggests in all cases similar expression levels of enzymes of similar activity. Following identical purification procedures from this relatively uniform starting point, however, the final yields of pure protein covered a wide range: the final purification yield of G6PD R393G was about 50%, similar to that for WT, but was much lower, at 6–7%, for R393H and R393E. The other three mutants gave yields of 24–31%. The purified G6PD WT and R393G showed specific activities of around 180 IU/mg, and the three mutants R393V, R393L and R393I also showed similar activities ranging from 166 to 181 IU/mg. Again G6PD R393H and R393E were distinctly different, with specific activities about 25% lower, at 130–135 IU/mg.

3.2. Steady-state kinetic parameters

Detailed kinetic measurements were made for the two clinical mutants and WT G6PD for comparison. The Dalziel [23] parameters of G6PD R393G and R393H (Table 2) were derived as described for the WT enzyme from primary and secondary plots of the initial rates [16,20]. The k_{cat} value ($1/\phi_o$) of G6PD WT (275 s^{-1}) was only slightly higher than those of G6PD R393G (232 s^{-1}) and G6PD R393H (192 s^{-1}), showing that the maximal catalytic efficiency is only moderately affected by the mutations. However, the K_m values of G6PD R393H for G6P (ϕ_{G6P}/ϕ_o)and $NADP^+$ (ϕ_{NADP+}/ϕ_o) were 190 μM and 16.5 μM respectively, about four-fold and two-fold higher than the values for the WT enzyme, implying that this mutation has significantly affected the binding of the substrates. By contrast,

Table 1
Yields and specific activities of recombinant human G6PD and six mutants

	WT	R393G	R393H	R393E	R393V	R393L	R393I
Total enzyme activity in the crude extract from 200 ml culture (IU)	260±17	240±19	200±16	195±15	190±17	200±16	190±18
Specific activity in the crude extract (IU/mg)	2.5±0.2	2.4±0.2	2.1±0.3	1.9±0.3	2.1±0.1	2.0±0.2	1.9±0.3
Total activity of purified enzyme (IU)	130±15	120±15	12±5	14±8	48±10	62±10	46±9
Specific activity of purified enzyme (IU/mg)	180±10	178±15	130±10	135±15	166±14	176±12	181±10
Yield (%)	50±5	50±5	6±3	7±4	25±5	31±5	24±5

The total activity in the crude extract reflects both the expression level and the specific activity for each protein. After purification, on the one hand, the specific activities reflect the true catalytic properties of each protein, and, on the other, the total activity at this point is a reflection of the extent of loss through instability during purification.

the K_m values for the glycine mutant were, within statistical error, unaltered.

3.3. Susceptibility to denaturation by heat or urea and to protease digestion

Structural instability of the mutant enzyme is thought to be the most frequent explanation for G6PD deficiency in man and might be expected to be demonstrable *in vitro*. At the physiological concentration of $NADP^+$ (around 10 μM), T_{50}, the temperature at which 50% of the original activity is retained after incubating at different temperatures for 20 min, was about 50 °C for WT and R393G. However, the T_{50} of R393H was only 40 °C under the same conditions, suggesting that this mutant is much more thermolabile (data not shown). G6PD WT and R393G were similarly quite stable in the presence of 1 M urea, and began to lose activity gradually (30–40% over 3 h) only when the concentration of urea was increased to 1.5 M (Fig. 1). In contrast, R393H was very susceptible to urea, and lost about 80% of its activity even after incubating with 0.5 M urea for only 10 min. For all three enzymes, $NADP^+$ (10 μM) offered protection against urea denaturation, but this was clearly only partial in the case of R393H. Compared with G6PD WT and R393G, G6PD R393H was also more susceptible to proteolysis both by trypsin and by chymotrypsin (Fig. 2). Addition of $NADP^+$ again seemed to counteract the susceptibility of all three enzymes to proteolytic digestion in a concentration-dependent manner.

