Structural and functional properties of mutant Arg203Pro from yeast phosphoglycerate kinase, as a model of phosphoglycerate kinase-Uppsala

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A pathological variant of human phosphoglycerate kinase, phosphoglycerate kinase-Uppsala, associated with chronic nonspherocytic hemolytic anemia has been found to differ from the normal enzyme by substitution of an arginine at position 206 (corresponding to position 203 in yeast) by a proline. In order to understand the structural and functional consequences of this mutation, the corresponding mutant in yeast phosphoglycerate kinase was constructed. The three-dimensional structure of this mutant was resolved at 2.9 Å. Although the overall structure is not modified, small local changes were observed. The kinetic parameters of the mutant were not found to be greatly affected, the catalytic constant being lowered by only 10–20%. The most significant difference when compared with the wild-type enzyme is a decrease in stability by about 3 kcal/mol. The physiological implications of this instability are discussed.

Keywords: enzyme activity/pathological mutation/phosphoglycerate kinase/stability/structure

Introduction

Phosphoglycerate kinase is the first enzyme of the glycolytic pathway which generates ATP. This enzyme has been very well conserved during evolution as shown by the homologies in the known phosphoglycerate kinase sequences. Furthermore, the three-dimensional structures of the horse (Banks et al., 1979), pig (Harlos et al., 1992), muscle, yeast (Watson et al., 1982) and Bacillus stearothermophilus (Davies et al., 1993) enzymes determined by X-ray crystallography are very similar.

In man, inherited deficiency in phosphoglycerate kinase is generally associated with hemolytic anemia and often with mental disorders. Several unrelated families with phosphoglycerate kinase deficiency have been reported and, among them, phosphoglycerate kinase-Uppsala (Yoshida and Watanabe, 1972; Fuji and Yoshida, 1980). This pathological variant, associated with chronic nonspherocytic hemolytic anemia, differs from the normal enzyme only by the substitution R → P at position 206 in the aminoacid human enzyme sequence (Fuji and Yoshida, 1980). This mutation is located near the hinge region, suggesting that the hinge bending motion required to bring the two substrates in close contact in a water-free environment for catalysis (Banks et al., 1979) might be restricted in the mutant. In the known yeast enzyme structure Arg 203 points towards the solvent, whereas in other PGK structures this conserved arginine interacts through hydrogen bonds with CO of the residue corresponding to K227 in the yeast enzyme located in the C-domain. Indeed, in the pathological variant, a conserved proline residue is adjacent to proline 206; a Pro–Pro sequence in this region could be expected to affect the flexibility of the protein. A decrease in the enzyme activity of phosphoglycerate-Uppsala has been reported by Fuji and Yoshida (1980). The three-dimensional structure of the human enzyme is unknown, but there are only 14 amino acid differences between human and horse phosphoglycerate kinase, suggesting a close structural similarity. Taking into account the close similarity between yeast and mammalian enzymes, a mutant of yeast phosphoglycerate kinase was constructed in order to mimic the variant PGK Uppsala by replacing the corresponding arginine, R203 in yeast, by a proline. The three-dimensional structure was resolved at 2.9 Å and the stability and functional properties of the mutant were studied.

Materials and methods

Preparation of the mutant enzyme

The mutation was introduced in yeast phosphoglycerate kinase (PGK) by site-directed mutagenesis at position 203, which corresponds to 206 in the human enzyme, according to the procedure described by Minard et al. (1990). Mutant R203P was purified as previously reported (Minard et al., 1989). The purified protein was precipitated and stored in ammonium sulfate. Before use, each sample was centrifuged, and the protein pellet was desalted against an appropriate buffer solution. Protein concentration was determined by the absorbance at 280 nm using the extinction coefficient reported by Büchler (1955): ε = 0.49 ml/mg cm.

