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Mutations in the tetramer interface of human glucose-6-phosphate dehydrogenase reveals kinetic differences between oligomeric states

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ABSTRACT

Glucose-6-phosphate dehydrogenase (G6PDH) catalyzes the oxidation of glucose-6-phoshate to 6-phospho-gluconolactone with the concomitant reduction of $NADP^+$ to NADPH. In solution, the recombinant human G6PDH is known to be active as dimers and tetramers. To distinguish between the kinetic properties of dimers and tetramers of the G6PDH is not trivial. Steady-state kinetic experiments are often performed at low enzyme concentrations, which favor the dimeric state. The present work describes two novel human G6PDH mutants, one that creates

four disulfide bonds among apposing dimers, resulting in a "cross-linked" tetramer, and another that prevents the dimer to dimer association. The functional and structural characterizations of such mutants indicate the tetramer as the most active form of human G6PDH.

INTRODUCTION

The enzyme Glucose-6-phosphate dehydrogenase, E.C. 1.1.1.49 (G6PDH) catalyzes the first step of the pentose phosphate pathway (PPP), which consists in the oxidation of glucose-6-phoshate (G6P) to 6-phospho-gluconolactone with the concomitant reduction of $NADP^+$ to NADPH. G6PDH plays a central role in cellular homeostasis by supplying NADPH for several biosynthetic processes and to maintenance of redox balance. G6PDH deficiency is the most common human enzymatic defect disorder; it causes hemolytic anemia triggered by exposure of red blood cells to oxidant agents (1). Despite this negative effect, G6PDH deficiency confers some level of resistance against malaria, being found in higher frequency in regions where malaria is endemic (2). Although G6PDH is considered a "housekeeping" enzyme, its expression level, intracellular translocation and post-translation (phosphorylation) modification have been demonstrated to be regulated, both positively and negatively, by different signaling factors (revised by Stanton, 2010 (3)). Altered G6PDH regulation can affect cellular metabolism and propagation, with direct implication to cancer (4), metabolic syndrome and vascular diseases progression (5).

The active forms of the human G6PDH are associated to dimeric and tetrameric states, but not to monomers. Equilibria exist between tetramers to dimers and dimers to monomers, but not between tetramers directly to monomers. Moreover, NADPH shifts the equilibrium from active dimers to inactive monomers (6). The equilibrium between dimer and tetramer is affected by ionic strength, pH and enzyme concentration; pH below 7.2, low ionic strength, high protein concentration favor the tetrameric state (7). Additionally, the binding of a structural $NADP^+$ to a site different from the catalytic one, also favors the tetrameric conformation (8). Crystallographic structures of recombinant human G6PDHs present the protein at the tetrametric state, even when crystals were grown at pH 5.8 (9).

Usually standard G6PDH steady-state kinetic experiments are performed at low enzyme concentration by measuring the fluorescence or absorbance relative to NADPH production. Once G6PDH dimer is predominant at low protein concentration, one can assume

that data retrieved from such kinetic experiments are related to the dimeric form of the enzyme. In order to compare kinetic parameters between dimers and tetramers it would be necessary to establish a "cross-linked" G6PDH tetramer that would resist dissociation once diluted during the kinetic experiments.

In the present work we describe the design of two new G6PDH mutants, one that prevents the tetramer formation and another that stabilizes the tetramer by the introduction of four disulfide bridges between adjacent dimers. The kinetic characterization of those mutants indicates relevant differences between dimers and tetrames of human G6PDH.

MATERIALS AND METHODS,

Site-directed mutagenesis and preparation of recombinant G6PDHs

The cloning of the expression vector pET_SUMO_HsΔG6PDH encoding for a short construction of HsG6PDH (HsΔG6PDH, residues 29 to 511) with a N-terminal His-SUMO tag was described elsewhere (10). The single HsΔG6PDH mutants A277C and E347A (HsΔG6PDH_A277C and HsΔG6PDH_E347A, respectively) were generated using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene), following the manufacturer's instructions and with the oligonucleotides: 5'-GGCCATGGAGAAGCCCTGCTCCACCAACTCAG-3' and 5'-CTGAGTTGGTGGAGCAGGGCTTCTCCATGGCC-3' for A277C and 5'-CTATGTGGAGAATGCGAGGTGGGATGGGG-3' and 5'- CCCCATCCCACCTCGCATTCTCCACATAG-3' for E347A (nucleotides underlined indicate the mutation positions). Mutations were verified by automated sequencing of both mutant constructions in a HITACH 3130X Genetic Analyzer (Applied Biosystems).

