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Properties of the recombinant glucose/galactose dehydrogenase from the extreme thermoacidophile, *Picrophilus torridus*

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In *Picrophilus torridus*, a euryarchaeon that grows optimally at 60 °C and pH 0.7 and thus represents the most acidophilic thermophile known, glucose oxidation is the first proposed step of glucose catabolism via a non-phosphorylated variant of the Entner–Doudoroff pathway, as deduced from the recently completed genome sequence of this organism. The *P. torridus* gene for a glucose dehydrogenase was cloned and expressed in *Escherichia coli*, and the recombinant enzyme, GdhA, was purified and characterized. Based on its substrate and coenzyme specificity, physicochemical characteristics, and mobility during native PAGE, GdhA apparently resembles the main glucose dehydrogenase activity present in the crude extract of *P. torridus* DSM 9790 cells. The glucose dehydrogenase was partially purified from *P. torridus* cells and identified by MS to be identical with the recombinant GdhA. *P. torridus* GdhA preferred $NADP^+$ over NAD^+ as the coenzyme, but was nonspecific for the configuration at C-4 of the sugar substrate, oxidizing both glucose and its epimer galactose (K_m values 10.0 and 4.5 mM, respectively). Detection of a dual-specific glucose/galactose dehydrogenase points to the possibility that a 'promiscuous' Entner–Doudoroff pathway may operate in *P. torridus*, similar to the one recently postulated for the crenarchaeon *Sulfolobus solfataricus*. Based on Zn^{2+} supplementation and chelation experiments, the *P. torridus* GdhA appears to contain structurally important zinc, and conserved metal-binding residues suggest that the enzyme also contains a zinc ion near the catalytic site, similar to the glucose dehydrogenase enzymes from yeast and *Thermoplasma acidophilum*. Strikingly, NADPH, one of the products of the GdhA reaction, is unstable under the conditions thought to prevail in *Picrophilus* cells, which have been reported to maintain the lowest cytoplasmic pH known (pH 4.6). At the optimum growth temperature for *P. torridus*, 60 °C, the half-life of NADPH at pH 4.6 was merely 2.4 min, and only 1.7 min at 65 °C (maximum growth temperature). This finding suggests a rapid turnover of NADPH in *Picrophilus*.

With a growth optimum pH of ≈ 0.7 and the ability to grow even at molar concentrations of sulfuric acid at 60 °C, *Picrophilus torridus* and *P. oshimae* are the most acidophilic thermophiles known to date [1]. These organisms belong to the order of *Thermoplasmales* within the *Euryarchaeota*. Of note, the intracellular pH of *Picrophilus* cells of 4.6 is far lower than usually found in other thermoacidophilic organisms, i.e. > 6.0

Abbreviations
ADH, alcohol dehydrogenase; LADH, liver alcohol dehydrogenase; ORF, open reading frame; YADH, yeast alcouol dehydrogenase.

[2]. As a consequence, it is expected that the cellular enzymes and metabolism of *P. torridus* carry distinct features that are due to the low cytoplasmic pH.

Glucose dehydrogenase is the first enzyme in a variant of the Entner–Doudoroff pathway, involving nonphosphorylated intermediates, which is utilized as the central hexose catabolic pathway in several members of the thermoacidophile group [3], in particular in *Sulfolobus solfataricus* [4] and *Thermoplasma acidophilum* [5], and is suggested to be present also in *P. torridus* as indicated by genome-sequencing data [6]. Glucose dehydrogenase catalyses the oxidation of glucose to gluconate via gluconolactone, using NAD^+ and $NADP^+$ as cofactors:

$$\text{Glucose} + \text{NAD(P)}^+ \text{Gluconate} + \text{NAD(P)H} + \text{H}^+$$

In their primary structure, archaeal glucose dehydrogenases show the typical GXGXXG/A fingerprint motif found in most $NADP^+$-binding proteins [7] and all known representatives belong to the medium-chain dehydrogenases/reductases. On the basis of the three-dimensional structure of the glucose dehydrogenase from *T. acidophilum* it was shown that, although only distantly related by amino acid sequence, structural homology to the eukaryotic medium-chain alcohol dehydrogenases (ADHs) exists, i.e. to horse liver alcohol dehydrogenase (LADH) and yeast alcohol dehydrogenase (YADH) [8]. In the crystal structures of all these dehydrogenases one catalytic and one structural zinc ion have been detected, and the role of the latter has been well examined in YADH [9]. By contrast, little is known about the effect of zinc on archaeal glucose dehydrogenases. In this study we report on the cloning and expression of the glucose dehydrogenase gene of *P. torridus* in *Escherichia coli*, the biochemical characterization of its product and the effect of zinc ions on the pH and temperature stability of the protein.