3.4. Exposure of hydrophobic areas

Fig. 3 shows the ratio of the fluorescence of WT and mutants, which were incubated with different concentrations of urea for 2 h, to the fluorescence of these enzymes in the absence of urea. The fluorescence signals of WT and the two mutant enzymes increased and reached a maximum after incubating with 3 M urea for 30 min (data not shown) or at 2 M for 2 h, although each enzyme showed a different maximum peak height at these urea concentrations. Without urea, the fluorescence signal of R393H was about 31.4% ($\delta F_0/\delta F_{max}$) of the maximum fluorescence signal observed after incubating the mutant with 2 M urea for 2 h. The much lower corresponding figures for WT (19%) and R393G (9%), suggest that a larger hydrophobic area is accessible to ANS in R393H in the absence of urea.

3.5. Dissociation constant of "structural" $NADP^+$ and stability

In a recent study [22] we have found that "structural" $NADP^+$ may be removed from G6PD to form an active apoenzyme. The addition of $NADP^+$ quenches the intrinsic fluorescence of the apoenzyme, making it possible to estimate a dissociation constant for the tight binding of the "structural" $NADP^+$. Similar experiments were carried out with the two mutant enzymes, and, from the fluorescence titration, the dissociation constant of R393H was around 500 nM, almost 14 times higher than that of the WT enzyme (37 nM), indicating that binding of "structural" $NADP^+$ in this mutant is considerably impaired. In this case the use of relatively high coenzyme concentrations allowed the K_d to be readily estimated from a Scatchard plot (not shown) of the data. However, for G6PD R393G the dissociation constant of "structural" $NADP^+$ was much lower, necessitating an iterative fit of the data, since binding to the enzyme significantly depletes the concentration of free $NADP^+$. An excellent fit was obtained for a K_d value of 53 nM, on the assumption of 97.2% quenching of the fluorescence of the free enzyme. (For 12 points the value of F_{calc}/F_{actual} was randomly scattered either side of 1.000, with a maximum value of 1.005 and a minimum value of 0.997). This figure for K_d is only moderately elevated relative to that for the normal enzyme.

Previous studies showed that "structural" $NADP^+$ is important for the stability and integrity of the active enzyme [6,24–28]. In the current study, the WT apoenzyme lost activity rapidly when incubated at 37 °C: after 30 h at 37 °C, only 10% enzyme activity remained [22]. Compared with the WT enzyme, G6PD R393H without $NADP^+$ was even less stable, losing all activity after 20 h, in agreement with the original observations of Beutler et al. [9] and Filosa et al. [11]. However, under the conditions used, no significant difference in stability between R393G and WT could be observed. In the presence of 10 μM $NADP^+$, all the enzymes appeared to be more stable, but there was still a clear differentiation between the two mutants: WT and R393G remained active for 1 month at 37 °C, whereas R393H lost all activity after 9 days of incubation (data not shown).

4. Discussion

G6PD deficiencies are usually recognized through haematological studies, and the classification of clinical severity is based on the levels of G6PD activity found in red cells.

Table 2
Dalziel steady-state kinetic parameters

Enzyme	ϕ_o (s)	ϕ_{NADP+} (μM s)	ϕ_{G6P} (μM s)	$\phi_{NADP+G6P}$ (μM^2 s)	$\phi_{NADP+G6P}/\phi_{NADP+}$(μM)	$\phi_{NADP+G6P}/\phi_{G6P}$(μM)	k_{cat} (s^{-1})	K_{mNADP+} (μM)	K_{mG6P} (μM)	k_{cat}/K_{mNADP+} (μM^{-1} s^{-1})	k_{cat}/K_{mG6P} (μM^{-1} s^{-1})
WT	0.00366±0.00023	0.0259±0.0041	0.191±0.021	1.61±0.22	58.0±0.68	7.77±0.68	275±18	7.07±1.13	52±4	39.7±7.5	5.31±0.69
R393G	0.0043±0.00032	0.0402±0.0092	0.288±0.018	3.47±0.59	84.8±4.6	12.0±1.21	232±16	9.31±2.4	67.1±13	25.8±3.2	3.52±0.50
R393H	0.0052±0.0005	0.0853±0.0067	0.992±0.11	12.1±0.57	142.0±9.5	12.4±1.3	192±18	16.5±2.7	190±27	13.9±2.3	1.02±0.15

The table shows the four constants of the reciprocal initial-rate equation [23] as estimated for wild-type G6PD and for the two mutant enzymes R393G and R393H. The corresponding of k_{cat} values and the true K_m values for both substrates are also listed. The parameters in each case were obtained from three independent experiments and from different enzyme preparations.

