

Active site of Zn²⁺-dependent *sn*-glycerol-1-phosphate dehydrogenase from *Aeropyrum pernix* K1

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Summary The enzyme *sn*-glycerol-1-phosphate dehydrogenase (Gro1PDH, EC 1.1.1.261) is key to the formation of the enantiomeric configuration of the glycerophosphate backbone (*sn*-glycerol-1-phosphate) of archaeal ether lipids. This enzyme catalyzes the reversible conversion between dihydroxyacetone phosphate and glycerol-1-phosphate. To date, no information about the active site and catalytic mechanism of this enzyme has been reported. Using the sequence and structural information for glycerol dehydrogenase, we constructed six mutants (D144N, D144A, D191N, H271A, H287A and D191N/H271A) of Gro1PDH from *Aeropyrum pernix* K1 and examined their characteristics to clarify the active site of this enzyme. The enzyme was found to be a zinc-dependent metalloenzyme, containing one zinc ion for every monomer protein that was essential for activity. Site-directed mutagenesis of D144 increased the activity of the enzyme. Mutants D144N and D144A exhibited low affinity for the substrates and higher activity than the wild type, but their affinity for the zinc ion was the same as that of the wild type. Mutants D191N, H271A and H287A had a low affinity for the zinc ion and a low activity compared with the wild type. The double mutation, D191N/H271A, had no enzyme activity and bound no zinc. From these results, it was clarified that residues D191, H271 and H287 participate in the catalytic activity of the enzyme by binding the zinc ion, and that D144 has an effect on substrate binding. The structure of the active site of Gro1PDH from *A. pernix* K1 seems to be similar to that of glycerol dehydrogenase, despite the differences in substrate specificity and biological role.

Keywords: glycerophosphate backbone, metalloenzyme, zinc.

Introduction

The Archaea comprise a phylogenetically distinct group (domain) that diverged from the Bacteria and Eukarya at an early stage of evolution (Woese et al. 1990, Nelson et al. 2000). *Aeropyrum pernix* K1 (JCM no. 9820) is an aerobic hyperthermophilic archaeon for which the complete genome sequence has been determined (Faguy et al. 1999, Kawarabayasi

et al. 1999). *Aeropyrum pernix* is the only hyperthermophile known to obtain energy exclusively through aerobic respiration of complex organic matter (Sako et al. 1996). One characteristic feature of archaea is that their cellular membrane has an ether linkage between the glycerol backbone and the hydrocarbon residues. The core lipid of the phospholipids and glycolipids in archaeal cells is *sn*-2,3-dialkylglycerol, which has a polar head group in the *sn*-1 position. In contrast, the major lipids of eukaryotic and eubacterial cells consist of *sn*-1,2-diacylglycerol, which has a polar head group in the *sn*-C-3 position (Zhang and Poulter 1993). The enzyme *sn*-glycerol-1-phosphate dehydrogenase (Gro1PDH) is key to the formation of the enantiomeric configuration of the glycerophosphate backbone (*sn*-glycerol-1-phosphate) of archaeal ether lipids, and catalyzes the reversible conversion between dihydroxyacetone phosphate (DHAP) and glycerol-1-phosphate (Gro1P) using either NADH or NADPH as a coenzyme (Zhang and Poulter 1993, Nishihara and Koga 1995). We have found and characterized a hyperthermostable Gro1PDH from *A. pernix* (Han et al. 2002) that exhibits maximum activity at 96 °C.

From the homology modeling of Gro1PDH (Daiyasu et al. 2002), aspartic acid (Asp or D) and histidine (His or H) residues are speculated to be located at the active site and related to zinc ion (Zn²⁺) binding. Until now, however, there has been no evidence concerning the roles of these residues. Information on the active site and catalytic mechanism of Gro1PDH will lead to an initial understanding of the formation mechanism for polar lipids in archaea, and of the difference between archaea and bacteria in evolution.

According to the results of sequence alignment, the Gro1PDH isolated from *A. pernix* seems to have a different three-dimensional structure to that of glycerol-3-phosphate dehydrogenases, which have a role in bacteria corresponding to that of Gro1PDH in archaea (Nishihara and Koga 1995, Han et al. 2002). Glycerol dehydrogenases (GroDH), which allow the use of glycerol as a carbon source (May and Sloan 1981), have been isolated from several different organisms (Scharschmidt et al. 1983, Spencer et al. 1989). Despite its different biological role and substrate specificity, Gro1PDH ex-

hibits a sequence similarity to GroDH. Therefore, the design of the Gro1PDH mutants was performed on the basis of the sequence homology between Gro1PDH and GroDH. Analysis of the crystal structures of family III metal-dependent GroDHs showed that residues D123, D173, H256 and H274 are important in the orientation of the glycerol that forms ligands to the coordinated Zn^{2+} (Ruzhenikov et al. 2001). These amino acids are conserved in the Gro1PDH from *A. pernix*, and correspond to D144, D191, H271 and H287, respectively. It has also been reported that Gro1PDH from *Methanobacterium thermoautotrophicum* contains Zn^{2+} (Koga et al. 2003). In this study, we prepared mutant enzymes by site-directed mutagenesis and examined their characteristics to clarify the active site of Gro1PDH.

