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Purification and characterization of thermostable glucose isomerase from *Clostridium thermosulfurogenes* and *Thermoanaerobacter* strain B6A

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Glucose isomerases produced by *Thermoanaerobacter* strain B6A and *Clostridium thermosulfurogenes* strain 4B were purified 10–11-fold to homogeneity and their physicochemical and catalytic properties were determined. Both purified enzymes displayed very similar properties (native M_r 200000, tetrameric subunit composition, and apparent pH optima 7.0–7.5). The enzymes were stable at pH 5.5–12.0, and maintained more than 90% activity after incubation at high temperature (85 °C) for 1 h in the presence of metal ions. The *N*-terminal amino acid sequences of both thermostable glucose isomerases were Met-Asn-Lys-Tyr-Phe-Glu-Asn and were not similar to that of the thermolabile *Bacillus subtilis* enzyme. The glucose isomerase from *C. thermosulfurogenes* and *Thermoanaerobacter* displayed pI values of 4.9 and 4.8, and their $k_{cat.}$ and K_m values for D-glucose at 65 °C were 1040 and 1260 min^{-1} and 140 and 120 mM respectively. Both enzymes displayed higher $k_{cat.}$ and lower K_m values for D-xylose than for D-glucose. The *C. thermosulfurogenes* enzyme required Co^{2+} or Mg^{2+} for thermal stability and glucose isomerase activity, and Mn^{2+} or these metals for xylose isomerase activity. Crystals of *C. thermosulfurogenes* glucose isomerase were formed at room temperature by the hanging-drop method using 16–18% poly(ethylene glycol) (PEG) 4000 in 0.1 M-citrate buffer.

INTRODUCTION

D-Glucose (D-xylose) isomerase (EC 5.3.1.5) is an intracellular enzyme found in a number of bacteria which can utilize xylose as a carbon substrate for growth (Chen, 1980*a*). Although the physiological function of the enzyme *in vivo* is isomerization of D-xylose to D-xylulose, this enzyme also converts D-glucose into D-fructose *in vitro* (Takasaki *et al.*, 1969). The latter activity of the enzyme is used in industry for production of high-fructose corn syrup, and glucose isomerase is one of the largest volume commercial enzymes used today (Antrim *et al.*, 1979; Bucke, 1980). Owing to the industrial significance of the enzyme, glucose isomerases from various micro-organisms have been studied, and their catalytic and physicochemical properties have been reviewed (Chen, 1980*b*). Immobilization techniques and a continuous isomerization process with the enzyme have also been described (Schnyder, 1974; Jorgensen *et al.*, 1988).

Most commercially available glucose isomerases are isolated from mesophilic micro-organisms, including *Streptomyces*, *Actinoplanes* and *Flavobacterium* species. These enzymes are generally thermostable, and are utilized in the immobilized form to enhance enzyme half-life (Verhoff *et al.*, 1985). These enzymes require metal ions for their activity and stability, and the pH optima for enzyme activity are in the range 7.5–9.0. The reaction temperature used in the current industrial process for sweetener production is limited to 60 °C because of by-product and colour formation during reaction at high temperature and alkaline pH (Bucke, 1977). Reaction temperatures greater than 60 °C have the advantage of faster reaction rates, higher equilibrium concentrations of fructose, and decreased viscosity of the substrate and product stream. Therefore thermostable glucose isomerases with neutral or slightly acidic pH optima have potential industrial application.

Thermophilic micro-organisms are known to produce intrinsically thermostable enzymes which have been evolved and adapted to the extreme environment of their natural habitat (Amelunxen & Murdock, 1978). Advantages of using these thermostable and thermophilic enzymes in industrial processes were proposed (Zeikus, 1979; Ng & Kenealy, 1987). Our laboratory has reported on purification and biochemical characterization of thermostable β-amylase and amylopullulanase from *Clostridium thermosulfurogenes* and *C. thermohydrosulfuricum* respectively (Saha *et al.*, 1988; Shen *et al.*, 1988). In spite of the large number of studies on glucose isomerases from various enzyme sources, nothing is known about the detailed biochemical or molecular-genetic properties of glucose isomerases from thermoanaerobic bacteria.

We report here on the biochemical characterization of thermostable glucose isomerase purified from *C. thermosulfurogenes* strain 4B and *Thermoanaerobacter* strain B6A, and on the crystallization of the pure enzyme from *C. thermosulfurogenes* strain 4B.

