Human glucose-6-phosphate dehydrogenase Lysine 205 is dispensable for substrate binding but essential for catalysis

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Abstract **By site-directed mutagenesis of the cloned human glucose-6-phosphate dehydrogenase cDNA, lysine 205 (the residue that after reacting with pyridoxal-5′-phosphate renders inactive enzyme) was mutated to threonine (K205T) to remove the amino group, or to arginine (K205R) to displace the position of the amino group, in order to analyze the role of its nucleophilic group in position ε. Compared to the wild-type enzyme, the K205T and K205R mutants retain a specific activity of 2.6 and 11.4%, respectively; their catalytic specificity (K_{cat}/K_m) is drastically decreased, whereas the K_m values for both substrates are only slightly increased. These findings in the light of the 3D structure of G6PD suggest that the ε-amino group of lysine 205 can favour a hydrogen bond within the active pocket essential for catalysis.**

Key words: Glucose-6-phosphate dehydrogenase; Mutagenesis; Enzymatic catalysis

1. Introduction

Glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) is a housekeeping enzyme, and in red cells it is the key enzyme for protection against oxidative damage by peroxide and other oxygen radicals. Genetically determined G6PD deficiency is responsible for a variety of haemolytic anaemias (reviewed in [1]). The primary structure of human G6PD was deduced from the nucleotide sequence of the respective cDNA [2], and insertion of this cDNA in eukaryotic [3] and prokaryotic [4] expression vectors leads to the production of G6PD with properties indistinguishable from those of purified human red cell G6PD [5]. Although there is a wealth of information on the genetic polymorphism of G6PD and on G6PD deficient variants, our understanding of the mechanism of action of the enzyme is rather limited. The three dimensional structure of G6PD from the bacterium *Leuconostoc mesenteroides* has recently been determined [6]. This molecule is sufficiently homologous to the human enzyme (33% identity) that is has been possible to deduce the major features of the human structure with a reasonable degree of confidence (C. Naylor and M. Adams, personal communication).

A lysine residue reactive with pyridoxal-5′-phosphate (PLP) has been previously identified in position 205 within a highly conserved peptide of G6PD [7]. Because the reaction between G6PD and PLP was associated with marked loss of enzyme activity, and because G6P prevented the reaction, it was suggested that this residue was near the G6P binding site. The importance of this residue was further corroborated by in vitro mutagenesis (Lys^{205} to Thr) of the cDNA coding for G6PD and its expression in *cos* cells [3]: no human G6PD activity could be detected in extracts from cells that expressed the mutant protein.

In this report we have produced in *E. coli* two mutant G6PD proteins with alternative residues at position 205 (K205T and K205R). From the properties of the two proteins we have been able to draw specific inferences on the role of the ε-amino group of Lys^{205} in the catalytic mechanism of G6PD.

2. Experimental

2.1. Plasmid Constructions

All DNA manipulations were carried out by standard procedures [8].

pKK233-2 G6PD-wild type, pKK233-2 G6PD-K205T and pKK233-2 G6PD-K205R. Full length G6PD cDNA was inserted into the inducible expression vector pKK233-2 [9] which contains the inducible *tac* promoter. Since this plasmid, like pPLG6PD which we have previously used [5], also contains an *Nco*I site immediately downstream of the promoter we inserted the same *Xho*I–*Nco*I (partial) fragment described previously [5] into pKK233-2 DNA that had been cleaved with *Hin*dIII (filled in) and *Nco*I. The resulting plasmid, pKK233-2 G6PD, gives expression of G6PD in *E. coli* that is about half as good as pPLG6PD [5], but the plasmid is more convenient to use since no temperature shifts are required. The construction of a G6PD cDNA containing a single base change that codes for a threonine residue at position 205 instead of the wild-type lysine residue has been described [3]. To incorporate this mutation into pKK233-2 G6PD a *Bst*EII–*Sma*I fragment containing the mutation was cloned into *Bst*EII–*Sma*I cleaved pKK233-2 G6PD to give pKK233-2 G6PD-K205T. The single base change to code for Arg instead of Lys at position 205 was carried out by standard procedures [8] using the mutagenic oligonucleotide 5′-CCTGGGCAGAGAGATGGT-3′ and a template of single-stranded DNA from G6PD cDNA cloned in M13. The sequence of the 866 bp of the mutated cDNA between *Bst*EII and *Kpn*I was determined and the *Bst*EII/*Kpn*I fragment cloned into the expression vector.

