Mutagenesis of catalytically important residues of cupin type phosphoglucose isomerase from Archaeoglobus fulgidus

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Keywords
Archaeoglobus fulgidus; cupin; phosphoglucose isomerase; mutagenesis

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(Received 18 July 2005, revised 13 September 2005, accepted 6 October 2005)

Phosphoglucose isomerase (PGI; EC 5.3.1.9) catalyzes the reversible aldose-ketose isomerization of glucose-6-phosphate to fructose-6-phosphate. PGI plays a central role in sugar metabolism of eukarya, bacteria and archaea, both in glycolysis via the Embden–Meyerhof pathway in eukarya and bacteria, and in the modified versions of this pathway found in archaea. PGI is also involved in gluconeogenesis, where the enzyme operates in the reverse direction [1–3]. Two lines of PGI have evolved independently: the PGI superfamily and the cupin type PGIIs (cPGIs) [2,4,5]. The PGI superfamily, which includes the vast majority of known PGIIs, has recently been divided into the PGI family, which is found in almost all bacteria and eukarya, and the PGI/PMI-family, which is predominantly found in crenarchaeota [5]. A cupin type PGI was first described for Pyrococcus furiosus (PfcPGI) [2,6]. To date, 14 members of this novel family, which is presumably of...
In order to assess the effect of mutations on the structure of the protein, a homology model of AfcPGI was constructed using the known structure of PfcPGI in complex with 6PG as a template [17]. Sequence alignments show that the key difference between PfcPGI and AfcPGI is in the N-terminal and C-terminal regions (Fig. 1). In comparison with AfcPGI, the N-terminal of PfcPGI is nine residues longer. In the structure of PfcPGI, these residues comprise a β-strand (β1) that undergoes domain swapping to interact with a β-sheet in the neighboring subunit in the dimer. Its absence in AfcPGI may be the primary reason for AfcPGI existing as a monomer enzyme as demonstrated by gel filtration [4] in contrast to PfcPGI, which is dimeric. AfcPGI also differs from PfcPGI by having an extension at the C-terminus of approximately 40 residues and so no attempt was made to model this region. Given the relatively low sequence homology between the two enzymes (15% identity), the model of AfcPGI was surprisingly easy to generate using the PfcPGI structure as a template and required only minor adjustments to the model to accommodate the altered residues. The principal differences between the two structures lie in the loop regions. Specifically, three connecting loops are longer in AfcPGI. These are two extra residues in the loop between β5 and β6, four extra between β9 and β10, and four extra between β11 and β12 (see [17] for definition of the secondary structure). The loop leading into β10 was modeled as a helical turn and the connection between β11 and β12 was modeled as an extension of the β-ribbon structure. One other slight adjustment was made to the main chain to ensure that the side chain of R41 was exposed to solvent.

As expected, the residues within the active sites of the PfcPGI crystal structure and the AfcPGI model are highly similar (Fig. 2). The coordination shell around the metal ion, comprising H80, H82, H136, as well as E93 (AfcPGI numbering), is totally conserved (Fig. 1, Table 1). This is commensurate with the metal ion having an important role in catalysis [17]. Also conserved are G79 and Y160, which both contact the phosphate group of the substrate, as well as T63 and Y95, which are within hydrogen bonding distance of O-5 and O-2 of the substrate, respectively. The residues that differ lie immediately beyond the substrate-binding residues, and these include S158 in place of H158, D61 in place of A69 and N150 in place of A150. Surprisingly, Y152 in PfcPGI, which has been postulated to promote a hydride shift by creating a hydrophobic region around the site of hydrogen transfer [17], is replaced by the smaller V152 in AfcPGI. The hydrophobic character of this region, however, is conserved by another amino acid replacement, that of A156 (in PfcPGI) to F156 (in AfcPGI). In the AfcPGI
Fig. 1. Multiple sequence alignment of amino acid sequences of the cPGI-family. The alignment was generated with CLUSTALX [29]. Accession numbers: PfcPGI, NP577925; AfcPGI, NP070323; MmcPGI, NP633992; StcPGI, NP461684; EmcPGI1, Q92MQ8; EmcPGI2, Q92UI1, and its putative homologues from Pyrococcus horikoshi, O59618; Pyrococcus abyssi, Q9UXW3; Thermococcus kodakaraensis, BAD85300; Methanosarcina barkeri, ZP00076760; Methanosarcina acetivorans, NP615780; M. burtonii, ZP00149312; Moorella thermoacetica, ZP00330916. The sites selected for mutagenesis are printed in bold.
model, the phenylalanine side chain occupies the space filled by Y152 in PfcPGI.

