Exchange of domains of glutamate dehydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus* and the mesophilic bacterium *Clostridium difficile*: effects on catalysis, thermoactivity and stability

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The glutamate dehydrogenase gene from the hyperthermophilic archaeon *Pyrococcus furiosus* has been functionally expressed in *Escherichia coli* under the control of the λ P_L promoter. The *P. furiosus* glutamate dehydrogenase amounted to 20% of the total *E. coli* cell protein, and the vast majority consisted of hexamers. Following activation by heat treatment, an enzyme could be purified from *E. coli* that was indistinguishable from the glutamate dehydrogenase purified from *P. furiosus*. Hybrid genes, that consisted of the coding regions for the homologous glutamate dehydrogenases from *P. furiosus* and the mesophilic bacterium *Clostridium difficile*, were constructed and successfully expressed in *E. coli*. One of the resulting hybrid proteins, containing the glutamate binding domain of the *C. difficile* enzyme and the cofactor binding domain of the *P. furiosus* enzyme, did not show a detectable activity. In contrast, the complementary hybrid containing the *P. furiosus* glutamate and the *C. difficile* cofactor binding domain was a catalytically active hexamer that showed a reduced substrate affinity but maintained efficient cofactor binding with the specificity found in the *Clostridium symbiosum* enzyme. Compared with the *C. difficile* glutamate dehydrogenase, the archaeal–bacterial hybrid is slightly more thermoactive, less thermostable but much more stable towards guanidinium chloride-induced inactivation and denaturation.

Keywords: glutamate dehydrogenase/heterologous expression/hybrid enzymes/*Pyrococcus furiosus*/thermostability

Introduction

The discovery of microbes that have the capacity to grow optimally at or around the boiling temperature of water (Stetter *et al.*, 1990) has greatly stimulated the analysis of the molecular mechanisms of adaptation of these hyperthermophilic organisms to their extreme environment. Considerable attention has been focused on protein thermostability that is generally accompanied by a stability towards chemical denaturants and proteolysis (Fontana, 1991). Theoretical considerations in combination with protein sequence comparisons have indicated that protein thermostability is a consequence of small but multiple changes distributed all over the protein (Jaenicke,

1991). Site-directed and random mutagenesis studies have been used to investigate the contribution of specific interactions to protein stability and have confirmed that only a few residues play a critical role in achieving thermal and chemical stability (Goldenberg, 1992). The recent elucidation of the 3-D structures of several proteins from thermophilic microbes and their comparison with proteins from mesophilic sources have advanced the understanding of the thermal stability of proteins. Although various different mechanisms have been identified by which the thermal stabilization of proteins may occur, an analysis of the structures of rubredoxin and aldehyde ferredoxin oxidoreductase from the hyperthermophilic *Pyrococcus furiosus* has indicated that they both share an increased number of electrostatic interactions in comparison with other proteins (Day *et al.*, 1992; Chan *et al.*, 1995). A large number of extra ion pairs was also observed in the structure of the glyceraldehyde-3-phosphate dehydrogenase from the thermophilic *Thermotoga maritima* (Korndörfer *et al.*, 1995). However, site-directed mutagenesis studies have so far failed to identify the specific ion pairs that contribute to the high thermostability of the latter enzyme (Tomschy *et al.*, 1994).