Results

Analysis of the amino acid sequence

Metabolic pathway reconstruction based on genome data suggested the presence of a nonphosphorylated variant of the Entner–Doudoroff pathway [6]. For its first enzyme, glucose dehydrogenase (EC 1.1.1.47), three open reading frames were identified in the annotated *P. torridus* genome, each coding for different proteins with similarity to glucose dehydrogenases of the medium-chain ADH family (data not shown). Based on similarity in the homologous genome region of the related archaeon *T. acidophilum* [10], we selected open reading frame PTO1070 (*gdhA*) for cloning and expression. The open reading frame codes for a protein of 359 amino acids (M_r 40 462), which corresponds by size to the purified enzyme as determined by SDS/PAGE (Fig. 1A). The degree of amino acid sequence similarity of GdhA and its homologues in *T. acidophilum* and *F. acidarmanus* is 60 and 57%, respectively.

Based on amino acid sequence similarity, *P. torrridus* glucose dehydrogenase could be assigned as a

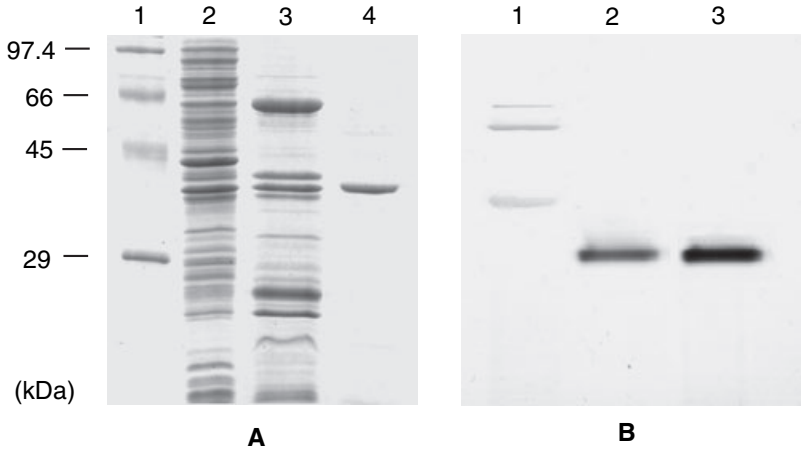

Fig. 1. SDS/PAGE and native PAGE analysis of *P. torridus* GdhA. (A) SDS/PAGE of the different steps in the purification of recombinant *P. torridus* GdhA. Lane 1, molecular mass marker; lane 2, *E. coli* pBAD_glucose dehydrogenase cellular extract; lane 3, heat-treated fraction; lane 4, GdhA pooled fractions after anion exchange chromatography. The molecular masses of the marker proteins are shown on the left. (B) Native PAGE, stained for glucose dehydrogenase activity. Lane 1, molecular mass marker containing ferritin (450 kDa), katalase (240 kDa) and cytochrome C (12.5 kDa); lane 2, recombinant GdhA; lane 3, cell-free extract of *P. torridus* grown on Brock's medium supplemented with 0.2% (w/v) yeast extract.

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Ta-Gdh (98) K C I N C R I G R Q D N C S I G D P
Pt-GdhA(97) K C V N C R I G R E D D C S D G D K
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Fig. 2. Amino acid sequence alignment of the structural Zn binding region of *T. acidophilum* and *P. torridus* glucose dehydrogenase. The residues involved in zinc coordination according to John *et al.* [8] are boxed. The numbers in brackets indicate the amino acid position in the sequence.

member of the medium-chain alcohol/polyol dehydrogenase/reductase branch of the superfamily of pyridine-nucleotide-dependent alcohol/polyol/sugar dehydrogenases [11]. Members of this group are characterized by conserved structural and catalytic zinc binding and nucleotide-binding sites. The crystal structure of the glucose dehydrogenase from *T. acidophilum* has been reported and the residues involved in zinc binding have been identified [8]. While in the structural homologue, horse LADH, the structural zinc is ligated to four cysteine residues that are highly conserved throughout the structural zinc-containing ADHs, the enzymes from *T. acidophilum* as well as *P. torridus*, which share 60% amino acid sequence identity, carry only three cysteine residues in this region. The fourth ligand has been established in *T. acidophilum* as Asp115, and the amino acid alignment shows that *P. torridus* GdhA also has Asp at this position (Fig. 2). In addition, the residues reported to be involved in Zn^{2+} coordination in the catalytic zinc-binding region of the *T. acidophilum* glucose dehydrogenase [8] were also found in the primary structure of the *P. torridus* enzyme. The GXGXXG/A fingerprint motif, characteristic for pyridine nucleotide-binding proteins is also present, together with Asp and His residues at positions 213 and 215 (*P. torridus* glucose dehydrogenase numbering), which are reported to explain the dual cofactor specificity of the enzyme from *T. acidophilum*.

