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Clinical mutants of human glucose 6-phosphate dehydrogenase: impairment of NADP⁺ binding affects both folding and stability

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Key words: Glucose 6-phosphate dehydrogenase deficiency; Structural NADP⁺; Dissociation constant; Refolding; Stability

Abbreviation: G6PD, glucose 6-phosphate dehydrogenase; CD, circular dichroism; Gdn-HCl, guanidinium hydrochloride; Arg, L-Arginine monohydro-chloride; DTT, dithiothreitol

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Abstract

Human glucose 6-phosphate dehydrogenase (G6PD) has both the “catalytic” NADP⁺ site and a “structural” NADP⁺ site where a number of severe G6PD deficiency mutations are located. Two pairs of G6PD clinical mutants, G6PD_{Wisconsin} (R393G) and G6PD_{Nashville} (R393H), and G6PD_{Fukaya} (G488S) and G6PD_{Campinas} (G488V), in which the mutations are in the vicinity of the “structural” NADP⁺ site, showed elevated K_d values of the “structural” NADP⁺, ranging from 53 nM to 500 nM compared with 37 nM for the wild-type enzyme. These recombinant enzymes were denatured by Gdn-HCl and refolded by rapid dilution in the presence of L-Arg, NADP⁺ and DTT at 25 °C. The refolding yields of the mutants exhibited strong NADP⁺- dependence and ranged from 1.5% to 59.4% with 1000 μM NADP⁺, in all cases lower than the figure of 72% for the wild-type enzyme. These mutant enzymes also displayed decreased thermostability and high susceptibility to chymotrypsin digestion, in good agreement with their corresponding melting temperatures in CD experiments. Taken together, the results support the view that impaired binding of “structural” NADP⁺ can hinder folding as well as causing instability of these clinical mutant enzymes in the fully folded state.

1. Introduction

The pentose phosphate pathway (PPP) enzyme glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49; MIM# 305900) provides NADPH, vital, amongst other roles, in protecting cells against oxidative stress. The deficiency of G6PD in human red blood cells affects more than 400 million people and can cause severe disease phenotypes, mainly neonatal jaundice and haemolytic anaemia [1-3]. In the light of the resolved 3-D structure of human G6PD [4-6], extensive studies have been carried out to elucidate the molecular mechanisms underlying G6PD deficiency, which potentially include protein instability, decreased catalytic efficiency, and altered dimerisation [7-9]. Among these molecular defects, the low levels of G6PD activity in red blood cells have been mainly attributed to the instability of the protein, since, over 3-4 months in circulation, the non-nucleate mature erythrocytes cannot synthesize new enzyme to replenish lost activity. However, instability alone cannot account convincingly for low levels of G6PD activity also sometimes found in cases with reticulocytosis, where, by definition, the enzyme will have been recently synthesised.

Recently, several papers have also reported that G6PD mutations in different locations could affect the refolding ability *in vitro*, correlating with decreased stability of the enzymes but almost unaltered kinetic properties [10-12]. If these results also correlate with the ability of the enzyme to fold *in vivo*, it would suggest that, with some clinical mutations, even young erythrocytes would have depleted G6PD levels. If variants cannot fold with efficiency comparable to that of the WT enzyme, this also leaves them both prone to aggregation and susceptible to protease degradation *in vivo*.

Human G6PD has two NADP⁺ binding sites per subunit. One is the “catalytic” site, and the other is the “structural” site. Although the tightly bound NADP⁺ at this latter site is not directly involved in the enzyme-catalysed reaction, previous studies have shown that many severe Class I G6PD deficiency mutations are located in the dimer interface and close to the “structural” NADP⁺ site [5]. The auxiliary cofactor molecule is thought to be crucial for the stability and integrity of

active enzyme [13]. Furthermore, it has also been reported that NADP⁺ is required for monomer hybridization and refolding [10,11,14,15,16].

Several clinical mutations close to the “structural” NADP⁺ binding site, e.g. G6PD_{Wisconsin} (nt1177 C→G, R393G), G6PD_{Nashville} (nt1178 G→A, R393H), G6PD_{Fukaya} (nt1462 G→A, G488S) and G6PD_{Campinas} (nt1463 G→T, G488V), have been found to affect the binding capacity for the additional NADP⁺ molecule [9,13], although R393 mutations are located in the dimer interface and also affect the dimerization process. Whether impaired binding of “structural” NADP⁺ might also damage the folding of the mutant enzymes is still uncertain. Recently, we have developed a reproducible, high-yield refolding protocol, and it is clear that the presence of the coenzyme is critical in recovering the majority of the G6PD in its correctly folded, active form [14]. This protocol is, therefore, applied here to test these four clinical mutants in which binding of “structural” NADP⁺ is known to be impaired. In addition, tests of stability and susceptibility to protease digestion and CD experiments were carried out to further elucidate the importance of the “structural” NADP⁺.

