Purification and characterization of glucose dehydrogenase from the thermoacidophilic archaeobacterium *Thermoplasma acidophilum*

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Glucose dehydrogenase was purified to homogeneity from the thermoacidophilic archaeobacterium *Thermoplasma acidophilum*. The enzyme is a tetramer of polypeptide chain M_r 38 000 $\pm$ 3000, it is catalytically active with both NAD⁺ and NADP⁺ cofactors, and it is thermostable and remarkably resistant to a variety of organic solvents. The amino acid composition was determined and compared with those of the glucose dehydrogenases from the archaeobacterium *Sulfolobus solfataricus* and the eubacteria *Bacillus subtilis* and *Bacillus megaterium*. The N-terminal amino acid sequence of the *Thermoplasma acidophilum* enzyme was determined to be :

(S/T)-E-Q-K-A-I-V-T-D-A-P-K-G-G-V-K-Y-T-T-I-D-M-P-E

INTRODUCTION

The thermoacidophilic archaeobacteria *Sulfolobus solfataricus* and *Thermoplasma acidophilum* metabolize glucose via a modified Entner–Doudoroff pathway involving non-phosphorylated intermediates (De Rosa *et al.*, 1984; Budgen & Danson, 1986; Danson, 1988). The first enzyme of this unusual metabolic route is glucose dehydrogenase (EC 1.1.1.47), which catalyses the reaction:

The enzyme can accept both NAD⁺ and NADP⁺ on the same protein molecule and is one of a number of dual-cofactor-specific dehydrogenases found in the thermophilic archaeobacteria (reviewed in Danson, 1988). Other examples include isocitrate dehydrogenase (Danson & Wood, 1984), malate dehydrogenase (Grossebuter *et al.*, 1986) and malic enzyme (Bartolucci *et al.*, 1987).

In the present paper we report the purification and characterization of glucose dehydrogenase from *Tp. acidophilum*. Our findings are compared with those reported for the enzyme from *S. solfataricus* (Giardina *et al.*, 1986) and from the eubacteria *Bacillus megaterium* (Jany *et al.*, 1984) and *Bacillus subtilis* (Lampel *et al.*, 1986), all of which can utilize both NAD⁺ and NADP⁺. The comparisons are discussed with respect to current ideas on the phylogenetic relationship of *Thermoplasma* to *Sulfolobus* within the archaeobacterial Kingdom and of the archaeobacteria to the eubacteria.

EXPERIMENTAL

Materials

Chemicals used were analytical grade or the finest grade commercially available. NAD⁺ (grade I) and NADP⁺ (grade I) were from Boehringer, Mannheim,

W. Germany, and Matrex Gel Red A was from Amicon Corp., Lexington, MA, U.S.A. *Thermoplasma acidophilum* (D.S.M. 1728) was grown at 55 °C (pH 2.0) as described by Christiansen *et al.* (1975).

Assay of glucose dehydrogenase

Glucose dehydrogenase was assayed spectrophotometrically at 55 °C in 50 mM-sodium phosphate buffer, pH 7.0, containing 0.4 mM-NADP⁺ and 50 mM-glucose. The reaction, in a final volume of 1 ml, was started with enzyme, and the increase in A_{340} was monitored with time. Specific activity is expressed as μmol of NADPH produced/min per mg of protein. The NAD⁺-dependent glucose dehydrogenase activity was measured in the same way, NAD⁺ being substituted for NADP⁺; the concentration of NAD⁺ in the assay varied according to the experiment and is specified in the text.

Purification of glucose dehydrogenase

Tp. acidophilum cells (6 g wet wt.) were resuspended in 40 ml of 100 mM-Tris/HCl buffer, pH 8.0, containing 1 μg of DNAase/ml. The suspension was homogenized at 4 °C in a hand-held Pottenheim homogenizer, a procedure that effected cell lysis. Debris was removed by centrifugation at 110 000 g (r_{av} 6.1 cm) for 60 min. To the supernatant (37 ml) was added methanol to a total volume of 200 ml and, after 10 min, the resultant precipitate was collected by centrifugation at 13 000 g (r_{av} 11.9 cm) for 15 min. The pellet was resuspended in 40 ml of 35 mM-sodium phosphate buffer, pH 7.0, and the suspension was clarified by centrifugation at 110 000 g (r_{av} 6.1 cm) for 60 min.