Complementary to studying the role of specific residues, hybrid proteins in which large parts of the polypeptide chain are exchanged between homologous proteins offer the possibility of investigating the role of larger substructures in conferring functionality and stability to a protein. This approach has been applied to bacterial 3-isopropylmalate dehydrogenase from *Thermus thermophilus* and the archaeal glyceraldehyde-3-phosphate dehydrogenase from *Methanothermobacter fervidus*, in which parts of mesophilic counterparts were introduced (Bi *et al.*, 1990; Onodera *et al.*, 1994). In addition, hybrid enzymes have been made between the bacterial *Thermoactinomyces intermedius* phenylalanine dehydrogenase and *Bacillus stearothermophilus* leucine dehydrogenase (Kataoka *et al.*, 1994). In all cases, novel dehydrogenases with characteristics from both parental enzymes were generated that allowed the localization of regions involved in the thermostability and binding of substrate and cofactor. We have extended this approach of producing hybrid proteins by exchanging domains in glutamate dehydrogenase (GDH). GDH is a well studied enzyme catalyzing the oxidative deamination of L-glutamate to α -ketoglutarate and ammonia, accompanied by the reduction of a single molecule of NAD⁺ or NADP⁺ (Smith *et al.*, 1975). Primary sequences of GDH are available from a wide variety of organisms and have been used to arrange GDHs into two different families (Benachou-Lahfa *et al.*, 1993). In general, GDH is a hexameric enzyme that consists of identical subunits. Recently, the 3-D structure of the hexameric GDH from *Clostridium symbiosum*, belonging to family I, has been solved (Baker *et al.*, 1992). Each subunit can be divided into two domains: domain I forms the core of the enzyme and binds the substrate, while domain II points outwards and binds the cofactor.

The most thermostable GDH is found in the hyperthermophilic archaeon *P. furiosus* with a half-life of ~12 h at 100°C

Table I. Universal and gene-specific primers used for construction and DNA sequencing of the hybrid *gdh* genes

Primer	Sequence	Position		
BG26	5'-GGATAACAATTTACACAGG-3'	pUC19	354-373	S
BG27	5'-GTTTCCCAGTCACGAC-3'	pUC19	492-476	A
BG18	5'-TGTCTTGCCCTTGAGGG-3'	<i>P.furiosus gdh</i>	629-645	A
BG56	5'-TATACCTGCATCCACCAATGCTAAGAGGC-3'	<i>P.furiosus gdh</i>	550-531	A
BG57	5'-TATACCTGCAATTTGGTGGTTCTTTAGG-3'	<i>C.difficile gdh</i>	539-557	S
BG58	5'-TATACCTGCTGGTGGATCACTTGAAGAA-3'	<i>P.furiosus gdh</i>	543-563	S
BG59	5'-TATACCTGCAAAGATCCACCAATTCAACTG-3'	<i>C.difficile gdh</i>	547-533	A
BG65	5'-CCACAAATAATGGCATG-3'	<i>P.furiosus gdh</i>	448-464	S
BG66	5'-AGCTATAGAAGAAGTG-3'	<i>P.furiosus gdh</i>	888-903	S

The position of the primers is indicated in the sequence of pUC19 (Sambrook *et al.*, 1989) or that of the different *gdh* genes numbered from first base of the initiation start codon (Lyerly *et al.*, 1991; Eggen *et al.*, 1993). S, sense primer; A, antisense primer.

(Consalvi *et al.*, 1991). Remarkably, GDH may comprise up to 20% of the total cell protein in *P.furiosus* and plays an important role in the removal of reduction equivalents (Kengen and Stams, 1994). We have cloned and characterized the *gdh* gene from *P.furiosus* and found it to encode a GDH belonging to family II (Eggen *et al.*, 1993). Recently, we described the overexpression of the *P.furiosus gdh* gene in *Escherichia coli* and the formation of an active GDH in this host (Eggen *et al.*, 1994). Here we present the characterization of the *P.furiosus* GDH produced by *E.coli* and a comparison with the enzyme isolated from *P.furiosus*. To study the role of the different domains in catalytic activity and stability, hybrid proteins were constructed between GDH from *P.furiosus* and the phylogenetically very distant mesophilic bacterium *Clostridium difficile* that share 52% amino acid sequence identity and both belong to the family II GDHs. Two hybrids were generated that contained either the substrate or the cofactor binding domain of *P.furiosus* or *C.difficile* GDH. The functionality, catalytic activity and both the chemical and thermal stabilities of these hybrids were compared with those of the parental enzymes produced in *E.coli*. An active archaeal-bacterial hybrid GDH was obtained with new properties.