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 Life Technologies (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.

NADP⁺ and NADPH were obtained from Apollo Scientific Ltd (UK), and 2'5' ADP-Sepharose 4B was from GE Healthcare. EDTA-free protease inhibitor cocktail was from Roche. Glucose 6-phosphate, guanidinium hydrochloride (Gdn-HCl), L-arginine monohydrochloride, dithiothreitol (DTT), polyethylene glycol (PEG, MW 3350), Trizma base, and TLCK-treated

chymotrypsin, all of reagent grade or better, were from Sigma. Spectrophotometric and fluorimetric measurements were made throughout with a Cary Bio50 spectrophotometer and a Hitachi F-4500 fluorimeter. Protein secondary structure was examined with a J810 spectropolarimeter from JASCO (UK).

2.2. Construction, expression and purification of recombinant proteins

The recombinant proteins, G6PD R393G, R393H, G488V and G488S, corresponding to the clinical mutants G6PD Wisconsin, Nashville, Campinas and Fukaya were expressed in *E. coli* and purified to homogeneity as previously reported [9,13].

2.3. Kinetic characterization of G6PD wild type and mutant enzymes

Steady-state kinetic studies and the thermostability tests on the recombinant enzymes were carried out as previously reported [8,9,13,17]. In brief, reaction mixtures contained 0.01 M MgCl_2 in 0.1 M Tris-HCl buffer, pH 8.0 with varying amounts of sugar phosphate and coenzyme in a total volume of 1ml. NADP^+ concentrations ranged from 1 to 75 μM and G6P concentrations from 10 to 1000 μM . Reaction was initiated by adding enzyme, typically in 10 μl , to give a linear fluorescence increase for at least the first 2 min.

2.4. Thermostability tests

Purified wild-type and mutant enzymes, freed from loosely bound NADP^+ by serial dilution using Centricon YM-50, were adjusted to 0.01 mg/ml protein concentration. NADP^+ was added back to give concentrations of 1 μM , 10 μM , 100 μM and 1 mM. All samples were incubated at various temperatures (25 °C – 65 °C at 5 °C intervals) for 20 min and subsequently cooled to 4°C. Residual activity was expressed as a percentage of the activity for the same sample measured at 25 °C before incubation.

2.5. *Protease digestion of G6PD wild type and mutant enzymes*

Chymotrypsin was used to probe proteolytic sensitivity of G6PD WT and mutants. G6PD samples were diluted to a final protein concentration of 0.01 mg/ml. Chymotrypsin (0.05 mg/ml) and different amounts of NADP⁺ from 1 μ M to 1000 μ M 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.

2.6. *Determination of dissociation constant of “structural” NADP⁺*

To determine the dissociation constant of “structural” NADP⁺, as described elsewhere [9,13], in brief, the purified enzyme, from which any loosely bound NADP⁺ had previously been removed by serial dilution, was incubated at room temperature with 1 mM glucose 6-phosphate for 30 min before removing NADPH by passing down a Sephadex G-75 column pre-equilibrated and eluted with the usual 0.1 M Tris-HCl buffer at pH 7.6. The “stripped” G6PD (5 nM subunits) was titrated with a concentrated solution of NADP⁺ added with a Hamilton syringe. Protein fluorescence at 345 nm was monitored after exciting at 290 nm. The power setting was 950w and the slit 10/10nm. The resulting data were fitted to the equation for a single binding constant with the help of the GOSA software package (Global Optimisation by Simulated Annealing) from Bio-Log (Toulouse, France).

2.7. *Protein unfolding and refolding*

Purified recombinant G6PD wild type and mutant enzymes (2-4 mg/ml) were reduced and denatured by 20 mM DTT and 4 M Gdn-HCl in 50 mM Tris-HCl, pH 7.6 at 30 °C for 2 hours. The unfolded proteins were diluted rapidly into the optimised refolding buffer [14] containing 500 mM L-Arg, 50 mM NaCl, 10 mM DTT, 40 mM PEG 3350 and varying concentrations of NADP⁺ in 50 mM Tris, pH 8.0. The refolding mixture was kept at 25 °C and the regain of enzyme activity was followed.