Unfolding–refolding transition as assessed by circular dichroism

Unfolding–refolding studies were carried out in 50 mM Tris-HCl buffer (pH 7) containing 50 μM EDTA and 1 mM DTT at 20°C. For the unfolding, the protein (2 μM final concentration) was incubated for 15 h in Gdn.HCl of concentration varying from 0 to 6 M. For the refolding, a solution of 100 μM phosphoglycerate kinase was preincubated for 15 h in 6 M Gdn.HCl before dilution to the desired final concentration of Gdn.HCl. Measurements were performed after 15 h of incubation. The final concentration of protein was 2 μM. The circular dichroism spectra were recorded with a Mark V dichrograph (Jobin Yvon) using a 2 mm light-path cell.

Analysis of the transition curves

The experimental data were fitted according to the following equation introduced by Aune and Tanford (1969):

\[ f_u = c^u(K_0 + c^u), \]

using the simplex procedure with a personal computer. In this equation, \( f_u \) is the fraction of unfolded protein, \( c \) the denaturant...
concentration, n a cooperativity index and \( K_0 \) the constant of unfolding in water. Transition curves were analyzed by using the linear dependence of \( \Delta G_u \) upon denaturant concentration according to Pace (1986):

\[
\Delta G_u = \Delta G_0 + mc,
\]

with \( \Delta G_u = -RT\ln K_u \) \( [K_u = f_j/(1 - f_j)] \), \( m \) being the dependence of \( \Delta G_u \) on the denaturant concentration and \( \Delta G_0 \) the variation of free energy in the absence of denaturant. In order to take into account the solvent effect on signals, the following equation was used:

\[
y_c = y_n + s_n c + \left[ \frac{e^{\Delta G_0 - mcRT}}{1 + e^{\Delta G_0 - mcRT}} \right] (A + (s_d - s_n)c)
\]

where \( y_c \) is the experimental signal in the presence of \( c \) molar Gdn.HCl, \( y_n \) is the signal of the native form, and \( s_n \) and \( s_d \) are the solvent effects on the native and denatured signals, respectively.

**Enzyme assay**

The specific activity was determined using the coupled assay with glyceraldehyde-3-phosphate dehydrogenase, according to Bücher (1955), slightly modified by Betton et al. (1984). The catalytic parameters were determined in 20 mM triethanolamine buffer (pH 7.5) containing 50 mM sodium sulfate at 25°C; all the experiments were carried out at a fixed concentration of free Mg\(^{2+} \) ions, namely 1 mM, according to Scopes (1978). The Michaelis constant for 3-phosphoglycerate (3-PG) was determined at saturating concentration of ATP (5 mM), and the Michaelis constant for ATP was determined at saturating concentration of 3-PG (10 mM). The activation by sulfate ions, namely 1 mM, according to Scopes (1978).

**Crystallization**

Crystallization of R203P mutant was carried out by the hanging drop method. Protein was dialyzed overnight in the chosen buffer, then concentrated by centrifugal filtration to concentrations varying from 0 to 150 mM, the ATP and 3PG concentrations being 1 and 2 mM, respectively.

**Crystallization**

Crystallization of R203P mutant was carried out by the hanging drop method. Protein was dialyzed overnight in the chosen buffer, then concentrated by centrifugal filtration to concentrations of 10–30 mg/ml, in Centronex (Amicon) and Ultrafree centrifugal cells (Millipore).

Among many possible buffer conditions, sodium pyrophosphate (Bücher, 1955; Watson et al., 1971) gave the thicker crystals. Finally, a pH range from 5.5 to 7 was chosen. The current buffer composition was the following: 20 mM Na\(_2\)P\(_2\)O\(_7\), 1 mM EDTA, 1 mM DTT, 1% dioxane. The precipitant used for crystallization was about 18°C. Crystals grew to their full size at a concentration of 3-PG (10 mM). The activation by sulfate ions, namely 1 mM, according to Scopes (1978).