The supernatant from the methanol extraction was subjected to ion-exchange chromatography at 25 °C on the Pharmacia f.p.l.c. system with the Mono Q anion-exchanger in 35 mM-sodium phosphate buffer, pH 7.0. Protein was eluted with a linear gradient of 0–0.4 M-NaCl. Fractions containing enzyme at a specific activity

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of ≥ 3 units/mg were pooled and applied to a column (6 cm $\times$ 1 cm) of Matrex Gel Red A, previously equilibrated in 50 mM-sodium phosphate buffer, pH 7.0. The column was washed with 25 ml of this same buffer, and the glucose dehydrogenase was then eluted with the phosphate buffer containing 5 mM-NADP⁺; fractions containing enzyme at a specific activity ≥ 20 units/mg were pooled. This enzyme was subjected to chromatofocusing on the Pharmacia f.p.l.c. Mono P column previously equilibrated in 25 mM-Bistris buffer, pH 7.1; protein was eluted with Polybuffer 74, that is with a decreasing pH gradient of pH 7.0 to pH 4.0. Pooled fractions containing enzyme were then gel-filtered on the Pharmacia f.p.l.c. Superose 12 column in 10 mM-NH₄HCO₃. Fractions containing enzymic activity were subjected to SDS/polyacrylamide-gel electrophoresis, and those showing only a single protein band were pooled for amino acid analysis and sequencing.

SDS/polyacrylamide-gel electrophoresis

Gel electrophoresis in 0.1 % (w/v) SDS was carried out with 10 % (w/v) polyacrylamide gels according to the method of Laemmli (1970).

Amino acid analysis

Two independently purified samples of glucose dehydrogenase were reduced and S-carboxymethylated with iodoacetic acid (Perham, 1978), and then hydrolysed *in vacuo* in 6 M-HCl containing 0.1 % phenol and 0.1 % thioglycolic acid at 110 °C for 24 h, 48 h and 72 h. Values for tryptophan were obtained after hydrolysis at 100 °C for 24 h in 3 M-mercaptoethanesulphonic acid. Analyses were performed on a Rank Hilger Chromaspek J180 amino acid analyser.

N-Terminal amino acid sequencing

N-Terminal sequencing was performed on an Applied Biosystems 470A gas-phase sequencer coupled to a 120A phenylthiohydantoin analyser. Samples of glucose dehydrogenase were evaporated to dryness after the final stage of the purification procedure, namely after gel filtration in 10 mM-NH₄HCO₃. These samples were re-dissolved in distilled water and re-dried several times before sequencing was performed.

RESULTS

Purification of glucose dehydrogenase

A summary of the purification of glucose dehydrogenase from *Tp. acidophilum* is given in Table 1. A 4000-fold increase in the purity of the enzyme was achieved. The preparation was found to be homogeneous as judged by electrophoresis on SDS/polyacrylamide gels; comparison of the electrophoretic mobility with those of standard proteins run simultaneously gave a polypeptide chain M_r of 38000 ± 3000 . The M_r of the native enzyme was determined by gel filtration on the Pharmacia f.p.l.c. Superose 12 column with the following standard proteins as calibration markers: *Escherichia coli* β -galactosidase (M_r 448000), pig heart fumarase (M_r 194000), rabbit muscle fructose-bisphosphate aldolase (M_r 158000), pig heart citrate synthase (M_r 98000), pig heart mitochondrial malate dehydrogenase (M_r 67000) and rabbit muscle phosphoglycerate mutase (M_r 64000). The M_r of the glucose dehydrogenase was thus found to be 155000 ± 9000 , indicating that the enzyme is a tetramer ($n = 4.1 \pm 0.4$).

