Structural Evidence for a Hydride Transfer Mechanism of Catalysis in Phosphoglucose Isomerase from Pyrococcus furiosus*

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Phosphoglucose isomerase (PGI) catalyzes the interconversion of glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P), which are substrates of glycolysis, the interconversion of glucose 6-phosphate (G6P) and fructose 6-phosphate; PAB, 5-phosphoarabinonate; PfPGI, Pyrococcus furiosus phosphoglucose isomerase.

In the Euryarchaeota species Pyrococcus furiosus and Thermococcus litoralis, phosphoglucose isomerase (PGI) activity is catalyzed by an enzyme unrelated to the well known family of PGI enzymes found in prokaryotes, eukaryotes, and some archaea. We have determined the crystal structure of PGI from Pyrococcus furiosus in native form and in complex with two active site ligands, 5-phosphoarabinonate and gluconate 6-phosphate. In these structures, the metal ion, which in vivo is presumed to be Fe2+ is located in the core of the cupin fold and is immediately adjacent to the C1-C2 region of the ligands, suggesting that Fe2+ is involved in catalysis rather than serving a structural role. The active site contains a glutamate residue that contacts the substrate, but, because it is also coordinated to the metal ion, it is highly unlikely to mediate proton transfer in a cis-enediol mechanism. Consequently, we propose a hydride shift mechanism of catalysis. In this mechanism, Fe2+ is responsible for proton transfer between O1 and O2, and the hydride shift between C1 and C2 is favored by a markedly hydrophilic environment in the active site. The absence of any obvious enzymatic machinery for catalyzing ring opening of the sugar substrates suggests that pyrococcal PGI has a preference for straight chain substrates and that metabolism in extreme thermophiles may use sugars in both ring and straight chain forms.

The reaction catalyzed by PGI is an aldose-ketose isomerization in which a hydrogen atom is transferred between the C1 and C2 positions of the substrate. A second hydrogen, in the form of a proton, also moves between the O1 and O2. The carbon-bound hydrogen can move by one of two mechanisms, a hydride shift or a proton transfer via a cis-enediol intermediate (6). In isomerases that contain a metal ion at the catalytic center the mechanism appears to be a hydride shift, e.g. xylose isomerase (7), whereas isomerases without a metal cofactor use the cis-enediol mechanism (6). Conventional PGI does not require a metal for activity and, accordingly, operates via an acid-base mechanism involving a cis-enediol intermediate (see Ref. 6). In mammalian PGIs, an active site glutamate (Glu-357) is the base catalyst for isomerization, and His-388 is the acid catalyst for ring opening (8–10). Along with its enzymatic properties, PGI from mammalian sources exhibits the properties of a cytokine (11, 12). Given the structural differences between the two types of PGI, the catalytic mechanism of the novel PGI is likely to be distinct from that of conventional PGI. One question is whether the metal ion in the cupin fold mediates a hydride transfer mechanism or whether it serves a structural role.

Although the recent crystal structure of the enzyme from P. furiosus confirmed the cupin-based fold (4), in the absence of any data from crystals in complex with either substrate or inhibitor, little could be inferred regarding the active site or of the residues responsible for catalysis. To decipher the catalytic mechanism of these novel PGIs, we have determined the crystal structure of PGI from P. furiosus (PfPGI) in complex with the inhibitors 5-phosphoarabinonate (PAB) and gluconate 6-phosphate (6PG). These structures provide evidence for a hydride mechanism of hydrogen transfer.

Phosphoglucone isomerase (PGI) catalyzes the interconversion of glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P), which are substrates of glycolysis, the interconversion of glucose 6-phosphate and fructose 6-phosphate; PAB, 5-phosphoarabinonate; PfPGI, Pyrococcus furiosus phosphoglucose isomerase.
TABLE I