**Data processing**

Data processing was done using the MOSFLM program (Leslie et al., 1986), leading to the integration and reduction of 6375 unique reflections with an \( R_{sym} \) of 0.03, where

\[
R_{sym} = \Sigma_{hkl} \Sigma_i \langle F_i \rangle - 1/\Sigma_{hkl} \Sigma_i |F_i|
\]

Only 66% of the diffraction data could be treated, possibly owing to crystal sliding during data collection, and no useful data could be obtained for the remainder of the asymmetric unit. Since we expected that the mutant domains' structures should be close to those of the wild-type phosphoglycerate kinase, a solution of the molecular structure was searched by the molecular replacement method (Rossman and Blow, 1962). When the rotation and translation functions ALMN and TFSGEN of the CCP4 program package were used (Crowther, 1972; Tickle, 1985; CCP4, Daresbury Laboratory, Warrington, UK), with a yeast PGK model (Watson et al., 1982) using one domain at a time, no outstanding solution appeared. Finally, the correct full solution was obtained using the program AMORE of Navaza (1987). In this case, the molecule was not split and all the data were taken into account. At this stage, the reliability index was 0.37 and the correlation factor was 0.57 at 2.9 Å resolution:

\[
R = \Sigma_{hkl} |F_{o} - 1F_{c}|/\Sigma_{hkl} F_{o}, C = |<F_o>F_c - <F_c>F_o|/|<F_o>F_c - <F_o>F_c|
\]

**Results**

In this preliminary model, eight amino-acid residues, mostly arginines and lysines, and including Arg 203, clashing with...
203 Arg -> Pro Mutant PGK

Fig. 1. (A) Superposition of the C° backbones of wild-type yeast phosphoglycerate kinase (dotted line) and mutant R203P (full line). The position of the mutation is indicated by an arrow. (B) Structure of wild-type phosphoglycerate kinase indicating the secondary structures and the position of the mutation (RASMOL: Sayle and Milner-White, 1995).

residues of symmetrically related molecules, were replaced by alanine residues. The model was then subjected to one cycle of the simulated annealing protocol of program XPLOR (Brünger et al., 1987; Brünger, 1988) using data in the 8–2.9 Å resolution range. This led to a $R$ factor of 0.23. The resulting model was then subjected alternatively to steps of model building on an Evans and Sutherland graphical terminal using the program FRODO (Jones, 1982) and weighted $2|F_o| - |F_c|$.
maps (Ten Eyck, 1977), and to three cycles of conventional conjugate gradient refinement using XPLOR. The final $R$ factor was 0.17, with an r.m.s. deviation from the ideal bond length of 0.015 Å and from the ideal bond angle of 3.6°. No water molecule was introduced in the model structure, which consists of non-hydrogen atoms (Table II).

Fig. 2. Electron densities around the C203 mutation site contoured at 2σ level. The map was calculated with $2F_o - F_c$ amplitudes. (A) Peptide bond P203-P204 in a cis configuration; (B) peptide bond P203-P204 in a trans configuration.

Fig. 3. Local geometry of the residues around residue 203. Hydrogen bonds are indicated by arrows. (A) R203PyPGK; (B) WTyPGK; (C) hPGK.
Properties of mutant Arg203 Pro

Fig. 4. Unfolding–refolding transition of mutant R203P induced by Gdn.HCl at pH 7 and 20°C. The black squares correspond to the unfolding and the white squares to the refolding. The dashed curve corresponding to that of the wild-type enzyme (from Ballery et al., 1993) is shown for comparison.