The expression and purification of both HsΔG6PDH mutants followed the protocol previously stablished for HsΔG6PDH (10). Briefly, *E. coli BL21 (DE3)* cells harbouring either HsΔG6PDH_A277C or HsΔG6PDH_E347A vectors were grown in ZYM5052 autoinduction medium (11) for 48h at room temperature and 250 rpm. Cells were harvested, lysed by sonication, clarified by centrifugation and submitted to metal affinity chromatography (Ni-NTA, Invitrogen). The His-SUMO tag was cleaved by addition of ULP-1 protease nickel charge resin and HsΔG6PDH mutants were recovered at resin wash step. Both HsΔG6PDH mutants were then concentrated and submitted to a final size exclusion chromatography step.

Size exclusion chromatography

The preparative and analytical size exclusion chromatography were performed using the columns Superdex 200 16/60 (GE Healthcare) and Superdex 200 10/30 (GE Healthcare), respectively, in an AKTA purifier FPLC system (GE Healthcare). The proteins were applied at 4 mg/mL (71 µM) on preparative column. The fractions of the main peak were collected, concentrated and subjected to analytical chromatography. For HsΔG6PDH, two different dilutions were applied: 4 mg/mL (71 µM) and 0.4 mg/mL (7.1 µM). While for HsΔG6PDH_E347A and HsΔG6PDH_A277C, each one was applied at 4 mg/mL (71 µM) and 0.4 mg/mL (7.1 µM), respectively. The proteins were eluted in 50 mM Tris-HCl pH 8.0 and 150 mM NaCl. The analytical column was calibrated using a molecular weight (MW) protein markers kit (MWGF200, Sigma-Aldrich): β-amilase (200 kDa), Alcohol Dehydrogenase (150 kDa), Albumin (66 kDa), Carbonic anhydrase (29 kDa) and Cytochrome C (12.4 kDa). The void volume (Vo) was determined by Blue Dextran. The MW markers chromatography was performed at the same conditions of HsΔG6PDH. The calibration curve (Fig. S1) was done following manufacturer's recommendations.

Protein crystallization and structure determination

The first crystals of HsΔG6PDH_A277C were obtained at 291 °C by the hanging-drop vapor diffusion method, following the crystallization conditions published elsewhere (12). The crystal used to solve the structure was grown from drops made of well buffer containing 100 mM Tris-HCl pH 9.0, 200 mM $MgCl_2$, 13% PEG 4000 and 20% glycerol and a protein solution comprised of 20 mg/mL HsΔG6PDH_A277C, 2mM $NADP^+$, 10mM G6P, 50 mM Tris-HCl pH 8.0 and 150 mM NaCl. The crystal was flash-frozen and collected at 100 K, at X06DA beamline from the Swiss Lightsource (Villigen, Switzerland), using a PILATUS 2M-F detector (DECTRIS). Diffraction data were indexed and integrated using XDS (13), and scaled by SCALA (14). The structure was solved by molecular replacement, using the HsΔG6PDH structure (PDB: 2BH9) as search model, using MOLREP (15). Restrained refinement was performed using REFMAC (16) and manual rebuilding in Coot (17). The diffraction data and refinement statistics are shown at table 1.