Data collection and refinement statistics

| Data set | Native | PAB | 6PG |
|----------|--------|-----|-----|
| Data collection | | | |
| Molarity of soak (mM) | 6 | 4 | 4 |
| Length of soak | 4 h | 4 h | 4 days |
| Resolution of data (Å) | 1.8 | 1.7 | 1.8 |
| No. of unique reflections | 130,434 | 103,907 | 89,107 |
| Complete (relative) | 92.1 (83.4)* | 92.5 (88.2)* | 93.5 (90.9)* |
| I > 2σ (outer shell) | 18.5 (2.7)* | 12.5 (3.3)* | 8.8 (2.7)* |
| Refinement | | | |
| Number of water molecules | 249 | 126 | 202 |
| Rmerge* | 19.7 | 22.3 | 22 |
| Rwork (%) | 19.4 | 22.1 | 21.8 |
| Refmac (%) | 24.9 | 26.4 | 26.6 |
| R.m.s. deviations from ideal stereochemistry | | | |
| Bond lengths (Å) | 0.011 | 0.013 | 0.016 |
| Bond angles (°) | 1.47 | 1.54 | 1.72 |
| B factors: | | | |
| Mean B factor (main chain) (Å²) | 23.3 | 28.1 | 32.1 |
| R.m.s. deviation in main chain B factor (Å²) | 0.57 | 0.65 | 0.65 |
| Mean B factor (side chains and waters) (Å²) | 26.6 | 31 | 35 |
| R.m.s. deviation in side chain B factors (Å²) | 1.74 | 1.86 | 1.57 |
| Ramachandran plot: | | | |
| Residues in most favoured region (%) | 93.6 | 94.6 | 93.6 |
| Residues in additionally allowed regions (%) | 6.1 | 4.8 | 6.1 |
| Residues in generously allowed regions (%) | 0.3 | 0.6 | 0.3 |
| Residues in disallowed regions (%) | 0 | 0 | 0 |
| PDB accession no. | 1QXJ | 1QXR | 1QY4 |

* Numbers in parentheses are for the outer shell of data.

RESULTS

Native Structure Determination—Previously, we reported on crystals of PfPGI grown from polyethylene glycol 4000 that belonged to space group P2₁ (13). Subsequently, a second crystal form was obtained over wells containing 25% polyethylene glycol 4000 (w/v) and 0.2 M ammonium acetate and buffered with 0.1 M sodium acetate, pH 5.6. This is similar to the previous condition, except that ammonium acetate was used in place of magnesium chloride. The crystals belong to space group P1 with cell dimensions a = 36.0 Å, b = 39.7 Å, c = 74.7 Å, α = 73.7°, β = 78.5°, and γ = 72.6° and diffract to at least 1.7 Å. The asymmetric unit in this crystal form is a dimer of PfPGI (corresponding to a solvent content of 45%; Ref. 21), whereas for the published native structure it is one subunit of the dimer (4). The structure was solved by molecular replacement, and the statistics of the final refined model at 1.8 Å resolution are shown in Table I. All residues are visible in the electron density except two at the C terminus. The final model contains residues 1–187 and 249 water molecules.

Structure Description.—The fold of PfPGI has been described previously (4). Briefly, the structure is dominated by the cupin domain, which is a sandwich of two β sheets (Fig. 1) in the shape of a barrel. The secondary structure was calculated using the DSSP program (22) and is shown in Fig. 1c. This varies slightly from that described for the previous structure because of marginal differences in the assignment of β strands. The structure begins with a segment that is separate from the cupin domain, comprising a β strand (β1) followed by a β hairpin (β2 and β3). In the cupin domain, one sheet comprises β4, β5, β6, β13, and β17 (sheet 1), and the second sheet comprises β9, β11, β12, and β14 (sheet 2). Located between β4 and β5 are two segments of α helical structure (α1 and α2). A third helix, α3, is between β15 and β16. There are additional β hairpins that are not part of the cupin sandwich; β5 and β6 form a connection...
Inhibitor Structures of P. furiosus Phosphoglucose Isomerase

The structure of PGI from P. furiosus. a, ribbon representation of the monomer in which the molecule is color ramped blue to red in the N to C direction. α helices and β strands are labeled individually. The metal ion, presumed to be Ni²⁺ but labeled M, is represented as an orange sphere. The figure was produced using MOLSCRIPT (30) and Raster3D (31). b, ribbon representation of the dimer where one monomer is colored red and yellow and the other monomer is dark blue and light blue. For the respective monomers, the yellow and light blue regions correspond to the cupin fold. The metal atoms are represented as orange spheres. The figure was produced using MOLSCRIPT (30) and Raster3D (31). c, the sequence of PPGI and secondary structure notation used in this paper. The coloring scheme for the secondary structure is the same as was used for panel a. Arrows denote β strands, and zigzags represent helices.

between the two sheets of the sandwich, and β15 and β16 form the C terminus of the protein.

