The hyperthermophilic crenarchaeon *Aeropyrum pernix* contains phosphoglucose isomerase (PGI) activity. However, obvious homologs with significant identity to known PGIIs could not be identified in the sequenced genome of this organism. The PGI activity from *A. pernix* was purified and characterized.Kinetic analysis revealed that, unlike all known PGIIs, the enzyme catalyzed reversible isomerization not only of glucose 6-phosphate but also of epimeric mannose 6-phosphate at similar catalytic efficiency, thus defining the protein as bifunctional phosphoglucose/phosphomannose isomerase (PGI/PMI). The gene *pgi/pmi* encoding PGI/PMI (open reading frame APE0768) was identified by matrix-assisted laser desorption ionization time-of-flight analyses; the gene was overexpressed in *Escherichia coli* as functional PGI/PMI. Putative PGI/PMI homologs were identified in several (hyper)thermophilic archaea and two bacteria. The homolog from *Thermoplasma acidophilum* (Ta1419) was overexpressed in *E. coli*, and the recombinant enzyme was characterized as bifunctional PGI/PMI. PGI/PMIs showed low sequence identity to the PGI superfamily and formed a distinct phylogenetic cluster. However, secondary structure predictions and the presence of several conserved amino acids potentially involved in catalysis indicate some structural and functional similarity to the PGI superfamily. Thus, we propose that bifunctional PGI/PMI constitutes a novel protein family within the PGI superfamily.

Phosphoglucose isomerase (PGI; EC 5.3.1.9) catalyzes the reversible isomerization of glucose 6-phosphate to fructose 6-phosphate. PGI plays a central role in sugar metabolism of eukarya and bacteria, and Archaea both in glycolysis via the Embden-Meyerhof pathway in eukarya and bacteria and in its modified versions found in Archaea. PGI is also involved in gluconeogenesis where the enzyme operates in the reverse direction (for the literature see Refs. 1–3). PGIIs from the domains of eukarya and bacteria are well studied enzymes. A variety of PGIIs have been purified and biochemically characterized, and the encoding genes have been cloned and sequenced (e.g., Refs. 4–11). Crystal structures have been determined for the eukaryotic PGIIs from pig, rabbit, human, and from the bacterium *Bacillus stearothermophilus*, and conserved amino acids proposed to be involved in substrate binding and/or catalysis have been identified (12–15, 17, 18, 20–25). The eukaryal and bacterial PGIIs belong to the PGI superfamily defined by its two conserved signature patterns [DEN][S]-[LIVM]-G-G-R-[FY]-S-[LIVMT]-[FY]-[DS]-[STA]-[PSAC]-[LIVMA]-G-[GS]-X-[LIVM]-[LIVMFYW]X-[FY]-[DN]-Q-X-G-V-E-X-Y-K. To date this superfamily includes more than 300 PGI sequences (see www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF00342) (26) from bacteria and eukarya but only three from the archaeal domain. These include the PGI from the hyperthermophilic euryarchaeon *Methanococcus jannaschii* (MjPGI). This PGI has recently been characterized as the first archaeal member of the PGI superfamily.2

So far little is known about other archaeal PGIIs. Recently, two other euryarchaeal PGIIs have been characterized, one from the hyperthermophilic euryarchaeon *Pyrococcus furiosus* (2, 27) and from closely related *Thermococcus litoralis* (28). Both PGIIs belong to the cupin superfamily (29) and, thus, represent a convergent branch of PGI evolution. The cupin superfamily is present in all three domains of life, eukarya, bacteria, and Archaea, and comprises a group of structurally conserved but functionally diverse proteins ranging e.g. from mannose 6-phosphate isomerases and epimerases involved in formation of bacterial cell wall carbohydrates to nonenzymatic storage proteins in plant seeds and transcription factors (29, 30).

So far PGIIs of any crenarchaeota have not been characterized, and the encoding genes have not been identified. Recently, cell extracts of the aerobic crenarchaeon *Aeropyrum pernix* have been shown to contain high PGI activities together with other enzymes of a modified Embden-Meyerhof pathway.3 Several unusual enzymes of this pathway, i.e. an archaeal ATP-dependent repressor, open reading frame, and kinase glucokinase (31), a non-regulatory ATP-dependent 6-phosphofructokinase of the phosphofructokinase-B family (32), and a non-regulatory pyruvate kinase (33), have been characterized. Despite the fact that *A. pernix* contained high PGI activity, a

2 Rudolph, Hansen, and P. Schönheit, Arch. Microbiol., in press.
3 P. Schönheit, unpublished information.
gene with significant similarity to either the PGI superfamily or the cupin-type PGIs could not be identified in the completely sequenced genome of *A. pernix* (34). This suggests that the *A. pernix* PGI could be significantly different from all known PGI s analyzed so far.