MATERIALS AND METHODS

Chemicals, organisms and growth conditions

Medium components and all other chemicals were reagent grade. *C. thermosulfurogenes* strain 4B (Schink & Zeikus, 1983) and *Thermoanaerobacter* strain B6A (Weimer *et al.*, 1984) were routinely grown at 60 °C in anaerobic 26 ml pressure tubes or in 1-litre round-bottom flasks that contained a phosphate-buffered tryptone–yeast extract (TYE) medium (Zeikus *et al.*, 1980) supplemented with 1% xylose. For large-scale enzyme preparation, cultures were grown at 60 °C in a 14-litre Biostat E fermentor (B. Braun Biotech, Bethlehem, PA, U.S.A.) containing 10 litres of TYE medium with 2% xylose, and the culture pH was maintained at 5.5 by on-line control with 1 M-NaOH. Cells were

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harvested in late-exponential growth phase with a Pellicon cell harvester (Millipore Corp., Bedford, MA, U.S.A.) and washed with 50 mM-Mops buffer (pH 7.0) containing 10 mM-$MgSO_4$ and 1 mM-$CoCl_2$ before freezing.

Enzyme assays

Glucose isomerase activity was measured by incubating a 1 ml reaction mixture that contained 0.8 M-glucose, 10 mM-$MgSO_4$, 1 mM-$CoCl_2$ and an enzyme preparation in 100 mM-Mops buffer (pH 7.0 measured at room temperature). For assay of xylose isomerase activity, the reaction mixture (1 ml) contained 70 mM-xylose, 10 mM-$MnSO_4$ and the enzyme preparation in 100 mM-Mops buffer (pH 7.0). After 30 min incubation at 65 °C, 1 ml of 0.5 M-$HClO_4$ was added to stop the reaction, and the mixture was further diluted 50- and 10-fold with double-distilled water and assayed for fructose and xylulose respectively by the cysteine/carbazole/sulphuric acid method (Dishe & Borenfreund, 1951). Fructose-isomerizing activity was assayed under the same reaction conditions as in the glucose isomerase assay, except that 0.4 M-fructose instead of 0.8 M-glucose was used in the reaction mixture. After 30 min incubation, the reaction was terminated by placing the assay tubes on ice, and the amount of glucose formed was estimated with a glucose analyser (model 27; Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). One unit of isomerase activity is defined as the amount of enzyme that produced 1 μmol of product/min under the assay conditions. Protein concentration was determined by the method of Lowry *et al.* (1951), with BSA as the standard.

Purification of glucose isomerase

All the procedures were performed under aerobic conditions at 4 °C unless otherwise stated.

(i) Preparation of cell extract. Cells of *Thermoanaerobacter* or *C. thermosulfurogenes* (50 g) were suspended in 200 ml of 50 mM-Mops buffer (pH 7.0) containing 10 mM-$MgSO_4$ and 1 mM-$CoCl_2$. The cells were disrupted by two passages through a French pressure cell (American Instrument Co., Silver Spring, MD, U.S.A.) at 18000 lb/in^2 (124 MPa). The cell debris was removed by centrifugation at 12000 *g* for 20 min, and the supernatant was used as the crude enzyme preparation.

(ii) Heat treatment. The cell extracts of *Thermoanaerobacter* and *C. thermosulfurogenes* were heated for 15 min at 85 °C and 80 °C respectively, and cooled to 4 °C. The soluble fractions were recovered after centrifugation at 12000 *g* for 20 min.

(iii) $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was added to the heat-treated extracts to give 65% saturation, and the precipitate was removed by centrifugation (20000 *g* for 30 min). More $(NH_4)_2SO_4$ was added to the supernatant to give 85% saturation. This precipitate was collected, dissolved in 50 mM-Mops buffer (pH 7.0) containing 5 mM-$MgSO_4$ and 0.5 mM-$CoCl_2$, and dialysed overnight against the same buffer.

(iv) Column chromatography. The above enzyme preparations were loaded on to DEAE-Sepharose CL-6B columns (4.0 cm × 32 cm), previously equilibrated with 50 mM-Mops buffer (pH 7.0) containing 5 mM-$MgSO_4$ and 0.5 mM-$CoCl_2$. The columns were washed with the same buffer and then eluted with a linear NaCl salt gradient (0–0.5 M) in the same buffer. The activity-peak fractions were pooled and concentrated by ultrafiltration (YM 30 membrane; Amicon Co., Danvers, MA, U.S.A.). The enzyme solutions were divided into three portions, and each portion was applied on to a Superose-12HR (Pharmacia, Piscataway, NJ, U.S.A.) gel-filtration column and eluted with the same buffer by using a Pharmacia f.p.l.c. system.