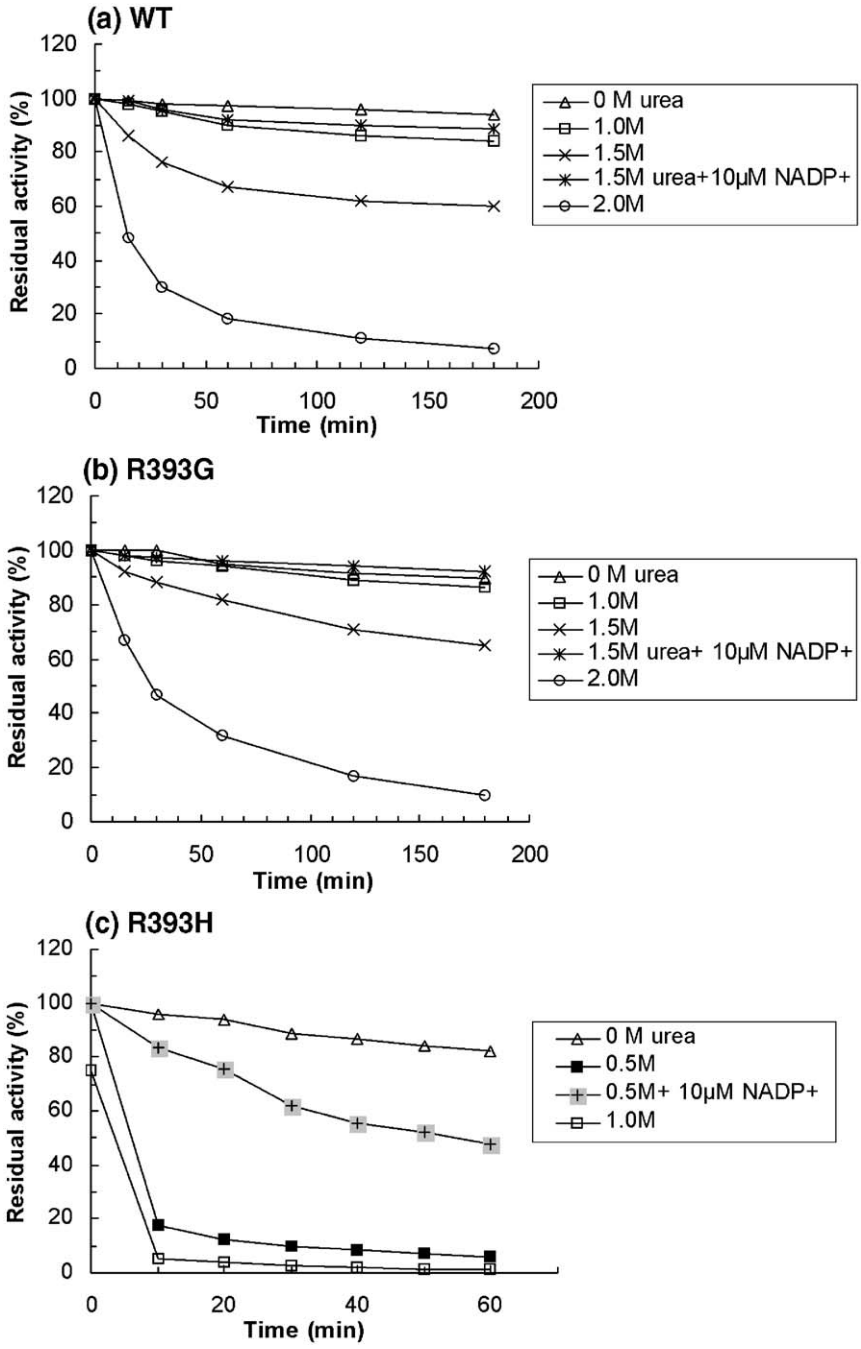

Fig. 1. Stability of G6PD WT, R393G and R393H incubated with different concentrations of urea in the absence or presence of $NADP^+$.