Results

Structure of mutant R203P

The main result of this study is that the R203P mutation does not introduce any major change in the structure while this mutant has a crystal packing different from that of the wild-type protein. The overall features of both molecules are the same (Figure 1A). The differences observed in residue 51, and segments 126–136, 249–252, 292–298 and 380–382, which have been remodeled, may be attributed to close molecular crystalline contacts. In the same way, a slight displacement of helices IV, IX and X, may be due to crystal packing (Figure 1B). The overall r.m.s. deviation between yeast wild-type PGK and mutant R203P backbone is 0.87 Å, and when discarding the flexible loops and using 382 Ca, the r.m.s. value decreased to 0.68 Å (Table III). A comparison of the different PGK molecular structures after superposition of the C-domain by fitting segments of the backbone included in the region 185–415 was performed by calculating the angular rotation of the N-domain of a PGK with regard to each of the others. As shown in Table III, the N-domain angular rotation following superposition of the C-domain of the mutant compared with the wild-type enzyme is small, much smaller than the corresponding rotation between yeast and mammalian PGKs and even with the binary complex of pig PGK with 3-phosphoglycerate. This situation reflects a rather large degree of freedom in the angular motion as indicated by the weak energy barrier in the domain motion (C. Guilbert and D. Perahia, personal communication).

The global examination of the 2IF0l - 1F0l map shows a reinforcement of electron density along the y-axis, particularly in the core of both domains, blurring somewhat the β-sheets of the N-domain. This may be due to series termination effect, and incompleteness of the data. This is less observed on the outsides of the molecule where turns, helices and β-strands are often well contrasted.

The examination of the polypeptide chain conformation of the known wild-type yeast PGK structure in the region 201–205 including Arg203 shows that the peptide bond R203–P204 is in a trans configuration. In the R203P mutant, this part of the model did not fit well to the map. A better fit was obtained with the corresponding peptide bond P203–P204 in a cis configuration (Figure 2). It should be noted that arginine 203 side chain in pig (Harlos et al., 1992), horse muscle (Banks et al., 1979) and B. stearothermophilus (Davies et al., 1993) PGK is hydrogen bonded to the carbonyl of the main chain at position 227 (yeast numbering) and not far from O51 of the residue equivalent to Asp229. This region appears different in the currently known structure of wild-type yeast PGK, owing to the trans configuration R203 protruding outside towards crystal symmetrical neighboring molecules (Figure 3). In this case, and also in the case of R203P mutant, Asp229 is located at a distance of 3.2 Å from the NH205 of the main chain. For pig and horse PGKs, this distance between Asn equivalent to Asp229 in yeast PGK and NH205 is 2.8 Å. Figure 3 shows that the mutation R203P does not change the geometry of the region surrounding position 203 relatively to wild-type yeast, pig, horse and B. stearothermophilus enzymes. Furthermore, Pro203 does not contract any interaction with neighboring residues.

Stability of mutant R203P

The unfolding–folding transition as followed by the variations in ellipticity at 220 nm, which reflect the variations in secondary structure is presented in Figure 4. The transition is completely reversible. For comparison, the transition corresponding to the wild-type enzyme (Ballery et al., 1993) is also reported in Figure 4. The cm value decreases from 0.82 for the wild-type to 0.58 for the mutant. Furthermore, the transition for the mutant is less cooperative (n = 7) than that of the wild-type protein (n = 11) and ∆G0 value decreases by 3 kcal/mol. Table IV summarizes these results, which clearly show that mutant R203P is less stable than the wild-type enzyme.

Kinetic parameters of the mutant enzyme

The specific activity of the mutant enzyme was found to be 1658 UI/mg whereas that of the wild-type was 1990 UI/mg.
In contrast to what has been reported by Fujii and Yoshida (1980), the intrinsic activity of the yeast mutant R203P is not lowered to a large extent, since it represents about 87% of the activity of the wild-type enzyme.

The apparent kinetic parameters were determined for the backward reaction. Michaelis constant and $V_m$ values for ATP and 3-phosphoglycerate (3-PG) are reported in Table V, for both wild-type PGK and R203P mutant. The apparent $K_m$ values for both substrates are not greatly affected by the mutation. The $V_m$ values are lowered only by 10–20% compared with that reported by Fujii and Yoshida (1980) on the human mutant enzyme which retains only 30% of the activity of the normal enzyme. In the yeast mutant enzyme, no such decrease in enzyme activity was observed, the most significant change being the loss in stability compared with the wild-type protein.