Expression and purification of variants

Of the 16 invariant residues (Fig. 1), eight residues T63, G79, H80, H82, E93, Y95, H136, and Y160, were found at the active site in the model of AfcPGI (Fig. 2) and thus were selected for mutagenesis (Tables 1 and 2). These residues were mutated to change a polar side chain to a non polar of similar size, to reduce the length of the side chain by conservation of its respective functional group, or to introduce a more spacious side chain instead of a conserved glycine. Wild-type AfcPGI and the mutants were expressed in transformed *Escherichia coli* BL21(DE3) cells and purified by a single affinity chromatography step on Ni-NTA-superflow, as previously described for wild-type AfcPGI [4]. CD-spectra of the respective AfcPGI mutant proteins did not reveal significant

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**Table 1.** Catalytically important residues of *Archaeoglobus fulgidus* cupin type PGI (AfcPGI) according to the AfcPGI model and the equivalent residues from *Pyrococcus furiosus* cPGI. The proposed function of the respective residues was deduced from the structure of PfcPGI in complex with 6-phosphogluconate [17]. The mutations in AfcPGI as well as their effects on enzymatic properties are listed.

<table>
<thead>
<tr>
<th>AfcPGI</th>
<th>PfcPGI</th>
<th>Proposed function</th>
<th>AfcPGI mutants</th>
<th>AfcPGI: effect of mutations on</th>
</tr>
</thead>
<tbody>
<tr>
<td>T63</td>
<td>T71</td>
<td>Hydrogen bonds substrate O-3 and O-5</td>
<td>T63A</td>
<td>Catalysis</td>
</tr>
<tr>
<td>G79</td>
<td>G87</td>
<td>Binding of substrate phosphate group</td>
<td>G79A, G79L</td>
<td>Catalysis, thermostability</td>
</tr>
<tr>
<td>H80</td>
<td>H88</td>
<td>Coordinates divalent metal ion binding of substrate phosphate group</td>
<td>H80A, H80D</td>
<td>Catalysis, metal binding</td>
</tr>
<tr>
<td>H82</td>
<td>H90</td>
<td>Coordinates divalent metal ion</td>
<td>H82A</td>
<td>Metal binding</td>
</tr>
<tr>
<td>E93</td>
<td>E97</td>
<td>Coordinates divalent metal ion and compensates its positive charge, hydrogen bonds O-2, stabilizes transition state by binding to O-1A</td>
<td>E93A, E93D</td>
<td>Catalysis, metal binding</td>
</tr>
<tr>
<td>Y95</td>
<td>Y99</td>
<td>Hydrogen bonds O-2, stabilizes transition state</td>
<td>Y95F, Y95K</td>
<td>Catalysis, thermostability</td>
</tr>
<tr>
<td>H136</td>
<td>H136</td>
<td>Coordinates divalent metal ion, hydrogen bonds E97</td>
<td>H136A</td>
<td>Catalysis, metal binding</td>
</tr>
<tr>
<td>Y160</td>
<td>Y160</td>
<td>Binding of substrate phosphate group</td>
<td>Y160F</td>
<td>Catalysis</td>
</tr>
</tbody>
</table>
changes in the overall secondary structure (not shown). However, limited local changes could not be excluded due to the detection limit of this method used.