In this communication we report the purification and characterization of PGI activity of the cyanobacterium *A. pernix*. Surprisingly, unlike all known PGIs, the purified enzyme catalyzed the isomerization of both glucose 6-phosphate and epimeric mannose 6-phosphate as a similar catalytic efficiency, thus deriding the enzyme as a novel bifunctional phosphoglucoisomerase/phosphomannomannose isomerase (PGI/PMI). The encoding gene *pgi*/*pmi* was identified in the genome of *A. pernix* and functionally expressed in *Escherichia coli*. Putative homologues were found in several archaeal and two bacterial genomes. The homolog of the euryarchaeon *Thermoplasma acidophilum* was functionally expressed in *E. coli* and characterized as bifunctional PGI/PMI. Sequence comparison and phylogenetic analyses indicate that these bifunctional PGI/PMIs constitute a novel protein family within the PGI superfamily.

**MATERIALS AND METHODS**

**Growth of *A. pernix***—*A. pernix* K1–A. pernix* K1 (DSM 11879) was grown aerobically at 90 °C in a 100-L Biostat fermenter on a complex medium as described (35) except that artificial sea water was used instead of Biomaris water, and 1 g of starch was added per liter. Cells were grown and harvested after 17 h at the late exponential growth phase.

**Purification of PGI Activity (PGI/PMI) from *A. pernix***—Because the enzyme was not sensitive to oxygen, all steps of the purification procedure were carried out in the presence of oxygen at 4 °C. During the purification procedure, the PGI activity of bifunctional PGI/PMI was followed as described below. Cell extracts were prepared from 214 g (wet weight) of frozen cells that were suspended in 100 ml of Tris/HC1, pH 7.5, containing 2 mM dithioerythritol, 5 mM EDTA (buffer A). Cells were disrupted by passing through a French pressure cell at 1.3 × 10⁸ pascals. Cell debris and unbroken cells were removed by centrifugation for 90 min at 100,000 × g at 4 °C. The 100,000 × g supernatant was applied to DEAE-Sepharose (100 ml) that had been equilibrated with buffer B (50 mM sodium phosphate, pH 7.0). After ultracentrifugation (90,000 × g for 60 min) the solutions were heat-precipitated for 45 min at 90 °C (ApPGI/PMI) and 75 °C (TaPGI/PMI), centrifuged again (5,000 × g for 30 min), and extensively dialyzed against buffer B. Recombinant ApPGI/PMI and TaPGI/PMI were purified by phenyl-Sepharose and gel filtration on Superdex 200 HiLoad 16/60 to apparent homogeneity as described above for the native ApPGI/PMI. TaPGI/PMI eluted from phenyl-Sepharose and at 84–93 ml from the gel filtration column.

**Molecular Mass Determination**—Molecular mass determinations were performed by both gel filtration on Superdex 200 (150 mM NaCl, 50 mM Tris/HC1, pH 7.5) and by analytical ultracentrifugation. For analytical ultracentrifugation the TaPGI/PMI was dialyzed against 20 mM Tris/HC1, pH 7.0. Centrifugation was done at 20 °C in a Beckman Optima XL-A analytical ultracentrifuge equipped with a Titan AN 50 rotor and absorption optics. Sedimentation velocity experiments were performed for an approximation of the molecular mass at 50,000 rpm, and sedimentation-diffusion equilibrium runs were performed at 10,000 rpm for determining the molecular mass in a 150-μl volume. Inulin was used as the apparent molecular mass and an approximated molecular mass were evaluated from the velocity and shape of the sedimenting boundary by fitting the time-dependent concentration profiles calculated with Lamm’s differential equation for a single sedimenting species to the measured data using the program package AKKUPROG (40). When the measured concentration profile of sedimentation-diffusion equilibrium runs remained unchanged for 12 h, we assumed equilibrium to be attained. AKKUPROG was used to calculate the apparent molecular mass by fitting the ideal distribution for a single species to the measured concentration profiles. The partial specific volume of the protein was calculated from the amino acid sequence.

**Kinetic Assays and Determination of Kinetic Parameters**—Activity of PGI/PMI s of *A. pernix* and *T. acidophilum* was measured both as PGI or PMI activity. PGI activity was used to determine the pH and the temperature optima of the enzymes. pH optima were determined using Tris/HC1, ethanolamine, CHES, and CAPS at 100 mM each. For the determination of enzyme activity the following standard conditions were used: 1 mg of ApPGI/PMI, 2.5 μg of TaPGI/PMI, 50 mM, pH 7.6, 5 μg of enzyme. PGI activity (GEI → F6P) was determined in both directions. The formation of glucose 6-phosphate (G6P) was investigated by coupling it to the reduction of NADP⁺ either via yeast (20–50 °C) or via hyperthermophilic Thermotoga maritima glucose 6-phosphate dehydrogenase (50–95 °C) (41). The standard assay I 1 mg of ApPGI/PMI, 5 μg of TaPGI/PMI, 50 mM, pH 7.6, 2.5 μg of NADP⁺, 5 μg of glucose 6-phosphate dehydrogenase (from Klebsiella pneumoniae), which was ex-
pressed and purified as previously described (42). The standard assay II contained 100 m M Tris/HCl, 0.3 m M NADH, 0.1–15 mM G6P, and 0.6 units of mannitol-1-phosphate dehydrogenase. At temperatures higher than 50 °C a discontinuous assay was used as previously described (2). PMI activity of PGI/PMIs was analyzed in the direction of F6P formation as described above with the modified standard assays I or II using 0.1–15 mM mannose 6-phosphate (M6P) as substrate. At 50 °C and below F6P formation was detected using modified standard assay II. Above 50 °C, F6P formation was detected by using modified standard assay I with the addition of 1 units of hyperthermophilic \textit{P. furiosus} PGI (2). The following substances were tested for potential inhibitory effect on PGI activity: erythrose 4-phosphate, 6-phosphogluconate, and phosphate. The date points given in the figures are mean values of at least three experiments; S.E. are given. Kinetic data were fitted to the Michaelis-Menten equation; the curves drawn represent best fits to the Michaelis-Menten equation (see Fig. 1) or to first order exponential decay equations (see Fig. 2, C and D) with the MicroCal™ software 5.0 using the Levenberg-Marquardt algorithm; respective S.E. are given.