Enzyme kinetics

G6PDH activity was monitored by measurement of NADPH absorbance at 340 nm, recorded in an Envision microplate reader (Perkin-Elmer). The assays were performed at 25 °C, in 96 well microplates, final reaction volume of 0.2 ml, in reaction buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl). The apparent-Km for G6P was determined measuring NADPH formation rates at different concentrations of G6P (1000, 500, 250, 125, 62.5, 31.25, 15.625 and 7.8125 µM) and a fixed $NADP^+$ concentration (200 µM). The K_m^{app} for $NADP^+$ was determined at different concentrations of $NADP^+$ (200, 100, 50, 25, 12.5, 6.25, 3.125 µM) and a fixed G6P concentration (1 mM). A calibration curve of NADPH absorbance value in different concentrations (200, 100, 50, 25, 12.5, 6.25, 3.125 µM) was used to calculate the NADPH formation velocity from the absorbance measurements. The resultant curves were fitted to Michaelis-Menten equation to determine the K_m^{app} and maximum velocity. For DHEA inhibition assay, the enzyme activity was measured in varying concentrations of inhibitor (80, 40, 20, 10, 5, 2.5 and 1.25 µM, 2 % DMSO) with saturating substrate condition (1 mM G6P and 200 µM $NADP^+$). The IC_{50} values were calculated by fitting the normalized activity measured (% Activity) at different inhibitors concentration to the sigmoidal curve (Eq. 1). For enzyme activity normalization, reaction velocity in the absence of inhibitor or substrate were used as 100 and 0 %, respectively. In all assays, the enzyme concentration used was 10, 2.5 and 20 nM for HsΔG6PDH, HsΔG6PDH_A277C and HsΔG6PDH_E347A, respectively. The data analysis was done using GraphPad Prism 5 (GraphPad Software).

$$\%\ \text{Activity} = \frac{100}{1 + 10^{(\text{X} - \text{Log}\text{IC}_{50})}} \quad \text{Eq. 1}$$

X: logarithm of inhibitor concentration.

Thermal Stability

The thermal stability of HsΔG6PDHs was assessed by differential scanning fluorimetry using SYPRO Orange (Sigma-Aldrich) as fluorescent dye (18). 25 µL of 5 µM of protein with 5X of SYPRO Orange (in 50 mM Tris-HCl pH 8.0 and 150 mM NaCl) was dispensed in a 96-well microplate. The data were taken on a 7500 PCR Real-Time System (Applied Biosystems), measuring the SYPRO Orange fluorescence (filters recommended by manufacturer) in a temperature range of 20 °C to 96 °C, in steps of 1 °C/ minute. The melting temperature (T_M) was retrieved by fitting the normalized fluorescence (% Fluorescence) to sigmoidal curve (Eq.

2), using GraphPad Prism 5. For data normalization, it was considered as 0 % the lowest fluorescence intensity value before the denaturation, and 100 %, the highest value after denaturation. All assays were performed in triplicate.

$$\% \text{ Fluorescence} = \frac{100}{1 + 10^{(\text{Log}T_M - X).n}} \quad \text{Eq. 2}$$

X: logarithm of temperature; n: Hill slope.

RESULTS AND DISCUSSION

HsG6PDH mutants design

According to Au and co-authors (19), the tetramer interface of the wild type HsG6PDH buries only 706 $Å^2$ per monomer and involves 15 residues, out of which 11 are charged residues. The tetramer is formed by the interaction of dimer AB to CD, and four salt bridges (K275(chain-A)/E347(chain-D), K275(chain-B)/E347(chain-C), E287(chain-A)/K290(chain-D) and E287(chain-B)/K290(chain-C)) contribute to stabilize the tetrameric state (Fig. 1). The electrostatic nature of the tetramer interface explains its sensitive to pH, ionic strength and protein concentration. In order to disrupt the tetramer the residue glutamic acid 347 was mutated to an alanine (E347A). Inspection of residues in the tetramer interface leads us to hypothesize that mutation of alanine 277 to a cysteine would bring its sulfur atom in a favorable orientation to form a disulfide bond with cysteine 294.