Metal coordinating residues

Proteins from the cupin superfamily, including cPGIs, typically contain a divalent metal ion that is essential for catalytic function [4,7,15,17,19,20,24]. In the hydride transfer mechanism described for PfcPGI, the divalent metal mediates proton transfer between O-1 and O-2 [17], whereas, in an alternative mechanism proposed for the same enzyme, it stabilizes the negative charge of the cis-enediol intermediate [15,17]. Commensurate with a role for the metal ion in catalysis of cPGIs, the four metal coordinating residues are completely conserved in all known cPGIs [4]. Substitution of any one of the four metal-coordinating residues to alanine in TlcPGI resulted in both an almost complete loss of bound metal and catalytic activity [7]. However, the precise function of the four metal binding residues in metal binding and catalysis could not unambiguously be resolved. Analyses of the respective positions in AfcPGI via mutagenesis (H80A, H80D, H82A, E93A, E93D, and H136A) revealed a more complex picture because neither metal content nor catalytic activity was completely lost in any of these mutants (Table 3). However, our data appear to rule out a structural role for the metal ion in cPGIs, as has been postulated for PfcPGI [16], because none of the mutations of the metal coordinating residues reduced the thermostability of the protein (Tables 1 and 3).

Compared with His-tagged wild-type AfcPGI, which contained, per mole of enzyme, 0.39 moles of Ni²⁺ and traces of Fe²⁺ (0.03 mol·mol⁻¹·enzyme⁻¹) [25], almost no Fe²⁺ (< 0.01 mol·mol⁻¹·enzyme⁻¹) could be measured in the wild-type AfcPGI.

Table 2. Primers used to introduce mutations in the afcpgi gene. Mutated bases are underlined.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>T63A</td>
<td>5'-GCTGAGATACGACTACGGTTATACGGCCAAAC-3'</td>
<td>5'-GGTTTGCGGTATAAACCCTGCAGTGCTATCCACGC-3'</td>
</tr>
<tr>
<td>G79A</td>
<td>5'-GAGAGCATTAAAACTTACGGCCACATCTACCGGA-3'</td>
<td>5'-CCCGGATGAGTGGCGAATGTATGGCTCTCC-3'</td>
</tr>
<tr>
<td>G79L</td>
<td>5'-GAGAGGATTAAAACTTACGGCCACATCTACCGGA-3'</td>
<td>5'-CCCGGATGAGTGGCGAATGTATGGCTCTCC-3'</td>
</tr>
<tr>
<td>H80A</td>
<td>5'-CATTTAAACTTACGGCCACTATCCGGAAGTATGGA-3'</td>
<td>5'-CAACTTCCGGAATGATGGCGGCATAGTTAATG-3'</td>
</tr>
<tr>
<td>H80D</td>
<td>5'-CATTTAAACTTACGGCCACTATCCGGAAGTATGGA-3'</td>
<td>5'-CAACTTCCGGAATGATGGCGGCATAGTTAATG-3'</td>
</tr>
<tr>
<td>H82A</td>
<td>5'-CATTTACGGCCCTACTATGGCTCAGGGAAGTATGGA-3'</td>
<td>5'-CAACTTCCGGAATGATGGCGGCATAGTTAATG-3'</td>
</tr>
<tr>
<td>E93A</td>
<td>5'-GGCTTTACCTATCTACGGCCATCTCTGCTCTTG-3'</td>
<td>5'-CAAGACCTTGGTAAACTCCGAGATGTAAGGCC-3'</td>
</tr>
<tr>
<td>E93D</td>
<td>5'-GGCTTTACCTATCTACGGCCATCTCTGCTCTTG-3'</td>
<td>5'-CAAGACCTTGGTAAACTCCGAGATGTAAGGCC-3'</td>
</tr>
<tr>
<td>Y95F</td>
<td>5'-CCTATCCGAACTTCTGCGGTTTACAGGGAGGAG-3'</td>
<td>5'-CTTCCCAAGACCTGAAACTTCCGAGATGGAAGG-3'</td>
</tr>
<tr>
<td>Y95K</td>
<td>5'-CCTATCCGAACTTCTGCGGTTTACAGGGAGGAG-3'</td>
<td>5'-CTTCCCAAGACCTGAAACTTCCGAGATGGAAGG-3'</td>
</tr>
<tr>
<td>H136A</td>
<td>5'-CAAAACTCAGCGTCGACATTAACCGCACTG-3'</td>
<td>5'-CAAGACCTTGGTAAACTCCGAGATGTAAGGCC-3'</td>
</tr>
<tr>
<td>Y160F</td>
<td>5'-CAGGAAATCCAGCTCACTTCCAAGACTTGACAGC-3'</td>
<td>5'-CTTCCCAAGACCTGAAACTTCCGAGATGGAAGG-3'</td>
</tr>
</tbody>
</table>