Cloning and expression of the *P. torridus* glucose dehydrogenase gene

Primers were constructed using the data of the complete *P. torridus* genome sequence and gene amplification was accomplished by PCR with genomic DNA as template. The product was cloned in pCR4_TOPO and subsequently in pBAD/*Myc* for expression. Presumably because of the presence of rare codons in the coding sequence of GdhA (most notably the Arg codon AGG with 3.3%), initial expression experiments in the *E. coli* strain TOP 10 carrying pBAD-glucose dehydrogenase showed no detectable level of GdhA expression (data not shown). This made necessary the use of an expression strain supplying tRNAs for these codons, and the *E. coli* Rosetta strain was tested as such a host. As an alternative, another expression vector was constructed, p24-glucose dehydrogenase, which was obtained by cloning the *gdhA* gene in the T7 promoter-regulated vector pET24d. However, expression from this construct in *E. coli* Rosetta resulted in abundant inclusion body formation.

Although inclusion body formation was also observed in cell-free extracts of *E. coli* Rosetta carrying the plasmid pBAD-glucose dehydrogenase, a high level of glucose dehydrogenase activity could be detected after induction with 0.2% D-arabinose. The activity observed in the recombinant cells (10 $U \cdot mg^{-1}$) was 700-fold higher than that in negative controls (0.014 $U \cdot mg^{-1}$). Also, a higher level of expression was observed, when the expressing *E. coli* cells were grown at 30 °C compared with 37 °C (not shown).

Purification and characterization of the recombinant glucose dehydrogenase

The *P. torridus* glucose dehydrogenase GdhA was purified from *E. coli* Rosetta transformed with pBAD-glucose dehydrogenase in a three-stage process, which is summarized in Table 1. The thermostability of the enzyme permits the use of heat treatment as a first step in the purification. By subsequent anion exchange and size-exclusion chromatography we purified the enzyme to electrophoretic homogeneity. The isolated enzyme had a specific activity of 252 $U \cdot mg^{-1}$ and gave a single band on SDS/PAGE with a M_r corresponding to the size predicted from sequence analysis (Fig. 1A). Gel filtration of the purified GdhA indicated a tetrameric structure ($M_r \approx 160\,000$), which was not affected by the absence of NAD^+ or $NADP^+$ (data not shown).

The recombinant *P. torridus* glucose dehydrogenase was active with glucose and galactose and both $NADP^+$ and NAD^+ as cosubstrates, displaying approximately 20-fold higher activity with $NADP^+$. Kinetic analysis, accomplished by the direct linear plot

Table 1. Purification of recombinant *P. torridus* glucose dehydrogenase.

Enzyme fraction	Total protein (mg)	Total activity (U)	Specific activity ($U.mg^{-1}$)	Yield (%)	Purification (fold)
Cell-free extract	72	720	10	100	1
Heat treated	26	689	26.5	96	2.6
Source Q	1.56	301	193	42	19
Superdex 200	0.6	151	252	20	25

method under optimal conditions and saturating concentration of the cosubstrate, resulted in apparent K_m values of 10 (± 1) mM for glucose (at 5 mM $NADP^+$ as the cosubstrate) and 1.12 (± 0.2) mM for $NADP^+$ (at 50 mM glucose). The precise determination of the K_m for NAD^+ was not possible, as we were unable to reach saturation of the enzyme.

A broad range of aldose sugars was tested as potential substrates for GdhA. The enzyme was significantly active only with D-galactose, reaching 74% of the activity with D-glucose with a K_m of 4.5 (± 0.6) mM, when $NADP^+$ was used as a cosubstrate. None of the C2 and C3 epimers of D-glucose or derivatives (D-mannose, D-allose, D-glucosamine, 2-deoxy-D-glucose, glucose-6-phosphate) and none of the aldopentoses (D-xylose, L-arabinose, D-ribose) tested showed activity above 2% both with $NADP^+$ and NAD^+ as cosubstrates.

In the standard assay system (10 min assay), the highest rate of glucose oxidation was measured at 55 °C. At the optimum growth temperature for *P. torridus* of 60 °C, GdhA displayed 88% of its maximal activity. The pH optimum of the pure enzyme was determined to be pH 6.5, but at the physiological pH of 4.6 found in the cytoplasm of *Picrophilus* cells it showed merely 10% of its maximal activity. Also, incubation at 60 °C (the optimum growth temperature of *P. torridus*) and pH 4.6 in McIlvaine or acetate buffer without supplementation of Zn^{2+} for 1 h led to almost complete loss of enzyme activity. Thermal inactivation kinetics followed at pH 6.5 without the addition of Zn^{2+} to the buffer showed a $t_{1/2}$ of 5 min at 70 °C and > 3 h at 65 °C.