2.2. Bacterial cultures, enzymes purification and enzymes assays

E. coli strain DR612 [*pgi*::*Tn*10, *Δ(Zeb)HB351*] was transformed with the expression plasmids pKK233-2 G6PD, pKK233-2 G6PD-K205T and pKK233-2 G6PD-K205R (selectable with ampicillin). 200 ml of Superbroth medium [10] containing ampicillin (50 μg/ml) were inoculated with 2 ml of an overnight culture of bacteria, and incubated at 37°C with shaking. After incubation for 90 min, cells were induced with 4 mM isopropyl-1-thio-*b*-D-galactopyranoside for 14 h. Cells were then collected by centrifugation (5,000 × *g* at 4°C for 12 min) and resuspended in 60 ml of extraction buffer. The three recombinant G6PDs were purified as described previously [5].

Enzymatic assays to obtain the K_m value of G6PD for NADP were conducted in duplicate or triplicate using a Perkin-Elmer LS-3B spectrofluorimeter. The precautions to be taken when enzyme kinetic studies are carried out by fluorimetry reported by Dalziel [11] and by Engel and Dalziel [12] were taken into account. The other enzymatic assays were performed measuring the increase in absorbance at 340 nm ac-

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cording to previously published procedures [13]. The buffer used in the kinetic studies was always 0.25 M Tris-Borate, pH 8.0 [14].

Pyridoxal-5′-phosphate inactivation experiments were carried out at 22°C exactly as described previously [15] except that 75 mM HEPES, pH 7.5 was used instead of the original phosphate buffer. Assays of activity at the indicated times were carried out at 30°C in 70.5 mM HEPES buffer, pH 8, 7 mM $MgCl_2$, 0.18 mM NADP and 1.8 mM glucose-6-phosphate (0.6 mM NADP and 3 mM glucose-6-phosphate for the mutants K205T and K205R).

3. Results

Although mutagenessis of lysine 205 and subsequent expression in *cos* cells had already demonstrated the importance of this residue for G6PD activity [3], this expression system was not ideal for isolation of mutant enzymes, because of the presence of endogenous monkey G6PD. We resorted therefore to a prokaryotic expression system (see [4]), using as vector the plasmid pKK233-2 and as host cell the *E. coli* mutant DR612 [16], which is deficient in G6PD because of a phage μ insertion into the gene *zwf* encoding G6PD. In cell extracts from cultures containing the plasmid carrying mutations K205T or K205R we detected G6PD activity of about 2 and 10%, respectively, when compared to parallel cultures containing the wild type G6PD cDNA. By using a protocol worked out previously for wild type recombinant G6PD [5] we purified these mutant proteins. The yield was about 75% for both, and a single band was obtained on SDS-gels.

The enzymatic properties of these mutagenized G6PD species were then analysed in some detail (Table 1). The most significant deviation from normal G6PD was observed in the specific activity of K205T, which is about 40-fold lower (5.9 IU/mg). The K_m values for both G6P (290 μM) and NADP (56 μM) were higher than normal, indicating a decrease of more than 4 times in the affinity of the mutant K205T for both natural substrates. By contrast, in the mutant K205R we observbed a 9-fold decrease in specific activity (25.2 IU/mg), an increase of nearly two fold in the K_m for G6P (121 μM), but a normal affinity for NADP.

The binding of the coenzyme by both mutants was explored further by analyzing the kinetics of inhibition by NADPH. The K_i obtained (Table 1) did not differ substantially from that obtained in parallel with the wild-type G6PD. This result was unexpected in view of the fall in the affinity for NADP of K205T, and it suggests that this mutation, rather than affecting the binding of NADP(H) as such, modifies subtly the minimal structure and/or the electronic requirements for the forward catalysis of NADP by virtue of its drastic effect on the G6P binding site.

Because Camardella et al. [7,15] had shown that the PLP-reactive residue in G6PD is lysine 205, we expected that our mutants would no longer react with PLP. To our surprise, when K205T and K205R were incubated with PLP we observed gradual inactivation to a plateau level of about 50% and 35%, respectively, as against 50% for the wild type enzyme (see Fig. 1). Unlike with the wild-type enzyme, G6P failed to protect the mutant enzymes from inactivation. By contrast, in both K205T and K205R we found that 0.2 mM NADP protects against inactivation by PLP.

In order to assess the effect of replacing 205^{Lys} with either Thr or Arg on the molecular activity, k_{cat} was calculated the mutants enzymes. We found that a 200-fold increase in the k_{cat} for