Materials and methods

Bacterial strains and media

Escherichia coli TG1 [*supE hsd* Δ 5 *thi* (*lac-proAB*) F' (*traD* 35 *proAB*⁺ *lacI*^q *lacZ* Δ M15); Gibson, 1984] was cultivated in LB-based medium at 37°C. *Escherichia coli* K12 Δ H1 Δ trp [M72 Sm^R*lacZ*am Δ bio-*uvrB* Δ trpEA2(λ Nam7-Nam53-cl857 Δ H1); Bernard *et al.*, 1979] was used in combination with expression plasmids containing the λ P_L promoter and was cultivated in the same medium.

Enzymes, chemicals and primers

Restriction enzymes and DNA modifying enzymes were purchased from Life Technologies Inc. and Pharmacia LKB Biotechnologies. Biochemicals of the highest purity were obtained from Boehringer and Fluka. Oligonucleotides were purchased from Pharmacia LKB Biotechnologies and are listed in Table I.

DNA isolation and manipulation

Small-scale plasmid DNA isolations, ligations, restrictions, DNA purification, agarose gel electrophoresis and transformation of *E.coli* were all performed as described by Sambrook *et al.* (1989). The plasmids pUC18 and pUC19 and the bacteriophages M13mp18 and M13mp19 (Yannisch-Perron *et al.*, 1985) were used as cloning vectors. The expression vector pPLc2833 is a derivative of pPLc28 (Remaut *et al.*,

Fig. 1. Construction of hybrid genes. The physical maps of the parental plasmids pLUW401, containing the 1.6 kb HindIII-HincII fragment with the *P.furiosus gdh* gene (black), and pLUW420, containing the 1.6 kb EcoRI-SphI fragment with the *C.difficile gdh* gene (white). Relevant restriction sites are indicated: H, HindIII; C, HincII; E, EcoRI; S, SphI; B, BspMI.

1981) containing additional *Pst*I-SalI-XbaI-SalI-XbaI-BamHI restriction sites in between the unique BamHI and HindIII sites immediately downstream of the P_L promoter in pPLc2833. The hybrid genes were sequenced according to the dideoxy chain termination method (Sanger *et al.*, 1977) using the T7 sequencing kit (Pharmacia). Universal and gene-specific oligonucleotides were used as primers (Table I).

Construction of the expression plasmids for wild-type and hybrid *gdh* genes

A 1.6 kb HindIII-HincII fragment containing the *P.furiosus gdh* gene was isolated from pLUW400 (Eggen *et al.*, 1994). Sticky ends were filled in using the Klenow fragment of *E.coli* DNA polymerase. The fragment was cloned into the blunted EcoRI restriction site of pPLc2833. The orientation of the *P.furiosus gdh* gene under the control of the P_L promoter was confirmed by restriction analysis, and the construct was denoted pLUW410. A 1.6 kb EcoRI-SphI chromosomal fragment containing the *C.difficile gdh* gene (Lyerly *et al.*, 1991; kindly provided by Dr Lisa Barosso, Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA) was cloned into an EcoRI-SphI linearized pUC18 vector to position the *gdh* gene under the control of the *lacUV5* promoter, resulting in pLUW420. Hybrid