Addition of $ZnCl_2$ to the assay buffer at up to 5 mM had no effect on GdhA activity. Also, no effect was observed with 5 mM NaCl, $MgCl_2$, $MnCl_2$ or $CaCl_2$. EDTA added at up to 10 mM caused no loss of activity. However, the addition of $ZnCl_2$ to the incubation buffers showed a marked effect on the stability of the enzyme at both high temperature and acidity. This effect was the same across the range of $ZnCl_2$ concentrations tested, i.e. from 0.05 to 1 mM. The influence of Zn^{2+} on the pH stability of GdhA is most evident after incubation (1 h, 55 °C) at pH 3.5, where, in the presence of the metal ion at 0.1 mM, there was 96% residual activity, opposed to only 5% in its absence (Fig. 3). The long-term stability of GdhA at elevated temperatures was also considerably improved by the addition of Zn^{2+} (Fig. 4). At 0.1 mM Zn^{2+}, incubation at 70 °C for 3 h did not result in loss of activity. The specificity of Zn^{2+} in stabilizing GdhA was confirmed by incubating the enzyme for 30 min at 75 °C in the presence of 1 mM NaCl, $MgCl_2$ or $CaCl_2$, where

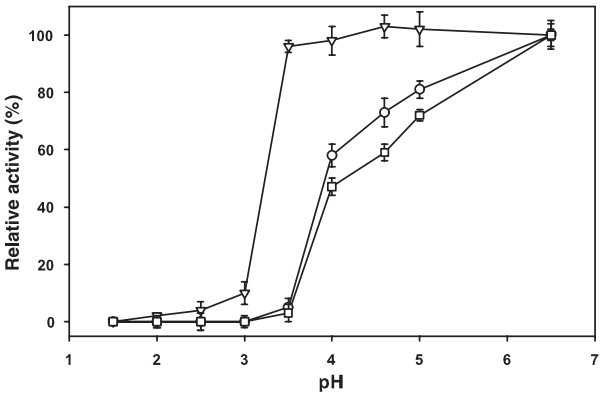

Fig. 3. pH stability of GdhA. GdhA at 2.9 mg·mL^{-1} was diluted 25-fold in incubation buffer at the specified acidity and incubated for 1 h at 55 °C. The activity is expressed as percent of the activity after incubation at pH 6.5. The buffers used were: 50 mM glycine HCl in the range pH 1.5–3.3, 50 mM sodium acetate for pH 3.5–5.5, 50 mM phosphate for pH 6–7 and 50 mM Tris for pH 7.5–8.5. (○) no $ZnCl_2$, (▽) 0.1 mM $ZnCl_2$, (□) 10 mM EDTA.

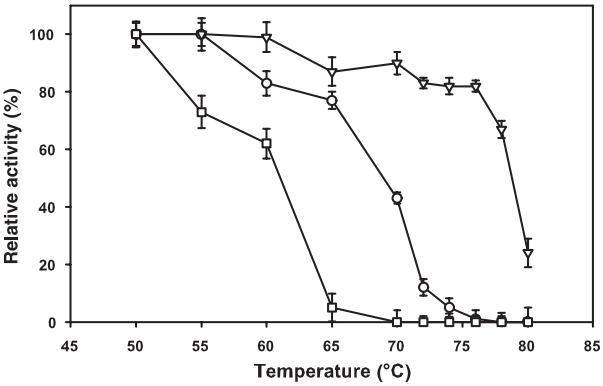

Fig. 4. Temperature stability of GdhA. The purified enzyme (at concentration 0.3 mg·mL^{-1}) was incubated for 30 min in McIlvaine buffer at the specified temperatures with (▽) and without (○) the addition of $ZnCl_2$ at 0.1 mM or in the presence of EDTA at 10 mM (□) and the residual activity measured under optimal conditions. Residual activity is expressed as percent of the activity after incubation at 50 °C (221 U·mg^{-1}).

the remaining activity did not differ from that of the sample incubated in the absence of salts (data not shown). Also, EDTA completely abolished the stabilizing effect of Zn^{2+}. When the enzyme was incubated with $ZnCl_2$ and EDTA supplied at different molar ratios (1 : 10 and 10 : 1) at high temperature (70 °C, 30 min incubation at pH 6.5) or acidity (pH 3.6, 1 h incubation at 55 °C), the remaining activities did not differ from the activities of the respective controls incubated with $ZnCl_2$ or EDTA alone. At an equal molar ratio of EDTA and Zn^{2+} in these assays EDTA complexed the metal ion completely, resulting in the

same residual activity as after incubation with EDTA, i.e. 0 and 3% for the assays at 70 °C and pH 3.6, respectively.