Table 3. Kinetic constants, apparent nickel concentrations at half maximal velocity (K₉₀,₅ V) and apparent melting temperature of wild-type and mutant cupin type phosphoglucose isomerase from Archaeoglobus fulgidus. The kcat and Km values were deduced from Michaelis–Menten curves at saturating Ni²⁺ concentrations, the K₉₀,₅ V values from reactivation curves were obtained after previous EDTA treatment. *

<table>
<thead>
<tr>
<th>Mutant</th>
<th>kcat (s⁻¹)</th>
<th>Km (F6P, mM)</th>
<th>kcat/Km (mλ⁻¹·s⁻¹)</th>
<th>Km (Ni²⁺, μM)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>32.4 ± 1.9</td>
<td>0.25 ± 0.05</td>
<td>130</td>
<td>36 ± 4</td>
<td>98</td>
</tr>
<tr>
<td>T63A</td>
<td>1.9 ± 0.1</td>
<td>0.22 ± 0.04</td>
<td>8.6</td>
<td>35 ± 3</td>
<td>98</td>
</tr>
<tr>
<td>G79A</td>
<td>0.32 ± 0.04</td>
<td>0.5 ± 0.1</td>
<td>0.64</td>
<td>30 ± 3</td>
<td>87</td>
</tr>
<tr>
<td>G79L</td>
<td>0.07 ± 0.02</td>
<td>2.0 ± 0.2</td>
<td>0.035</td>
<td>32 ± 4</td>
<td>77</td>
</tr>
<tr>
<td>H80A</td>
<td>0.43 ± 0.07</td>
<td>20.3 ± 5.3</td>
<td>0.02</td>
<td>75 ± 8</td>
<td>98</td>
</tr>
<tr>
<td>H80D</td>
<td>0.42 ± 0.06</td>
<td>0.39 ± 0.05</td>
<td>1.1</td>
<td>350 ± 50</td>
<td>98</td>
</tr>
<tr>
<td>H82A</td>
<td>8.0 ± 1.0</td>
<td>2.0 ± 0.2</td>
<td>4.0</td>
<td>57 ± 7</td>
<td>98</td>
</tr>
<tr>
<td>E93A</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>–</td>
<td>98</td>
</tr>
<tr>
<td>E93D</td>
<td>0.06 ± 0.01</td>
<td>2.9 ± 1.2</td>
<td>0.02</td>
<td>370 ± 45</td>
<td>98</td>
</tr>
<tr>
<td>Y95F</td>
<td>0.22 ± 0.02</td>
<td>0.36 ± 0.05</td>
<td>0.61</td>
<td>32 ± 3</td>
<td>69/96*</td>
</tr>
<tr>
<td>Y95K</td>
<td>0.68 ± 0.04</td>
<td>0.18 ± 0.08</td>
<td>3.8</td>
<td>34 ± 4</td>
<td>98</td>
</tr>
<tr>
<td>H136A</td>
<td>30.8 ± 1.5</td>
<td>0.19 ± 0.04</td>
<td>162</td>
<td>55 ± 8</td>
<td>97</td>
</tr>
<tr>
<td>Y160F</td>
<td>11.6 ± 1.0</td>
<td>1.4 ± 0.4</td>
<td>8.3</td>
<td>36 ± 5</td>
<td>98</td>
</tr>
</tbody>
</table>