genes were constructed according to a method described by Tomic *et al.* (1990) (Figure 1). Parts of the *P.furiosus* and *C.difficile* *gdh* genes encoding either domain I or II of GDH were amplified by PCR using 100 ng pLUW401 (pUC19 containing the *P.furiosus* *gdh* gene on a 1.6 kb *HindIII*-*HincII* fragment) or pLUW420 as template and, for each reaction, 100 ng universal vector primer and 200 ng of a gene-specific primer with a *Bsp*MI site (Table I). Restriction endonuclease *Bsp*MI digests DNA at a short distance from its recognition site, allowing the formation of complementary overhanging ends on the PCR products that may be ligated. In addition to the template and primers, the 100 µl reaction mixtures contained 0.2 mM dNTPs, *Taq* DNA polymerase buffer, 5 mM MgCl₂, 5 U *Taq* DNA polymerase and were subjected to 30 cycles of amplification (1.5 min at 92°C, 2.5 min at 50°C and 3.5 min at 72°C) on a programmable Ori-Block (New Brunswick Scientific). As determined by agarose gel electrophoresis, the amplification of the *P.furiosus* glutamate and cofactor binding domains resulted in the expected 0.6 and 1.0 kb products, respectively. Amplification of the *C.difficile* glutamate and cofactor binding domains also resulted in the expected 0.6 and 1.0 kb products, respectively. Each product was digested with *Bsp*MI and *HindIII*, *HincII*, *Eco*RI or *Sph*I that cut in the polylinker sequence (Figure 1). A three-point ligation of the 5' *P.furiosus* *gdh* gene fragment and the 3' *C.difficile* *gdh* gene fragment into *HindIII*-*Sph*I linearized pUC19 resulted in pLUW440. A three-point ligation of the 5' *C.difficile* *gdh* gene fragment and the 3' *P.furiosus* *gdh* gene fragment into *Eco*RI-*HincII* linearized pUC18 resulted in pLUW450. All constructions were performed in *E.coli* TG1.

Purification of GDH

GDH was purified from *P.furiosus* as described previously (Consalvi *et al.*, 1991). To isolate *P.furiosus* GDH from *E.coli*, strain K12ΔH1Δ*trp* harboring pLUW410 was cultured at 28°C until an OD₆₀₀ of 0.5 was reached and incubated for another 2 h at 42°C. Subsequently, the culture was centrifuged for 10 min at 5000 r.p.m. and 4°C, medium was discarded and cells were resuspended in 20 mM potassium phosphate buffer, pH 6.5, 1 mM EDTA (buffer A) containing 1 µg/ml DNase. The suspension was sonified using a Branson sonifier and cell debris was removed by centrifugation at 13 000 r.p.m. for 10 min. The resulting supernatant was incubated for 10 min at 90°C and precipitated proteins were removed by centrifugation for another 10 min at 13 000 r.p.m. The sample was dialyzed against buffer A and applied to a 60 ml Matrex gel Red A column (Amicon) equilibrated with buffer A. After extensive washing with buffer A, GDH was eluted from the column using a step gradient of 120 ml of 0.10, 0.25, 0.35 and 1.00 M NaCl in buffer A. The fractions 0.25–1.00 M were pooled, concentrated and dialyzed against 0.1 M sodium phosphate buffer, pH 7.0, and contained pure GDH as judged by SDS-PAGE (12% polyacrylamide).

To purify *C.difficile* and the *P.furiosus*-*C.difficile* hybrid GDH, *E.coli* TG1 cultures harboring either pLUW420 or pLUW440 were centrifuged for 10 min at 5000 r.p.m. and 4°C. The medium was discarded and the cells were resuspended in 10 mM potassium phosphate buffer, pH 7.2, 1 mM EDTA (buffer B) containing 1 µg/ml DNase. The cells were broken in a French press under 1000 atmospheres pressure and cell debris was removed by centrifugation at 5000 r.p.m. for 20 min. Crude extracts were dialyzed against buffer B and applied to a Q Sepharose column (Pharmacia) equilibrated with buffer B. The column was washed until a stable baseline was recorded.

The elution of GDH was performed by applying a gradient of 0.0–1.0 M NaCl in buffer B. Active fractions were pooled and dialyzed against buffer B. Ammonium sulfate was added up to 40% saturation and the precipitated proteins were removed by centrifugation for 10 min at 13 000 r.p.m. The sample was loaded onto a phenyl Sepharose column (Pharmacia) equilibrated with 40% ammonium sulfate saturated buffer B. After extensive washing using equilibration buffer, elution was performed by a 40–0% ammonium sulfate gradient in buffer B. Active fractions were pooled, dialyzed against 1.0 mM potassium phosphate buffer, pH 6.8 (buffer C), and applied to a hydroxyl apatite column equilibrated with buffer C. After extensive washing, elution was performed by applying a 1.0 mM–1.0 M gradient of potassium phosphate, pH 6.8. Active fractions were pooled and dialyzed against 0.1 M sodium phosphate buffer, pH 7.0. To obtain pure *P.furiosus* GDH produced by *E.coli* without heat incubation, the protocol described for the *C.difficile* GDH purification was followed. The purity of the samples was analyzed by SDS-PAGE.