The purified enzyme was considerably stable in the presence of organic solvents: overnight incubation (14 h) at room temperature with 50% (v/v) of acetone, methanol or ethanol did not result in a detectable loss of activity. In addition, in the presence of 20% ethanol, 30% methanol and 40% acetone (v/v/v) in the reaction assay, GdhA still displayed half of its maximal activity.

The influence of adenine nucleotides, inorganic phosphate and pyrophosphate and downstream products of the Entner–Doudoroff pathway on enzymatic activity was tested (at 5 and 20 mM) in order to investigate whether GdhA was regulated by metabolites or the energy status of the cell. The enzyme was inhibited by ATP and the inhibition displayed Michaelis–Menten kinetics in a noncompetitive mode with respect to the cofactor $NADP^+$. At saturating glucose concentration (50 mM), the K_i was determined to be 5.9 (± 1.1) mM. Pyruvate, phospho*enol*pyruvate, 3-phosphoglycerate, 2-phosphoglycerate, as well as P_i and PP_i did not affect the activity when added to the standard assay at 5 or 20 mM.

Identification of the native glucose dehydrogenase in *P. torridus*

In order to identify the native GdhA in *P. torridus* cells, we determined the pH and temperature optima for the glucose dehydrogenase activity in crude extracts. Both optima (55 °C and pH 6.5) were in concert with the optima of the recombinant enzyme. Further evidence in support of the identity of the recombinant enzyme reported here with the enzyme present in *P. torridus* cells is the ratio of enzymatic activity with NAD^+ and $NADP^+$ as cosubstrates, which was $\approx 1:20$ in both cases, as well as the ratio of D-glucose/D-galactose oxidation rates (Table 2).

Table 2. Comparison of some properties of the native *P. torridus* glucose dehydrogenase activity with the recombinant GdhA.

Parameter	Glucose/galactose dehydrogenase	Recombinant GdhA activity in crude *P. torridus* extract
Temperature optimum (°C)	55	55
pH optimum	6.5	6.5
$NADP^+/NAD^+$ ratio of glucose oxidation activity	20.1	19.4
D-Glucose/D-galactose ratio of dehydrogenase activity	1.43	1.35

Also, upon native PAGE and subsequent zymogram staining for glucose dehydrogenase activity the recombinant enzyme was indistinguishable from the cell-free *P. torridus* band (Fig. 1B). Finally, the protein conferring the main glucose dehydrogenase activity in *P. torridus* cells was partially purified by a two-step chromatographic purification (36-fold), giving a preparation of the enzyme that had a specific activity of 68.5 $U \cdot mg^{-1}$. The most prominent band on a SDS/PAGE gel after this purification corresponded by size with the recombinant protein (not shown); it was recovered from the gel, tryptically digested and the resulting peptides were subjected to mass spectroscopy [12]. This protein was identified as PTO1070 (GdhA) in the *P. torridus* database with a protein score of 540, peptide X_{corr} values up to 5.7 and a sequence coverage by amino acids of 54.6%.

Effect of temperature and pH on the stability of NADPH

Because NADPH is not stable at high temperature or low pH [13], it was important to determine its degradation rate under the conditions present in the cytoplasm of *Picrophilus*. The kinetics of NADPH degradation was followed by measuring the rate of decrease of its absorbance at 340 nm over the pH range 3.6–7.0 and at 40, 60 and 80 °C. The measured half-life of NADPH at the optimal growth conditions for *P. torridus* (60 °C, pH 4.6) was 2.4 min and at 65 °C (maximum temperature that supports growth), the half-life was 1.7 min. Also, the reaction order of NADPH degradation with respect to pH was determined by plotting the logarithm of the obtained rate constants ($\log k_1$) vs. pH (not shown). The obtained reaction order value of 0.56 corresponds well with the one reported by Wu *et al.* (0.59) [13] and was constant across the temperatures tested.

Discussion

The functionality of the nonphosphorylated variant of the Entner–Doudoroff pathway has been shown in the thermoacidophilic archaea *Sulfolobus solfataricus* [4] and *Thermoplasma acidophilum* [5], as well as in *Thermoproteus tenax* [14,15]. Genome based metabolic pathway reconstruction has suggested its presence also in *P. torridus* [6]. The cloning, expression and purification of *P. torridus* glucose dehydrogenase, reported here, permits biochemical analysis of the enzyme, which is the first protein of this extreme acidophile to be studied after expression of its gene in a heterologous host.