The two monomers in the dimer are essentially identical; they can be superimposed with a root mean square deviation between all main chain atoms of 0.30 Å. The only significant structural differences occur in two regions as follows: 1) residues 18–20, which form the connection between the β1-β2-β3 segment and the main body of the molecule; and 2) residues 45–46 (α2-β5 connection). Both monomers also superimpose very closely with the published native structure (4); the root mean square deviation in common main chain atoms is 0.50 Å for monomer A and 0.41 Å for monomer B. The principal structural differences also lie in the 18–20 region.

Dimer Interface—As determined by gel filtration, the native form of PPGI is a dimer (1, 2), and in the crystal form used for our structure determination the protein crystallized as a dimer in the asymmetric unit (Fig. 1b). The dimer interface is formed by the packing of sheet 1 against its partner from the other monomer. Interestingly β1 of monomer A is a member of sheet 1 from monomer B and vice versa, creating a six-stranded sheet. This is another example of so-called domain swapping (23) that likely contributes to the overall stability of the enzyme. The interface region is populated almost exclusively with hydrophobic residues, predominantly valines, leucines, and phenylalanines. There is one electrostatic interaction, between Lys-3 and Asp-65, which is repeated at each edge of the interface. Because Lys-3 is part of β1, this “intermolecular” contact is actually mediated via residues that belong to the same monomer.

Iron Binding Site—Members of the cupin family typically contain a metal ion, which in many cases is essential for function (for review, see Ref. 5). The metal binding site is located in the core of the cupin barrel, and, in the majority of cases, the metal ion is coordinated by three histidines and one glutamate. The crystal structure of native PPGI as reported previously (4) did not contain any bound metals, but this was attributed to the use of EDTA during protein purification. Zn²⁺ and Mn²⁺ were introduced through crystal soaking experiments, and this confirmed the presence of a metal binding site in the same location as observed in other members of the cupin family (4). In our structure, a strong peak of electron density is observed in the protein core surrounded by the same constellation of residues, i.e. His-88, His-90, His-136, and Glu-97 (Fig. 2). Two additional coordination positions are occupied by apparent water molecules. The coordination distances around the metal in both monomers range between 2.1 and 2.3 Å; the closest coordination is to an oxygen of Glu-97 (2.1 Å).

The identity of this metal depends on both the source of protein and its purification procedure. Analysis of the recombinant protein by atomic absorption spectroscopy showed 0.39 mol of nickel and 0.04 mol of iron per mol of protein.² These two atoms are very similar in mass and are indistinguishable in the electron density maps. Accordingly, we modeled this density as nickel and, after refinement, the resulting Fo–Fc difference density in this region was flat in monomer A, and there was only a slight peak of negative density in molecule B, showing that this was a reasonable interpretation. Because both metal binding sites were close to fully occupied, the protein must have chelated additional metal ions from impurities in the buffers used for crystallization. The native enzyme in vivo presumably binds iron, and, given the reducing environment in which P. furiosus is found, this must exist in the reduced state i.e. ²⁺. This is supported by metal depletion experiments, which show that the activity of EDTA-treated PPGI can be restored by the addition of Fe²⁺.² Irrespective of the identity of the metal ion, the catalytic mechanism (presented below) is the same.