2.8. Circular dichroism analysis

Far-UV CD spectra (200-260 nm) of G6PD wild type and mutants (0.15 mg/ml) were recorded at 25 °C on a JASCO J-810 CD spectropolarimeter with a thermal Peltier temperature controller using a 2 mm path length cuvette. All reported spectra are averages of multiple scans with appropriate baseline subtraction. The thermostability of G6PD WT and mutant enzymes was also studied by following the change in CD at 222 nm as temperature was increased at 2 °C /min.

3. Results

3.1. Kinetic properties of human G6PD wild type and mutants

Table 1 shows the kinetic properties of human G6PD wild type and mutant enzymes. The detailed kinetic properties of G6PD clinical mutants G488S and G488V have not been reported previously. Here, detailed kinetic studies were carried out for these two clinical mutants in order to compare with published data for WT G6PD and R393 mutants [9]. The purified recombinant mutant enzymes, G488V and G488S, have specific activities at 160 IU/mg and 175 IU/mg respectively, quite similar to 180 IU/mg for WT enzyme, but a little higher than the R393H (130 IU/mg) mutant. The k_{cat} values ($1/\phi_o$) of G6PD G488S and G488V were 254 s⁻¹ and 212 s⁻¹, only slightly lower than that of the WT (275 s⁻¹), but comparable to R393G (232 s⁻¹) and R393H (192 s⁻¹), indicating that the maximal catalytic efficiency was only moderately affected by the mutations. In addition, the K_m values of G6PD G488V and G488S for NADP⁺ (ϕ_{NADP^+}/ϕ_o) were 10.7 μM and 11.8 μM respectively, about 1.5-fold higher than the value for the WT enzyme, implying that the mutations had some slight negative effects on coenzyme binding at the catalytic site. By contrast, the K_m values for the substrate G6P for these mutants were unaltered within statistical error (approx.

50 μM). In comparison, the K_m for G6P increased almost four-fold (190 μM) in R393H and to a much smaller extent (67 μM) in R393G.

3.2. Thermostability of G6PD WT and mutant enzymes

The stability of G6PD WT and mutant enzymes was examined with increasing temperatures in the presence of different concentrations of NADP^+ . R393G was as stable as the WT, whereas the other mutants R393H, G488V and G488S were all quite unstable (Figure 1). The transition temperature, $T_{1/2}$, is defined for each enzyme as the temperature at which 50% of its original activity is retained under current experimental conditions. At the physiological concentration of NADP^+ (around 10 μM), $T_{1/2}$ for both WT and R393G was above 50 $^{\circ}\text{C}$, about 10 $^{\circ}\text{C}$ higher than the values for R393H (42 $^{\circ}\text{C}$), G488V (37 $^{\circ}\text{C}$) and G488S (41 $^{\circ}\text{C}$). However, all the enzymes became more stable when the added concentrations of NADP^+ increased, and this NADP^+ -dependent stabilisation is more obvious for the R393H, G488V and G488S mutants.

3.3. Susceptibility to chymotrypsin digestion

The susceptibility of G6PD wild type and the mutant enzymes to chymotrypsin was examined in the presence of different concentrations of NADP^+ . Figure 2 shows that the mutant enzymes G488S and especially G488V were very unstable in the presence of 0.05 mg/ml chymotrypsin at room temperature. At 10 μM NADP^+ , T_{50} , the time at which 50% of the original activity is retained after incubating with chymotrypsin, was only 8 min and 5 min respectively for G488S and G488V, a much shorter time than 50 min for the WT enzyme. When the concentration of NADP^+ increased, the mutant enzymes became more resistant to chymotrypsin; T_{50} increased to 32 min for G488S and 8 min for G488V in the presence of 1000 μM NADP^+ , but was still much shorter than 80 min for the WT enzyme under these conditions. However, the mutant enzymes showed more marked protection by NADP^+ against chymotrypsin compared with the WT enzyme. As to the other mutant enzymes, R393G, like the WT enzyme, was quite stable compared with the

other mutants whereas R393H was very vulnerable to chymotrypsin, showing a similar pattern to G488S.

3.4. Dissociation constant of “structural” NADP⁺

A fluorescence titration approach [9,13] was applied to determine the dissociation constants of the WT and mutant enzymes for “structural” NADP⁺. The figures for the WT, R393G, R393H, G488V and G488S were 37 nM, 53 nM, 500 nM, 480 nM, and 273 nM, respectively, as previously reported [9,13].