Materials and methods

Construction of mutated Gro1PDH

Site-directed mutation of Gro1PDH was performed by the polymerase chain reaction (PCR) method using the wild-type Gro1PDH gene (Mullis et al. 1986). Synthetic oligonucleotides were designed to produce the desired point mutations: D144N (Asp144→asparagine (Asn or N)), 5'-ACCGTGGCTAGCCACAACGGGATAACATCG-3'; D144A (Asp144→alanine (Ala or A)), 5'-ACCGTGGCTAGCCACGCCGGGATAACATCG-3'; D191N, 5'-TCGCTGGCTTCGGAAACCTGATAGGCAAG-3'; H271A, 5'-GCAAGCGGCAGTGAAGCCCTCTTCGCCAC-3' and H287A, 5'-GATGGCCTACCTCGCCGTAAGAAGTGGAG-3'. The mutated positions are indicated in bold. These substituted codons are used frequently in *Escherichia coli*. The D191N/H271A double mutant was constructed from a single mutant plasmid (D191N) as a template. All mutants were transformed with the pET11a vector system into the host, *E. coli* BL21 (DE3) (Han et al. 2002). The transformant cells were grown in 2YT medium containing ampicillin ($100 \mu\text{g ml}^{-1}$) at 37°C . After incubation with shaking at 37°C until the optical density at 600 nm reached 0.6–1.0, induction of the protein was carried out by adding isopropyl β -D-thiogalactopyranoside to a final concentration of 1 mM, and shaking for 4 h at 37°C . The complete DNA sequence of each mutated gene was verified with an automated DNA sequencing system (ABI PRISM 310, Applied Biosystems, Foster City, CA), and only the expected differences from the wild type at the mutation sites were observed.

Preparation and purification of mutated Gro1PDH

Each Gro1PDH mutant was expressed and purified by the same methods as previously reported for the wild-type Gro1PDH (Han et al. 2002). Cells were harvested by centrifugation, frozen at -80°C , and disrupted by sonication in 50 mM Tris-HCl buffer (pH 8.0). The homogenate was heated at 85°C for 30 min and centrifuged. The supernatant was dialyzed against 50 mM Tris-HCl buffer (pH 8.0), and the dialyzed sample was purified by chromatography on a HiTrap Q column (Pharmacia, Uppsala, Sweden), a HiLoad Phenyl Sepharose column (Pharmacia) and then a HiLoad Superdex column (Pharmacia). The homogeneity of each Gro1PDH mu-

tant was confirmed by SDS-PAGE (12%). The protein concentration was determined with Coomassie protein assay reagent (Pierce Chemical Company, Rockford, IL) with bovine serum albumin as the standard.

Thermal denaturation measurement by circular dichroic spectroscopy

Circular dichroism (CD) spectra were recorded with a J-820 spectrometer (Jasco, Tokyo, Japan). Wavelength scans were collected in 0.1 nm increments from 250 to 200 nm at 20 nm min^{-1} and 25°C , and temperature scans were collected in 0.2°C increments from 20 to 95°C with a 12 s per point mean time at 222 nm. Baseline buffer spectra were subtracted from sample spectra.

Enzyme assay

Enzyme activity was measured spectrophotometrically with a J-520 spectrophotometer (Jasco) by monitoring the decrease in the NAD(P)H concentration in 50 mM Tris-HCl buffer (pH 8.0) containing 70 mM KCl, 2.1 mM DHAP and 0.32 mM NADH (or 0.32 mM NADPH) at 65°C . The reaction was initiated by the addition of 10 μl of enzyme solution. The total volume of the reaction mixture was 1.2 ml. The kinetic constants of Gro1PDH from *A. pernix* were obtained by activity measurements in the direction of DHAP reduction, with substrate concentrations ranging from 0.1 to $10 K_m$. Each individual rate measurement was performed in triplicate and the kinetic mechanism was determined by the damped nonlinear least-squares method (Marquardt-Levenberg Method) according to the following equation (Menke 1989, Press et al. 1992, Han et al. 2002):

$$V = \frac{V_{\max}[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B]} \quad (1)$$

where A is the coenzyme (NAD(P)H), B is DHAP, K_{ia} is the dissociation constant for NAD(P)H, K_a is K_m for NAD(P)H, and K_b is K_m for DHAP.

Analysis of bound metal ions

The bound metals of the enzyme were analyzed by inductively coupled plasma atomic emission spectroscopy (IRIS AP; TJA Solutions, Franklin, MA). The wild-type enzyme and double mutant D191N/H271A were dialyzed against 50 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. The dialyzed enzyme, which contained 0.35 mg ml^{-1} protein, was used for the analysis, and the amount of zinc was calculated with a zinc standard solution.

Materials

The cloned Gro1PDH gene from *A. pernix* was used as a template for constructing mutant products (Han et al. 2002). The DNA primers were synthesized by Hokkaido System Science (Sapporo, Japan). All other chemicals and reagents were of commercially available analytic grade.

Results

Selection of mutation sites

There was no information about the structure and active site residues of Gro1PDH. Sequence alignment (see Figure 1) suggested a similar three-dimensional structure between Gro1PDH and GroDH despite their different substrate specificities (Ruzheinikov et al. 2001). The structure of GroDH has been solved (Ruzheinikov et al. 2001), and most of the active site residues are conserved in Gro1PDH (Han et al. 2002). The crystal structures of family III metal-dependent GroDHs showed that the amino acids D123, D173, H256 and H274 are important in the orientation of the glycerol that forms ligands to the enzyme-bound Zn^{2+} at the active site (Ruzheinikov et al. 2001). These amino acids are conserved in Gro1PDH from *A. pernix*, corresponding to D144, D191, H271 and H287, respectively (see Figure 1). Six mutant Gro1PDHs (D144N, D144A, D191N, H271A, H287A and D191N/H271A) were constructed by site-directed mutagenesis.