Oligomeric state of HsΔG6PD mutants

Preparative size exclusion chromatography of HsΔG6PD at 4 mg/mL returns two peaks with elution volumes equivalent to tetrameric and dimeric states (Fig. 2A). When samples collected from the peak corresponding to the tetrametic state are pooled together, re-concentrated to 4 mg/mL and loaded into an analytical column, we observed an asymmetrical peak with elution volume corresponding to a trimeric state (Fig.2B solid line). This effect may be related to the rapid tetramer-dimer equilibrium, generating an intermediate apparent MW. The same effect was already observed for HsG6PDH at 1 mg/mL by Wang *et al*. (8). However, if the same sample is loaded at lower concentration (0.4 mg/mL), a single peak appears with an elution volume characteristic of the dimeric state (Fig. 2B, dashed line). When the same procedure is applied to the HsΔG6PD_ E347A mutant no signal of tetramer is observed in the

preparative column, but only the dimer (Fig. 2C). Moreover, when samples collected from the dimeric peak are concentrated to 4 mg/ml and loaded in the analytical column the mutant remains as in the dimeric state (Fig. 2D). For the mutant HsΔG6PD_A277C, an increase in the tetramer fraction at the preparative size exclusion chromatography is observed when compared to HsΔG6PD (Fig. 2E) and the mutant remains as a tetramer even after a 10-fold dilution (Fig. 2F). A minor dimeric fraction of HsΔG6PD_A277C is always observed in the preparative run, most probably due to troubles in disulfide bridge formation in bacterial cellular environment.

Structure of HsΔG6PD_A277C

Crystals for the mutant HsΔG6PD_A277C were obtained in the same crystallization condition described for HsΔG6PD (12). The protein crystallized in space group *F222*, with one monomer in the asymmetric unit. The tetramer is formed by the crystallographic 222 symmetry. One G6P is bound to the active site, at the same orientation of previous characterized complex. The present structure revealed a right-handed disulfide bond between cysteine 277 and 294, as clearly seen in the electron density (Fig. 3). The monomer structure is highly conserved with the published HsΔG6PD:G6P structure (PDB: 2BHL), with an average RMSD of 0.39 Å and 0.71 Å for the main chain and side chain, respectively.

Catalytic properties of HsΔG6PD mutant

The measurement of steady state kinetic parameters require the enzyme to be in a concentration much lower than its substrates, usually in the nanomolar range. Under such condition, human G6PDH will be predominantly in dimeric state, but a minority of tetramers might remain. In order to access the catalytic proprieties of distinct oligomeric states from HsΔG6PD, we stablished two new mutants that can be considered to exist exclusively as dimers or tetramers at low protein concentration conditions. Comparison of kinetic parameters among mutants and wildtype G6PDH shows a slight raise in K_m values for the HsΔG6PD_A277C (Table 2). While a pronounced increase is observed for k_{cat} parameter. These alterations lead to a gain in catalytic efficiency of HsΔG6PD_A277C, as observed by comparison of its turnover rates to that of HsΔG6PD_E347A and wildtype.

An important feature of the human G6PDH is its uncompetitive inhibition by steroids like dehydroepiandrosterone (DHEA). Although that was first described in 1960 (20), the

precise location of the steroid binding site remains unknown. DHEA inhibition was assessed for the mutants and no significant difference on IC50 values were observed (Table 2). That suggests the steroid inhibition site was not affected by A277C neither E347A mutations and it must exist independently of the ability of G6PDH to form tetramers.

Stability

The differential scanning fluorimetry assay shows a considerable gain of thermal stability for the HsΔG6PD_A277C in comparison to HsΔG6PD_E347A and the wildtype (Table 3). The melting temperature of the HsΔG6PD_A277C is higher than ten degrees when compared to the other proteins here evaluated. This is an additional feature of this G6PDH mutant that may be explored towards the development of improved enzymatic reagents.

CONCLUSION

The functional and physiological significance of the interconversion of dimer-tetramer of active HsG6PDH is elusive. The measurement of the tetramer enzymatic activity by steady-state kinetics is hampered by the fact that the protein dilution in the assay favors the dimeric state. Here we present HsG6PDH mutants that display just one oligomeric state, enabling the study of each form independently. The point mutation E347A prevents the tetramer formation even at high protein concentration, while A277C mutation results in the formation of an interchain disulfide bridge with cysteine 294. This last single mutation leads to the establishment of four disulfide bonds in the tetramer interface, making it stable at low protein concentration. The comparison of the kinetic proprieties of the new mutants indicates that the tetramer is at least fourfold more efficient than the dimer. Moreover, the incorporated disulfide bonds make the tetrameric mutant less susceptible to thermal denaturation. Lastly, the designed mutants are valuable tools for further studies to elucidate the role of the oligomeric state in the function of HsG6PDH.