Electrophoresis and M_r determination

SDS/PAGE was performed as described by Laemmli (1970). Native PAGE was performed without SDS, and Tris/HCl buffer (pH 8.6) was used during polyacrylamide-gel preparation. Protein bands were made visible by Coomassie Brilliant Blue G-250 staining.

M_r values of purified glucose isomerases were determined by gel filtration on a Superose-12HR column in a f.p.l.c. system, and Blue Dextran (M_r 2000000), apoferritin (443000), alcohol dehydrogenase (150000) and BSA (66200) were used as M_r standards. The subunit M_r values were estimated by SDS/PAGE with low-range protein M_r standards (Bio-Rad Laboratories, Richmond, CA, U.S.A.): phosphorylase (M_r 97400), BSA (66200), ovalbumin (42700), carbonic anhydrase (31000) and soybean trypsin inhibitor (21500).

Two different pH ranges of Servalyt-Precotes isoelectric-focusing gels (Serva Co., Heidelberg, Germany; pH 3–10 and pH 3–6) were used for pI determination. An Ultrapore isoelectric-focusing apparatus (Pharmacia LKB Biotechnology) was used, and the gels were stained with Serva Blue W.

Amino acid compositions and sequence determination

Samples were prepared by washing the purified glucose isomerase preparations with double-distilled water five times with a Centricon-30 (Amicon) filtration device to remove metal salts from the enzyme solution. The samples were hydrolysed in a gas phase for 24 h by using 5.7 M-HCl. Amino acid composition analysis was performed with a Pico-Tag amino acid analyser (Waters Associates, Milford, MA, U.S.A.), and the *N*-terminal amino acid sequences were identified by a protein sequencer model 477A (Applied Biosystems, Foster City, CA, U.S.A.) with an on-line phenylthiohydantoin analyser (Applied Biosystems) in the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University.

Metal ion effects on enzyme activity and stability

To prepare a metal-ion-free enzyme solution, glucose isomerase purified from *C. thermosulfurogenes* was treated with 5 mM-EDTA at 60 °C for 1 h, and then washed with double-distilled water five times in an Amicon Centricon-30 filtration device at 4 °C. The effect of metal ions on enzyme thermal stability was determined by measuring residual glucose isomerase activity under optimum assay conditions after a 15 min preincubation at 80 °C in the presence of various metal ions (1 mM). The effect of metal ions on enzyme activity was determined at 55 °C, where the enzyme was stable without metal cofactors during the reaction period.

Protein crystallization

The hanging-drop vapour-diffusion method was used to crystallize the purified glucose isomerase from *C. thermosulfurogenes* in Linbro tissue-culture multi-well plates (Flow Laboratories, McLear, VA, U.S.A.). The protein solution used to prepare crystals contained glucose isomerase (15 mg/ml) in 50 mM-Mops buffer (pH 7.0) with 10 mM-$MgSO_4$ and 1 mM-$CoCl_2$. The reservoir solution (1 ml) in each well contained a series of different poly(ethylene glycol) (PEG) 4000 concentrations (5–30%) in 0.1 M-citrate buffer (pH 5.5) containing 0.2 M-Li_2SO_4. Equal volume (6 μl) of protein and reservoir solutions were mixed to make a droplet on a silicone-treated glass microscope cover slip, and then the cover slips were sealed on to the corresponding well with high-vacuum grease. Duplicate samples of the plates were incubated at 4 °C and 20 °C until crystals appeared.

Table 1. Summary of glucose isomerase purification steps

(*a*) *Thermoanaerobacter* strain B6A

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)
Cell-free extract	2820	1410	0.5	1.0
Heat treatment (85 °C, 15 min)	725	1310	1.8	3.6
$(NH_4)_2SO_4$ fractionation	254	585	2.3	4.6
DEAE-Sepharose anion exchange	94	385	4.1	8.2
Superose-12 gel filtration	38	181	4.8	9.6

(*b*) *C. thermosulfurogenes*

Step	Total protein (mg)	Total activity (unit)	Specific activity (units/mg)	Purification (fold)
Cell-free extract	2640	820	0.3	1.0
Heat treatment (80 °C, 10 min)	920	690	0.8	2.7
DEAE-Sepharose anion exchange	118	342	2.9	9.7
Superose-12 gel filtration	46	156	3.4	11.3