Amino acid analysis and N-terminal sequence

The amino acid composition of the glucose dehydrogenase is given in Table 2 and is expressed as residues per 100 residues. The compositions of glucose dehydrogenases from the thermoacidophilic archaebacterium *S. solfataricus* (Giardina *et al.*, 1986) and from the eubacteria *B. subtilis* (Hones *et al.*, 1987) and *B. megaterium* (Jany *et al.*, 1984) are given for comparison. We have compared the amino acid compositions in Table 2 by using the composition divergence of Harris & Teller (1973):

$$\text{Composition divergence} = \sqrt{\sum_{i=1}^{18} (X_{i,A} - X_{i,B})^2}$$

where $X_{i,A}$ and $X_{i,B}$ are the normalized values of amino acid i in proteins A and B, expressed as residues per 1000 residues. The two archaebacterial enzymes showed a composition divergence of 47, whereas values of 69 and 62 were found between the glucose dehydrogenase of *Tp. acidophilum* and those of *B. subtilis* and *B. megaterium*.

Table 1. Purification of glucose dehydrogenase from *Tp. acidophilum*

Experimental details are given in the text. The protein in the fractions after Mono P chromatofocusing could not be determined accurately owing to the presence of Polybuffer 74. The enzymic activity is that assayed with NADP⁺ as cofactor.

Purification step	Volume (ml)	Total enzyme (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Overall purification (fold)
Cell extract	80	160	2080	0.08	100	
Methanol extraction	80	130	800	0.16	81	2
Mono Q chromatography	16	99	20	5.0	62	63
Matrex Gel Red A	4	80	2.8	28.6	50	358
Mono P chromatofocusing	2	54	—	—	34	—
Superose 12 gel filtration	10	32	0.1	320	20	4000

Table 2. Amino acid composition of glucose dehydrogenase from *Tp. acidophilum*: comparison with glucose dehydrogenases from another archaeobacterium and two eubacteria

Amino acid	Amino acid composition (residues/100 residues)			
	<i>Tp. acidophilum</i> enzyme*	<i>S. solfataricus</i> enzyme†	<i>B. subtilis</i> enzyme‡	<i>B. megaterium</i> enzyme§
Cm-Cys	0.3±0.1	N.D.	0	0
Asp	13.3±0.3	12.9	10.0	9.6
Thr	4.9±0.3	4.6	4.6	5.0
Ser	5.6±1.0	6.3	4.2	7.6
Glu	11.5±0.4	9.6	10.3	10.7
Pro	4.2±0.2	4.3	5.4	3.8
Gly	9.0±0.3	9.3	10.0	9.2
Ala	7.9±0.5	5.8	11.2	9.5
Val	7.6±0.5	8.4	8.1	9.2
Met	2.1±0.1	1.9	3.4	4.2
Ile	6.4±0.3	7.8	8.1	7.6
Leu	6.2±0.2	9.3	5.4	5.3
Tyr	3.3±0.1	2.6	3.5	3.1
Phe	4.1±0.1	3.9	3.5	3.1
His	1.9±0.1	1.4	1.2	1.1
Trp	0.4±0.1	N.D.	1.2	1.5
Lys	6.7±0.3	7.0	8.4	7.2
Arg	4.6±0.1	4.9	1.5	2.3

* Values for threonine and serine were obtained by extrapolation to zero time of hydrolysis. Values for valine and isoleucine are from 48 h and 72 h values only. Tryptophan was determined by hydrolysis in 3 M-mercaptoethanesulphonic acid. All other values are means ± S.E.M. for duplicate samples, taken after 24 h, 48 h and 72 h hydrolyses in 6 M-HCl, of two preparations of the enzyme.

† Data taken from amino acid compositions reported by Giardina *et al.* (1986). Cysteine and tryptophan contents were not determined (N.D.).

‡ Data taken from the DNA-derived sequence (Hones *et al.*, 1987).

§ Data taken from Jany *et al.* (1984). Values for asparagine and glutamine are added to those of aspartate and glutamate, respectively.

|| Carboxymethylcysteine.

respectively. The equivalent values between the *S. solfataricus* glucose dehydrogenase and the two eubacterial enzymes are 88 and 87 respectively. As discussed by Black & Harkins (1977), these values may suggest a significant homology between the two archaeobacterial dehydrogenases, whereas the values found between archaeobacterial and eubacterial enzymes are too high to infer any relationships, the correlation between sequence differences and composition divergences above a value of 50 breaking down as a consequence of balanced mutations. Interestingly, the two eubacterial glucose dehydrogenases, which have a sequence identity of 80% (Hones *et al.*, 1987), also showed a composition divergence of 47.