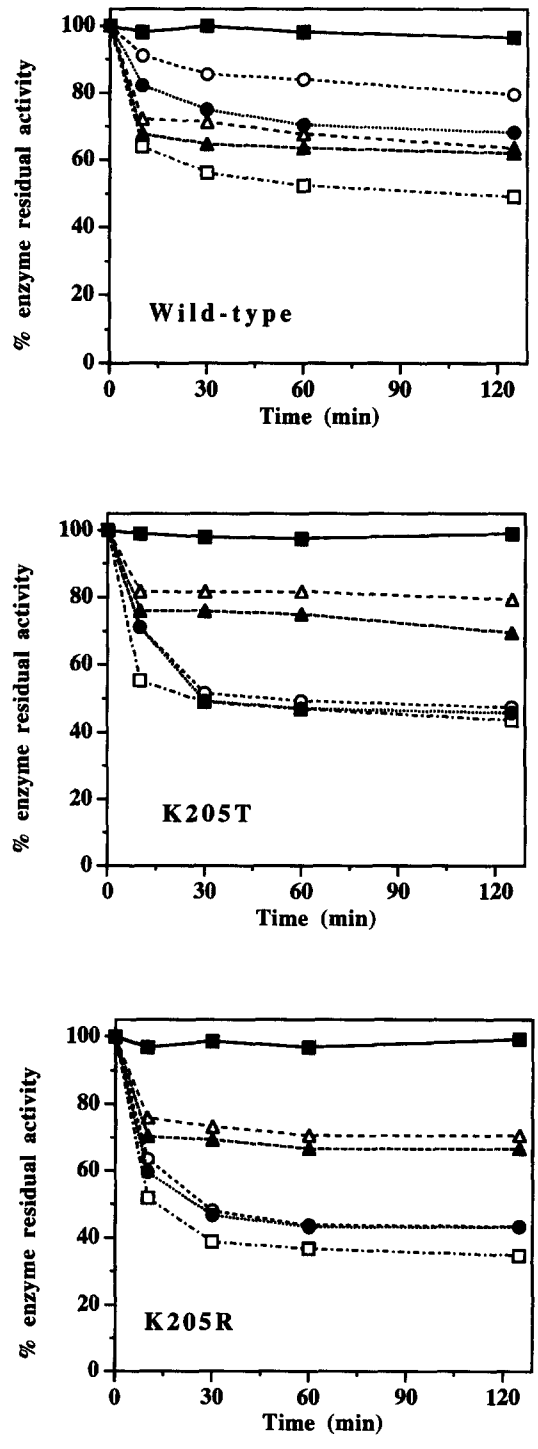

Fig. 1. Change in G6PD activity upon incubation of wild-type recombinant G6PD, K205T and K205R with pyridoxal-5′-phosphate. The incubation was carried out in 75 mM HEPES, pH 7.5, containing 6.75% glycerol, 0.15% β-mercaptoethanol, 0.75 mM EDTA and 7 μM NADP. Initial enzyme activities at zero time were between 350 and 450 mIU/ml. All the incubation mixtures contained 1 mM pyridoxal-5′-phosphate except the control which had no additions (■). Additions to the standard mixtures are as follow: none (□), 1 mM glucose-6-phosphate (○), 0.2 mM glucose-6-phosphate (●), 1 mM NADP (△), 0.2 mM NADP (▲).

Table 1
Specific activity and kinetic constants obtained experimentally from the purified wild-type, and mutants K205T and K205R of G6PD made in *E. coli*

Experimental constants	Wild-type	K205T	K205R
Specific activity (IU/mg)	220.0 ± 21[a]	5.9 ± 1.3 (5)	25.2 ± 6.5 (3)
K_m^{G6P} (μM)	69 ± 3[a]	290 ± 17 (4)	121 ± 14 (2)
K_m^{NADP} (μM)	12 ± 2[a]	56 ± 5 (3)	15 ± 2 (2)
K_i^{NADPH} (μM)	14 ± 3[a]	12 ± 3 (3)	13 ± 4 (2)

The buffer used to carried out the assays was 0.25 M Tris-borate, pH 8. The number in parentheses represents the number of duplicated experiments made to calculate the given mean.
[a] From [5].

K205R and a 400-fold increase for K205T. This corresponds to a retardation in the catalytic turnover from 15 μs to 3.0 ms in K205R and to 6.6 ms in K205T (Table 2). The loss of specificity of the mutants is best expressed by the relative values of their k_{cat}/K_m ratios [17]. Taking this ratio as 1 for the wild-type G6PD, the value for the K205T mutant is of the order of 10^{-4}, and for the K205R mutant it is of the order of 10^{-3} (Table 2).

4. Discussion

Site-directed mutagenesis of Lys^{205} to Thr or Arg (K205T and K205R) and subsequent purification to homogeneity of these G6PD mutants has enabled us to explore the role of this residue in the catalytic function of the enzyme. We first replaced the ε-amino group naturally present at position 205 of the human G6PD with a smaller and non-ionic polar group (carried by threonine), and thus substantially modified the chemical properties of the protein domain reported previously to be involved in the binding of G6P [7]. The mutant K205R produces a more subtle change in the G6PD active centre, since arginine retains the amino group, but this is displaced by approximately 1.5 Å from its original position and, additionally, the pK_a of the side chain is increased in approximately two units.