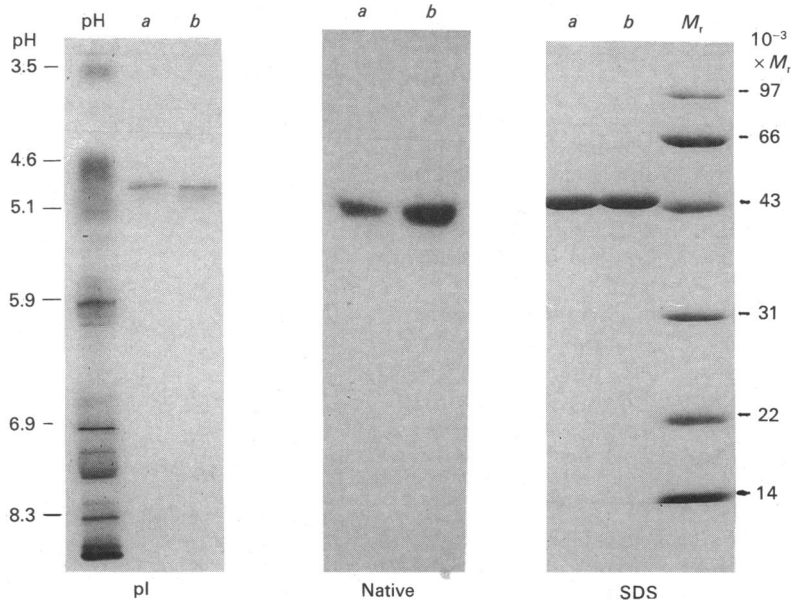

Fig. 1. Electrophoretic analysis of glucose isomerases purified from *Thermoanaerobacter* (*a*) and *C. thermosulfurogenes* (*b*)

pI: isoelectric-focusing gel electrophoresis with Servalyt-Precotes gel (pH range 3–10). Native: native PAGE with Tris/HCl buffer (pH 8.6). SDS: SDS/PAGE with a 12% gel. pH: pH separation of protein standards. M_r: M_r separation of protein standards.

RESULTS

Purification and molecular properties

The protocols used for glucose isomerase purification from *C. thermosulfurogenes* and *Thermoanaerobacter* strain B6A achieved a 11–10-fold purification and 19–13% yield from cell extracts (see Table 1). An $(NH_4)_2SO_4$ fractionation was used to purify the enzyme from *Thermoanaerobacter*, because it removed a certain protein that was not well separated by the following steps. In the final purification step, only a single major protein peak was detected by Superose-112 gel filtration with f.p.l.c. The purified enzymes were considered to be homogeneous by the detection of single bands on SDS/PAGE, native PAGE, and Serva Precoat isoelectric-focusing gels (Fig. 1).

The M_r of purified glucose isomerase was determined by Superose-12 gel filtration with f.p.l.c. Glucose isomerases from *C. thermosulfurogenes* and *Thermoanaerobacter* strain B6A displayed an identical M_r of 200000. SDS/PAGE analysis showed a single band for both enzymes with an M_r of 50000, indicating that both enzymes were composed of homo-tetrameric subunits. pI values of *C. thermosulfurogenes* and *Thermoanaerobacter* glucose isomerase were 4.9 and 4.8 respectively.

The amino acid compositions of the purified glucose isomerases are compared in Table 2. These enzymes had similar amino acid

Table 2. Amino acid compositions of thermostable glucose isomerase from *C. thermosulfurogenes* and *Thermoanaerobacter* strain B6A

Abbreviation: n.d., not determined.

Amino acid	Composition (mol %)	
	C. thermosulfurogenes	*Thermoanaerobacter*
Asx	9.5	7.9
Glx	7.3	3.6
Ser	4.0	3.6
Gly	9.1	8.5
Hos	2.0	2.3
Arg	5.9	6.0
Thr	4.4	4.9
Ala	13.9	15.4
Pro	5.3	4.7
Tyr	4.3	4.9
Val	4.3	3.7
Met	1.7	2.7
Ile	4.3	3.9
Leu	7.1	7.9
Phe	8.8	11.3
Lys	7.6	8.1
Trp	n.d.	n.d.
Cys	n.d.	n.d.
Total	100	100
Hydrophilic	50.3	45.5
Hydrophobic	49.7	54.5

compositions, except for glutamine and glutamate, methionine and phenylalanine. The enzyme was hydrophobic, and alanine was the most abundant residue in both enzyme molecules. The total amount of hydrophobic residues in the enzyme molecule was slightly higher in *Thermoanaerobacter* than in *C. thermosulfurogenes*.