Expression of the mutants

The mutant enzymes were constructed, expressed and purified as described under Materials and methods. The amount of the expressed enzyme was unaffected by the mutations. The chromatographic patterns during purification and the yields of the expressed mutants were similar to those of the wild-type Gro1PDH. The purified mutant enzymes each migrated as a single band on SDS-PAGE, with an apparent molecular mass of 38 kDa, the same as that of the wild type.

Thermal stability of the mutants

The CD spectrum of the wild type in the far-UV region was examined from 20 to 95 °C. The thermal stabilities of the wild type and mutants were compared by CD. Circular dichroism at 222 nm of the mutants was measured from 20–95 °C (see Figures 2a and 2b). Some mutants exhibited a slight decrease in ellipticity at 220 nm at temperatures greater than 70 °C. These results indicate that major conformational changes of the wild type and mutants were unaffected by temperatures between 20 and 70 °C.

Characteristics of the mutants

All enzyme assays were performed at 65 °C because of the thermostability of the substrates and enzymes. Residues D144 and D191 were changed to their amidated forms (D144N and D191N), and H271 and H278 were changed to alanine (H271A and H278A) by site-directed mutagenesis. Mutant D144N exhibited a 13-fold increase in activity on NADH-dependent DHAP reduction. The activity of D144A also increased. The activity of D191N, H271A and H287A decreased. It has been reported that the catalytic reaction of Gro1PDH from *A. pernix* follows an ordered bi-bi mechanism (Han et al. 2002). Analysis of DHAP reduction was performed by bi-substrate kinetic analysis with NAD(P)H and DHAP because of the low Gro1P oxidation activity (Nishihara and Koga 1997). The experimental data for the mutants could be fitted to

the equation for an ordered bi-bi mechanism. The kinetic constants K_a (K_m for NAD(P)H), K_{ia} (dissociation constant for NAD(P)H), K_b (K_m for DHAP) and k_{cat} were determined (see Tables 1 and 2). The kinetic parameters for NADH and DHAP of D144N differed from those of the wild type. The k_{cat} value increased 12-fold compared with that of the wild type and the K_a value was also increased. Based on the k_{cat}/K_a value, D144N was more effective than the wild type. The dissociation constant for NADH was decreased greatly by this mutation. A threefold increase in the k_{cat} value was observed for the D144A mutant, but this mutant was less effective than the wild type when the k_{cat}/K_a values were compared.

The D191N, H271A and H287A mutations decreased k_{cat} . The k_{cat} value for H271A decreased markedly, to about one tenth that of the wild type. The kinetic constants for H287A were similar to those for the wild type, although the k_{cat} value was lower for the mutant enzyme, and there was a 140-fold decrease in the dissociation constant K_{ia} . Comparison with the active site structure of GroDH (Ruzheinikov et al. 2001) suggests that residues D191 and H271 are located in the vicinity of the Zn^{2+} ion in the active site of Gro1PDH. Therefore, we designed the D191N/H271A double mutant and found it had no enzyme activity.

The kinetic constants for D144N and D144A were also determined with NADPH as the coenzyme (see Table 2). The k_{cat} and K_b values for D144N and D144A increased significantly when determined with NADH as the coenzyme (see Table 1).

Metal ions bound to Gro1PDH

Atomic absorption analysis showed that Gro1PDH contained 0.81 mol of Zn^{2+} per monomer enzyme protein, and small amounts (less than 0.05 mol of metal ion per monomer enzyme protein) of magnesium and manganese. The enzyme did not contain other divalent cations, such as calcium, cobalt, molybdenum, nickel or copper. The effect of the metal-chelating reagent EDTA on the activity of Gro1PDH was examined after the enzymes had been dialyzed against 50 mM EDTA in Tris-HCl buffer (pH 8.0) for 12 h at 4 °C and then against 50 mM Tris-HCl buffer (pH 8.0). Exposure to EDTA led to a total loss of activity and a loss of Zn^{2+} to less than 0.05 mol Zn^{2+} per monomer protein for these enzymes. Activity was restored by incubation in 50 mM Tris-HCl buffer (pH 8.0) containing $ZnCl_2$ at 4 °C for 1 h (Figure 3), whereas activity was not restored by incubation in $MnCl_2$ solutions (data not shown). Figures 3a and 3b show the restored activity of the wild-type and mutant forms of Gro1PDH following incubation in a $ZnCl_2$ solution. The zinc reactivation curves for the wild type and all mutants were sigmoid, and the effect depended on the zinc concentration. The specific activity abruptly increased until 0.5 mM $ZnCl_2$ was added, and it was suppressed in the wild type when the $ZnCl_2$ concentration exceeded 1.0 mM. Enzyme activity increased in D144N and D144A until 0.5 mM $ZnCl_2$ was added, similar to the response of the wild type, indicating that this mutation had no effect on the zinc binding affinity of this enzyme. However, the specific activities of the D191N and H287A mutations increased until 0.75 mM $ZnCl_2$ had been added, with lower maximum specific

