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Energy cost for the proper ionization of active site residues in 6-phosphogluconate dehydrogenase from *T. brucei*

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ABSTRACT

The catalytic mechanism of 6-phosphogluconate dehydrogenase requires the inversion of a Lys/Glu couple from its natural ionization state. The pK_a of these residues in free and substrate bound enzymes has been determined measuring by ITC the proton release/uptake induced by substrate binding at different pH values. Wt 6-phosphogluconate dehydrogenase from *Trypanosoma brucei* and two active site enzyme mutants, K185H and E192Q were investigated. Substrate binding was accompanied by proton release and was dependent on the ionization of a group with pK_a 7.07 which was absent in the E192Q mutant. Kinetic data highlighted two pK_a, 7.17 and 9.64, in the enzyme–substrate complex, the latter being absent in the E192Q mutant, suggesting that the substrate binding shifts Glu192 pK_a from 7.07 to 9.64. A comparison of wt and E192Q mutant appears to show that the substrate binding shifts Lys185 pK_a from 9.9 to 7.17. By comparing differences in proton release and the binding enthalpy of wt and mutant enzymes, the enthalpic cost of the change in the protonation state of Lys185 and Glu192 was estimated at ≈6.1 kcal/mol. The change in protonation state of Lys185 and Glu192 has little effect on Gibbs free energy, 240–325 cal/mol. However proton balance evidences the dissociation of other group(s) that can be collectively described by a single pK_a shift from 9.1 to 7.54. This further change in ionization state of the enzyme causes an increase of free energy with a total cost of 1.2–2.3 kcal/mol to set the enzyme into a catalytically competent form.

1. Introduction

In most cases, enzymatic function requires substantially altered pK_a in the specific residues involved in the catalytic mechanism [1]. These pK_a changes are due to the local environment of the residue, and the energy cost of these changes is paid either during protein folding or by substrate/ligand binding. Many studies have focused on the effect of substrate on pK_a change and energy gains in terms of catalytic efficiency but little attention has been devoted to the energy cost of these changes. For example, it has been shown that for *Trypanosoma cruzi* trans-sialidase the presence of substrate reduced the energy required to put the catalytic couple Tyr/Glu into the correct ionization state by about 8 kcal/mol [2]. In 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44) a similar inversion of the "natural" ionization state of the Lys/Glu couple has been suggested [3], and this inverted ionization has been used to model the catalytic pathway [4]. However, nothing is known about the amount of binding energy spent in facilitating the change in the ionization state of the two residues. Such a type of evaluation has practical interest yielding to a significant piece of the energetic balance required for structure-based drug design.

6PGDH catalyzes the reversible oxidative decarboxylation of 6-phosphogluconate (6PG), producing ribulose 5-phosphate (Ru5P) and CO_2 with a concomitant reduction of NADP to NADPH. The kinetic mechanism is rapid equilibrium random on the basis of a complete kinetic characterization of sheep liver, *Candida utilis* and *Trypanosoma brucei* enzymes [4–6]. The pH dependence of kinetic parameters indicates a general acid–general base chemical mechanism, and site-directed mutagenesis studies of sheep liver enzyme [6–8] suggest that lysine and glutamate are most likely to be the general base and general acid, respectively. In this mechanism, a general base is required in order to accept a proton from the 6PG 3-hydroxyl group concomitant with a hydride transfer from 6PG C-3 to the coenzyme and the formation of 3-keto 6-phosphogluconate. Reduction in the nicotinamide ring is accompanied by rotation around the *N*-glycosidic bond to such an extent that the ring occupies the position formerly occupied by the 1-carboxylate of the substrate [9], and this movement could facilitate decarboxylation. Indeed, it has been observed that NADPH promotes 3-keto-2-deoxy-6- phosphogluconate decarboxylation, an analog of the true intermediate [10,11]. The 3-keto-6-phosphogluconate intermediate is decarboxylated to produce Ru5P enediol with the general base used to protonate the carboxyl oxygen. A general acid is needed to facilitate the Ru5P enediol tautomerization to the keto product (Fig. 1).

Abbreviations: 6PG, 6-phosphogluconate; 6PGDH, 6-phosphogluconate dehydrogenase; ITC, isothermal titration calorimetry; Ru5P, ribulose-5-phosphate; wt, wild type; TEA, triethanolamine hydrochloride; DTNB, 5,5-dithiobis-2-nitro benzoic acid

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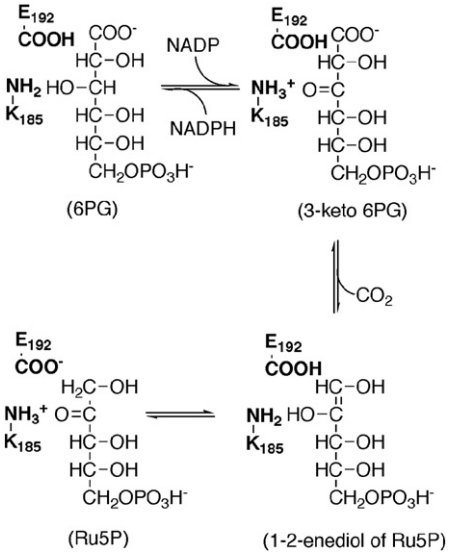

Fig. 1. Mechanism of 6PGDH-catalyzed reaction with the two main amino acid residues involved.