**Temperature Dependence and Thermal Stability**—The temperature dependence of enzyme activity was measured between 20 and 96 °C in 50 mM sodium phosphate with F6P as substrate (5 mM). The thermostability of the purified enzyme (1 µg of enzyme, pH 7.0) was tested in sealed vials that were incubated at temperatures between 70 and 110 °C for 2–120 min. The vials were then cooled on ice for 10 min, and residual enzyme activity was tested and compared with the controls (unheated samples).

### Table I

| Molecular and kinetic properties of bifunctional PGI/PMI purified from \textit{A. pernix} and of purified recombinant PGI/PMI from \textit{T. acidophilum} | Units | \textit{A. pernix} | \textit{T. acidophilum} |
|---|---|---|---|
| Apparent molecular mass |  |  |  |
| Native enzyme | kDa | 45 ± 5 | 48 ± 5 |
| Analytical ultracentrifugation | kDa | ND a | 67 ± 4 |
| Subunit (SDS-PAGE) | kDa | 36.112 | 35.157 |
| Calculated molecular mass (subunit) | kDa | 36 | a |
| Sedimentation coefficient s20 °C | s | ND | 4.8 ± 0.2 S |
| pH optimum | °C | 7.6 ± 0.2 | 7.6 ± 0.2 |
| Tm | °C | >100 | 86 ± 2 |
| T opt | °C | >98 | 75 ± 2 |
| Arrhenius activation energy | kJ/mol | 39.3 ± 1.4 |  |
| V max F6P | mM | 0.44 ± 0.10 | 0.20 ± 0.03 |
| | (50 °C) | 0.21 ± 0.05 |  |
| V max G6P | mM | 3.5 ± 0.5 | 0.72 ± 0.20 |
| V max M6P | mM | 1.1 ± 0.3 | 0.25 ± 0.03 |
| V max F6P | mM | 195 ± 15 | 57 ± 2 |
| | (50 °C) | 59 ± 3 | 83 ± 6 |
| V max G6P | mM | 226 ± 26 | 75 ± 3 |
| V max M6P | mM | 209 ± 22 |  |
| Ki Erythrose 4-phosphate | mM | 35 ± 4 | 164 ± 26 |
| 6-Phosphogluconate | mM | 59 ± 2 | 210 ± 8 |

a ND, not determined.

**Fig. 1.** Rate dependence of bifunctional PGI/PMI on mannose 6-phosphate concentration. \textit{A. pernix}, \textit{T. acidophilum}. S.E. are given.
Circular Dichroism (CD) Spectroscopy—CD spectroscopy analyses were performed on Jasco J-715 CD spectrometer. Spectra were recorded in 0.1-mm cuvettes and corrected for the signal of the solvent (10 mM sodium phosphate, pH 7.0). At least five spectra were averaged. Secondary structure analysis and assignments to different secondary structure types were performed by the experimentally established spectra-structure correlation using the Varselec option of Dicroprot (43). The program Predict Protein was used for sequence-based secondary structure predictions (cubic.bioc.columbia.edu) (44, 45). Heat-induced unfolding of PGIs were analyzed in temperature gradient experiments using closed cuvettes (0.1 cm). The protein samples were dialyzed against 10 mM sodium phosphate buffer, pH 7.0, and the protein concentrations were set to 100 μg/ml. The temperature of the samples was raised at a rate of 1 °C/min (40–100 °C). Protein unfolding was followed by changes of the α-helical CD ellipticity at 221 nm. The observed ellipticity at a given temperature (Φ) was corrected for the temperature-dependent base line, giving Φ'. The corrected values of the folded state Φ' and the unfolded state Φ' were used for calculating the fraction of folded protein (x): 
\[ x = (\Phi' - \Phi')/(\Phi' - \Phi') \]
Spectra were recorded before and after each temperature gradient. The spectra as well as the ellipticity of the unfolded state was obtained at pH 2 and 12 when complete thermal unfolding was not obtained up to 100 °C. Temperature gradient experiments were performed in triplicate; the respective S.E. are given.