A. pernix	1: MLYHSVAGSRSSAAGVAGLYTSFHRIDLPRTIVVGGVLDKAGGYVSGVAQRGSYVLVVS	60
M. thermo	1: -----MDPRKIQLPREIYTGPGVIEDTGRICRDLRFEGRAMV-VT	39
P. abyssi	1: -----MHLMEFPREVI L GKNLVPEVNNVIKRLKLESPLGV-VY	37
S. solfa	1: -----MNVKEHVISLP RR V F V G H D I V Y D I S I Y F S Q L G V T P P F - L I V T	41
B. stearo	1: -----MAAERVFISPAKYVQ GKNVITKIANYL-EGIGNKTVV-IAD	39
E. coli	1: -----MPHLALLISKGAIMDRIIQS PGKYIQGADVINRLGEYL-KPLXERWLIV-VGD	50
S. pombe	41: RFYAPIETETPHKVGVEFEESKDRIFTS P QKYVQGRHAFTRSYMYVKKWATKSAVV-LAD	99
A. pernix	61: GPTVSSKYFERLRASLEAEGLTVGLKIIRDATVETAEEVAREALESRIEVAAGLGGGKSI	120
M. thermo	40: GPRTLQIAGEAAIESLQAEGFEVDQVTVDATMASVRNVQDGLDGVSV--VLGVGGGKVI	97
P. abyssi	38: GPVTKKIAGESVKKAIRDE-FDVYSITVKKAHIGEVEKVEAKIRDYNIKWAIIVAGGGSSII	96
S. solfa	42: GTKYTKKIADKVIEINLPKDA-KYEVVEIDSATLDDVYVVEEVIKRI SP S L L L G I G G G K V I	100
B. stearo	40: EIVWKIAGHTIVNELKKGNI AAEVVFVSGEASRNEVERIANIARKAEAAIIVIGVGGGKTL	99
E. coli	51: KFVLGFAQSTVEKSKFDAGLVEIAPFGGECSONEIDRLRGIETAQCGAILGIGGGKTL	110
S. pombe	100: QNVWNICANKIVDSLSQNGMTVTKLVFGGEASLVELDKLRK-QCPDDTQVIIGVGGGKTM	158
A. pernix	121: DVAKYASKRAGSVFVSIPTVASHDGITSPFSSL-KGFDK-PI-SRPKAPAEAI IIDVDVI	177
M. thermo	98: DVAKMSATLEGLHFISVPTAASHDGIASPRASI-RNGEG-TA-SLEASSPIGVIADTEII	154
P. abyssi	97: DVTKLASYRSGIPFISFP T T A S H D G I A S A N A S I - R G I E A - K T - S I K A R P P I A V I A D I E V I	153
S. solfa	101: DVTKYAAFRNSLEFVSIPTSPSHDGITSPFASL-KGLQK-PV-SVKAKEPLAI IADIEIL	157
B. stearo	100: DTAKAVADELDAYIVIVPTAASDTAPT SALSVIYSDDGVFESYRFYKKNPDLVLDTKII	159
E. coli	111: DTAKALAHFMGPVAIAPTIASTDAPCSALSVIYTDGEFDRYLLLPNNPNMIVVDTKIV	170
S. pombe	159: DSAKYIAHSMNLP S I I C P T T A S S D A A T S S L S V I Y T P D G Q F Q K Y S F Y P L N P N L I F I D T D V I	218
*		
A. pernix	178: AEAPRRYNIAGFGDLIGKYTAVLDWRLAHKLR--EYYGEYAASLALLSAKHVSQYAEET	235
M. thermo	155: SRAPFRLLASGCADII SNYTAIMDWKLAHRLN--ERYSESAAALSMTAKMIIKSADAI	212
P. abyssi	154: KTAPRRYLAAGVGDVINSITAVRDWKLAKLKG--EYFSEYAAALSMSAKMVIRDAEII	211
S. solfa	158: SLSPRRLINAGIGDTIGKIIAVRDWKLAAKLRG--EYYGDYASLALMSAKHAFQCTKII	215
B. stearo	160: ANAPPRLASGIADALATWVEARSVIKSGGKTMAGGIPTIAAEAI A E K C E Q T L F K Y G K L A	219
E. coli	171: AGAPARLLAAGIGDALATWFEARACSRSGATTMAGGKCTQAALALAE LCYNTLLEEGEKA	230
S. pombe	219: VRAPVRFLISGIGDALSTWVETESVIRSNSTSFAGGVASIAGRYIARACKDTLEKYALSA	278
*		
A. pernix	236: ALGTREGYRVL-LEALVSSGVSMCIAGSTRPASGSEHLFAHAL--HIVARNKPLHGEAVG	292
M. thermo	213: KEGLEESARLA-VKSLISSGIAISIAGSSRPASGSEHKFSHAL--DMIAPKPALHGEQCG	269
P. abyssi	212: RLGNDGVRKV- IKALISSGVAMSIAGSSRPASGAEHLFSHAL--DLLLLDKPALHGEQTG	268
S. solfa	216: NKDIKYGVRML-MEALISSGVAMGAGSTRPASGSEHLFAHAV--ELIHPEGILHGEIVG	272
B. stearo	220: YESVKAKVVTPALEAVVEANTLLSGLGFESGGLA AAAHAIHNGFTALEGEIHHLTHGEKVA	279
E. coli	231: MLAAEQHVVTPALERVIEANTYLSGVGFESGGLA AAAHAVHNGLTAI-PDAHYYHGEKVA	289
S. pombe	279: ILSNTRGVCTEAFENVVEANTLMSGLGFENGGLA AAAHAIHNGMTAIGHPVHRLMHGEKVA	338
* * *		
A. pernix	293: VGTIMMAYLHGKNWRRIRGLLKT V G A P T N A K E I G V E D D E V V E A L T I A A R I R P E R Y T I L G E	352
M. thermo	270: VGTIMMHLHGGDWQFIRDALARINAPTTAAELGIDPEYII EALTMAHNIRRERYTILGD	329
P. abyssi	268: IGTIIMAYLHGINWRKIKETLKT V G A P T S A Y E L G I D P E I I E A L T I A H K I R P E R Y T I L G D	328
S. solfa	273: LGTIIMAYLHGINWKIIRNR L K K I G F P V K A K D L G S D E E V I K A L T I A H T I R P E R Y T I L G D	332
B. stearo	280: FGTLVQLALEEHSQQEIER Y I E L Y L S L D L P V T L E D I K L K D A S R E D I L K V A K A A T A E G E T I	339
E. coli	290: FGTLTQLVLENAPVEE I E T V A A L S H A V G L P I T L A Q L D I K E D V P A K M R I V A E A C A E G E T I	349
S. pombe	339: YGTLVQVVL EDWPLEDFNNLASFMACKHLPI T L E E L G I P N V T D E E L L M V G R A T L R P D E S I	398
A. pernix	353: KGLTREAAEALARKTGVI-	370
M. thermo	330: RGLTREAAERLAKITEVI-	347
P. abyssi	329: EGLTREAAEKAAKITGVI-	346
S. solfa	333: RGLTWSSAEKIARVTKIID	351
B. stearo	340: HN-AFNVTADDVADAI FAADQYAKAYKEKHKR-----	370
E. coli	350: HNMPGGATPDQVYAALLVADQYGRFLQ-EWE-----	380
S. pombe	399: HNMSKKNFNPQIADAIKAVDSYSQKWQEQGTGWTFRFRLPPSRHSPHLTDIHP	450