3.5. Refolding of G6PD WT and mutants

Although the mutations in these clinical variants affect the binding capacity for “structural” NADP⁺, which has been reported to be important for the stability and integrity of folded, active enzyme, it has not been clear until now whether these mutants are also impaired in their folding properties. Investigation of this aspect of the defective proteins depends on a reliable procedure for obtaining reproducible levels of folding for human G6PD *in vitro*. Unfolding and refolding experiments on the mutant enzymes were therefore carried out according to the method recently established for the WT enzyme [14]. As with WT G6PD, refolding of the mutant enzymes was slow, taking about 7 days to reach the maximum yield (data not shown). Without added NADP⁺, the refolding yields were low, with only around 10% for the WT and R393G, and undetectable activity for R393H, G488S and G488V (Figure 3). In the presence of 200 μ M NADP⁺, the final recovery yield of R393G was 56.2%, a little lower than 69% for the WT. As for G488S, the yield increased significantly to about 16.4%. However, even with this level of NADP⁺, the mutants R393H and G488V still regained hardly any activity. The recovery yields increased further when higher concentrations of NADP⁺ were used, and this NADP⁺-dependent pattern was predictably more prominent for the mutants G488S, G488V and R393H. With 1000 μ M NADP⁺ the recovery yields only increased marginally for the WT enzyme (from 69% to 72%) and R393G (from 56.2% to

59.4%). In contrast, the refolding yields increased about two fold for R393H (from 1.8% to 3.5%), G488V (from 0.6% to 1.5%) and G488S (from 16.4% to 29.8%). However, even in this case, the recovery yields of R393H and G488V were still very low (Figure 3).

3.6. Secondary structure of G6PD WT and mutant enzymes

Since the mutant enzymes have low binding capacity for “structural” NADP⁺ and impaired refolding ability, it was necessary to check whether this reflected a wider structural disruption rather than a local defect. The secondary structures of the clinical mutants were examined by measuring the CD spectra in the far UV region (200-260nm). The CD spectra of the mutant enzymes were comparable in pattern and intensity to the native enzyme (Figure 4), indicating that the mutations did not significantly alter the secondary structures of the proteins once folded.

Although the secondary structures of the WT and the mutant enzymes are similar, the stability of these structures showed significant differences at elevated temperatures. The melting temperatures (midpoint of the transition), the temperatures at which half of the secondary structure is disrupted, were around 55.5 °C for WT and R393G, higher than 50 °C for R393H. As to the G488 mutants, G488V was very unstable, losing half of the secondary structure at 45.5 °C, even lower than G488S at 48.5 °C (Figure 5).

4. Discussion

It has long been proposed that human G6PD has a second “structural” NADP⁺ binding site in addition to the “catalytic” site [18-22]. Confirmation from the 3-D crystal structure of human G6PD_{Canton} [5], reveals that the “structural” NADP⁺ is tightly bound between the β sheets in the dimer interface and the C-terminus, well separated from the catalytic coenzyme binding site. Remarkably, more than 40 severe G6PD Class I deficiency mutations, e.g. R393, G488, and P489 mutations [23-28], are located in a distinct region which is close to the “structural” NADP⁺ and

interface dimerization sites, attracting inescapable attention to the roles of the auxiliary NADP⁺ and the dimer interface. Previous study showed that mutation of Asp421 in the dimer interface to neutral (A or N) and positively charged (K or R) residues could increase the thermostability and reduce the NADP⁺-dependent stabilising effect by fixing the enzyme conformation with a strong electrostatic attraction, indicating that the compactness and integrity of the dimer interface is crucial for the NADP⁺-independent stability of the enzyme [29].

Recent studies, in which it has become possible to measure the dissociation constant for the tightly-bound “structural” NADP⁺ [9,13], have shown that some naturally-occurring mutations in the cluster mentioned above cause dramatically decreased affinity for the auxiliary coenzyme molecule. From the 3-D structure, Gly 488 is located in the β O- α O turn of the C-terminus, important in positioning Arg 487 to interact with the adenine and the 2'-phosphate of “structural” NADP⁺ directly $\approx 3.01 \text{ \AA}$ (Figure 6). If Gly 488 was mutated to Val or Ser, the larger side chains of these two amino acid residues might impede the binding of “structural” NADP⁺, leading the clinical mutants G6PD_{Campinas} (G488V) [23] and G6PD_{Fukaya} (G488S) [24] to show severe deficiency. The determined K_d values for G488S and G488V were in fact 273 nM and 480 nM, respectively, about 7 and 13-fold higher respectively than the figure for wild type enzyme (37 nM) [13]. As for Arg 393, it is at the end of the sheet β L in the dimer interface and the atoms N η 1 and N η 2 of this residue interact with the nicotinamide amide oxygen of the “structural” NADP⁺ (2.99 \AA) (Figure 6). The decreased binding capacity for “structural” NADP⁺ in the mutant enzymes G6PD_{Wisconsin} (R393G) [25] ($K_d \sim 53$ nM) and G6PD_{Nashville} (R393H) [26] ($K_d \sim 500$ nM), could also account for the disease phenotypes [9].