Measurements on red cell extracts may give a reasonably accurate indication of alterations in K_m values, but decreased absolute levels of activity may reflect either decreased specific activity of the mutant G6PD or else impaired folding or stability, resulting in a lower concentration of active enzyme protein. To resolve such ambiguities and obtain a clearer view of the molecular basis of the enzymopathy, it is valuable to be able to compare the purified enzymes, and recombinant expression provides a route to substantial quantities of pure protein for such studies.

In the present study we have focused attention on two severe Class I mutants, G6PD Wisconsin (R393G) and G6PD Nashville (R393H), in which the substituted residue is at a site well removed from the catalytic site but close to the binding site [6] for "structural" $NADP^+$ between the G6PD dimer interface and the C-terminus. It was hoped that detailed study of these two naturally-occurring mutants, and of other mutations at the same site, might illuminate the role of Arg 393 in the structure and function of h-G6PD and yield further evidence regarding the significance of the "structural" $NADP^+$ molecule.

The similar levels of recombinant expression of wild-type G6PD and all six mutants studied clearly indicate that the problem in the clinical cases does not lie in impaired folding.

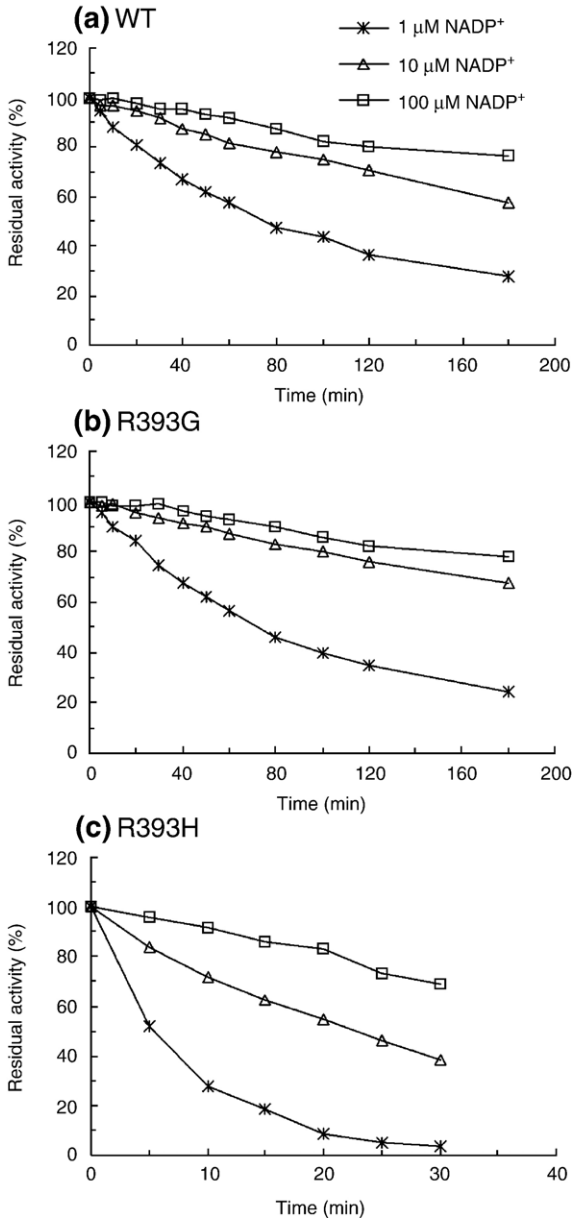

Fig. 2. Susceptibility of G6PD WT, R393G, and R393H to trypsin in the presence of different concentrations of $NADP^+$.