It is well known that the codon usage of *E. coli* is highly biased. In particular, arginine AGA and AGG codons are extremely rare, which often affects the heterologous expression of archaeal proteins, where these are the major codons for arginine [16]. In our cloning and expression experiments, supplying minor arginine tRNAs in the expression host improved the heterologous production level of *P. torridus* GdhA from undetectable to $\approx 10\ U{\cdot}mg^{-1}$ in crude cellular extracts of the recombinant *E. coli* Rosetta (pBAD-glucose dehydrogenase) strain. When a T7 promoter-based expression vector was used, a large proportion of the *P. torridus* protein was found as inclusion bodies. Placing the *gdhA* gene under the control of the *araB* promoter allowed us to optimize the expression in *E. coli* and to obtain a substantial amount of soluble, active glucose dehydrogenase.

Surprisingly, we observed that the purified enzyme was inactivated completely after incubation for 1 h at conditions thought to be physiological for a cytoplasmic enzyme of *P. torridus* (60 °C and pH 4.6). This finding prompted us to look for stabilizing factors that could have been lost during the purification process. Our results indicate the critical importance of Zn^{2+} for the stability of GdhA. The resistance of GdhA against inactivation at high temperature as well as its stability at low pH were considerably increased in the presence of $ZnCl_2$, and this effect was abolished by the chelating agent EDTA. However, the addition of Zn^{2+} did not affect the specific activity of the enzyme, and even high concentrations of EDTA (20 mM) could not decrease the activity of GdhA in the standard assay. This is in contrast to the effect of EDTA on the glucose dehydrogenase from *Sulfolobus solfataricus*, where at a 10 mM concentration the reported decrease in activity was 60% [17]. These observations may be due to a very stable coordination of Zn^{2+} in the catalytic site of the *P. torridus* protein, whereas the enzyme may contain an additional structural zinc which is not bound as tightly. This may also be the case for the glucose dehydrogenase from *T. acidophilum*, which shares a high degree of amino acid sequence similarity (60% identity) with the homologous enzyme of *P. torridus*. Based on the conservation of the zinc-binding sequences of both enzymes (see Fig. 2), including the cysteine and aspartate residues involved in coordination of the metal ions, the structural basis of zinc binding in *P. torridus* GdhA is probably similar to the situation found in *T. acidophilum* glucose dehydrogenase, whose crystal structure has been solved. John *et al.* [8] have shown that in *T. acidophilum* glucose dehydrogenase the catalytic and nucleotide-binding domains are separated by a deep active site cleft, the putative catalytic zinc being at the bottom of the cleft and a lobe containing the structural zinc at the mouth of the cleft and thus exposed to the solvent [8]. Gradual depletion of the enzyme first of the structural and then of the catalytic zinc has also been reported for YADH [9], a member of the medium-chain alcohol/polyol dehydrogenase family that bears structural similarity with the *T. acidophilum* glucose dehydrogenase.

Active GdhA from *P. torridus* has a tetrameric quaternary structure which is found in most archaeal and some eukaryotic ADHs [18–20]. It has been argued previously that the role of the structural zinc is to stabilize the quaternary structure of *T. acidophilum* glucose dehydrogenase [8]. However, no change in the quaternary structure of *P. torridus* GdhA destabilized by EDTA treatment was observed (data not shown), indicating that in *P. torridus* GdhA the structural zinc is only responsible for stabilizing the tertiary structure of the enzyme.

Interestingly, the recombinant GdhA has a pH optimum of 6.5, which is 1.9 pH units higher than the normal intracellular pH of *Picrophilus*. At the cytoplasmic pH reported for *Picrophilus* cells, i.e. pH 4.6, GdhA displayed merely 10% of its maximum activity. We are not aware of any $NAD(P)^+$-dependent dehydrogenases with a pH optimum of around pH 4.5 for the oxidation reaction.

The glucose (galactose) dehydrogenase activity measured in *P. torridus* crude cellular extracts turned out to have very similar characteristics with the recombinant protein, i.e. pH and temperature optima, $NADP^+/NAD^+$ and glucose/galactose activity ratios (Table 2). Also, after zymogram staining of proteins separated on a native PAGE gel for glucose dehydrogenase activity, the purified recombinant enzyme was undistinguishable from the band obtained with the *P. torridus* crude extract. In support, the glucose dehydrogenase active protein purified from *P. torridus* cells was found to be identical with the recombinantly expressed one by mass spectroscopy. Thus we assume that the GdhA protein indeed represents the prominent glucose dehydrogenase activity in *P. torridus* cells under the growth conditions employed in this study. Considering the presence of two additional putative glucose dehydrogenase ORFs in the *P. torridus* genome however, further experiments are needed to unravel the physiological roles of these enzymes in *P. torridus*.