The *N*-terminal sequences of the first seven residues from the two thermostable glucose isomerases are identical (Met-Asn-Lys-Tyr-Phe-Gln-Asn), and they are different from those of thermolabile enzymes from *Escherichia coli* (Met-Gln-Ala-Tyr-Phe-Asp-Gln; Schellenberg *et al.*, 1984) and *Bacillus subtilis* (Met-Ala-Gln-Ser-His-Ser-Ser; Wilhelm & Hollenberg, 1985).

Physicochemical properties

The purified glucose isomerases are active at high temperature up to 80 °C, and displayed very similar apparent pH optima for enzyme activity at pH 7.0–7.5 (Figs. 2 and 3). Both glucose isomerases were stable in the broad pH range 5.5–12.0, and were readily denatured at pH values lower than 5 (Fig. 3).

Further physicochemical studies were limited to just one enzyme, since the biochemical properties of both glucose isomerases were nearly identical. The effect of temperature on the stability of *C. thermosulfurogenes* glucose isomerase is shown in Fig. 4. EDTA-treated enzyme was stable for 1 h at 60 °C, but it was readily denatured at 70 °C. In the presence of metal ions (5 mM-$MgSO_4$ and 0.5 mM-$CoCl_2$) the enzyme was stable for 1 h at 85 °C. The half-life of the glucose isomerase in 50 mM-phosphate buffer (pH 7.0) containing 5 mM-$MgSO_4$ and 0.5 mM-$CoCl_2$ was 198 h at 60 °C and 42 h at 70 °C (results not shown). The effect of various metals on thermal stability of EDTA-treated glucose isomerase from *C. thermosulfurogenes* is shown in Table 3. The enzyme required Co^{2+} and/or Mg^{2+} for high thermal stability, and Mn^{2+} was less effective to protect the enzyme from heat denaturation at 80 °C. Other metal ions examined in this study did not enhance thermostability of the

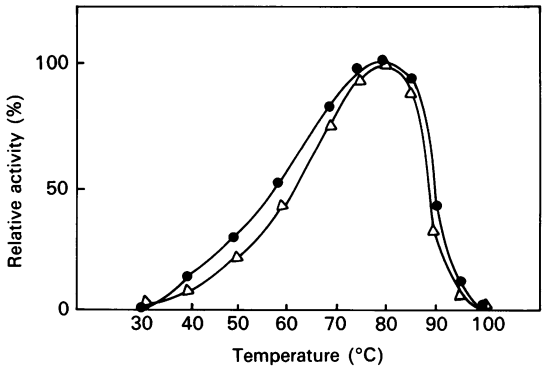

Fig. 2. Effect of temperature on glucose isomerase activity

Symbols: ●, *C. thermosulfurogenes*; △, *Thermoanaerobacter*. The 100 % activities correspond to 7.1 and 8.8 units/mg for *C. thermosulfurogenes* and *Thermoanaerobacter* glucose isomerases respectively.

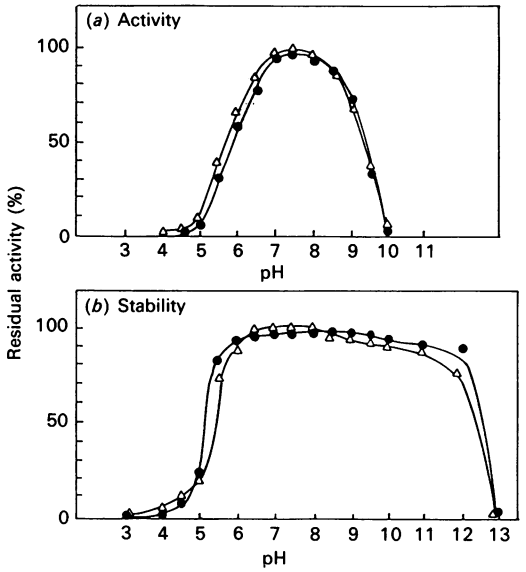

Fig. 3. Effect of pH on thermostable glucose isomerase activity (*a*) and stability (*b*)

Symbols: ●, *C. thermosulfurogenes*; △, *Thermoanaerobacter*. Enzyme activities were assayed in glycylglycine buffer at pH 3.0–4.5 and 8.5–11.0, sodium acetate buffer at pH 4.5–6.5 or Mops buffer at pH 6.5–8.6. Enzyme stabilities were assayed in these buffer solutions with 5 mM-$MgSO_4$ and 0.5 mM-$CoCl_2$ after treatment at 60 °C for 1 h. The 100 % activities correspond to 4.5 and 5.3 units/mg for *C. thermosulfurogenes* and *Thermoanaerobacter* respectively.

enzyme, and Cu^{2+} and Zn^{2+} showed an inhibitory effect. Treatment of the enzyme with 25 mM-dithiothreitol did not affect glucose isomerase activity (results not shown).