In view of the fact that mature red blood cells lack a nucleus and cannot synthesize proteins, structural instability of the mutant enzyme is a likely cause of haemolytic anaemia associated with G6PD-deficiency. Such instability, however, may affect not only the half-life of active enzyme once formed but also the proportion of newly-synthesised enzyme molecules that are able to achieve the

correctly folded state. The results reported here clearly show that the four mutations mentioned, which would appear in the first instance to exercise their deleterious effect at the molecular level by interfering with binding of the “structural” NADP⁺, also impair the refolding process *in vitro* and thus, in all probability, the formation of active enzyme *in vivo*. Importantly, the decreased refolding yields of the mutant enzymes (Figure 3) correlate well with the corresponding lower binding capacity for “structural” NADP⁺, strongly suggesting that such NADP⁺ is actively involved in the refolding process under current experimental conditions.

By contrast, the steady-state kinetic studies showed that the mutant enzymes had quite similar catalytic efficiency, k_{cat} , to WT G6PD and nearly unaltered K_m values for the substrates (Table 1). Likewise, the secondary structure of the enzymes examined by the CD spectra in far UV region showed that there is no significant difference in the secondary structure between the WT and the mutant enzymes (Figure 4).

The mutant enzymes with low affinity for “structural” NADP⁺, e.g. R393H, G488S and G488V, were more susceptible to chymotrypsin digestion compared with WT or mutant enzymes with relatively high binding affinity (Figure 2). Addition of NADP⁺ again seemed to counteract the vulnerability of all enzymes to proteolytic digestion in a concentration-dependent manner, which is more prominent for the mutant enzymes. In keeping with these results, mutants with low affinity for “structural” NADP⁺ all also showed decreased thermostability (Figure 1). The transition temperature, $T_{1/2}$, of the WT enzyme is about 10 °C, or even more, higher than the mutants R393H, G488V and G488S in the presence of different concentrations of NADP⁺. Once again, the low stability of G6PD mutants could be compensated to a certain extent by adding high concentrations of NADP⁺, and this NADP⁺-dependent effect was more significant for the mutant enzymes. The difference in the thermostability between the WT and mutant enzymes was further confirmed by secondary structure change at elevated temperatures (Figure 5). Secondary structures of WT and R393G began to disrupt at about 52 °C, whereas R393H, G488S and G488V began to lose their secondary structures at 47 °C, 45 °C, and 42 °C, respectively, indicating that although the native

secondary structures of the mutant enzymes are very similar to the WT, the mutant enzymes with low binding affinity for “structural” NADP⁺ are less stable, tending to lose the compact structures at relatively low temperatures.

All of the four G6PD deficiency mutants discussed above reportedly belong to Class I, with less than 5% G6PD activity in the red blood cells, accompanying the disease phenotype of chronic non-spherocytic haemolytic anemia. However, so far no clinical data for G488S and only limited amounts of information about the other three mutants are available. As to G6PD Wisconsin (R393G), although it is classified as Class I, the biochemical properties studied here did not show significant difference from the wild type enzyme, raising a question as to whether the observed Class I phenotype was dependent on environmental challenge or on the individual genetic background. In contrast, the other three mutants, i.e. R393H, G488V and G488S, all exhibited dramatically decreased stability and impaired refolding capacity, and needed high concentrations of NADP⁺ to compensate these defects, correlating well with their corresponding disease phenotypes. Among these mutants, G488V seems most severe. Considering the difficulty of obtaining adequate G6PD deficient human blood samples, the recombinant proteins are still quite useful for evaluating the relative severity of the mutations based on measurements of stability, folding capacity, catalytic efficiency, and affinity for substrates, etc.

In summary, it seems likely that “structural” NADP⁺ in human G6PD plays important roles in both attaining and maintaining the native state. Folding defects and instability, caused by mutations located around the “structural” NADP⁺ site, are thus responsible for the disease phenotypes of the mutant enzymes. However, it must be borne in mind that *in vitro* refolding studies cannot fully reflect the situation *in vivo*. Also, the possible role of the catalytic NADP⁺ in the folding of G6PD still needs to be investigated.