On the other hand the results of the purification procedure do provide immediate evidence that two of the mutants, R393H and R393E are considerably less stable than the others. Although R393 is not close to the active site, it was also necessary to check whether the mutations had affected kinetic properties, since a small structural perturbation might markedly impair catalytic efficiency, as in the case of G6PD R454C (G6PD Union) and R454H (G6PD Andalus) [20]. The specific activities of the purified proteins (Table 1) show, however, only a moderate decrease in R393H and R393E, with the figures for the other mutants close to that for wild-type G6PD. A more detailed kinetic comparison of the two clinical mutants with normal G6PD (Table 2) reveals that the K_m values for coenzyme (ϕ_{NADP+}/ϕ_o) and sugar phosphate (ϕ_{G6P}/ϕ_o) of the R393G enzyme are 9.3 μM and 67 μM, very similar to the corresponding figures for WT enzyme (7.07 μM and 52 μM). On the other hand, the K_m values of 16.5 μM and 190 μM for $NADP^+$ and G6P found for R393H are considerably higher, in agreement with the original report of a substantially increased K_m for G6P with $G6PD_{Nashville}$ [11]. G6PD WT and R393G have quite similar catalytic efficiencies under saturating conditions, whereas the k_{cat} value of G6PD R393H (192 s^{-1}) was about 30% lower than that of the WT enzyme (275 s^{-1}). This modest decrease in catalytic efficiency is, however, clearly unlikely to be the predominant cause of the severe disease phenotype.

A distinctive characteristic of mature non-nucleate erythrocytes is their inability to synthesize proteins to replace damaged and aged enzyme molecules. Therefore, instability of the protein is usually the key problem in G6PD deficiency. The initial indications of instability in R393H from Table 1 were convincingly borne out by a series of studies on the purified protein, showing that, even in the presence of a physiological concentration of $NADP^+$, the mutant R393H is much more susceptible than the WT or R393G to denaturation by heat or urea and also to proteolysis. The study with ANS suggests that this mutant enzyme has a looser structure, making more hydrophobic surfaces accessible to the dye probe. In striking contrast, in all these studies the second clinical mutant, R393G, behaved quite similarly to the normal enzyme.

Details from the 3-D structure of human $G6PD_{Canton}$ imply that the "structural" $NADP^+$ is important in maintaining the stability and integrity of the active enzyme [6]. Modeling shows that Arg 393 is close to, and may interact with, the "structural" $NADP^+$ [6], a view supported by details from the newly-solved crystal structures of G6P and $NADP^+$ complexes of a truncated G6PD mutant [29]. Arg 393 is at the end of the sheet βL in the dimer interface (Fig. 4) and the atoms Nη1 and Nη2 of this residue interact with the nicotinamide amide oxygen of the "structural" $NADP^+$. It has also been proposed that the side chain of Arg 393 could likely form hydrogen bond or charge interactions with other amino acids in/around the extensive β sheets which form the dimer interface. Replacement of this

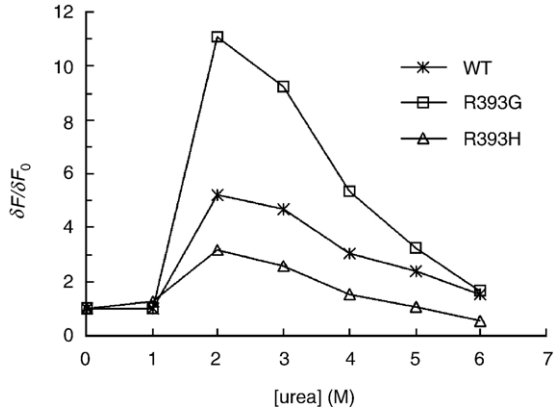

Fig. 3. Different hydrophobic exposure areas of G6PD WT, R393G, and R393H after incubation in different concentrations of urea for 2 h. δF and δF_0 are the fluorescence signals in the presence and absence of urea respectively.