Sequence Handling—Protein sequences were extracted from the NCBI, GenBank™, and Pfam data bases. Sequence alignments were constructed with the neighbor-joining (NJ) method of ClustalX using the GONNET matrix (46). Phylogenetic trees were constructed using

**Fig. 2. Effect of temperature on the specific activity and thermostability of PGI/PMI from A. pernix and T. acidophilum.**

A. ApPGI/PMI temperature dependence. B, TaPGI/PMI temperature dependence. C, ApPGI/PMI thermostability at 100 °C (●) and 110 °C (■). D, TaPGI/PMI thermostability at 70 °C (●), 80 °C (▲), and 90 °C (▽). S.E. are given.
the neighbor-joining option of ClustalX, the maximum likelihood (ML) method of PROML (Phylip, version 3.6), and the maximum parsimony (MP) method of PROTPANS (Phylip, version 3.6). Confidence limits were estimated by 100 bootstrapping replicates. The PGI signature (MP) method of PROTPARS (Phylip, version 3.6). Confidence limits were estimated by 100 bootstrapping replicates. The PGI signature

**RESULTS**

**Purification of PGI Activity of A. pernix and Its Identification as Bifunctional PGI/PMI—**Extracts from *A. pernix* grown on media with peptone and starch exhibited PGI activity (0.27 units/mg at 75 °C). This PGI activity was purified about 800-fold using five chromatographic steps to a specific activity of 41 units/mg at 75 °C. This PGI activity was purified about 800-fold using five chromatographic steps to a specific activity of 41 units/mg at 75 °C. The recombinant protein was purified by five chromatographic steps to a specific activity of 75 units/mg at 75 °C.

**PMI** differs from all known PGIs that show strict substrate specificity for G6P and F6P and did not use M6P. Rather, M6P has been shown to be an inhibitor of conventional PGIs (e.g. Refs. 1, 47, and 48).

**Identification of the Gene pgg1pim** Encoding Bifunctional PGI/PMI of *A. pernix* and Functional Overexpression in *E. coli*—By MALDI-TOF spectroscopy analysis of purified PGI/PMI from *A. pernix* ORF APE0768 was identified as putative *pgg1pim* gene. ORF APE0768 contains 1008 bp coding for a polypeptide of 335 amino acids with a calculated molecular mass of 36.112 kDa. The ORF was cloned and expressed in *E. coli*. The recombinant protein was purified by one heat treatment and two chromatographic steps to apparent homogeneity. The purified recombinant protein showed bifunctional PGI/PMI activity, thus proving ORF APE0768 to represent the functional *pgg1pim* gene. The recombinant PGI/PMI showed almost identical properties as the enzyme purified from *A. pernix*.

**Putative PGI/PMI Homologs and Functional Overexpression of PGI/PMI from *T. acidophilum*—**Putative homologs of the *A. pernix* PGI/PMI were identified in the genomes of eight (hyper)thermophilic Archaea and bacteria: *Pyrobaculum aerophilum, T. acidophilum, Thermoplasma volcanium, Ferroplasma acidarmanus, Sulfolobus tokodaii, Sulfolobus solfataricus, Aquifex aeolicus, Anaeroecoccus thermophilum*. An alignment of ApPGI/PMI and its putative homologs exhibited significant amino acid sequence identity (17–70%) (see Fig. 5 and “Discussion”). One putative PGI/PMI, the gene product of ORF Ta1419 of the thermophilic euryarchaeon *T. acidophilum*, was characterized. ORF Ta1419 contains 933 bp coding for a polypeptide of 310 amino acids with a calculated molecular mass of 35.157 kDa. The ORF was cloned and expressed in *E. coli*. The recombinant protein was purified by one heat treatment and two chromatographic steps. The purified protein also showed bifunctional PGI/PMI activity and was characterized in detail (see “Catalytic Properties and Substrate Specificity”).

**Characterization of PGI/PMIs from *A. pernix* and *T. acidophilum*—**The biochemical and biophysical properties of PGI/PMIs from *A. pernix* (ApPGI/PMI) and from *T. acidophilum (T. acidophilum* (Fig. 3). Thermal unfolding of PGI/PMI from *A. pernix* (○) and *T. acidophilum* (■) as monitored by CD spectroscopy at 221 nm.

**Fig. 4.** CD-spectra of PGI/PMI from *A. pernix* (○) and *T. acidophilum* (■). Mean molar ellipticity per residue (θ[221]°) is given.

the neighbor-joining option of ClustalX, the maximum likelihood (ML) method of PROML (Phylip, version 3.6), and the maximum parsimony (MP) method of PROTPANS (Phylip, version 3.6). Confidence limits were estimated by 100 bootstrapping replicates. The PGI signature

**RESULTS**

**Purification of PGI Activity of *A. pernix* and Its Identification as Bifunctional PGI/PMI—**Extracts from *A. pernix* grown on media with peptone and starch exhibited PGI activity (0.27 units/mg at 75 °C). This PGI activity was purified about 800-fold using five chromatographic steps to a specific activity of 200 units/mg with a yield of 3%. The purified protein was electrophoretically homogenous, as indicated by one band on SDS-PAGE. The purified enzyme catalyzed the reversible isomerization of G6P to F6P, i.e. showed PGI activity. The V[n]max values (at 80 °C) with G6P and F6P were 225 and 195 units/mg; the corresponding K[m] values were 3.5 and 0.44 mM, respectively (see also Table I). Surprisingly, the enzyme also catalyzed the isomerization of M6P to F6P at almost similar catalytic efficiency; with M6P the V[n]max and K[m] values were 209 units/mg and 1.1 mM, respectively. Thus, the enzyme of *A. pernix* exhibited bifunctional activities as PGI and PMI, and it was, therefore, assigned as bifunctional PGI/PMI. The PGI/PMI from archaea, eukarya, and Archaea