Figure 1. Amino acid alignment of several glycerol dehydrogenases (GroDHs) together with *sn*-glycerol-1-phosphate dehydrogenase (Gro1PDH) of *Aeropyrum pernix* K1. Archaeal Gro1PDH: *Methanobacterium thermoautotrophicum* (M. thermo; 370 aa); *Pyrococcus abyssi* (P. abyssi; 346 aa); and *Sulfolobus solfataricus* (S. solfa; 351 aa). Bacterial or eukaryotic GroDH: *Bacillus stearothermophilus* (B. stearo; 370 aa); *Escherichia coli* (E. coli; 380 aa); and *Schizosaccharomyces pombe* (S. pombe; 450 aa). Amino acid residues that are identical in all sequences are shaded in dark gray, and residues that are identical in all Gro1PDHs are shaded in light gray. Asterisks indicate amino acid residues changed by site-directed mutagenesis. The box indicates the NAD(P)H binding site.

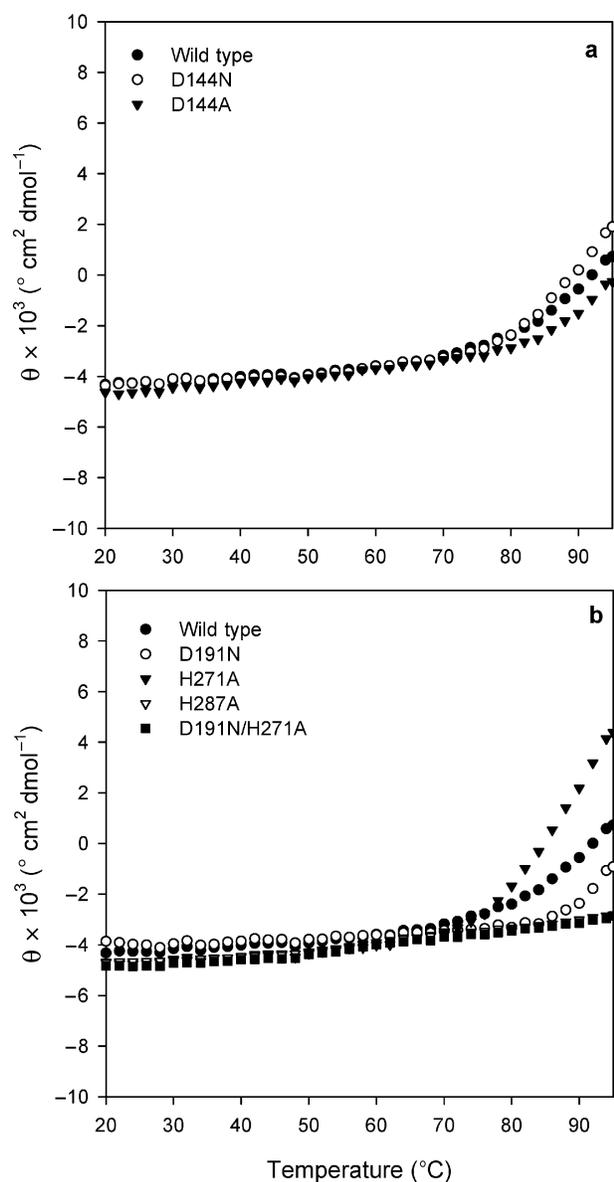


Figure 2. Thermal unfolding curves for the wild type and mutant *sn*-glycerol-1-phosphate dehydrogenase (Gro1PDH) (0.21–0.34 mg ml⁻¹) at 222 nm of CD. Abbreviation: θ = ellipticity.

activities. The curves for both mutants are shifted to the right compared with those for the wild type, indicating that these mutations decrease the apparent affinity of the enzyme for zinc. For the H271A mutant, activity was not restored by incubation in a ZnCl₂ solution up to 2.0 mM (see Figure 3). Atomic absorption analysis showed that the D191N/H271A double mutant contained less than 12% Zn²⁺ (0.09 mol Zn²⁺ per monomer enzyme protein) compared with the wild type.