The results from testing the substrate specificity of the purified recombinant GdhA indicate a relatively strict range of substrates. Nevertheless, the enzyme was considerably active with D-galactose, and it displayed approximately twofold increased affinity

for this substrate ($K_m = 4.5$ mM) compared with D-glucose. In this context, it is noteworthy that a 'promiscuous' Entner–Doudoroff pathway was recently postulated to operate in *S. solfataricus* by Lamble *et al.* [17], who suggested that in this organism the utilization of glucose and galactose is carried out by the same enzymes, which lack facial selectivity [17,21]. Based on the observed activity of GdhA with galactose, such a promiscuity cannot be excluded in *P. torridus*. In accordance, the growth of *P. torridus* in Brock's medium supplemented with 0.2% yeast extract was significantly improved in the presence of galactose (data not shown).

Highly significant when considering the extremely acidophilic lifestyle of *P. torridus*, and in particular the low cytoplasmic pH in the cells of the genus *Picrophilus* [2], is the observation that one of the products of the dehydrogenase reaction, NADPH, is unstable at elevated temperatures and low pH values [13]. At the conditions considered to be physiological in the cytoplasm of *Picrophilus* (pH 4.6 and 60 °C), NADPH showed dramatically decreased stability ($t_{1/2} = 2.4$ min), the most important factor being the hydronium ion concentration. Near neutrality, which is typical for the cytoplasm of most organisms, NADPH is much more stable, e.g. at 55 °C and pH 6.5 NADPH has a half-life of nearly 50 min (data not shown). This observation implies a high turnover rate of NADPH in *P. torridus*. Further studies are needed in order to elucidate how the metabolism of this organism has adapted to this circumstance.

Because of the unusually low intra- and extracellular pH of *Picrophilus* cells and their milieu, respectively, certain enzymes from this organism may bear a promising biotechnological potential. In addition, comparative studies with the related *Thermoplasma* give an opportunity to obtain insight into the mechanisms of protein adaptation to high acidity.

Experimental procedures

Strains and growth conditions

Picrophilis torridus DSM 9790 was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ) and was grown aerobically at 60 °C and pH 0.7 in Brock's medium supplemented with 0.2% (w/v) yeast extract, as described in Schleper *et al.* [1]. The medium contained (per L): 1.32 g $(NH_4)_2SO_4$, 0.28 g KH_2PO_4, 0.25 g $MgSO_4.7H_2O$, 0.07 g $CaCl_2.2H_2O$, 0.02 g $FeCl_3.6H_2O$, 1.8 mg $MnCl_2.4H_2O$, 4.5 mg $Na_2B_4O_7.10H_2O$, 0.22 mg $ZnSO_4.7H_2O$, 0.05 mg $CuCl_2.2H_2O$, 0.03 mg $Na_2MoO_4.2H_2O$, 0.03 mg $VOSO_4.2H_2O$, 0.01 mg $CoSO_4$. The pH was adjusted with concentrated H_2SO_4.

Escherichia coli XL1-Blue was used as a general host for DNA manipulations. For expression of the recombinant glucose dehydrogenase, *E. coli* Rosetta (Novagen, Madison, WI, USA) was used. These strains were cultivated in Luria–Bertani medium at 37 °C. When necessary, 50 mg·L^{-1} ampicillin and/or 34 mg·L^{-1} chloramphenicol were added to the medium to maintain plasmids.

Cloning of the *P. torridus* glucose dehydrogenase gene and expression in *E. coli*

The candidate *P. torridus* ORFs coding for glucose dehydrogenase were identified in the genome sequence [6], using the ERGO software package (Integrated Genomics, Chicago, IL, USA). Genomic DNA from *P. torridus* was used as a template for PCR amplification of the glucose dehydrogenase gene (*Pt-gdh*), using Pfu DNA polymerase (Promega, Madison, WI, USA) and the following primers: sense, 5′-GGCGTTCATAACCCTTGTTACCTCTTCA-3′ and antisense, 5′-CGTCATGCCATCAACGTCCTTGTAGAAT-3′. The PCR product obtained was purified from an agarose gel (Gel Extraction Kit, Qiagen, Hilden, Germany), incubated with *Taq* DNA polymerase in the presence of 0.2 mM dATP and cloned in the pCR4 TOPO vector (Invitrogen), yielding plasmid pCR-glucose dehydrogenase. In order to construct an expression vector for *Pt-gdh*, pCR-glucose dehydrogenase was subjected to *Nco*I restriction and the *Pt-gdh*-containing fragment was ligated with pBAD*myc* (Invitrogen), placing it under the control of the arabinose-inducible *araB* promoter. The resulting expression vector, named pBAD-glucose dehydrogenase, was introduced into *E. coli* Rosetta, and the recombinant cells were cultured in Luria–Bertani medium containing 50 mg·L^{-1} ampicillin and 34 mg·L^{-1} chloramphenicol at 37 °C. The expression vector pET24d was obtained from Novagen.