Crystals of glucose isomerase from *C. thermosulfurogenes* usually appeared on the next day in a hanging drop in the well with the reservoir solution containing 16–18 % PEG 4000 at room temperature. Fig. 5 shows crystals of glucose isomerase which exhibited a long and flat shape, with 0.7 mm in length × 0.1 mm in width. The preliminary results (results not shown) of X-ray-diffraction analysis confirmed that the crystal is a protein crystal and is large enough for detailed analysis for future studies on the three-dimensional structure of the enzyme.

Catalytic properties

The kinetic features of the purified glucose isomerases on three

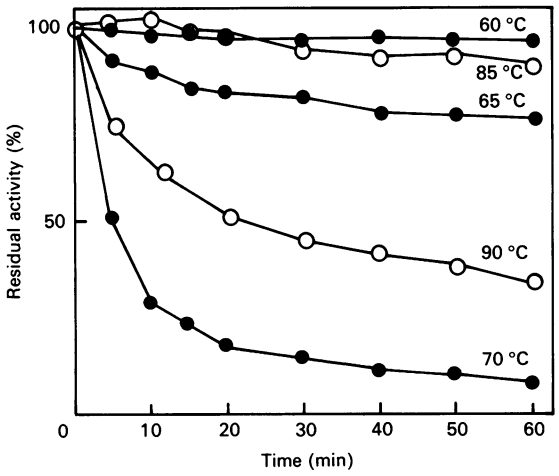

Fig. 4. Thermostability of EDTA-treated glucose isomerase of *C. thermosulfurogenes* in the absence (●) or presence (○) of metal ions

Residual activities were assayed after preincubation of the EDTA-treated enzyme in 50 mM-Mops buffer (pH 7.0) with or without 5 mM-$MgSO_4$ and 0.5 mM-$CoCl_2$ at indicated temperatures for various time periods. The 100 % activity corresponds to 4.9 units/mg of the pure enzyme.

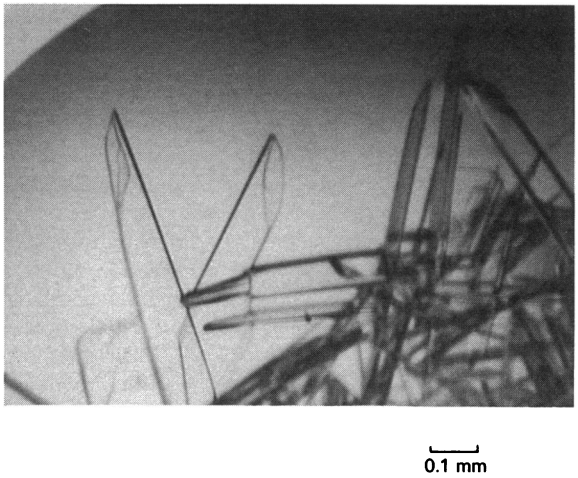

Fig. 5. Crystals of glucose isomerase from *C. thermosulfurogenes*

different substrates were determined at 65 °C, and the results are compared in Table 4. K_m and $V_{max.}$ values were obtained from Lineweaver–Burk plots of specific activities at various substrate concentrations. Glucose isomerases from two different thermoanaerobic bacteria displayed very similar apparent K_m, $V_{max.}$ and $k_{cat.}$ values for glucose, fructose and xylose. Both enzymes displayed lower K_m values for xylose than for glucose or fructose, and the K_m values for fructose were about 2-fold lower than those for glucose. The apparent $V_{max.}$ and $k_{cat.}$ values of these enzymes with xylose as substrate were approx. 3-fold higher than those with glucose, and approx. 6-fold higher than those with fructose.