| Organism          | Apparent molecular mass (kDa) | Native Subunit | Reference |
|-------------------|-------------------------------|----------------|-----------|
| PGI superfamily   |                               |                |           |
| *A. pernix*       | 45ᵃ (αₙ)                     | 36.1ᵇ          | This work |
| *T. acidophilum*  | 67ᵃ (αₙ)                     | 35.2ᵇ          | This work |
| PGI-family        |                               |                |           |
| *M. jannaschii*   | 89ᵇ (αₙ)                     | 45ᵇ            | (2)       |
| *B. stearothermophilus* | 172ᵃ (αₙ)                   | 59.2ᵇ          | (51)      |
| *L. lactis*       | ND                            | 56ᵇ            | (60)      |
| *E. coli*         | 120ᵇ (αₙ)                    | 59ᵇ            | (10)      |
| Human             | 132ᵇ (αₙ)                    | 63ᵇ            | (61)      |
| *P. furiosus*     | 132ᵇ (αₙ)                    | 67ᵇ            | (9)       |
| *Saccharomyces cerevisiae* | 119ᵇ (αₙ)       | 61.2ᵇ          | (16)      |
| Cupin-superfamily |                               |                |           |
| *P. litoralis*    | 43ᵃ (αₙ)                     | 23.3           | (2)       |
|                   |                               | 21.5           | (28)      |

ᵃ Determined by gel filtration analysis.
ᵇ Calculated molecular mass.
ᶜ Determined by analytical ultra centrifugation.
ᵈ Determined by SDS-PAGE.

| Organism          | Apparent molecular mass (kDa) | Native Subunit | Reference |
|-------------------|-------------------------------|----------------|-----------|
| PGI superfamily   |                               |                |           |
| *A. pernix*       | 45ᵃ (αₙ)                     | 36.1ᵇ          | This work |
| *T. acidophilum*  | 67ᵃ (αₙ)                     | 35.2ᵇ          | This work |
| PGI-family        |                               |                |           |
| *M. jannaschii*   | 89ᵇ (αₙ)                     | 45ᵇ            | (2)       |
| *B. stearothermophilus* | 172ᵃ (αₙ)                   | 59.2ᵇ          | (51)      |
| *L. lactis*       | ND                            | 56ᵇ            | (60)      |
| *E. coli*         | 120ᵇ (αₙ)                    | 59ᵇ            | (10)      |
| Human             | 132ᵇ (αₙ)                    | 63ᵇ            | (61)      |
| *P. furiosus*     | 132ᵇ (αₙ)                    | 67ᵇ            | (9)       |
| *Saccharomyces cerevisiae* | 119ᵇ (αₙ)       | 61.2ᵇ          | (16)      |
| Cupin-superfamily |                               |                |           |
| *P. litoralis*    | 43ᵃ (αₙ)                     | 23.3           | (2)       |
|                   |                               | 21.5           | (28)      |

ᵃ Determined by gel filtration analysis.
ᵇ Calculated molecular mass.
ᶜ Determined by analytical ultra centrifugation.
ᵈ Determined by SDS-PAGE.
FIG. 5. Multiple sequence alignment of amino acid sequences of the PGI/PMIs from A. pernix and T. acidophilum and its putative homologs from P. aerophilum, S. solfataricus, S. tokodaii, T. volcanium, T. acidophilum, F. acidarmanus, A. thermophilum, and A. aeolicus (for accession numbers see Fig. 6. The alignment was generated with ClustalX (46). The signature pattern of the PGI/PMI family S-Y-S-G-[NT]-T-[ESTIL]-E-T-[LIV] is highlighted by a green box. The predicted secondary structure for the ApPGI/PMI according to the program Predict Protein (cubic.bioc.columbia.edu) (44, 45) is given above the sequences, helices are indicated as bars, and β-sheets are indicated as arrows. Residues of PGI/PMI sequences that correspond to equivalent residues in PGIs are marked as follows. Residues proposed to be important for substrate binding and/or catalysis of PGIs as deduced from x-ray structures (14, 21, 22) are shaded yellow, and the two updated signature patterns of the PGI family, [DENSA]-X-[LIVM]-[FY]-[GAI]-[PSTACMV]-[LIVMSAC]-[GSA] and [GSA]-X-[LIVM]-[FY]-[GAI]-[PSTACMV]-[LIVMSAC]-[GSA], are highlighted by gray boxes. The respective PGI/PMI residues are printed red (conserved), green (functionally conserved), or blue (not conserved); only those residues are marked that correspond to catalytic important ones and to those within the PGI signature patterns. Residues that correspond to variable residues of the PGI signature patterns are printed in black.
FIG. 6. Phylogenetic relationship of enzymes from the PGI superfamily as constructed by the neighbor-joining algorithm of ClustalX (46). The numbers at the nodes are bootstrapping values according to neighbor-joining (first), maximum likelihood (second), and maximum parsimony (third). Only values greater than 60 are given. NCBI accession numbers or SwissProt identifiers: Aper, A. pernix BAA79746;
(TaPGI/PMI) were characterized (Table I).