detected in any of the mutants, whereas 40–60% lower Ni$^{2+}$ contents were found for the H80A, H82A, and H136A and about 80% lower Ni$^{2+}$ contents were found for the H80D, E93A and the E93D mutants (mol Ni$^{2+}$:mol enzyme$^{-1}$): H80A (0.15 mol Ni$^{2+}$:mol enzyme$^{-1}$), H80D (0.09 mol Ni$^{2+}$:mol enzyme$^{-1}$), H82A (0.23 mol Ni$^{2+}$:mol enzyme$^{-1}$), E93A (0.11 mol Ni$^{2+}$:mol enzyme$^{-1}$), E93D (0.08 mol Ni$^{2+}$:mol enzyme$^{-1}$), and H136A (0.18 mol Ni$^{2+}$:mol enzyme$^{-1}$). These data suggest that a mutation in the metal coordination site leads to a decrease, but not a complete loss, in Ni$^{2+}$ binding ability. For a more detailed evaluation of these mutants with regard to metal binding ability, we determined the respective apparent Ni$^{2+}$ affinities ($K_{Ni,0.5V}$) as recently described for wtAfcPGI (Table 1) [4]. Following EDTA treatment and subsequent dialyses with H$_2$O, wild-type AfcPGI and all mutants constructed showed no catalytic activity. Activity could be restored in almost all mutants by the addition of Ni$^{2+}$ at saturating fructose-6-phosphate (F6P) concentrations. From the respective Ni$^{2+}$-dependent hyperbolic reactivation curves (not shown), both the respective Ni$^{2+}$ concentrations at half maximal velocity ($K_{Ni,0.5V}$) as well as the respective $k_{cat}$ values at saturating Ni$^{2+}$ concentrations were deduced. However, the E93A mutant did not show any catalytic activity at all, whereas in contrast to all other AfcPGI mutants of this study, the H80A, H80D, H82A, E93D, and H136A mutants exhibited higher $K_{Ni,0.5V}$ values when compared with wild-type AfcPGI (Table 1). This indicates a reduced Ni$^{2+}$ binding ability of H80A, H80D, H82A, E93A, E93D, and H136A mutants, which goes along with the lower Ni$^{2+}$ contents found for these mutants (see above). The respective $K_{Ni,0.5V}$ values were about two-fold higher for the H80A, H82A and the H136A mutants, compared with wtAfcPGI.

The data suggest that the loss of one interaction in the metal coordination sphere indeed had a significant impact on metal binding but did not abolish the metal binding ability of the AfcPGI mutants completely. Moreover, the geometry of the metal binding site appears to be quite important, as the H80D as well as the E93D mutant showed, in addition to the lowest metal content determined (see above), by far the highest $K_{Ni,0.5V}$ values measured, which were about 10 times higher compared with wtAfcPGI.