GDH activity assay

GDH activity was assayed using a double-beam Kontron Uvikon 860 spectrophotometer at 340 nm and 25°C. The 1 ml standard assay reaction mixtures (reverse reaction, unless mentioned otherwise) contained 100 mM potassium phosphate buffer, pH 7.6, and 0.75 mM α-ketoglutarate, 75 mM ammonia and 100 µM NADPH for *P.furiosus* GDH; 4 mM α-ketoglutarate, 600 mM ammonia and 0.2 mM NADH for *C.difficile* GDH; and 6 mM α-ketoglutarate, 900 mM ammonia and 0.2 mM NADH for the hybrid GDH. Fixed substrate concentrations for glutamate oxidation (forward reaction) were 6 mM L-glutamate and 0.25 mM NADP for *P.furiosus* GDH; 50 mM L-glutamate and 4 mM NAD for *C.difficile* GDH; and 60 mM L-glutamate and 6 mM NAD for the hybrid GDH. The amount of enzyme was varied to obtain a rate of ~0.03 Δ absorbance/min. We define 1 U of enzyme activity as the amount of enzyme which produces 1 µmol of product per minute under standard conditions. Kinetic parameters were calculated by a nonlinear regression analysis of the data using ENZFITTER (Leatherbarrow, 1987). For each calculation, at least eight velocity/substrate data pairs were used. Substrate specificity was studied by measuring GDH activity in the presence of 20 and 100 mM D-glutamate, glycine, L-leucine, L-threonine, L-asparagine, L-valine, L-proline, L-aspartate, L-glutamine or L-norvaline and 5 and 25 mM oxaloacetate, glyoxylate, 2-oxobutylate, 2-oxo-L-methyl-pentanoate, pyruvate or 2-oxovalerate.

Protein analysis

Protein concentrations were determined using Bradford reagent (Bio-Rad, Veenendaal, The Netherlands) with bovine serum albumin as a standard. Protein concentrations of pure samples were determined using the following extinction coefficients at 280 nm, calculated as described by Gill and von Hippel (1989): $\epsilon_m = 82\,500\text{ M}^{-1}\text{cm}^{-1}$ for *P.furiosus* GDH, $\epsilon_m = 66\,000\text{ M}^{-1}\text{cm}^{-1}$ for *C.difficile* GDH and $\epsilon_m = 86\,910\text{ M}^{-1}\text{cm}^{-1}$ for the hybrid GDH. Protein bands on SDS-PAGE gels were scanned and quantified using an Image Analysis System (Visionary, Fotodyne) equipped with ImageQuant (Molecular Dynamics) software. UV absorbance spectra were recorded at 20°C on a Perkin Elmer Lambda 16 spectrophotometer. The electrophoretic separation of proteins under native conditions was performed on 8% running gels. Apparent molecular masses were determined by an analytical gel filtration using a Superose 12

column (Pharmacia) as described previously (Consalvi *et al.*, 1991). Immunodetection was performed using antibodies raised against purified pyrococcal GDH (Eggen *et al.*, 1993). Protein microsequencing was carried out by cyclic Edman degradation on an Applied Biosystems amino acid sequencer model 477A (Eurosequence, Groningen, The Netherlands) using protein samples dialyzed against H₂O.