The effect of sulfate ions on the activity was studied; the activation–inhibition pattern of PGK by sulfate has been considered to be related to the hinge bending motion (Mas et al., 1988). Furthermore, it has been postulated by Fujii and Yoshida that the mutation in PGK-Uppsala, which is adjacent to the hinge region, could affect this property. As displayed in Figure 5, mutant R203P is also activated by sulfate ions up to 70 mM sulfate, where the activity reaches a maximum value, and then for higher sulfate concentrations the activity decreases. The corresponding profiles for the wild-type and mutant enzyme are similar, whereas for several mutants the activation effect is suppressed (Sherman et al., 1990). The only difference is a slight shift of the curve for the mutant towards higher sulfate concentrations. The optimum activity of the wild-type enzyme was obtained for 50 mM sulfate, and a lower decrease in activity for high sulfate concentrations was observed for the mutant.

**Discussion**

In order to understand the functional abnormality of phosphoglycerate kinase-Uppsala in which a proline replaces an arginine residue, a corresponding mutant of the yeast enzyme was constructed, R203P. The three-dimensional structure of the yeast enzyme is known whereas that of the human

![Fig. 5. Effect of sulfate ions on the activity of wild-type and mutant PGKs. The white symbols correspond to R203P mutant and the black symbols to wild-type PGK.](https://academic.oup.com/peds/article-abstract/9/2/181/1584942/186)
enzyme remains unknown. Taking into account the structural similarities between the different PGKs whose threedimensional structure has been determined and the high conservation of this enzyme, the yeast mutant might provide a good model to analyze the possible constraints imposed by a Pro-Pro linkage and their possible consequences on the structurefunction relationships of this mutant.

The most significant difference between the mutant and the wild-type enzyme concerns the stability of the molecule, whereas the catalytic parameters are only slightly affected by the mutation, suggesting that the functional dynamics of the enzyme are not strongly perturbed. The decreased stability of the mutant has also been observed for human PGK-Uppsala (Fujii and Yoshida, 1980), whose rate of heat inactivation is higher than that of the normal human PGK. The difference of stability of the yeast wild-type and mutant PGK could be partially explained by the change in configuration of Pro204 in the mutant, neighboring Pro203 and introducing a rigidity in this region of the molecule. However, Pro204 is totally conserved in all known PGKs, although it is far from the active site, this strongly suggests that this region is crucial for stability and/or enzyme activity. Furthermore, in the known structures, with the exception of that of yeast PGK, X-P204 is in a cis configuration (Figure 3C) and R203 interacts through hydrogen bonds with CO of K211 in B.stearothermophilus PGK (1.6 Å resolution) corresponding to K227 in the yeast enzyme. It seems likely that in wild-type PGK (Figure 3B), this proline might be also in a cis configuration, although it appears to be trans, possibly owing to a modeling uncertainty in the yeast structure; consequently, R203 might contract equivalent hydrogen bonds with CO of K227. Therefore, the observed destabilization could result from the loss of such an interaction rather than from a change in configuration of Pro204. This kind of side chain–main chain hydrogen bonding is known to have a strong stabilizing effect (Serrano et al., 1992).

The instability resulting from the replacement of an arginine by a proline in PGK probably has important physiological implications. It could explain the severe erythrocyte enzyme deficiency observed in patients possessing the variant Uppsala. Even from the results reported by Fujii and Yoshida (1980), it can be deduced that the deficiency in PGK activity mainly results from the low average level in enzyme concentration in the cells rather than from a significant decrease in activity. The present work suggests that the low level of PGK is not due to a low expression level but to an intrinsic instability of the enzyme. The mutant enzyme, more easily unfolded, may become a good target for proteases, thus reducing its lifetime into the red cells.

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