All mutants within the metal coordination site exhibited a reduced catalytic activity when assayed immediately after purification using the standard assay at non saturating Ni$^{2+}$ concentrations; however, this was less pronounced than as described for similar mutations in TlcPGI [7]. However, possible overlapping influences regarding the loss of metal binding and/or catalysis require a more elaborate analysis. Assuming that at saturating Ni$^{2+}$ concentrations the respective metal binding site of wtAfcPGI and its mutants is completely occupied by Ni$^{2+}$, we used the $k_{cat}$ values measured at saturating Ni$^{2+}$ concentrations for a more detailed characterization of the mutants involved in metal coordination. At these conditions, effects on activity indicate an additional catalytic role of the residues involved in metal binding. The data indicate that with the exception of H136, the other three metal-coordinating residues (H80, H82 and E93) might play an additional role in catalysis as well (Tables 1 and 3). In comparison with wtAfcPGI, the H136A variant showed an 80% lower catalytic efficiency immediately after purification. However, when assayed at Ni$^{2+}$ saturating conditions, both $k_{cat}$ as well as $K_m$ of the H136A mutant were about the same as obtained for wtAfcPGI, suggesting that H136 is important for metal coordination, but has little impact on catalysis. Though an additional catalytic role for H82 besides metal coordination is not obvious from the model, the H82A mutant showed a 32-fold lower catalytic efficiency compared with wtAfcPGI. The situation might be different for H80 because, in addition to binding of the catalytic metal ion, this residue forms a hydrogen bond with the phosphate of the substrate and so the $\approx 16500$- and $\approx 120$-fold reduced catalytic activity might play an additional role in catalysis as well. The H80A variant showed an 80% lower catalytic efficiency immediately after purification.

Kinetic analyses of the E93A and E93D mutants suggest E93 to be a key residue of AfcPGI catalysis. The E93D variant exhibited a 6500-fold lower catalytic efficiency compared with wtAfcPGI. The situation might be different for H80 because, in addition to binding of the catalytic metal ion, this residue forms a hydrogen bond with the phosphate of the substrate and so the $\approx 16500$- and $\approx 120$-fold reduced catalytic efficiency for the H80A and H80D mutants, respectively, is commensurate with this dual function. These data will be further discussed in the context of residues contacting the phosphate group of the substrate (see below).

Residues contacting the phosphate group of the inhibitor

In the PfcPGI structure complexed with 6PG, the phosphate group of the inhibitor was hydrogen...
bonded by the side chains of Y52, H88, and Y160, and by the amide nitrogen of G87. In the AfcPGI model, these residues correspond to H80, Y160, and G79 but no corresponding phosphate-binding residue for Y52 is present. The loss of interactions with the phosphate group in both the H80A and Y160F mutants lowered catalytic activity. Their higher $K_m$ values might indicate a lower affinity for F6P, whereas the thermostability of the AfcPGI remained unchanged in both mutants (Fig. 3, Table 1). In contrast to the almost inactive H80A mutant (see above), the catalytic efficiency of the Y160F variant was reduced only about 16-fold when compared with wtAfcPGI.

Further, the introduction of amino acids containing side chains instead of G79 resulted in both a reduced catalytic efficiency and a reduced thermostability of the respective mutants when compared with wtAfcPGI (Table 1). The catalytic efficiency of the G79A mutant was 200-fold lower and that of the G79L mutant was 3700-fold lower, whereas the respective apparent melting temperatures were 10 and 20 °C lower (Fig. 3). Thus, the lack of side chain in this conserved glycine presumably allows both the close binding of the phosphate group of the substrate as well as to prevent local structural constraints upon folding of the enzyme.

Other inhibitor/substrate binding residues

Another crucial residue for catalysis is Y95, which forms a hydrogen bond to O-2 of glucose-6-phosphate (G6P) in the AfcPGI model. Both Y95F and Y95K mutations showed a significant lower catalytic efficiency. These were 200- and 40-fold lower, respectively, as compared with wtAfcPGI. Moreover, the Y95F, but not the Y95K mutant showed reduced thermostability as indicated by a biphasic melting curve with two apparent thermal transitions midpoints at 69 and 96 °C (Fig. 3, Table 1), which also affected the catalytic activity. The onset of the first thermal transitions (about 55 °C) was also reflected in a reduced Arrhenius activation energy at about the same temperature.