**Molecular Properties**—Apparent molecular masses of ApPGI/PMI and TaPGI/PMI, as analyzed by gel filtration, were 45 and 48 kDa, respectively. In addition, the molecular mass of TaPGI/PMI was determined by analytical ultracentrifugation. The measured concentration profile of sedimentation-diffusion equilibrium runs were best fit using a molecular weight of 67 kDa, which is significantly higher than the value (49 kDa) measured by gel filtration. A significant underestimation of molecular mass by gel filtration has also been reported for several hyperthermophilic proteins, e.g., pyrophosphatase from *Sulfolobus acidocaldarius* (49). SDS-PAGE revealed one subunit of ApPGI/PMI and TaPGI/PMI, each with apparent molecular masses of 36 and 35 kDa, respectively, indicating a dimeric structure for both PGIs/PMIs.

**Catalytical Properties and Substrate Specificity**—ApPGI/PMI and TaPGI/PMI exhibited bifunctional activities as PGIs and PMIs. In addition to G6P, M6P was isomerized to F6P at about the same catalytic rate. Kinetic properties of both enzymes were determined for both reaction directions for the PG activity (G6P and F6P) and in the direction of F6P formation for PMI activity. The rate dependence on all substrates of the enzymes followed Michaelis-Menten kinetics; the respective $K_m$ and $V_{max}$ values are given in Table I. The rate dependence of both enzymes on M6P concentration is shown in Fig. 1. $A$ and $B$. The respective $K_m$ values for all three substrates are 2–5-fold higher for the ApPGI/PMI (assay temperature 80 °C) as compared with those obtained for the TaPGI/PMI (assay temperature 50 °C) but were about identical for F6P when both enzymes were assayed at 50 °C, indicating a lower substrate for the ApPGI/PMI at higher temperatures.

The bifunctional PGIs/PMIs from *A. pernix* and *T. acidophilum* were inhibited by low concentrations of erythrose 4-phosphate and 6-phosphogluconate (Table I), i.e., effective inhibitors of PGIs of the PGI superfamily (10, 50–53). For the ApPGI/PMI a competitive inhibition with 6-phosphogluconate could be demonstrated, i.e., the addition of the inhibitor (50 μM) caused an apparent 4.3-fold increase of the $K_m$ value, whereas the respective $V_{max}$ values were almost identical in the presence and the absence of the inhibitor (61 and 59 units/mg, respectively, 50 °C). The pH optima of both ApPGI/PMI and TaPGI/PMI were at 7.6.

**Temperature Optimum and Thermostability**—The thermophilic properties of the PGI/PMIs were analyzed by following heat-induced unfolding as detected by CD spectroscopy at 221 nm, the temperature dependence of the specific activity, and the heat resistance of the proteins (Figs. 2 and 3). ApPGI/PMI activity showed a temperature optimum of about 98 °C (the highest temperature tested) and was extremely thermostable. Neither significant loss of enzymatic activity upon 120-min incubation at 100 °C nor unfolding could be detected up to 100 °C, indicating a melting temperature higher than 100 °C. At 110 °C the ApPGI/PMI still showed a half-life of about 35 min. The TaPGI/PMI also exhibited remarkable thermophilic properties. The activity of the enzyme showed a temperature optimum of 75 °C, which correlates with the onset of unfolding (about 75–78 °C). Above that temperature the heat resistance of the protein was lost. TaPGI/PMI was not inactivated upon incubation at 70 °C for 120 min; the half-life of the enzyme at 80 °C was about 30 min. The enzyme showed a melting temperature of 86 °C; at 90 °C almost complete unfolding as well as the complete loss of heat resistance could be detected.

**DISCUSSION**

In this report we describe bifunctional PGIs/PMIs from *A. pernix* and *T. acidophilum*. The enzymes represent the first characterized members of a novel bifunctional PGI/PMI family. **Bifunctionality and Physiological Function of PGI/PMI**—The ApPGI/PMI and the TaPGI/PMI exhibited very similar kinetic properties. The most striking feature of these enzymes is their bifunctionality, which is unique among the PGIs and PMIs described so far. All characterized PGIs and PMIs are highly specific for either G6P/F6P and M6P/F6P, respectively. In contrast, the PGI/PMIs described in this study used G6P, M6P, and F6P as substrates at almost equal rates and catalytic efficiency. The kinetic constants for G6P and M6P of these bifunctional enzymes were in the same range as described for PGIs and PMIs (e.g., Refs. 7, 9, 11, and 53). In contrast, M6P has been demonstrated to be a competitive inhibitor for several conventional PGIs (47, 54). Low rates of M6P formation from G6P via F6P could be demonstrated for the rabbit PGI; however, this (epimerase) activity proceeds at a 2 × 10^5-fold lower rate as compared with the isomerization rate of F6P (55). Thus, this reaction should be considered as a side reaction rather than being of physiological relevance. In contrast, the PGI/PMIs of this study catalyzed M6P conversion to G6P via fructose at similar rates as F6P isomerization. Neither *A. pernix* nor *T. acidophilum* contain an additional pgI nor a pmi gene in their genomes, which indicates that both enzymes have physiological roles as PGIs and as PMIs in these organisms. As reported for various conventional PGIs (10, 50–53), both the ApPGI/PMI and the TaPGI/PMI were inhibited by micromolar concentrations of erythrose 4-phosphate and 6-phosphogluconate, indicating a similar active site (see “Sequence Comparison”).