Expression of *Pt-gdh* under the control of *araB* promoter was induced for 4 h at 30 °C by the addition of 0.2% arabinose when the A_{600} of the growing culture reached 0.5. The cells from a 1-L culture were harvested by centrifugation (15 min 6000 ***g***), washed with 50 mM Tris–HCl buffer (pH 8.0) and lysed by double passage through a French Press Cell.

Purification of *P. torridus* glucose dehydrogenase

Cell lysate from *E. coli* Rosetta (pBAD-glucose dehydrogenase) was heated at 70 °C for 20 min, denatured protein was removed by centrifugation (15 min, 15 000 ***g***), and the supernatant was loaded onto a Source Q 15 anion exchange column (Amersham Pharmacia Biotech, Uppsala, Sweden). Proteins were eluted with a linear NaCl gradient (0–0.5 M) and the fractions containing glucose dehydrogenase activity

were pooled, concentrated (Amicon Ultra columns, Millipore Corp., Bedford, MA, USA) and dialysed against 50 mM Tris buffer pH 8.0. The pooled fractions were applied to a Superdex 200 gel filtration column (Amersham Pharmacia Biotech) and eluted isocratically. The active fractions were pooled and concentrated as in the previous step. The level of purification of the heterologously expressed protein at each step was monitored by measuring the specific glucose dehydrogenase activity and assessed by SDS/PAGE. Protein concentration was determined with the Bradford method using a Bio-Rad Protein Assay system (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as a standard.

Assay for glucose dehydrogenase activity and enzyme kinetics

Glucose dehydrogenase activity was assayed spectrophotometrically by measuring the increase of absorption at 340 nm and at 55 °C in phosphate buffer, pH 6.5, containing 2 mM $NADP^+$ (5 mM NAD^+) in a total volume of 1 mL. The reaction mixture was preincubated for 10 min at 55 °C and the reaction started by the addition of glucose at 50 mM final concentration. Specific activity is expressed as μmol of NADPH produced per min per mg of protein under the specified conditions. NAD^+-dependent glucose dehydrogenase activity was measured the same way, substituting NAD^+ for $NADP^+$. For determination of the pH optimum (at 55 °C, 10 min assay) and in pH stability testing, the following buffers were used: 50 mM glycine HCl in the range of pH 1.5–3.3, 50 mM sodium acetate for pH 3.5–5.5, 50 mM phosphate for pH 6–7 and 50 mM Tris/HCl for pH 7.5–8.5. In these assays, the glucose dehydrogenase activity was measured by monitoring the decrease of D-glucose (glucose determination kit, Sigma procedure no. 510).

To measure glucose dehydrogenase activity in *P. torridus* cell-free extracts, the cells of a growing culture were collected by centrifugation at 4 °C (20 min 6000 ***g***), lysed by sonification in 50 mM acetate buffer, pH 4.5 and the lysate was cleared by centrifugation for 20 min at 13 000 ***g***. Glucose dehydrogenase activity was visualized on a native PAGE by coupling the glucose-dependent $NADP^+$ reduction to NITRO BLUE tetrazolium formazan production (5-methyl phenazonium methyl sulfate was used as an intermediate hydrogen carrier). For activity staining the gel was soaked in 50 mM Tris/HCl containing 1 mM $NADP^+$, 50 mM glucose, 1 mM NBT, 0.025 mM phenazonium methyl sulfate for 10–15 min or until the appearance of a blue band. To normalize for the colour intensity, 30 mU glucose dehydrogenase were applied on each lane.

The rate of NADPH degradation was monitored with a Varian Cary 100 spectrophotometer (Varian, Mulgrave, Australia) in temperature-controlled cuvettes by following the decrease in absorbance at 340 nm, $[(A)_t]$. The reaction was started by adding NADPH at 0.5 mM (absorbance ≈ 2) after temperature equilibration of the buffer for 10 min. As the loss of absorbance followed first-order kinetics, the apparent rate constants of NADPH degradation (k_1) were determined by plotting log $(A)_t$ vs. time. The measurements were carried out in 50 mM acetate (pH 3.6–5.6) or 50 mM phosphate (pH 6–7) buffer at three different temperatures −40 °C, 60 °C and 80 °C.

Mass spectroscopy and protein identification

Coomasie stained polyacrylamide gel bands were digested with trypsin according to the protocol of Shevchenko *et al.* [12]. Tryptic peptides were separated by running water–acetonitrile gradients on Dionex-NAN75-15-03-C18-PM columns on an ultimate-nano-HPLC system (Dionex, Bavel, the Netherlands). Online ESI-MS/MS2 spectra were generated on a LCQ-DecaXP^{plus} mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Protein identification was done by analysis of MS2 spectra with the *P. torridus* protein database with SEQUEST/TURBOSEQUEST software (BioworksBrowser 3.1, Thermo Finnigan).

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