Sugar ring opening

In addition to isomerization, members of the PGI superfamily catalyse sugar ring opening of F6P or G6P. This goes along with the fact that the closed ring represents the predominant forms of glucose and fructose in solution (at 25 °C) [26]. Based on the crystal structure of rabbit and mouse PGI in complex with its substrate F6P or G6P [9,13], His388 (in rabbit PGI) is thought to act as an acid catalyst in ring opening. An equivalent ring-opening machinery could not be identified in the present PfPGI structures [15,17], thus raising the question whether PfcPGI acts specifically on G6P or F6P in their straight chain forms because the latter may be favored at the high growth temperatures of *Pyrococcus furiosus* (80–100 °C). However, the cPGI family comprises several mesophilic cPGIs including MmcPGI, StcPGI, EmcPGI1, and EmcPGI2 ([4], and T. Hansen, unpublished data). Thus it appears likely that at least some, if not all, cPGIs catalyse the sugar ring opening. Interestingly, the PGI/PMI from *P. aerophilum*, which exists at similar temperatures as *P. furiosus*, has the same ring-opening machinery as PGIs [14,27].

In the AfcPGI model, the nearest residue to the ring oxygen O-5 is T63, which forms a hydrogen bond with O-5, and with O-3 of G6P via a water molecule.
Changing this threonine to alanine (T63A variant) resulted in a 15-fold lower $k_{\text{cat}}$ whereas the $K_m$ for F6P as well as the thermostability remained about constant. These data are consistent with an important role for this residue although it is unlikely that a threonine would act directly in catalysis.

The characterization of some mutants indicates that some residues might have additional catalytic functions, which have not been identified yet. This is true for H82 (see above), as the mutagenic effect of H82A could not be completely understood. One might speculate that such residues might be involved in the ring-opening reaction, though H82 appears to be too remote for this reaction. Y95 might be another putative candidate, but still appears to be too remote from the substrate in the AfcPGI model or from the inhibitor in the PfcPGI structures [15,17]. Future structural analyses of cPGIs might help to address this question.

In summary, we have probed eight residues of AfcPGI that are strictly conserved within the cPGI family and which have been proposed to be involved in catalysis and/or metal binding by site-directed mutagenesis. The data clearly define the role of the respective residues in catalysis and/or metal binding. Among all residues probed, E93 was shown to be the key residue in cPGIs, which is in accordance with both mechanisms for this enzyme [17]. However, final proof of the catalytic mechanism (hydride or $\text{cis}$-enediol) remains open until detailed isotope exchange experiments, which are currently in progress.

**Experimental procedures**

**Modeling of AfcPGI**

A homology model for AfcPGI was constructed using the crystal structure of cPGI from *P. furiosus* [17]. In comparison with PfcPGI, AfcPGI has approximately 10 residues less at the N-terminus and a ~40 residue extension at the C-terminus (see Fig. 1). Hence, the first 10 residues of PfcPGI were not used in the homology modeling, nor was any attempt made to model the C-terminus de novo. The PfcPGI structure was displayed using the O program [28] and residues were mutated to correspond to the sequence of AfcPGI based on a sequence alignment prepared by CLUSTALX [29]. Deviations from the sequence alignment were permitted in order to accommodate insertions or deletions within loops rather than in the midst of $\beta$-strands or $\alpha$-helices. In the few instances where the replacement side chains clashed, these were altered by adjusting their torsion angles. As a final step, the stereochemistry of the model was regularized usingREFMAC [30]. The final model has a root mean square (RMS) deviation from ideality in bond distances of 0.006 Å and 1.0° in bond angles. As judged by Procheck [31], 78% of the residues lie in the most favored region of the Ramachandran plot, 15.4% in the additionally allowed region, 4.9% in the generously allowed region and 1.9% in the disallowed region. Of residues in the latter two categories, none are in the active site of the enzyme.