The effect of various metal ions on the activity of EDTA-treated enzymes from *C. thermosulfurogenes* was investigated (see Table 3). Co^{2+} or Mg^{2+} was required for glucose isomerase activity, whereas Mn^{2+} did not enhance enzyme activity. The addition of Co^{2+} and Mg^{2+} did not show a synergistic effect on glucose isomerase activity, and Mn^{2+} decreased the activation effect of Co^{2+} or Mg^{2+}. A minimum concentration of 5 mM-$MgSO_4$ or 0.5 mM-$CoCl_2$ was required to achieve maximum glucose isomerase activity (results not shown). For xylose isomerase activity, Mn^{2+} (1 mM), Co^{2+} (1 mM) or Mg^{2+} (1 mM) was required, and any combination of these metal ions showed a synergistic activation effect. Other metal ions had very little

Table 4. Comparison of kinetic properties of thermostable glucose isomerase from thermoanaerobes

Micro-organisms: 4B, *C. thermosulfurogenes*; B6A, *Thermoanaerobacter*. Enzyme activities were determined at 65 °C as described in the Materials and methods section.

	K_m (mM)		$V_{max.}$ (units/mg)		$k_{cat.}$ (min^{-1})	
Substrate	4B	B6A	4B	B6A	4B	B6A
Glucose	140	120	5.2	6.3	1040	1260
Fructose	60	50	2.5	2.8	500	560
Xylose	20	16	15.7	17.6	3140	3520

Table 3. Effect of metals on activity and thermal stability of EDTA-treated glucose isomerase from *C. thermosulfurogenes*

Enzyme activity was assayed at 55 °C, where the enzyme was stable during the reaction period without metal cofactors. Thermal stability (residual activity) was measured at 65 °C after incubation of these enzymes in the solution containing metal cofactor at 80 °C for 15 min.

	Isomerase activity (% of maximum)		Thermal stability
Metal	Glucose	Xylose	(% residual)
None	2	13	14
$MgSO_4$ (1 mM)	21	89	94
$MgSO_4$ (5 mM)	97	90	94
$CoCl_2$ (1 mM)	100	98	100
$MnSO_4$ (1 mM)	6	100	61
$FeSO_4$ (1 mM)	4	15	15
$NiSO_4$ (1 mM)	2	8	20
$BaCl_2$ (1 mM)	2	14	16
$CaCl_2$ (1 mM)	2	16	22
$ZnSO_4$ (1 mM)	0	0	0
$CuSO_4$ (1 mM)	0	0	0
$MgSO_4 + CoCl_2$ (1 mM)	98	117	111
$MgSO_4 + MnSO_4$ (1 mM)	16	113	86
$MnSO_4 + CoCl_2$ (1 mM)	35	120	78

effect on both glucose isomerase and xylose isomerase activities, and Zn^{2+} (1 mM) and Cu^{2+} (1 mM) totally inhibited enzyme activity on glucose and xylose.

DISCUSSION

To our best knowledge, this study represents the first detailed characterization of thermostable glucose isomerases purified from the thermoanaerobic bacteria. Initially, the general biochemical properties of glucose isomerase in crude cell extracts were compared from three different thermoanaerobes. As expected, the glucose isomerases in these thermophiles were very thermostable and required metal ions (Co^{2+} or Mg^{2+}) for enzyme activity and stability. The enzymes were also thermophilic, and they displayed maximal activity at 80 °C, but activity was not detected below 30 °C. The glucose isomerases from *C. thermosulfurogenes* and *Thermoanaerobacter* were purified because of enzyme requirements in industry (Antrim *et al.*, 1979; Bucke, 1977) and the desire to compare similar enzyme activities from thermoanaerobic species.

The enzymes from both micro-organisms were very stable under the conditions used during purification. Glucose isomerase was one of the major proteins (approx. 10% of total protein) in the cell extract, and purification of the enzymes was relatively simple. The abundance of this enzyme in the cell extracts may be due to the fact that the enzyme had a relatively low $k_{cat.}$ and high K_m, which is typical of xylose isomerase (Suekane *et al.*, 1978; Antrim *et al.*, 1979), and perhaps the organism needed to overproduce this enzyme, which may be rate-limiting for growth on xylose. The growth rate of these bacteria is faster on glucose than on xylose (Schink & Zeikus, 1983; Weimer *et al.*, 1984).