**Molecular and Thermophilic Properties**—The PGI/PMIs from the Archaea *A. pernix* and *T. acidophilum* were characterized as homodimers with subunits of about 36 kDa. When compared with conventional PGIs, PGI/PMIs (including the
Putative homologs) are significantly smaller (296–354 amino acids versus more than 401 amino acids (M. jannaschii for conventional PGIs (Table II; www.sanger.ac.uk/cgi-bin/Pfam)) and might represent the minimal core structure necessary for a phosphoglucone isomerase of the PGI superfamily. In contrast, the cupin type PGIs from Thermococcales have subunits of about 23 kDa and are significantly smaller (Table II) (2, 28). In accordance with the optimal growth temperature of A. pernix, the hyperthermophilic ApPGI/PMI is the most thermophilic enzyme described so far. In comparison, the PGIs from the hyperthermophiles M. jannaschii and P. furiosus had a temperature optimum of 89°C and above 96°C, respectively (2),2 P. furiosus PGI had a half-life of about 90 min at 100°C, whereas the A. pernix enzyme was heat-resistant at that temperature for up to 2 h. The TaPGI/PMI showed a temperature optimum of 75°C, which is above the growth optimum of the T. acidophilum (59°C).

Sequence Comparison—All PGIs characterized so far belong either to the PGI superfamily or to the cupin superfamily. All characterized PMIs belong to the cupin superfamily, which includes class I and class II PMIs; type I are bicupin PMIs characterized PMIs belong to the cupin superfamily, which either to the PGI superfamily or to the cupin superfamily. All T. acidophilum sequences exhibit significant changes from an updated version of the two PGI/PMI sequences as well, representing members of the ApPGI/PMI family. All ApPGI/PMI sequences exhibit significant changes from the corresponding residues of human PGI when aligned regions were compared. The protein architecture is very similar between mammalian and the bacterial PGI and comprises a large and a small domain, each with a central /-sheet surrounded by /-helices. The active site of PGI, which is located in a crevice between the domains close by the subunit boundary, comprises a novel convergent line of PMI evolution. Below PGI/PMIs were compared with the PGI superfamily, the conclusion being that PGI/PMIs represent a novel enzyme family within the PGI superfamily; at a first glance, the homology between ApPGI/PMI and TaPGI/PMI and conventional PGIs from the PGI superfamily is quite low (5–14% identity). However, at a closer look, including putative PGI/PMI homologs, some features of this PGI/PMIs become evident that strongly suggest a common origin of both conventional PGIs and PGI/PMIs.

Putative homologs of the ApPGI/PMI and the TaPGI/PMI were identified in the genomes of seven (hyper)thermophilic Archaea and bacteria: P. aerophilum, T. volcanium, F. acidarmanus, S. tokodaii, S. solfataricus, A. aeolicus, A. thermophilum. An alignment of ApPGI/PMI, TaPGI/PMI, and the putative homologs exhibited significant amino acid sequence identity (17–70%). 27 residues are completely conserved among these nine sequences (Fig. 5; for the accession numbers see Fig. 6). Although the coding function of the hypothetical ORFs has not be demonstrated, we expect these putative homologs of the ApPGI/PMI and TaPGI/PMI to encode for bifunctional PGI/PMIs as well, representing members of a the novel PGI/PMI family. All ApPGI/PMI sequences exhibit significant changes from the corresponding residues of human PGI when aligned regions were compared. The protein architecture is very similar between mammalian and the bacterial PGI and comprises a large and a small domain, each with a central /-sheet surrounded by /-helices. The active site of PGI, which is located in a crevice between the domains close by the subunit boundary, comprises a novel convergent line of PMI evolution. Below PGI/PMIs were compared with the PGI superfamily, the conclusion being that PGI/PMIs represent a novel enzyme family within the PGI superfamily; at a first glance, the homology between ApPGI/PMI and TaPGI/PMI and conventional PGIs from the PGI superfamily is quite low (5–14% identity). However, at a closer look, including putative PGI/PMI homologs, some features of this PGI/PMIs become evident that strongly suggest a common origin of both conventional PGIs and PGI/PMIs.
well (Table III). This implies that PGIs and PGI/PMIs might share a very similar catalytic mechanism. However, one might expect differences in substrate binding and/or the catalytic mechanism, which accounts for the extra PMI activity in PGI/PMIs.

The presence of functional conserved residues between PGIs and PGI/PMIs might indicate functional divergence from an ancestral PGI. To analyze the phylogenetic relationship of PGI/PMIs, of which ApPGI/PMI and TaPGI/PMI have been characterized, phylogenetic analyses were performed using three tree construction methods, NJ, ML, and MP. A phylogen including both characterized and putative PGIs/PMIs as well as PGIs from eukarya, bacteria, and Archaea based on a NJ tree is given in Fig. 5. The overall topology was achieved by the three methods used and is supported by good bootstrapping values. The most striking feature of this tree is its dichotomic structure. This topology goes along with two different enzyme functions as described above, separating the PGI superfamily into the PGI/PMI family and the PMI family. Within these families the topology is quite puzzling, which might be explained by assuming several independent lateral gene transfers between organisms of the three kingdoms. PGI has sometimes been described as a “workhouse” enzyme of sugar metabolism (14), and the uptake of pgi genes from environmental sources might be of advantage in improving the efficiency of sugar metabolism.