The *N*-terminal sequence of the *Tp. acidophilum* glucose dehydrogenase was determined for two preparations of the enzyme from cells grown independently from two separate freeze-dried cultures of the organism. The sequences obtained were identical:

(S/T)-E-Q-K-A-I-V-T-D-A-P-K-G-G-V-K-Y-T-T-I-D-M-P-E

To date, the only published sequences of glucose dehydrogenase are from the eubacteria *Bacillus megaterium* (Jany *et al.*, 1984) and *B. subtilis* (Lampel *et al.*, 1986). The *N*-terminal sequence of the *Tp. acidophilum* enzyme was compared with the complete sequence of the *B. subtilis* enzyme to find the region of maximum identity, giving insertions and deletions a penalty of one point.

The best alignment obtained was to residues 7–29 of the *B. subtilis* glucose dehydrogenase (Fig. 1); eight identities were found and, taking account of hydrophobicity and charge values, two conservative changes are observed.

Catalytic properties and enzymic stability

The catalytic properties of the purified glucose dehydrogenase were similar to those reported for the enzyme in cell extracts (Budgen & Danson, 1986). Thus it is catalytically active with both NADP⁺ and NAD⁺ and shows a hyperbolic dependence of rate on the concentrations of both cofactors and of the substrate glucose. The data were analysed by the direct linear plot method of Eisenthal & Cornish-Bowden (1974), giving *K_m* values of 0.113 ± 0.011 mM for NADP⁺, > 30 mM for NAD⁺ and 10.0 ± 0.9 mM for glucose. The *K_m* value for NAD⁺ could not be defined precisely as we were unable to reach saturation of the enzyme. We have demonstrated that the enzyme activity observed with NAD⁺ is not due to contamination of this cofactor with NADP⁺. Thus NADP⁺-specific isocitrate dehydrogenase from pig heart gave no detectable activity with the NAD⁺ solution. Contamination with NADP⁺ would therefore be ≤ 0.2%, yet, when the reaction with the *Tp. acidophilum* glucose dehydrogenase is allowed to go to completion, > 90% of the NAD⁺ cofactor is reduced. Also, treatment of the NAD⁺ with alkaline phosphatase, to convert any NADP⁺ into NAD⁺, did not affect the activity with the archaeobacterial enzyme.

The ratio of enzymic activities with NADP⁺ and NAD⁺ remained constant throughout the purification, indicating that the two activities observed in unfractionated cell extracts can be accounted for by the one dual-cofactor-specific glucose dehydrogenase. Moreover, we have evidence that NADP⁺ and NAD⁺ bind at the same site on the enzyme; that is, by using the kinetic analysis outlined previously (Danson & Wood, 1984), the two cofactors competed with each other with *K_i* values equal to their *K_m* values.

The purified glucose dehydrogenase also catalysed the NAD(P)⁺-dependent oxidation of galactose, the activity with galactose and NADP⁺ being approx. 70% of that observed with glucose and the cofactor. No activity was observed with mannose or glucose 6-phosphate with either cofactor.

Both the purified glucose dehydrogenase and the enzyme in cell extracts were resistant to high temperatures and to organic solvents. At 55 °C, the optimum growth temperature of *Tp. acidophilum*, full catalytic activity was retained after 9 h, and at 75 °C the half-life was approx. 3 h. Incubation of the enzyme for up to 6 h at room temperature with 50% (v/v) methanol, acetone or ethanol, or with 4 M-urea, caused no appreciable loss of activity. In each case, the enzyme was incubated with the solvent or urea and was then diluted 20-fold into the

	1	5	10	15	20	25	30																								
<i>B. megaterium</i>	M	Y	K	D	L	E	G	K	V	V	I	-	T	G	S	S	T	G	L	G	K	S	M	A	I	R	F	A	T	E	
<i>B. subtilis</i>	M	Y	P	D	L	K	G	K	V	V	A	I	-	T	G	A	A	S	G	L	G	K	A	M	A	I	R	F	G	K	E
<i>Tp. acidophilum</i>							S	E	Q	K	A	I	V	T	D	A	P	K	G	G	V	K	Y	T	T	I	D	M	P	-	E