Discussion

The results of metal analysis and restoration of activity on addition of a zinc ion to the EDTA-treated enzyme indicate that

Table 1. Kinetic constants for the wild-type and *sn*-glycerol-1-phosphate dehydrogenase (Gro1PDH) mutants in dihydroxyacetone phosphate (DHAP) reduction with NADH as the coenzyme. These parameters were estimated by the Marquardt-Levenberg method (Menke 1989, Press et al. 1992) in experiments in which a fixed concentration of substrate or coenzyme and appropriate ranges of concentrations of the other reactants were used. Abbreviations: k_{cat} = turnover number; $K_a = K_m$ for NADH; $K_b = K_m$ for DHAP; K_{ia} = dissociation constant for NADH; and nd = not detected.

Kinetic parameter	Wild type	D144N	D144A	D191N	H271A	H287A	D191N/H271A
k_{cat} (min ⁻¹)	299.9 ± 23.4	3628.2 ± 380.5	1126.5 ± 158.6	196.2 ± 38.4	29.58 ± 16.6	176.7 ± 4.99	nd
K_a (mM)	0.0477 ± 0.02	0.415 ± 0.06	0.213 ± 0.06	0.296 ± 0.10	0.321 ± 0.31	0.036 ± 0.004	nd
K_b (mM)	0.177 ± 0.01	1.12 ± 0.20	1.12 ± 0.34	0.633 ± 0.32	1.09 ± 0.13	0.259 ± 0.026	nd
K_{ia} (mM)	0.180 ± 0.17	0.0017 ± 0.00	0.245 ± 0.07	0.227 ± 0.14	0.042 ± 0.02	0.0025 ± 0.00	nd
% Fit	98.6	99.7	99.8	99.1	98.57	99.8	99.8
SD	0.562	1.04	0.294	0.108	0.027	0.125	0.125

Table 2. Kinetic parameters for the wild-type, D144N and D144A *sn*-glycerol-1-phosphate dehydrogenases (Gro1PDH) in dihydroxyacetone phosphate (DHAP) reduction with NADPH as the coenzyme. These parameters were estimated with the Marquardt-Levenberg method (Menke 1989, Press et al. 1992) in experiments in which a fixed concentration of substrate or coenzyme and appropriate ranges of concentrations of the other reactant were used. Abbreviations: k_{cat} = turnover number; $K_a = K_m$ for NADPH; $K_b = K_m$ for DHAP; and K_{ia} = dissociation constant for NADPH.

Kinetic parameter	Wild type	D144N	D144A
k_{cat} (min^{-1})	90.42 ± 3.01	284.1 ± 50.8	210.4 ± 7.91
K_a (mM)	0.086 ± 0.01	0.165 ± 0.06	0.039 ± 0.006
K_b (mM)	0.050 ± 0.02	0.360 ± 0.03	0.218 ± 0.030
K_{ia} (mM)	0.257 ± 0.02	0.022 ± 0.01	0.013 ± 0.002
% Fit	99.8	99.7	99.7
SD	0.06	0.19	0.18

the binding of one zinc ion to the enzyme plays a decisive role in determining Gro1PDH activity. This enzyme also contains small numbers of magnesium and manganese ions that do not affect its activity. Therefore, Gro1PDH is a zinc-dependent metalloenzyme.

The thermostability of the enzyme at 20–70 °C was unaffected by the mutations (Figure 2), indicating that the mutations did not affect the overall structure and folding of the enzyme. By changing residue D144 to Asn or Ala, the activity of the enzyme was increased with NADH and NADPH as the coenzyme. From the kinetic data (see Tables 1 and 2), it is clear that the DHAP binding affinity was decreased and the k_{cat} value was increased by these mutations. These results indicate that D144 is not the catalytic residue, although it is likely to be close to the DHAP binding site, and its carboxyl side chain may contribute to the affinity of the enzyme for DHAP.

The study for the homology modeling of Gro1PDH (Daiyasu et al. 2002) indicates that D144 forms hydrogen bonds with the carboxyl oxygen at C2 of DHAP. Furthermore, the affinity of the coenzymes was also influenced by these mutations. Replacement of the carboxyl side chain on D144 appears to stimulate activity by removing the negative charge that affects coenzyme binding. In addition, the similar zinc affinity of D144N and D144A compared with that of the wild type (Figure 3a) indicates that D144 does not take part in the metal–environment interaction of this enzyme. The decrease in activity by high zinc concentrations of the zinc-deplete D144N and D144A mutants (Figure 3a) suggests that these mutants have a secondary zinc ion binding site and that excess zinc ions decrease the enzyme's activity (Larsen and Auld 1991, Gomez-Ortiz et al. 1997).