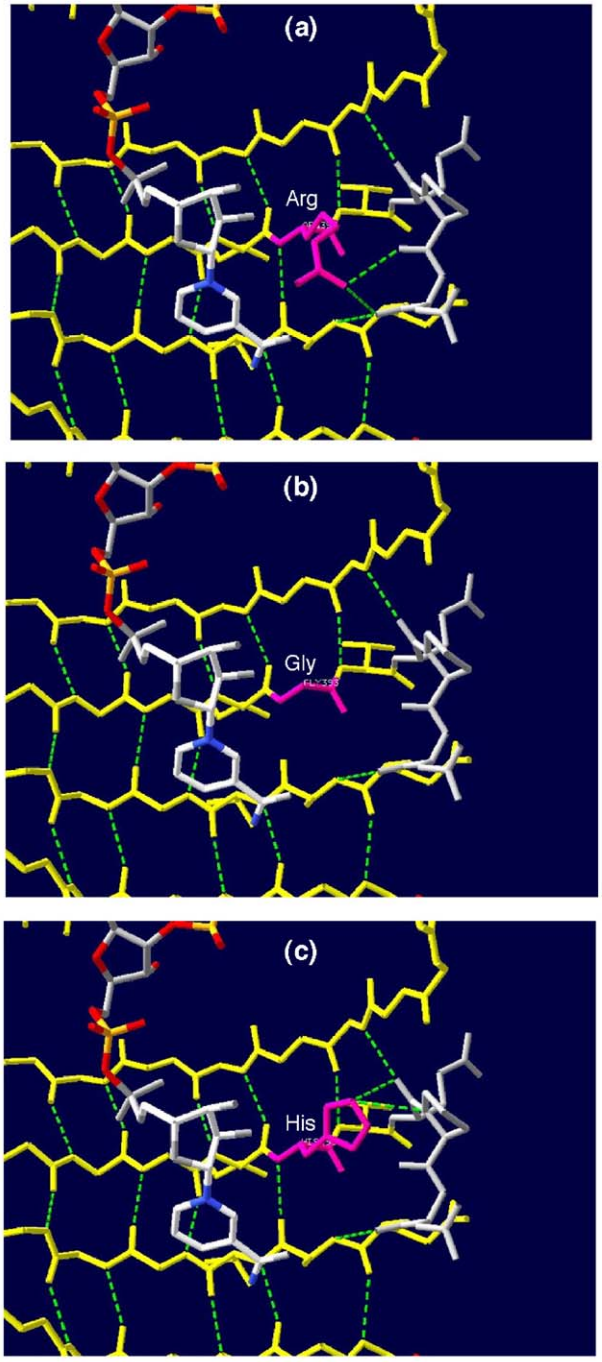

Fig. 4. Modeled 3-D structure of G6PD around amino acid 393. (a) WT, (b) R393G, and (c) R393H.

residue with Gly would prevent the interaction with the $NADP^+$ amide and the charge contacts would be disrupted as well (Fig. 4). This small change could affect the interplay between the dimer interface and the "structural" $NADP^+$, thus leading to instability of the mutant enzymes. The replacement with glycine thus might have been thought the more drastic of the two clinical mutations, but the overall pattern, including the results with E, V, L and I mutants, suggests that loss of the guanidino group's interactions in the various hydrophobic replacements is actually less damaging than replacement with a residue such as H or E which may form inappropriate hydrogen bond or charge interactions.

The ability to remove "structural" $NADP^+$ and make a functional apoenzyme [22] allowed us to look more closely at the importance of the additional coenzyme molecule in the properties of the clinical mutants. It is clear that in the absence of tightly bound $NADP^+$ the R393H apoprotein is intrinsically less stable than the apoprotein of normal G6PD, and this may well be accounted for by destabilisation of the dimer interface [10]. On the other hand, in all the stability tests, the inclusion of $NADP^+$ strongly stabilized the enzymes, and the K_d determination shows that the intrinsically unstable R393H protein is also markedly impaired in its ability to bind and retain "structural" $NADP^+$. It is thus not only less stable but also less able to be stabilized.