Fig. 1. Alignment of the *N*-terminal sequence of glucose dehydrogenase from *Tp. acidophilum* with those of *B. subtilis* and *B. megaterium* glucose dehydrogenases

The 24-amino acid-residue *N*-terminal sequence of *Tp. acidophilum* glucose dehydrogenase was determined and compared with the complete sequence of the enzyme from *B. subtilis* as described in the text. Identical residues between the sequences are boxed with continuous lines and conservative changes with broken lines. The sequence alignment of the *B. subtilis* and *B. megaterium* glucose dehydrogenases is taken from Hones *et al.* (1987). Residue numbers refer to the *B. subtilis* sequence.

assay mixture. Moreover, the enzyme was 50% active when assayed in 40% (v/v) methanol, 40% (v/v) acetone or 30% (v/v) ethanol, and 85% active in 4 M-urea.

DISCUSSION

The present paper reports the first purification to homogeneity and subsequent characterization of glucose dehydrogenase from the thermoacidophilic archaeobacterium *Tp. acidophilum*. The enzyme is strikingly similar to glucose dehydrogenase from another archaeobacterium, *S. solfataricus* (Giardina *et al.*, 1986), in its dual-cofactor-specificity, its oligomeric nature and its solvent and thermal stability. Moreover, comparison of their amino acid compositions indicates a possible sequence homology whereas any relationship between the archaeobacterial glucose dehydrogenases and those from *B. subtilis* and *B. megaterium* was below the level of detection by this method. The alignment of the *N*-terminal sequence of the enzyme from *Tp. acidophilum* with that from *B. subtilis* gave an identity of 33% (over 24 residues); a similar identity over the whole sequence would not have been detectable by the composition-divergence method. Small differences between the two archaeobacterial enzymes are found in the K_m and V_{max} values for the cofactors and in their sugar-specificities.

These observations are consistent with the idea that the archaeobacteria constitute a primary line of evolutionary descent and as such are phylogenetically distinct from the eubacteria and the eukaryotes (reviewed in Woese & Olsen, 1986; Woese, 1987). The proposed sequence similarity between the two archaeobacterial glucose dehydrogenases is worthy of note, especially as *Thermoplasma* and *Sulfolobus* are thought to be phylogenetically distant within the archaeobacterial Kingdom; that is, on the basis of rRNA sequence analysis (Woese & Olsen, 1986; Woese, 1987), and of hybridization homologies between rRNAs and nitrocellulose-bound DNAs (Klenk *et al.*, 1986), *Tp. acidophilum* is placed within the branch of the methanogens and halophiles whereas *S. solfataricus* is grouped in the second archaeobacterial branch with the Thermoproteales. The extreme halophile *Halobacterium cutirubrum* also probably possesses a dual-cofactor-specificity glucose dehydrogenase (Tomlinson *et al.*, 1974), and a detailed investigation of this enzyme is also a priority in these comparisons.

More definitive ideas on these relationships between the glucose dehydrogenases await full sequence analyses of the archaeobacterial enzymes. To this end, we have

obtained a 24-amino acid-residue *N*-terminal sequence of the *Tp. acidophilum* glucose dehydrogenase, from which oligonucleotide probes can be synthesized to effect the cloning and subsequent sequencing of the gene for this enzyme. Comparison of the archaeobacterial sequence with the whole sequence of the glucose dehydrogenases from *B. subtilis* and *B. megaterium* gave a tentative alignment to the *N*-terminal region of the eubacterial proteins.

Hones *et al.* (1987) have compared the sequences and various physicochemical properties of the glucose dehydrogenases from *B. subtilis* and *B. megaterium* with those of lactate dehydrogenase and have offered a tentative rationalization of the differential cofactor-specificities. It is believed that future comparisons between sequences of the eubacterial and archaeobacterial glucose dehydrogenases will add further valuable information to the investigation of cofactor preference in dehydrogenase enzymes.

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