The general base and general acid in the *T. brucei* enzyme are respectively, Lys185 and Glu192. Lys185 is expected to be protonated in the free enzyme and final complex with the product Ru5P, but non-protonated in the catalytically competent enzyme–6PG complex. A similar inversion of the ionization state occurs for Glu192. Ionization enthalpy of the lysine ε-amino group is one order of magnitude higher than that of glutamate, therefore a significant part of 6PG binding enthalpy must be spent in putting Lys185 and Glu192 into the correct ionization state to allow catalysis to proceed.

In this study, we measured the 6PG binding enthalpy in the wt enzyme and in two mutants, K185H and E192Q, in order to evaluate the energy required in putting these residues into the correct ionization state required for catalysis.

2. Materials and methods

2.1. Materials

Recombinant *T. brucei* 6PGDH was prepared and assayed as described previously [6]. Site-directed mutagenesis was performed with the QuikChange II mutagenesis kit (Stratagene) and as already reported [12]. Coenzymes, substrate and other reagents were from Sigma.

2.2. Kinetic parameters

Kinetic parameters were determined from a least-square fitting of the Michaelis–Menten equation. For 6PG K_m determination, NADP was at a concentration of 0.2 mM, while 6PG concentrations were varied between 6 and 50 μM. For NADP K_m determination, 6PG was at a concentration of 0.5 mM, while NADP concentrations varied between 5 and 40 μM. Duplicate measurements were performed using the buffers for the different pH ranges in combination at 10 mM each: triethanolamine hydrochloride (TEA)/NaOH pH 7.5–8.4, Hepes/NaOH pH 6.8–8.2, and Mes/NaOH pH 5.5–6.7, and two other sets of measurements were performed using the buffer separately at 50 mM [6]. Results were indistinguishable regardless of multiple or single buffer use.

2.3. Cysteine reactivity

The reactivity of cysteine residues was determined with the method of Ellman [13] based on the capacity of thiol groups to react with 5,5-dithiobis-2-nitro benzoic acid (DTNB), developing a spectrophotometrically measurable complex at 412 nm with a molar extinction coefficient of 13,600. The enzyme was freed from glycerol by gel-filtration in TEA/HCl buffer pH 7.5. The reaction was carried out for 10 min in the same TEA buffer, with 6 μM enzyme subunit and 0.1 mM DTNB.

2.4. pH dependent parameters

pK_a and pH independent values were determined using pH dependent parameters by numerically fitting the following equations:

$$Y_{obs} = Y/\left(1 + 10^{(pH - pK_a)}\right) \quad (1)$$

$$Y_{obs} = Y/\left(1 + 10^{(pK_a - pH)}\right) \quad (2)$$

$$Y_{obs} = Y/\left(1 + 10^{(pH - pK_{a1})} + 10^{(pK_{a2} - pH)}\right). \quad (3)$$

2.5. Isothermal titration calorimetry (ITC) studies

Isothermal titration calorimetry (ITC) studies were performed in a VP-ITC microcalorimeter (MicroCal). Before each experiment, the enzyme was dialyzed exhaustively and the titrant was diluted in dialysis buffer. Measurements were done as already reported [14] and the data were fitted using Origin™ software provided by the instrument manufacturer. All experiments were performed at least in duplicate in 50 mM buffer, containing 0.1 mM EDTA and 1.0 mM 2-mercaptoethanol. Several types of buffer were used: Mes (ΔH_{ion} 3.71 kcal/mol at 25 °C), Mops (ΔH_{ion} 5.21 kcal/mol at 25 °C), Hepes (ΔH_{ion} 5.03 kcal/mol at 25 °C), Tris (ΔH_{ion} 11.3 kcal/mol at 25 °C) and triethanolamine hydrochloride (ΔH_{ion} 8.028 kcal/mol at 25 °C), at a temperature of 20 °C. The ionization enthalpy of the buffers was corrected for temperature according to Fukada and Takahashi [15].

The buffer-independent enthalpy change (ΔH_o) was calculated from the relation

$$\Delta H_{obs} = \Delta H_o + nH^+ \Delta H_{ion}. \quad (4)$$

The number of hydrogen ions exchanged (nH^+) was calculated using the slope of the experimental value plots for ΔH_{obs} against the ΔH_{ion} of the buffer. Three buffers were used for each pH value.

ΔH_o is an additive property and can be analyzed as sum of individual components, i.e. for the wt:

$$\Delta H_0^{wt} = \Delta H_0^{K185} + \Delta H_0^{E192} \Delta H_0^{other} \quad (5)$$

where the term "other" refers to the residual ionization changes and to all the enthalpic binding contributions except K185 and E192. It is assumed that this term that includes the other bonds formed between substrate and enzyme, conformational changes and desolvation of reagents, does not change significantly between wt and mutants.

In a similar way the ΔH_o of the K185H and E192Q mutants is respectively:

$$\Delta H_0^{K185H} = \Delta H_0^{H185} + \Delta H_0^{E192} + \Delta H_0^{other} \quad (6)$$

$$\Delta H_0^{E192Q} = \Delta H_0^{K185} + \Delta H_0^{Q192} + \Delta H_0^{other}. \quad (7)$$

If the contribution of "other" does not change significantly between wt and mutants the contributions of Glu192 and "other" will be