The overall biochemical and physicochemical properties of the glucose isomerases purified from these two thermoanaerobic bacteria were similar, yet these species differ in sporulation and in saccharidase activities (Y.-E.-Lee, M. K. Jain, C. Lee & J. G. Zeikus, unpublished work). Different microbial glucose/xylose isomerases characterized previously vary in M_r from 80000 to 195000 and are composed of two or four identical subunits. M_r values (200000) and tetrameric subunit composition of both thermoanaerobic glucose isomerases are similar to the M_r-195000 enzyme present in *Lactobacillus brevis* (Yamanaka, 1968). Glucose isomerases characterized from *Streptomyces* species (Takasaki *et al.*, 1969; Kasumi *et al.*, 1981*b*), *Arthrobacter* (Henrik *et al.*, 1989), *Bacillus coagulans* (Danno, 1970*a*) and *Flavobacterium arborescens* (Boguslawski, 1983) display smaller M_r values (157000–183000) with tetrameric subunit compositions. The size and shape of *C. thermosulfurogenes* glucose isomerase crystals were very different from those reported previously from other enzyme sources (Yamanaka, 1968; Takasaki *et al.*, 1969; Danno, 1970*a*; Suekane *et al.*, 1978). Glucose isomerases from *E. coli* (Tucker *et al.*, 1988), alkalophilic *Bacillus* (Kwon *et al.*, 1987), *Actinoplanes missouriensis* (Gong *et al.*, 1980) and *Streptomyces olivochromogenes* (Suekane *et al.*, 1978) are dimers, with M_r values of 80000–120000.

The optimum pH for the glucose-isomerizing activity of *C. thermosulfurogenes* and *Thermoanaerobacter* was 7.0–7.5, which was similar to those of the enzymes from *B. coagulans* (Danno, 1970*b*) and *A. missouriensis* (Scallet *et al.*, 1974), and was lower than those (pH 8.0–10.0) of enzymes from *Streptomyces phaechromogenes* (Tsumura & Sato, 1965), *S. griseofuscus* (Kasumi *et al.*, 1981*a*) and *S. olivochromogenes* (Suekane *et al.*, 1978). The enzymes from both thermoanaerobes were stable within the pH range 5.5–11.0, and displayed pI values of 4.9 and 4.8.

Glucose isomerases are generally classified into two types of enzymes according to their thermal stability. *Lactobacillus brevis* glucose isomerase (Yamanaka, 1968) and *E. coli* xylose isomerase (Batt *et al.*, 1986), which are active at 37–50 °C, belong to the thermolabile type of glucose isomerase. Glucose isomerases from certain mesophilic microbial sources [e.g. *S. phaeochromogenes* (Takasaki *et al.*, 1969) and *A. missouriensis* (Scallet *et al.*, 1974)], however, can display quite high thermal stability. Glucose isomerases produced from *C. thermosulfurogenes* and *Thermoanaerobacter* are highly thermostable, and they belong to the latter type of glucose isomerase.

The requirement of Co^{2+} or Mg^{2+} for glucose-isomerizing activity and the requirement of Mn^{2+} for xylose-isomerizing activity of *C. thermosulfurogenes* enzyme was similar to that reported for *B. coagulans* enzyme (Danno *et al.*, 1967). Although *C. thermosulfurogenes* glucose isomerase required Co^{2+} or Mg^{2+} for its optimal thermostability, EDTA-treated enzyme was more thermostable than the enzymes from *E. coli* in the presence of these metals (Batt *et al.*, 1986).

These findings demonstrate that the two distinct thermoanaerobic bacteria produce highly thermophilic glucose isomerases with close similarity in physicochemical and catalytic properties. Because these bacteria have evolved in thermal hot-spring ecosystems (Schink & Zeikus, 1983; Weimer *et al.*, 1984) and are though to have a common phylogenic origin (Bateson *et al.*, 1989), one might expect that their glucose isomerases have similar properties, yet they represent distinct species. The high thermostability and the neutral optimum pH for enzyme activity of these glucose isomerases may provide practical advantages during the process of fructose production from glucose in the sweetener industry. However, further studies (e.g. enzyme immobilization, biocatalyst toxicity and large-scale process) are necessary to assess the biotechnological application of these new types of enzymes. At present, the molecular mechanism of thermophilicity (i.e. high temperature activity and stability) in these enzymes is not clear and remains to be solved. Cysteine disulphide bonds apparently do not contribute to protein thermostability, as evidenced by full activity in the presence of a reducing agent. The glucose isomerase from *C. thermosulfurogenes* appears suitable as a model for further studies on the structure–function relationship for enzyme thermophilicity, since it is readily crystallized. Further studies on the three-dimensional structure of the enzyme and protein modification via site-directed mutagenesis are required to explain the molecular mechanism of high thermophilicity in this glucose isomerase.

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