Our data show a substantial loss of catalytic activity in both mutants (they retain 2.5–11% of the specific activity of the wild-type enzyme), and a loss of the ability of G6P to protect against inactivation by PLP which is seen in the wild-type enzyme. These results confirm that lysine 205 is essential for activity because it is in or near the G6P-binding site. However, the fact that in both mutants the K_m values for glucose-6-phosphate are only moderately increased, in contrast with the thousand-fold fall of k_{cat}, provides new information. Specifically, we suggest that the replacement of Lys^{205} does not cause a major structural change in the region of G6P binding, but rather a subtle modification of the geometry, within the binding pocket, that is required for the catalytic step involving the reduction of NADP. The dramatic loss of catalytic efficacy by the K205R protein indicates that what is required for the normal activity of G6PD is not just the presence of an electrophilic group in position 205, but also its precise position. Since Lys^{205} in the active site of G6PD is not in a very hydrophobic location [6] it probably could not act as a proton donor during catalysis. Thus, Lys^{205} is essential for catalysis since it can hydrogen bond to other spatially close residues which, in turn, act as a base (proton donor for the leaving group) and/or to bind G6P. Comparable findings have been reported in triosephosphate isomerase [18] and in glutathione reductase [19], where the replacement of the electrophilic residues active in catalysis (His^{95} and His^{429}, respectively) by other amino acids with non-ionic polar groups resulted in enzymes that retained 0.25–1% of the wild-type activity, with much more marked loss in the k_{cat}/K_m constant than in the K_m values for the respective substrates.

Our data also provide a possible explanation for the fact that PLP-inactivation of the wild-type enzyme is never complete [7,15]. PLP has several reactive groups: the pyridinic nitrogen, the 3-hydroxyl and the 5-phosphate group can form hydrogen bonds with a variety of residues, and the 2-methyl group can form van der Waals contacts with hydrophobic residues. Thus, the Schiff base formed by PLP with Lysine 205 might produce a new conformation of the G6PD active site in which the aldehyde group of PLP and an amino group spatially close to position 205 make it still possible to bind G6P. As a result, the PLP-enzyme, which has lost the ε-amino group of Lysine 205, is still active, just like our mutants, which have lost Lysine 205 altogether. This explanation further supports the idea that Lys^{205} is more essential for catalysis than for G6P binding.

In contrast to G6P binding, the binding of the G6PD coenzyme appears not to be significantly affected in our mutants, based on the K_i^{NADPH} values. The increased K_m^{NADP} in K205T could be explained by an effect on the kinetic component of K_m, resulting from either the anomalous binding or the anomalous catalytic process of this mutant G6PD. A similar effect has been described in glutathione reductase, where a single mutation in the GSSG-binding pocket generates an increase in the K_m for the second substrate, NADPH [19]. In addition to the normal K_i^{NADPH} values, two additional lines of evidence suggest that Lys^{205} is not involved in the NADP binding site in a way that could account for the substantial loss of activity. (i) The mutant proteins were effectively purified by affinity chromatography on a resin with 2′5′ ADP as the active group, and their recovery and purity were similar to those obtained with the normal enzyme. (ii) The mutant enzymes are effectively protected by NADP against PLP inactivation.

This last phenomenon is of considerable interest. Since lysine 205 no longer exists in our mutants, PLP must react with another residue, which may be near the NADP binding site. In retrospect, this finding provides an explanation for the puzzling fact that the protection by G6P of the PLP-inactivation of the wild-type enzyme is not complete: about 20% of inactivation

Table 2
Catalytic parameters of wild-type and mutants K205T and K205R of G6PD

Forward reaction	Wild-type	K205T	K205R
k_{cat} ($min^{-1} \times 10^3$)	3,849 ± 860 (8)	9 ± 3 (5)	20 ± 6 (5)
$1/k_{cat}$ (μs)	15	6,660	3,000
k_{cat}/K_m^{G6P} ($min^{-1}/\mu M$)	55×10^3	31	164
k_{cat}/K_m^{NADP} ($min^{-1}/\mu M$)	320×10^3	0.16×10^3	1.35×10^3
k_{cat} (mutant/wild-type)	1	2.3×10^{-3}	5.2×10^{-3}
k_{cat}/K_m^{G6P} (mutant/wild-type)	1	5.6×10^{-4}	2.9×10^{-3}
k_{cat}/K_m^{NADP} (mutant/wild-type)	1	5.0×10^{-4}	4.2×10^{-3}

The buffer used to carried out the assays was 0.25 M Tris-borate, pH 8. The number in parentheses represents the number of duplicated experiments made to calculate the given mean.

still occurs in the presence of the substrate. Conversely, about 20% protection by NADP is seen in the wild-type, suggesting that an amino acid residue other than Lysine 205 reacts with PLP. In the mutants this other residue becomes, by default, the main PLP-reactive residue, and this may give us a new handle to identify the NADP-binding site.

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