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FIGURES LEGENDS

Figure 1: Tetramer organization of HsG6PDH. The tetramer is formed by the dimers AB and CD. In the boxes are shown the salt bridges which stabilize the tetramer. Also is highlighted the proximity between alanine 277 and cysteine 294 in the tetramer interface. (The figure was prepared using the structure of PDB entry 2BH9).

Figure 2: Size exclusion chromatography of HsΔG6PDH mutants. A, C and E: Preparative size exclusion of HsΔG6PDH, HsΔG6PDH_E347A and HsΔG6PDH_A277C, respectively. In grey, the fractions recovered and submitted to analytical size exclusion. B, D and F: Analytical size

exclusion of HsΔG6PDH, HsΔG6PDH_E347A and HsΔG6PDH_A277C, respectively. The apparent MW calculated for HsΔG6PDH_E347A, HsΔG6PDH_A277C and HsΔG6PDH at 4 mg/mL (71 μM) and 0.4 mg/mL (7.1 μM) were 109, 231, 157 and 100 kDa, respectively. The used calibration curve is at Supporting Information (Fig. S1). The theoretical MW of HsΔG6PDH monomer is 56.1 kDa. Dotted lines: HsΔG6PDH_A277C tetramer peak (11.58 mL) and HsΔG6PDH_E347A dimer peak (13.29 mL).

Figure 3: Disulfide bridge in the tetramer interface of the mutant HsΔG6PDH_A277C. A: The intermolecular disulfide bond formed by cysteines 277 and 294. B: Another orientation of the right handed disulfide bridge. The backbone of monomer A and D are shown in green and grey, respectively. In blue, $2F_o - F_c$ electron density map, contoured at 1 σ.

TABLES

Table 1: Diffraction data and refinement statistics of HsΔG6PDH_A277C.

Diffraction data statistics	
Wavelength (Å)	0.97643
Space group	F222
Unit-cell parameters (Å)	a = 59.10 / b = 177.82 / c = 216.47
Resolution (Å)	46.23 – 2.65
No. of reflections/unique reflections	155303/16889
Completeness (%)	100.0
R_{pim} (%)	7.0 (58.4)
Mean I/σ	17.2 (1.7)
$CC_{1/2}$	0.998 (0.579)
Refinement statistics	
R_{factor}/R_{free} (%)	18.9/23.4
Rmsd bond lengths (Å)	0.0154
Rmsd bond angles (°)	1.644
Number of atoms refined	
Protein	3862
Water	20
G6P	16
Glycerol	6
Mean B factor (Å^2)	58.67
PDB Entry	5UKW

The data in parentheses are for the reflections in the outer resolution shell 2.78–2.65 Å.

Table 2: Kinetic parameters of HsΔG6PDH mutants.

Protein	Variable substrate	K_m^{app} (μM)	k_{cat}^{app} (s^{-1})	k_{cat}^{app} / K_m^{app} ($\mu M^{-1}.s^{-1}$)	IC_{50}^{DHEA} (μM)
HsΔG6PDH	G6P	54 ± 3	25.0 ± 0.3	0.46 ± 0.02	11.2 ± 0.6
	$NADP^+$	9 ± 1	19.4 ± 0.7	2.6 ± 0.3	
HsΔG6PDH_A277C	G6P	96 ± 3	160 ± 2	1.67 ± 0.05	15.1 ± 0.7
	$NADP^+$	17 ± 2	150 ± 4	9 ± 1	
HsΔG6PDH_E347A	G6P	56 ± 3	21.2 ± 0.3	0.37 ± 0.02	12.3 ± 0.5
	$NADP^+$	10.1 ± 0.9	13.2 ± 0.3	1.3 ± 0.1	

* Fitted curves are shown at Supporting Material (Fig. S2).

Table 3: Melting Temperature of HsΔG6PDH mutants.

Protein	T_M (°C)
HsΔG6PDH	49.3 ± 0.8
HsΔG6PDH_A277C	62.2 ± 0.5
HsΔG6PDH_E347A	49 ± 1

* Raw data and fitted curves are shown at Supporting Material (Fig. S3).