Complex with 5-Phosphoarabinonate—PAB is a well known

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² T. Hansen and P. Schönheit, unpublished results.
competitive inhibitor in the conventional family of PGI enzymes that is presumed to mimic the cis-enediol intermediate of the reaction (19) (Fig. 3). Compared with G6P or F6P, it contains a carboxylate group in place of C2, O2, and C1; an atom directly equivalent to O1 is absent. To locate the active site of PfPGI and develop an understanding of the catalytic mechanism, we determined the structure of PfPGI in complex with PAB at 1.7 Å resolution (Table I). This structure was generated very easily after a single soaking experiment in 6 mM of PAB. An electron density map calculated using $F_o - F_c$ coefficients reveals the inhibitor molecule bound inside the cupin fold between the two sheets (Fig. 4a). The location is in agreement with the active sites in other enzymes containing a cupin fold e.g. oxalate decarboxylase (24). The binding of PAB to each monomer is essentially identical. The inhibitor is bound such that the carboxylate group contacts both the metal ion and Glu-97, thus demonstrating that Fe$^{2+}$ in the native enzyme in vivo is intimately involved with catalysis rather than simply serving a structural role. The carboxylate oxygens of PAB have been designated as O1A and O1B (Fig. 4b). Of the two water molecules coordinated to the metal ion, one has been displaced by O1A, whereas the other water molecule is still present and is within hydrogen-bonding distance of both O1A and O1B (Fig. 4b). O1A is also within hydrogen-bonding distance of the side chain hydroxyl group of Tyr-99 and His-88. Interestingly, O1B of the PAB molecule lies very close (2.1 Å) to the side chain carbonyl of Glu-97. This close proximity is permitted by the neutrality of the carbonyl group and by the fact that the negative charge of the carboxylate group of PAB will be compensated by the metal ion. At the other end of the inhibitor molecule, the phosphate group is coordinated through hydrogen-bonding interactions with the side chains of Tyr-160, Tyr-52, and His-88 as well as with the amide of Gly-87 and one water molecule. The intervening section of the inhibitor makes no direct contacts with the enzyme, with the exception of O4, which contacts Thr-71.

Complex with Gluconate 6-Phosphate—To determine the catalytic mechanism, the face of the substrate along which the hydrogen is transferred must be identified i.e. the configuration at C1 or C2 needs to be established unambiguously. PAB is less than ideal for this purpose because the equivalent to the C1 position is occupied by an oxygen, and the C2 position is trigonal planar in geometry. 6PG is an intermediate of the pentose phosphate pathway that inhibits both conventional and novel type PGIs (25). It is a better probe than PAB because, like G6P and F6P, it is a six-carbon molecule, whereas PAB is one carbon shorter (Fig. 3). It differs from straight chain G6P by having a carboxylate in place of the aldehyde group at C1. Most importantly, the tetrahedral shape of the density made assignment of the C2 position facile and shows that its hydrogen projects downwards into the active site rather than outward toward the solvent. Interestingly, this region of the active site is markedly hydrophobic in character, comprising Tyr-152, Ala-69, Ala-150, and, to a lesser extent, Phe-148 and Tyr-99.

As with PAB, the carboxylate oxygens of 6PG have been designated O1A and O1B. Examination of the ligand-enzyme contacts shows that the carboxylate group of 6PG binds some-
what differently to that of PAB, likely because of the longer length of 6PG. Most notably, both of the water molecules coordinated to the metal ion in the native structure have been displaced by O1A and O2. The inclusion of these atoms in the coordination shell of the metal ion may mimic the binding of O1 and O2 of the substrate molecules (Fig. 5b). The close contact seen between O1B and Glu-97 in the PAB-bound structure is lost, and, instead, Glu-97 is now much closer to the metal ion. In fact, in monomer B, the oxygen of Glu-97 is only 1.4 Å from the metal ion, which suggests that this interaction is essentially a covalent bond. The equivalent distance in monomer A is 1.7 Å, and, interestingly, this is the only instance where contacts vary between the two active sites in the dimer, albeit slightly and close to the coordinate error in a typical crystal structure. The only other significant difference with the PAB-bound structure is a new interaction between O1B of 6PG and His-158. The remaining enzyme-inhibitor contacts are essentially the same as those for PAB, including the phosphate group and the interaction between O5 (O4 in PAB) and Thr-71.

Conformational Changes upon Ligand Binding—In conventional PGI, a number of conformational changes associated with ligand binding have been described (9, 26, 27). These comprise a domain closure around the phosphate group and two helix movements that position a histidine and a lysine residue, respectively, both of which likely have a role in ring opening. A comparison of the native and both of the inhibitor-bound enzymes of PfPGI, however, reveals very little difference between the structures, thus precluding any similar induced fit mechanism of ligand binding occurring in the novel-type PGIs. The main chain atoms can be superimposed onto the native structure with a root mean square deviation of 0.12 Å for the PAB-bound structure and 0.18 Å for the 6PG-bound structure. The only significant shift is of His-158, which is relatively distant from the active site in both the native and PAB-bound
structures but has moved >2 Å toward the active site in the 6PG-bound structure where it interacts with both O1A and O1B (Fig. 5). The same interaction is not seen in the PAB-bound structure, because PAB is shorter than 6PG and because its carboxylate is positioned slightly differently. Thus, His-158 is probably important for positioning the O1 oxygen of the substrates.