Finally, in view of the key role of a low molecular weight additive in controlling both folding and subsequent stability of G6PD molecules, it may be asked whether this offers any prospect of clinical remediation. NADP⁺ itself is an unlikely candidate, since it is difficult to envisage efficient therapeutic delivery to the required site of action. Current information also indicates a high degree of specificity in binding at the structural NADP⁺ site. However, the possibility cannot be ruled out that a synthetic compound may be found with both a high affinity for this site and an ability to cross membranes. Its possible utility as a drug would then depend on not interfering with other NADP⁺ roles in the body.

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Table 1. Steady-state kinetic parameters of G6PD wild type, R393G, R393H, G488V and G488S. The table shows the specific activities of G6PD wild type and mutant enzymes, and their Dalziel parameters of the reciprocal initial-rate equation [30]. The corresponding 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.

Enzyme	Specific activity (IU/ mg)	ϕ_o (s)	ϕ_{NADP^+} (μMs)	ϕ_{G6P} (μMs)	$\phi_{\text{NADP}^+\text{G6P}}$ ($\mu\text{M}^2\text{s}$)	k_{cat} (s^{-1})	K_{mNADP^+} (μM)	K_{mG6P} (μM)
WT ^a	180 \pm	0.00366 \pm	0.0259 \pm	0.191 \pm	1.61 \pm	275 \pm	7.07 \pm	52 \pm
	10	0.00023	0.0041	0.021	0.22	18	1.13	4
R393G ^a	178 \pm	0.0043	0.0402	0.288	3.47	232	9.31	67.1
	8	± 0.00032	± 0.0092	± 0.018	± 0.59	± 16	± 2.4	± 13
R393H ^a	130 \pm	0.0052	0.0853	0.992	12.1	192	16.5	190
	9	± 0.0005	± 0.0067	± 0.11	± 0.57	± 18	± 2.7	± 27
G488V	160 \pm	0.00472 \pm	0.0502 \pm	0.253 \pm	2.77 \pm	212 \pm	10.7 \pm	53.8 \pm
	8	0.0002	0.0018	0.011	0.102	8.7	0.06	3.44
G488S	175 \pm	0.00397 \pm	0.0471 \pm	0.194 \pm	3.44 \pm	254 \pm	11.8 \pm	49.1 \pm
	9	0.0002	0.0013	0.006	0.067	5.5	0.4	3.2

a. The data for G6PD WT, R393G and R393H were from Wang *et al.* [9].

Figure 1. Thermostability of G6PD WT and mutant enzymes. G6PD wild type and mutant enzymes were incubated for 20 min at different temperatures together with various concentrations of added NADP^+ , and residual activities were measured. The transition temperature, $T_{1/2}$, at which 50% of original activity is retained under each set of experimental conditions was recorded.

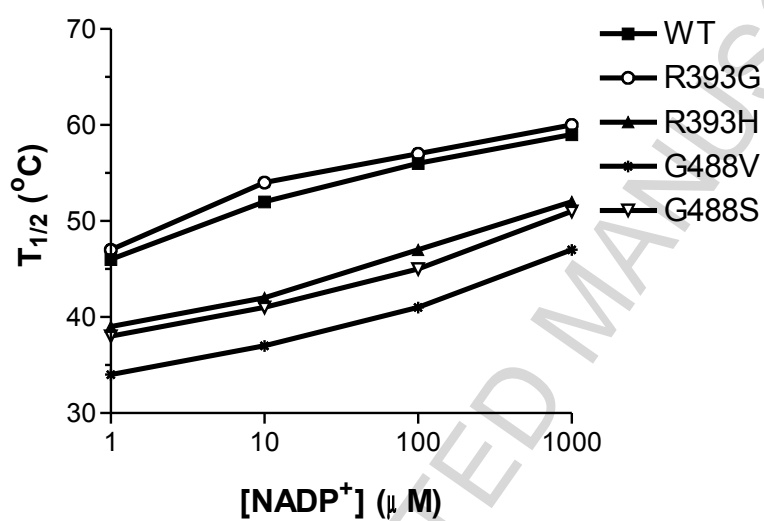


Figure 2. Susceptibility of G6PD WT and mutants to chymotrypsin in the presence of different concentrations of NADP⁺. G6PD WT and mutant enzymes (0.01 mg/ml) were incubated at room temperature with chymotrypsin (0.05 mg/ml) in the presence of different concentrations of NADP⁺ ranging from 1 μ M to 1000 μ M and corresponding residual enzyme activities were followed.

