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The interplay between protein stability and dynamics in conformational diseases: The case of hPGK1 deficiency

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ABSTRACT

Conformational diseases often show defective protein folding efficiency in vivo upon mutation, affecting protein properties such as thermodynamic stability and folding/unfolding/misfolding kinetics as well as the interactions of the protein with the protein homeostasis network. Human phosphoglycerate kinase 1 (hPGK1) deficiency is a rare inherited disease caused by mutations in hPGK1 that lead to loss-of-function. This disease offers an excellent opportunity to explore the complex relationships between protein stability and dynamics because of the different unfolding mechanisms displayed towards chemical and thermal denaturation. This work explores these relationships using two thermostable mutants (p.E252A and p.T378P) causing hPGK1 deficiency and WT hPGK1 using proteolysis and chemical denaturation. p.T378P is degraded ~30-fold faster at low protease concentrations (here, the proteolysis step is rate-limiting) and ~3-fold faster at high protease concentrations (where unfolding kinetics is rate-limiting) than WT and p.E252A, indicating that p.T378P is thermodynamically and kinetically destabilized. Urea denaturation studies support the decrease in thermodynamic stability and folding cooperativity for p.T378P, as well as changes in folding/unfolding kinetics. The present study reveals changes in the folding landscape of hPGK1 upon mutation that may affect protein folding efficiency and stability in vivo, also suggesting that native state stabilizers and protein homeostasis modulators may help to correct folding defects in hPGK1 deficiency. Moreover, detailed kinetic proteolysis studies are shown to be powerful and simple tools to provide deep insight into mutational effects on protein folding and stability in conformational diseases.

1. Introduction

Protein folding efficiency in vivo depends on a delicate balance between protein stability and dynamics (e.g. thermodynamic stability and folding/unfolding/misfolding rates) and the interaction of different protein states with machineries for protein folding, trafficking and degradation collectively known as the *protein homeostasis network* [1,2]. Mutations often alter protein folding and stability beyond the capability of the protein homeostasis network to fold them into their native state, leading to loss-of-function or gain-of-function conformational diseases [1–4]. Understanding these alterations in protein homeostasis caused by mutations is important to develop new pharmacological treatments targeting protein misfolding [1–4]. However, a comprehensive characterization of mutational effects on protein stability and dynamics, and their role in enzyme loss-of-function in conformational diseases is often lacking.

Phosphoglycerate kinase (PGK; EC 2.7.2.3) catalyzes the reversible phosphotransfer from 1,3-bisphosphoglycerate to ADP to produce 3-phosphoglycerate and ATP [5]. Two isoforms of PGK have been found in humans [6,7], namely hPGK1 and hPGK2. These two hPGK enzymes are structurally and functionally alike, sharing a two-domain and monomeric structure [8,9]. PGK has been a model system of a two-domain monomeric protein in folding/unfolding studies [9,10]. Interestingly, yeast (yPGK) and *E. coli* PGK (ecPGK) share virtually the same three-dimensional structure and conformational stability, but they largely differ in their unfolding kinetics and resistance to proteolysis, indicating divergent conformational dynamics [9].

Mutations in hPGK1 cause hPGK1 deficiency (OMIM ID:311800) and about 80% of the mutations cause single amino acid substitutions or small deletions [11,12]. Most mutations decrease protein thermal and kinetic stability leading to increased rates of aggregation and inactivation at physiological temperature, but the extent of kinetic destabilization differs significantly between mutants [11–13]. Kinetic stability changes according to the *Hammond postulate*, with the most destabilizing mutations (such as p.I47N and p.L89P) showing a more native-like denaturation transition state [13]. However, mutants such as p.E252A and p.T378P have half-lives towards thermal denaturation in the range of several days to months, and thus, the role of protein kinetic destabilization in some of the mutants causing human PGK1 deficiency remains unclear [13].