In summary, instability, enhanced by the low binding capacity for "structural" $NADP^+$, is clearly the main cause of clinical enzyme deficiency in R393H, although this mutant also shows some moderate kinetic impairment. In marked contrast, no significant difference in the functional properties between WT and R393G has been detected under the experimental conditions studied so far, and the detailed studies of this mutant protein provide no clue to the basis of a severe phenotype. Only one case of R393G has been reported to date with little accompanying clinical and haematological information, and it is impossible to assess whether the observed Class I phenotype was dependent on environmental challenge or the individual genetic background. The biochemical analysis of the recombinant enzyme inevitably raises a question over the clinical classification of this mutant, although it remains possible that subtle structural changes render the protein vulnerable to destructive influences that are present in the erythrocyte but missing in the artificial purity of our *in vitro* experiments.

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References

[1] H.N. Kirkman, S. Galiano, G.F. Gaetani, The function of catalase-bound NADPH, J. Biol. Chem. 262 (1987) 660–666.
[2] M.D. Scott, L. Zuo, B.H. Lubin, D.T. Chiu, NADPH, not glutathione, status modulates oxidant sensitivity in normal and glucose-6-phosphate dehydrogenase-deficient erythrocytes, Blood 77 (1991) 2059–2064.
[3] G.F. Gaetani, M. Rolfo, S. Arena, R. Mangerini, G.F. Meloni, A.M. Ferraris, Active involvement of catalase during hemolytic crises of favism, Blood 88 (1996) 1084–1088.