**Construction, production and purification of mutants**

The *afcpgi* gene, which has been cloned into a pET19b plasmid [4], was mutated using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The primers used to introduce the mutations are listed in Table 2. The sequence of the mutated *afcpgi* genes were confirmed by the method of Sanger et al. [23]. To produce AfcPGI and its variants, *E. coli* BL21(DE3) cells harboring the appropriate plasmid were grown in Luria–Bertani medium containing 100 μg/mL carbenicillin at 37 °C to an optical density at 600 nm of 0.8, and *cpgi* expressions were initiated by induction with 1 mM IPTG. After 4 h of further growth (D600 = 2.5–3.2), the cells were harvested by centrifugation at 4 °C, washed in 50 mM Tris/HCl, pH 7.0, containing 50 mM NaCl. Enzymes were purified to apparent homogeneity by Ni-NTA-superflow (Qiagen, Germany) as described for AfcPGI [4]. Following purification, AfcPGI variants were dialyzed with a 100-fold excess volume of 10 mM Tris/HCl pH 7.5.

**Analytical assays**

The purity of the respective preparation was checked by SDS/PAGE in 12% gels followed by staining with Coomassie Brilliant Blue R 250 according to standard procedures [32]. Protein concentrations were determined by the use of AfcPGIs molar extinction coefficient at 280 nm (38 830 M$^{-1}$cm$^{-1}$; 35 000 for variants carrying Y → F mutations) as calculated by the ExPASy ProtParam tool (http://us.expasy.org/tools/protparam.html). The iron and nickel content of the mutants were determined by atom absorption spectroscopy as recently described for AfcPGI [4,33]. To correct for unspecific metal binding by the respective His-tags, His-tagged G6P dehydrogenase from *T. maritima* [34] and His-tagged phosphoglucose isomerase from *P. aerophilum* [5] were dialysed and prepared as described above for the AfcPGI and its mutants and the respective metal content of the proteins was measured. The controls contained on average 0.02 Ni$^{2+}$ per enzyme molecule.

**Circular dichroism spectroscopy**

To detect possible changes in the secondary structure of the AfcPGI variants, circular dichroism (CD) spectroscopy analyses were performed using a JASCO J-715 CD-spectro-
of the mutant proteins to bind metal was analyzed by and 96 dependence of enzyme activity was measured between 20 protein concentration had to be used. The temperature and 1.1 U G6P dehydrogenase. For several mutants higher 100-fold excess of H2O. Following this treatment, enzyme samples was raised at a rate of 1

Determination of kinetic parameters

The kinetic constants of the wild-type His-tagged and the mutant His-tagged AfcPGIs were measured (unless otherwise stated) at 70 °C in the direction of G6P formation using glucose-6-phosphate dehydrogenase from T. maritima [34] as recently described for AfcPGI [4]. The following standard conditions were used: 100 mM Tris/HCl pH 7.4 (at 70 °C), 0.5 mM NADP⁺, 5 mM fructose-6-phosphate, and 1.1 U G6P dehydrogenase. For several mutants higher protein concentration had to be used. The temperature dependence of enzyme activity was measured between 20 and 96 °C in 50 mM sodium phosphate buffer with F6P as substrate (5 mM in Tris/HCl pH 7.4 at 70 °C). The ability of the mutant proteins to bind metal was analyzed by determination of the apparent Ni²⁺ affinity of wild-type AfcPGI and its variants, given as nickel concentrations at half maximal velocity (K_{N_{i,0.5}}) as recently described for AfcPGI [4]. According to this procedure, 10 mg of enzyme was incubated in the presence of 0.5 mM EDTA for 30 min at 70 °C, pH 7.4 in order to obtain metal-free enzymes. The proteins were subsequently dialysed five times against a 100-fold excess of H2O. Following this treatment, enzyme activity was restored by the addition of Ni²⁺ and determined according to the standard assay system as recently described for AfcPGI [4]. Kinetic data were fit to the Michaelis–Menten equation with the MicroCAL™ software using the Levenberg–Marquard algorithm.

Acknowledgements

The expert technical assistance of K. Lutter-Mohr and M. Kusche is gratefully acknowledged. We thank R. Hedderich and J. Koch (Max-Planck-Institut für terrestrische Mikrobiologie Marburg) for determination of the metal content. This work was supported by grants from the Deutsche Forschungsgemeinschaft (SCHO 316/9-1).

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