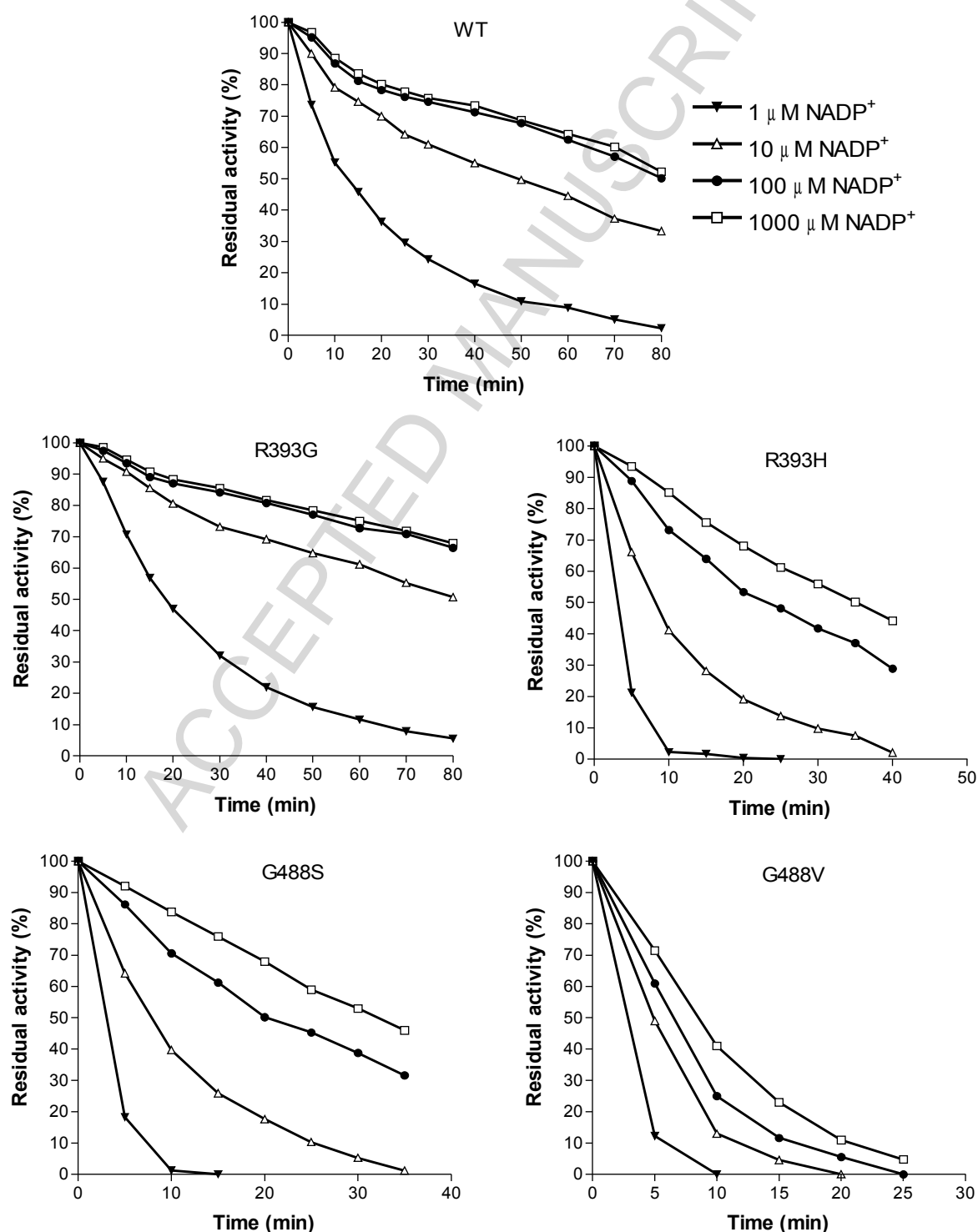


Figure 3. Refolding yields of G6PD WT and mutant enzymes in the presence of different concentrations of NADP⁺. G6PD WT and mutant enzymes were denatured in 4M Gdn-HCl and refolded in 50 mM Tris, pH 8.0 containing 500 mM L-Arg, 50 mM NaCl, 10 mM DTT, 40 mM PEG 3350 and varying concentrations of NADP⁺ at 25 °C. The regained enzyme activity was followed and the recovery yield was calculated based on the ratio of refolded enzyme activity to the activity before denaturation.