Glycerol dehydrogenase is a metalloenzyme containing a zinc or iron ion at the active center. It has been reported that Zn^{2+} is bound in a deep cleft, and is tetrahedrally coordinated through an ion–dipole interaction with amino acid residues D173, H256 and H274, and one water molecule (Ruzhenikov et al. 2001). Analysis of the conserved sequence in Gro1PDH and GroDH, and a study of homology modeling of Gro1PDH

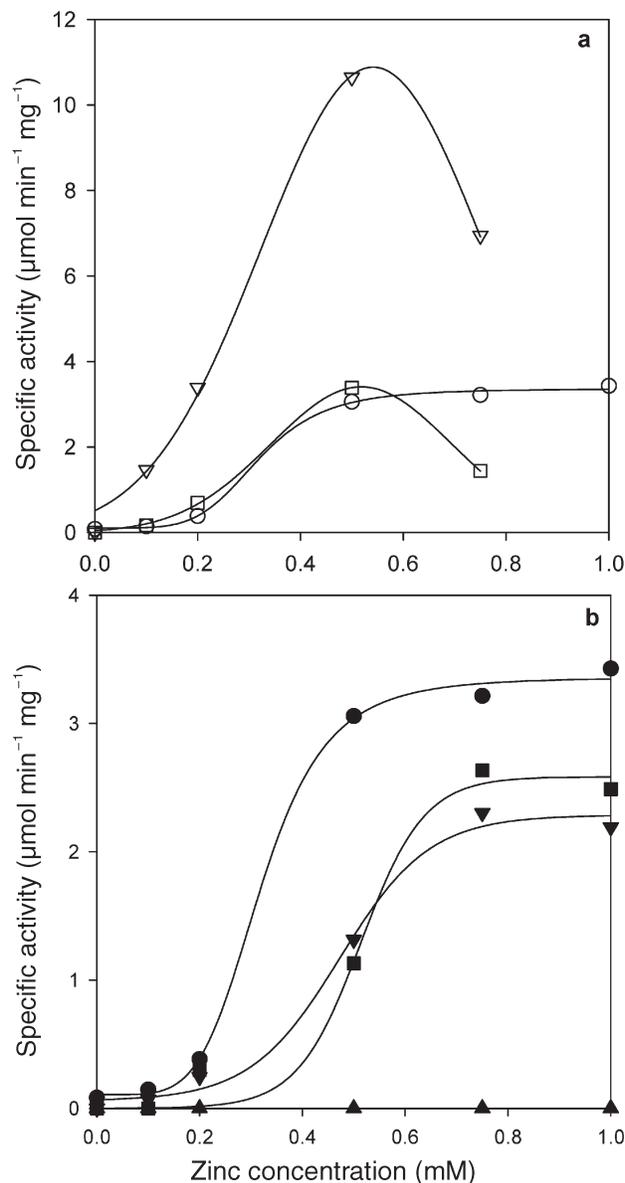


Figure 3. Restoration of activity by zinc ions in *sn*-glycerol-1-phosphate (Gro1PDH). The Gro1PDH was dialyzed against 50 mM EDTA (pH 8.0) for 24 h at 4 °C, and then against 50 mM Tris-HCl buffer/0.15 M NaCl (pH 8.0). Activity was measured after incubation in 50 mM Tris-HCl buffer/0.15 M NaCl (pH 8.0) containing ZnCl_2 at 4 °C for 1 h. (a) Wild type (○), D144N (▽) and D144A (□). (b) Wild type (●), D191N (▼), H271A (▲) and H287A (■).

(Daiyasu et al. 2002), showed that the three amino acid residues that bind zinc are completely conserved in *A. pernix* Gro1PDH. These residues are D191, H271 and H287. The H271 (H271A) mutation decreased the affinity for a substrate and decreased activity, which strongly suggests that this mutation modified the environment of the active site. Although the D191 and H287 mutations decreased the activity, the H287A mutant exhibited a binding affinity for the substrates similar to that of the wild type. The zinc activation curves for D191N and H287A were shifted to the right compared with that of the wild

type, indicating a small reduction in zinc binding. This indicates that the D191 and H287 mutations lower the apparent affinity of the enzyme for zinc, and D191 and H287 contribute ligands to the zinc ion. In addition, the simultaneous replacement of D191 and H271 (D191N/H271A) brought about a loss of both enzyme activity and the bound zinc ion. From the kinetic data obtained for D191N, H271A, H287A and D191N/H271A, and the restoration of their activity by Zn²⁺, it was apparent that the zinc ion is located at the active center and seems to coordinate with these three amino acid residues through an ion-dipole interaction.

The complete loss of enzyme activity and the zinc ion in the D191N/H271A mutant strongly indicates that these residues are not directly related to the catalytic mechanism, but that the coordinated Zn²⁺ plays an important catalytic role. Two of the three amino acid residues mutated in this study appear to be necessary for zinc binding; however, these residues are unrelated to substrate binding. The activity of H271A was not restored by Zn²⁺ (see Figure 3b), indicating that the environment of the zinc binding site of H271A is modified irreversibly on removal of the zinc ion, and that H271 plays an important role in stabilizing the conformation of the active site with the zinc ion.