**DISCUSSION**

**Catalytic Mechanism**—When considering a catalytic mechanism for PfPGI there are two possibilities, the hydrogen transfers as a proton via a catalytic base or directly as a hydride (6) (Fig. 6). Indeed, both mechanisms have been observed within the group of aldose-ketose isomerases, and, interestingly, in both classes of known structure either an aspartate or glutamate is located in the active site near the C1-C2 region of the substrate. In xylose isomerase, an aspartate (Asp-257) is presumed to polarize a water molecule that exchanges the proton between O1 and O2 (7), whereas both triose phosphate isomerase (TIM) and conventional PGI use a glutamate to abstract a proton from C1/C2 (9, 10, 28).

Which mechanism, then, operates in PfPGI? At first sight, the mechanism is the same as for “conventional” PGI, *i.e.*

![Fig. 5. The structure of the PfPGI in complex with 6PG at 1.8 Å resolution.](http://www.jbc.org/diagram)

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![Fig. 6. Alternative mechanisms for hydrogen transfer in aldose-ketose isomerase (after Ref. 6).](http://www.jbc.org/diagram)
proton transfer. In the crystal structure of PfPGI bound with 6PG, Glu-97 is close enough so that it could abstract a proton from C2 of G6P and donate it to C1 to form F6P (see Fig. 5) and vice versa for the reverse reaction. By analogy with mammalian PGIs, Glu-97 would then be equivalent to Glu-357, and, by stabilizing the negative charge of the enediolate intermediate, Fe$^{2+}$ would be equivalent to Arg-272. Further support to this mechanism is provided by the ease with which the substrate binds as the straight form of G6P, and O1 and O2 displace both water molecules from the coordination shell around Fe$^{2+}$. By withdrawing electron density from O2, Fe$^{2+}$ facilitates the movement of a proton from O2 to O1, creating a carbocation at C1. An atom of hydrogen in the form of a hydride then shifts from C2 to C1. A lone pair of electrons from O2 moves to form a double bond between O2 and C2, thus creating F6P. When the product leaves the active site, water molecules again occupy the coordination positions left vacant by O1 and O2. Note that, although Glu-97 is shown in this diagram, it does not play a direct role in this proposed mechanism of catalysis. Its role appears to be to counteract the positive charge of the inferred Fe$^{2+}$ ion and it does not mediate proton transfer.

The issue may be resolved by considering the impact of Fe$^{2+}$ on a glutamate. For Glu-97 to act as a base would require an upward shift of its pK$_a$ toward neutrality. The glutamate acting as a base catalyst in the reaction catalyzed by conventional PGIs has an apparent pK$_a$ of ~7 (25). But for such a shift to occur in a glutamate coordinated with Fe$^{2+}$ would be unusual because, in essence, Glu-97 is “protonated” by Fe$^{2+}$, which is a strong Lewis acid. This is especially the case given the very short distance between the e oxygen of Glu-97 and the metal ion seen in the 6PG-bound structure. In the absence of any other residues that could deprotonate the C2, an enediol mechanism for PfPGI is therefore considered unlikely.

Given this, we favor a hydride transfer mechanism for PfPGI as shown in Fig. 7 in the G6P to F6P direction. In this mechanism, the initial step is the binding of G6P, which is then coordinated to Fe$^{2+}$ by displacing the two water molecules from the coordination shell. The resulting Fe$^{2+}$-bound G6P facilitates the movement of a proton from O2 to O1, creating a carbocation at C1. An atom of hydrogen in the form of a hydride then shifts from C2 to C1. A lone pair of electrons from O2 moves to form a double bond between O2 and C2, thus creating F6P. When the product leaves the active site, water molecules again occupy the coordination positions left vacant by O1 and O2. Note that, although Glu-97 is shown in this diagram, it does not play a direct role in this proposed mechanism of catalysis. Its role appears to be to counteract the positive charge of the inferred Fe$^{2+}$ ion and it does not mediate proton transfer.