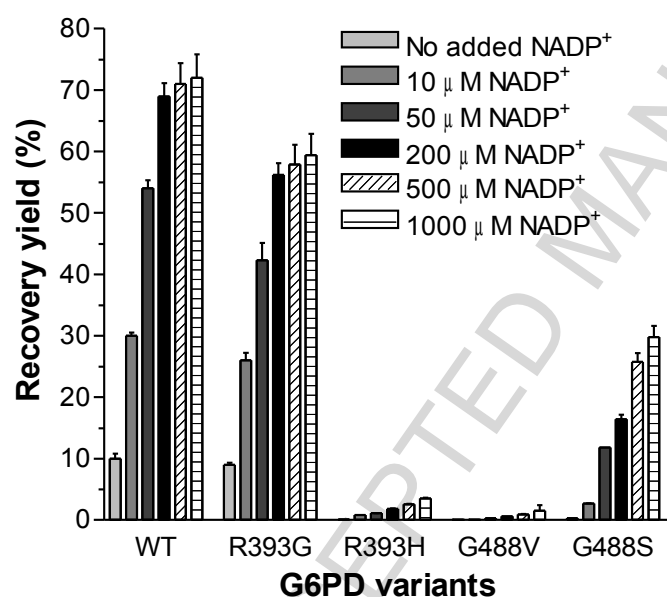


Figure 4. Far-UV CD spectra of G6PD WT and mutant enzymes. Protein concentration was 0.15 mg/ml and the light path was 2 mm.

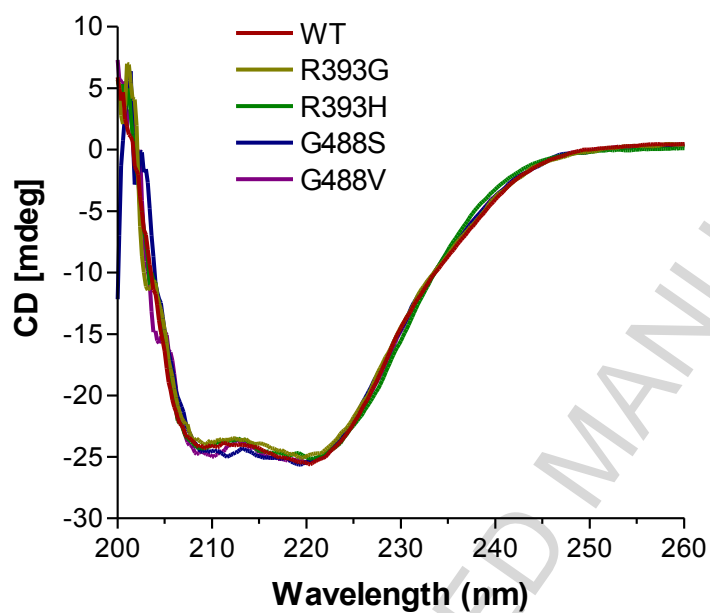


Figure 5. Changes in the CD signal at 222 nm with increase of temperature for G6PD WT and four clinical mutants.

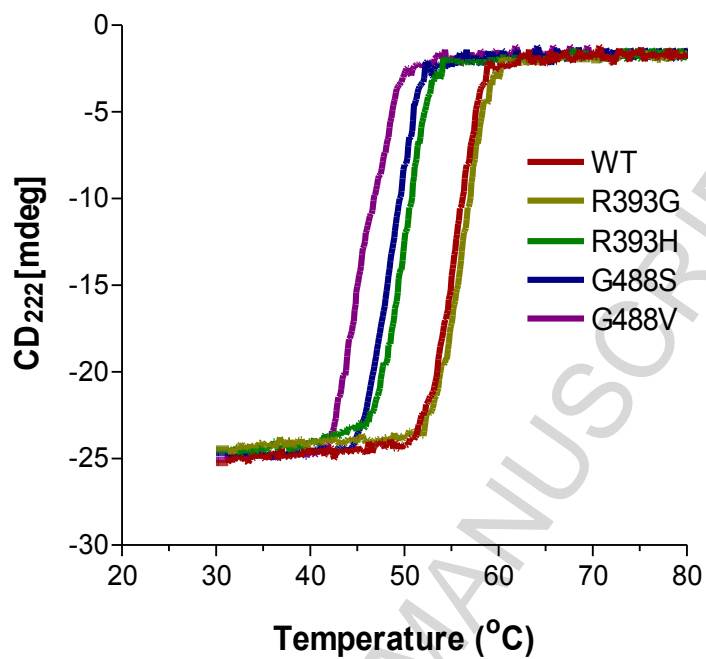


Figure 6. 3-D structure of dimer interface in human G6PD and the locations of “structural” NADP⁺, Arg393, Arg487 and Gly488.