Proteolysis provides an alternative way to study unfolding kinetics and conformational dynamics of proteins under native and denaturing

Abbreviations: DSP, differential scanning proteolysis; CD, circular dichroism; PGK, phosphoglycerate kinase; WT, wild-type; T_m, denaturation temperature

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conditions (i.e. in the presence of chemical denaturants such as urea; [14–16]). Proteolysis is very attractive because it does not require expensive and specialized equipment such as a spectropolarimeter or differential scanning calorimeter, or large amount of sample, which makes it suitable to characterize mutants associated to loss-of-function disease that often decrease protein yields or make protein purification a difficult task [17,18]. Recently, proteolysis has been combined with thermal scans (in differential scanning proteolysis or DSP; [15]) to measure proteolysis rates in a wide range of temperatures at which either proteolysis or unfolding kinetics may be rate-limiting, and thus, DSP may give access to valuable thermodynamic and kinetic information on the effects of mutations in protein stability (the present work).

In this work, the conformational stability and dynamics of WT and two-disease causing and *thermally* stable mutants [p.E252A, (c.755 A > C) and p.T378P, (c.1132 A > C)] of hPGK1 have been evaluated using a combination of detailed proteolysis analysis and equilibrium/kinetic experiments by chemical/thermal denaturation. The results obtained support that mutational effects on thermodynamic and kinetic stability of hPGK1 are factors contributing to loss-of-function in this conformational disease, thus suggesting that these alterations might be targeted pharmacologically to improve protein folding and stability in patients with hPGK1 deficiency.

2. Materials and methods

2.1. Protein expression and purification

Human PGK1 (hPGK1) enzymes were expressed and purified as previously described [13]. Purified hPGK1 enzymes were buffer exchanged to 20 mM HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid], 200 mM NaCl, 5 mM $MgCl_2$ or $CaCl_2$, pH 7.4. Thermolysin from *Bacillus thermoproteolyticus rokko* and subtilisin A from *Bacillus licheniformis* were purchased from Sigma-Aldrich, exchanged to 20 mM HEPES, 200 mM NaCl, 5 mM $CaCl_2$, pH 7.4. Proteins were flashed frozen in liquid nitrogen and stored at −80 °C. Protein concentration was measured based on protein primary sequences: $\varepsilon_{280\ nm} = 27960\ M^{-1}\cdot cm^{-1}$ (hPGK1), $\varepsilon_{280\ nm} = 66086\ M^{-1}\cdot cm^{-1}$ (thermolysin) and $\varepsilon_{280\ nm} = 23740\ M^{-1}\cdot cm^{-1}$ (subtilisin).

2.2. Proteolysis of hPGK1 variants analyzed by SDS-PAGE

Thermolysin and hPGK1 enzymes were incubated for 10 min at 25 °C in 20 mM HEPES, 200 mM NaCl, 5 mM $CaCl_2$, pH 7.4 and proteolysis was initiated by mixing them to a final concentration of 0.5 mg/ml hPGK1 and 0.1 mg/ml thermolysin. Aliquots were withdrawn at different times (1 min–24 h), and proteolysis was quenched by adding EDTA pH 8 (to a 20 mM final concentration) and mixed with an equal volume of Laemmli's buffer. Samples were resolved in 12% acrylamide gels and the bands corresponding to hPGK1 full length were scanned and analyzed using the ImageJ software (http://rsbweb.nih.gov/ij/).

2.3. Differential scanning proteolysis

The rates of proteolysis at multiple temperatures and protease concentrations were measured by differential scanning proteolysis (DSP; [15]). DSP experiments were carried out in VP-DSC microcalorimeter (GE Healthcare). Protein solutions for DSP contained 0.45 mg/ml (10 μM) of hPGK1 enzymes in 20 mM HEPES, 200 mM NaCl, 5 mM $CaCl_2$, pH 7.4 and were kept on ice prior to the experiments. During the cooling step of the instrument, thermolysin or subtilisin was added to a final concentration up to 0.6 mg/ml of protease to the solution containing hPGK1 and immediately loaded into the calorimeter. Thermal scans were routinely performed in the 2–70 °C range at 1.5 °C/min scan rate. Analysis of the DSP profiles was performed using a two-state kinetic model (see Supplementary material for details, and Eqs. S1–S3 therein). The observed rate constants of proteolysis (k_{obs}) were determined from the excess heat capacity vs. temperature profiles using:

$$k_{obs} = \frac{\nu \cdot C_{p(exc)}}{\Delta H - \langle H \rangle} \quad (1)$$

where k_{obs} is calculated using the excess heat capacity and the excess enthalpy at each temperature ($C_{p(exc)}$ and $\langle H \rangle$), ν and ΔH stand for the scan rate and the calorimetric enthalpy, respectively.

The rate constants k_{obs} for proteolysis were analyzed according to a N ⇄ X → F mechanism [15]. In this mechanism, N stands for the native state, X is a cleavable state (or ensemble of states) susceptible to undergo proteolytic attack by the protease to render the final (proteolyzed) state F. The observed rate constant (k_{obs}) for proteolysis is described by the following equation [15]:

$$k_{obs} = \frac{k_0 \cdot [P]}{1 + \left(\frac{k_0}{k_1}\right) \cdot [P]} \quad (2)$$

where [P] is the concentration of protease, k_0 and k_1 are the rate constants at very low and high protease concentrations, respectively.

The temperature dependence of rate constants was analyzed according to the linearized Arrhenius equation:

$$\ln k = \ln A - \frac{E_a}{RT} \quad (3)$$

where E_a is the activation energy, A is the pre-exponential factor, R is the ideal gas constant and T is the temperature in K.

To determine the effect of mutations on the thermodynamic stability (ΔΔG), it must be assumed that the rate of proteolysis k_p is not much affected by mutations and thus, $\Delta\Delta G_{unf}$ can be estimated using the k_0 values extrapolated to 25 °C using the following expression:

$$\Delta\Delta G_{unf} = -RT \ln \frac{k_{0(p.T378P)}}{k_{0(WT)}}. \quad (4)$$

2.4. Spectroscopic analyses

Far UV circular dichroism (CD) measurements were performed in a Jasco J-710 spectropolarimeter using 1-mm path-length cuvettes and 0.22 mg/ml (5 μM) hPGK1 protein. Spectra were acquired at a 50 nm/min scan rate and 4 scans were registered and averaged. Trp-fluorescence spectra were recorded in a Cary Eclipse spectrofluorometer (Varian), using 0.09–0.135 mg/ml (2–3 μM) hPGK1, 3-mm path length cuvettes, $\lambda_{exc} = 295$ nm and emission/excitation slits of 5 nm. All spectroscopic analyses were measured in 20 mM HEPES, 200 mM NaCl, 5 mM $MgCl_2$, 1 mM TCEP [Tris(2-carboxyethyl)phosphine hydrochloride], pH 7.4 at 25 °C and appropriate blanks were recorded and subtracted.

2.5. Equilibrium denaturation in the presence of urea

Urea solutions were daily prepared in 20 mM HEPES, 200 mM NaCl, 5 mM $MgCl_2$, pH 7.4, and their concentrations were measured by refractive index. Equilibrium experiments were performed by incubation of hPGK1 0.09–0.22 mg/ml (2–5 μM) in the presence of 0–6 M urea in 20 mM HEPES, 200 mM NaCl, 5 mM $MgCl_2$, 1 mM TCEP, pH 7.4 for at least 2 h at 25 °C to allow equilibration. Urea denaturation curves were analyzed assuming a two-state unfolding model ([13,19], see Eqs. S4 and S5). By applying the linear extrapolation method [20], this model provides the unfolding free energy changes extrapolated to the absence of urea (ΔG_U) as $\Delta G_U = C_m \cdot m$, where C_m is the urea concentration of half-denaturation and m is the slope of ΔG_U vs. urea concentration.