[4] L. Luzzatto, A. Mehta, T.J. Vulliamy, Glucose 6-phosphate dehydrogenase deficiency, in: C.R. Scriver, A.L. Beaudet, W.S. Sly (Eds.), The Metabolic and Molecular Bases of Inherited Disease, 8th ed., McGraw Hill, New York, 2001, pp. 4517–4553.
[5] E. Beutler, T.J. Vulliamy, Hematologically important mutations: glucose-6-phosphate dehydrogenase, Blood Cells Mol. Diseases 28 (2002) 93–103.
[6] S.W. Au, S. Gover, V.M. Lam, M.J. Adams, Human glucose-6-phosphate dehydrogenase: the crystal structure reveals a structural NADP+ molecule and provides insights into enzyme deficiency, Structure 8 (2000) 293–303.
[7] C.J. Kwok, A.C. Martin, S.W.N. Au, V.M.S. Lam, G6PDdb, an integrated database of glucose-6-phosphate dehydrogenase (G6PD) mutations, Hum. Mutat. 19 (2002) 217–224.
[8] E. Beutler, B. Westwood, A. Melemed, P. Dal Borgo, D. Margolis, Three new exon 10 glucose-6-phosphate dehydrogenase mutations, Blood Cells Mol. Diseases 21 (1995) 64–72.
[9] E. Beutler, W. Kuhl, T. Gelbart, L. Forman, DNA sequence abnormalities of human glucose-6-phosphate dehydrogenase variants, J. Biol. Chem. 266 (1991) 4145–4150.
[10] C.E. Naylor, P. Rowland, A.K. Basak, S. Gover, P.J. Mason, J.M. Bautista, T.J. Vulliamy, L. Luzzatto, M.J. Adams, Glucose 6-phosphate dehydrogenase mutations causing enzyme deficiency in a model of the tertiary structure of the human enzyme, Blood 87 (1996) 2974–2982.
[11] S. Filosa, V. Calabro, D. Vallone, V. Poggi, P. Mason, D. Pagnini, F. Alfinito, B. Rotoli, G. Martini, L. Luzzatto, et al., Molecular basis of chronic non-spherocytic haemolytic anaemia: a new G6PD variant (393 Arg→His) with abnormal KmG6P and marked in vivo instability, Br. J. Haematol. 80 (1992) 111–116.
[12] T.A. Weimer, F.M. Salzano, B. Westwood, E. Beutler, Molecular characterization of glucose-6-phosphate dehydrogenase variants from Brazil, Hum. Biol. 65 (1993) 41–47.
[13] S. Filosa, N. Giacometti, C. Wangwei, D. De Mattia, D. Pagnini, F. Alfinito, F. Schettini, L. Luzzatto, G. Martini, Somatic-cell selection is a major determinant of the blood-cell phenotype in heterozygotes for glucose-6-phosphate dehydrogenase mutations causing severe enzyme deficiency, Am. J. Hum. Genet. 59 (1996) 887–895.
[14] T.J. Vulliamy, J.S. Kaeda, D. Ait-Chafa, R. Mangerini, D. Roper, J. Barbot, A.B. Mehta, M. Athanassiou-Metaxa, L. Luzzatto, P.J. Mason, Clinical and haematological consequences of recurrent G6PD mutations and a single new mutation causing chronic nonspherocytic haemolytic anaemia, Br. J. Haematol. 101 (1998) 670–675.
[15] G. Vaca, E. Arambula, A. Esparza, Molecular heterogeneity of glucose-6-phosphate dehydrogenase deficiency in Mexico: overall results of a 7-year project, Blood Cells Mol. Diseases 28 (2002) 436–444.
[16] X.T. Wang, S.W.N. Au, V.M.S. Lam, P.C. Engel, Recombinant human glucose-6-phosphate dehydrogenase. Evidence for a rapid-equilibrium random-order mechanism, Eur. J. Biochem. 269 (2002) 3417–3424.
[17] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, New York, 1989.
[18] O. Landt, H.P. Grunert, U. Hahn, A general method for rapid site-directed mutagenesis using the polymerase chain reaction, Gene 96 (1990) 125–128.
[19] G. Sarkar, S.S. Sommer, The "megaprimer" method of site-directed mutagenesis, BioTechniques 8 (1990) 404–407.
[20] X.T. Wang, V.M.S. Lam, P.C. Engel, Marked decrease in specific activity contributes to disease phenotype in two human glucose 6-phosphate dehydrogenase mutants, G6PD(Union) and G6PD(Andalus), Hum. Mutat. 26 (2005) 284.
[21] G.V. Semisotnov, N.A. Rodionova, O.I. Razgulyaev, V.N. Uversky, A.F. Gripas, R.I. Gilmanshin, Study of the "molten globule" intermediate state in protein folding by a hydrophobic fluorescent probe, Biopolymers 31 (1991) 119–128.
[22] X.T. Wang, V.M.S. Lam, P.C. Engel, What is the role of the second, 'structural' $NADP^+$ binding site in human glucose 6-phosphate dehydrogenase? Properties of a native apoenzyme, Biochim. Biophys. Acta, Protein Struct. (submitted for publication).
[23] K. Dalziel, Initial steady state velocities in the evaluation of enzyme–coenzyme–substrate reaction mechanisms, Acta Chem. Scand. 11 (1957) 1706–1723.
[24] H.N. Kirkman, Glucose 6-phosphate dehydrogenase from human erythrocytes: I. Further purification and characterization, J. Biol. Chem. 237 (1962) 2364–2370.
[25] H.N. Kirkman, E.M. Hendrickson, Glucose 6-phosphate dehydrogenase from human erythrocytes: II. Subactive states of the enzyme from normal persons, J. Biol. Chem. 237 (1962) 2371–2376.
[26] A.E. Chung, R.G. Langdon, Human erythrocyte glucose 6-phosphate dehydrogenase: II. Enzyme–coenzyme interrelationship, J. Biol. Chem. 238 (1963) 2317–2324.
[27] L. Luzzatto, N.C. Allan, Different properties of glucose 6-phosphate dehydrogenase from human erythrocytes with normal and abnormal enzyme levels, Biochem. Biophys. Res. Commun. 21 (1965) 547–554.
[28] A. Bonsignore, I. Lorenzoni, R. Cancedda, A. De Flora, Distinctive patterns of NADP binding to dimeric and tetrameric glucose 6-phosphate dehydrogenase from human red cells, Biochem. Biophys. Res. Commun. 39 (1970) 142–148.
[29] M. Kotaka, S. Gover, L. Vandeputte-Rutten, S.W.N. Au, V.M.S. Lam, M.J. Adams, Structural studies of glucose-6-phosphate and NADP+ binding to human glucose-6-phosphate dehydrogenase, Acta Crystallogr. D: Biol. Crystallogr. 61 (2005) 495–504.