Recently, the PGI family has been divided into three major subfamilies: I, eukaryotic; II, cyanobacterial/chloroplast; III, bacterial (57, 58). Most PGI sequences fall into the subfamily I and the monophyly of the major eukaryotic groups is reflected in the topology of the reconstruction. However, several eubacterial clades (Zymomonas mobilis, Xanthomonas campestris, E. coli, Haemophilus influenzae) are interspersed between the eukaryotic groups. Their presence might be explained as a result of several independent lateral gene transfers from eukarya to its symbionts and parasites. PGI subfamily II comprises PGIs from chloroplasts, cyanobacteria, and amitochondriate protists. A common origin of these PGIs has been suggested (57). PGIs from various bacterial lineages are found in PGI subgroup III. In addition, PGIs from three euryarchaeota, M. jannaschii, Halobacterium NRC1, Haloarcula marismortui, were included in the bacterial cluster rather than in a separate cluster because they show a high degree of similarity to bacterial PGIs (35–48%), and these organisms might have obtained their pgi gene by a lateral gene transfer.2 Whereas the PGI family includes almost exclusively bacterial and eukaryal sequences (>300), the PGI/PMI family comprises nine sequences from thermophilic and hyperthermophilic species: four crenarchaeal sequences, three euryarchaeal sequences from Thermoplasmales, and two bacterial sequences. Thus, this family is presumably of archaeal or even crenarchaeal origin. The clustering within this group is supported mostly by good bootstrap values, subdividing it into two or three groups, A. pernix, P. aerophilum, Sulfolobales, Thermoplasmales, including two bacterial sequences. The two bacterial PGI/PMI sequences branch from the line leading to the Thermoplasmales according to all three tree construction methods used (NJ, ML, and MP); however, the position of the respective basal node differs. This suggests a common origin of these bacterial and the Thermoplasmales PGI/PMIs sequences. At least for the respective gene from Anaerocellum thermocellum, a lateral gene transfer has to be assumed since the PGIs from closely related Clostridium species (Clostridium thermohilum, Clostridium acetobutylicum, Clostridium perfringens, and Thermoanaerobacter tengcongensis) fall into the PGI subfamily III close to the PGIs from Bacillus species. Whether the PGI/PMI family originated in the early archaeal or even crenarchaeal evolution cannot be clarified due to the limited number of archaeal genomes sequences available so far. Thus, analysis of its distribution in other archaeal organisms will help to address this question. To date PGI/PMIs are present in all four crenarchaeal genomes available. In comparison, in euryarchaeota the presence of PGI/PMIs is limited so far to the Thermoplasmales; Thermococcales species have been shown to contain PGIs of the cupin type, representing a convergent line of PGI evolution.

According to NJ and MP tree reconstruction analysis, the Thermoplasmales group branches from the line leading to the Sulfolobales sequences, suggesting an exchange of pgipmi gene between ancestors of these organisms, a phenomenon that has been proposed to occur at high frequency between these organisms due to the common natural (acidic) biotopes (59). This would indicate an crenarchaeal origin of the PGI/PMI family, which is presumably a result of diversification of an ancestral PGI to PGI/PMI in the early (cren)archaeal evolution with the gain of the additional PMI function at the cost of a less regulatory potential as compared with two separately transcribed enzymes. On the other hand, ML analysis revealed two PGI/PMI groups, a crenarchaeal group and a Thermoplasmales group, suggesting an archaeal origin of this family, which would imply that all other euryarchaeota have lost their PGI/PMI sequences during evolution, which is unlikely. Also, because none of the available genomes contains both a pgi and a pgipmi gene, a scenario including an early gene duplication leading to PGIs and PGI/PMIs with a subsequent loss of one of the genes in all organisms appears unlikely as well.

In summary, the first characterized bifunctional PGI/PMIs from A. pernix and T. acidophilum belong to the PGI superfamily. Because of the bifunctional character of these enzymes and their low sequence identity to conventional PGIs and due to their separate phylogenetic clustering, bifunctional PGI/PMIs were established as a novel protein family within the PGI superfamily. Thus, this superfamily now comprises both a PGI family and a PGI/PMI family. The amino acid sequence S-Y-S-G-[NT]-T-[ESTIL]-E-T-[LIV] was derived as a specific signature pattern for PGI/PMI; it is not found in PGIs, which contain a lysine or an arginine at the second position of this pattern. Using the corresponding residues in PGIs, S-[KR]-[ST]-[GFS]-TSGNR-T-[ILATEQGP]-E-[TVPI]-[AILMRSG] was deduced as the signature pattern of the PGI family, and S-[YKR]-[ST]-[GFS]-TSGNR-T-[ILASTEQGP]-E-[TVPI]-[AILMRSG] was deduced as the signature pattern for the PGI superfamily comprising both PGI/PMIs and PGIs. Currently, further characterization of this novel family in particular structure/function analyses are in progress.

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