These results for the mutant enzymes are not in conflict with the active site data predicted by the homology modeling of Gro1PDH (Daiyasu et al. 2002). Despite the differences in substrate specificity and biological roles of the two dehydrogenases, the results of these experiments indicate that the overall structures of the active sites and catalytic mechanisms of Gro1PDH and GroDH are similar, and that these enzymes are related to the divergent evolution of archaea and bacteria. The utilization of glycerol as a carbon source by GroDH seems to have evolved from the the formation of the glycerophosphate backbone of ether lipids by Gro1PDH.

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References

Daiyasu, H., T. Hiroike, Y. Koga and H. Toh. 2002. Analysis of membrane stereochemistry with homology modeling of *sn*-glycerol-1-phosphate dehydrogenase. *Protein Eng.* 15:987–995.

Faguy, D.M. and W.F. Doolittle. 1999. Genomics: lessons from the *Aeropyrum pernix* genome. *Curr. Biol.* 9:R883–R886.

Gomez-Ortiz, M., F.X. Gomis-Ruth, R. Huber and F.X. Aviles. 1997. Inhibition of carboxypeptidase A by excess zinc: analysis of the structural determinant by X-ray crystallography. *FEBS Lett.* 400:336–340.

Han, J.-S., Y. Kosugi, H. Ishida and K. Ishikawa. 2002. Kinetic study of *sn*-glycerol-1-phosphate dehydrogenase from the aerobic hyperthermophilic archaeon, *Aeropyrum pernix* K1. *Eur. J. Biochem.* 269:969–976.

Kawarabayasi, Y., Y. Hiro, H. Horikawa et al. 1999. Complete genome sequence of an aerobic hyperthermophilic crenarchaeon, *Aeropyrum pernix* K1. *DNA Res.* 6:83–101.

Koga, Y., N. Sone, S. Noguchi and H. Morii. 2003. Transfer of Pro-R hydrogen from NADH to dihydroxyacetonephosphate by *sn*-glycerol-1-phosphate dehydrogenase from the archaeon *Methanobacterium thermoautotrophicum*. *Biosci. Biotechnol. Biochem.* 67:1605–1608.

Larsen, K.S. and S.D. Auld. 1991. Characterization of an inhibitory metal binding site in carboxypeptidase. *Biochemistry* 30:2613–2618.

May, J.W. and J. Sloan. 1981. Glycerol utilization by *Schizosaccharomyces pombe*: dehydration as the initial step. *J. Gen. Microbiol.* 123:183–185.

Menke, W. 1989. Discrete inverse theory. *In* *Geophysical Data Analysis*. Revised Edn. Academic Press, New York, pp 143–160.

Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn and H.A. Erlich. 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harbor Symp.* 51:263–273.

Nelson, K.H., I.T. Paulsen., J.F. Heidelberg and C.M. Fraser. 2000. Status of genome projects for nonpathogenic bacteria and archaea. *Nat. Biotech.* 18:1049–1054.

Nishihara, M. and Y. Koga. 1995. *sn*-Glycerol-1-phosphate dehydrogenase in *Methanobacterium thermoautotrophicum*: key enzyme in biosynthesis of the enantiomeric glycerophosphate backbone of ether phospholipids of archaeobacteria. *J. Biochem.* 117:933–935.

Nishihara, M. and Y. Koga. 1997. Purification and properties of *sn*-glycerol-1-phosphate dehydrogenase from *Methanobacterium thermoautotrophicum*: characterization of the biosynthetic enzyme for the enantiomeric glycerophosphate backbone of ether polar lipids of archaea. *J. Biochem.* 122:572–576.

Press, W.H., S.A. Teukolsky, W.T. Vetterling and B.P. Flannery. 1992. Numerical recipes in Fortran—the art of scientific computing. 2nd Edn. Cambridge University Press, Cambridge, pp 120–149.

Ruzhenikov, S.N., J. Burke, S. Sedelnikova, P.J. Baker, R. Taylor, P.A. Bullough, N.M. Muir, M.G. Gore and D.W. Rice. 2001. Glycerol dehydrogenase: structure, specificity, and mechanism of family III polyol dehydrogenase. *Structure* 9:789–802.

Sako, Y., N. Nomura, A. Uchida, Y. Ishida, H. Morri, Y. Koga, T. Hoaki and T. Maruyama. 1996. *Aeropyrum pernix* gen. Nov., sp. Nov., a novel aerobic hyperthermophilic archaeon growing at temperatures up to 100 °C. *Int. J. Syst. Bacteriol.* 46:1070–1077.

Scharschmidt, M., G. Pfeleiderer, H. Metz and W. Brummer. 1983. Isolation and characterization of the glycerol dehydrogenase from *Bacillus megaterium*. *Hoppe-Seyler's Z. Physiol. Chem.* 364:911–921.

Spencer, P., K.J. Brown, M.D. Scawen, T. Atkinson and M.G. Gore. 1989. Isolation and characterization of the glycerol dehydrogenase from *Bacillus stearothermophilus*. *Biochim. Biophys. Acta* 994:270–279.

Woese, C.R., O. Kandler and M.L. Wheelis. 1990. Towards a natural system of organisms: proposal for the domains archaea, bacteria, and eukarya. *Proc. Natl. Acad. Sci.* 87:4576–4579.

Zhang, D. and C.D. Poulter. 1993. Biosynthesis of archaeobacterial ether lipids. Formation of ether linkages by prenyltransferases. *J. Am. Chem. Soc.* 115:1270–1277.



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