In the catalytic mechanism proposed for xylose isomerase (7), Asp-257 abstracts a proton from a water molecule, and the resulting hydroxide shuttle a proton from O2 to O1 (in the xylose to xylulose direction). The resulting flow of electrons then induces a hydride shift between C2 and C1. The metal ion (in this case Mn$^{2+}$) stabilizes the hydroxide but also the transient negative charge on O2 (O1 in the reverse direction). Were this mechanism to operate in PfPGI, Glu-97 and Fe$^{2+}$ would be equivalent to Asp-257 and Mn$^{2+}$, respectively.

The issue may be resolved by considering the impact of Fe$^{2+}$ on a glutamate. For Glu-97 to act as a base would require an upward shift of its pK$_a$ toward neutrality. The glutamate acting as a base catalyst in the reaction catalyzed by conventional PGIs has an apparent pK$_a$ of ~7 (25). But for such a shift to occur in a glutamate coordinated with Fe$^{2+}$ would be unusual because, in essence, Glu-97 is “protonated” by Fe$^{2+}$, which is a strong Lewis acid. This is especially the case given the very short distance between the e oxygen of Glu-97 and the metal ion seen in the 6PG-bound structure. In the absence of any other residues that could deprotonate the C2, an enediol mechanism for PfPGI is therefore considered unlikely.

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molecules from the coordination shell. Fe$^{2+}$ then accepts a pair of electrons from O2, causing the oxygen to loosen the hold on its proton. The proton then bonds with O1 and, as a result, the carbon at C1 becomes electrophilic, leading to an intramolecular rearrangement comprised of a 1,2-hydride ion shift from C2 to C1. Finally, electron flow from O2 toward C2 creates a carbonyl at C2, thus forming F6P.

Contrary to the role proposed for glutamate residues in other aldose-ketose isomerases, the function of Glu-97 in this mechanism is simply to counterbalance the charge of the ferrous ion; its proximity to Fe$^{2+}$ precludes any proton exchange involving this residue. If this hypothesis is correct, the role of Asp-257 in xylose isomerase might be reconsidered.

A diagnostic of a hydride shift mechanism for catalysis is the absence of exchange of the transferred hydrogen with the solvent (as a proton). In xylose isomerase, such an exchange is prevented in part by a tryptophan residue (Trp-16), which forms a hydrophobic envelope around the site of hydrogen transfer (7). Similarly, in the complex of PPGI with 6PG, the tetrahedral density at the C2 position of the inhibitor indicates that the transferred hydrogen projects in toward the active site where it is framed by a markedly hydrophobic region. Tyr-152 is part of this region and may act in an analogous manner as Trp-16 in xylose isomerase. In addition, the active site pocket is relatively deep and highly complementary to the shape of the ligand, and, critically, the C1/C2 region of the inhibitor is partially buried (Fig. 8). Such a tight fit of the substrate would likely exclude water from the active site and so facilitate a hydride transfer between C1 and C2.

This is, therefore, a very simplistic mechanism in which the main role of the enzyme is not to exchange protons with the substrate but rather to create an appropriate environment for hydrogen transfer on each side of the substrate. Central to the mechanism is the presence of O1 and O2 within the coordination shell of Fe$^{2+}$. Given this, one can envision a transition state in a chair-like configuration that optimizes orbital overlap (Fig. 9). On one side, Fe$^{2+}$ mediates the movement of a proton between O1 and O2, and, on the other side, the hydrophobic environment formed in part by Tyr-152 promotes transfer of a hydride ion. Although our structural data are highly suggestive of this mechanism, its veracity can only be examined by isotope-exchange experiments that show that the transferred hydrogen does not exchange with the solvent.

In light of these observations, the belief that PAB mimics the catalytic machinery to facilitate ring opening begs the question as to whether PPGI has specificity for substrates in the straight chain form. If so, this is suggestive of the possibility that phosphorylated sugars in the open chain form are present in appreciable amounts in _P. furiosus_. In support of this, at the extreme temperatures in which _P. furiosus_ exists, the equilibrium would increasingly favor the open chain forms.

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