Effect of guanidinium chloride (GdmCl) on spectral properties

The enzymes (80 µg/ml) were incubated at 20°C for 24 h in 20 mM phosphate, pH 7.0, in the presence of increasing concentrations of GdmCl ranging from 0.0 to 7.2 M. UV absorbance spectra were monitored at 20°C in a Perkin Elmer Lambda 16 spectrophotometer. Fluorescence properties were studied at 20°C in a Perkin Elmer LS50B fluorimeter. Excitation was performed at 295 nm and emission was monitored at 300–400 nm. Far-UV CD spectra (210–250 nm) were recorded at 20°C in a Jasco J500A spectropolarimeter using a 0.2 cm cuvette. Fluorescence emission at 342 nm and observed ellipticities at 222 nm were normalized according to Pace (1986).

Thermostability

Enzyme thermostability was studied at 63, 65, 67 and 69°C for *C.difficile* GDH and hybrid GDH (5–50 µg/ml) and at 90 and 105°C for *P.furiosus* GDH (50 µg/ml). The enzymes were incubated in 20 mM sodium phosphate, pH 7.0, in sealed glass tubes for 0–24 h. Residual enzyme activity (forward reaction in the case of *C.difficile* GDH and hybrid GDH) was assayed at 25°C with respect to a control kept at room temperature. Slopes of first-order inactivation plots yielded rate constants for inactivation. These rate constants were used to calculate the free energy of the transition state for thermal inactivation ($\Delta G^\ddagger$) according to Segel (1975).

Results

Expression of the *P.furiosus* *gdh* gene in *E.coli*

High level overproduction of the *P.furiosus* GDH was obtained by cloning the *P.furiosus* *gdh* gene under the control of the λ P_L promoter on the expression vector pPLc2833 and introducing the resulting plasmid pLUW410 into *E.coli* Δ H1 Δ trp that carries a temperature-sensitive *cl857* repressor. Incubation at 42°C inactivates the *cl857* repressor and the *P.furiosus* *gdh* gene is expressed. The *P.furiosus* GDH produced in this system amounted to up to 20% of the total cell protein in *E.coli* and showed a subunit molecular mass of 46 kDa, similar to GDH isolated from *P.furiosus* cells (Figure 2, lanes 2 and 3). Heat incubation of the cell-free extract at 90°C for 10 min and the subsequent removal of precipitated *E.coli* proteins resulted in highly purified *P.furiosus* GDH (Figure 2, lane 4). The residual traces of *E.coli* proteins were removed by bioaffinity chromatography on a Matrex gel Red A column (Figure 2, lane 5). The enzyme showed a strong cross-reaction with antibodies raised against purified pyrococcal GDH, and native PAGE and subsequent immunoblotting revealed that at least 90% of the GDH was in the hexameric conformation (results not shown). N-terminal sequencing (results not shown) indicated that >98% of the *P.furiosus* GDH produced in *E.coli* contained its N-terminal methionine that is also present in the majority of GDH purified from *P.furiosus* (Maras *et al.*, 1994).

Total GDH activity of the incubated cell-free extract samples increased ~3-fold during the heat treatment, indicating an activation of *P.furiosus* GDH or the inactivation of inhibitors

Fig. 2. SDS-PAGE of *P.furiosus*, *C.difficile* and hybrid GDH. Lane 1, broad range molecular weight marker (Bio-Rad); lane 2, GDH isolated from *P.furiosus*; lanes 3–5, *E.coli* harboring pLUW410 cell-free extract (lane 3), supernatant after heat incubation (lane 4) and Matrex gel Red A fraction (lane 5).

present in the cell-free extract. The latter possibility was excluded because the addition of cell-free extract from an *E.coli* culture containing pPLc2833 to the reaction mixture did not affect the specific activity of purified *P.furiosus* GDH. *Pyrococcus furiosus* GDH purified from *E.coli* without heat treatment showed an apparent molecular mass of 260 kDa on a gel permeation column. Heat treatment of this 260 kDa fraction increased its activity ~10-fold, indicating that *P.furiosus* GDH is present as partially active hexamers in the *E.coli* cell-free extract which attained a fully active conformation upon incubation at 90°C. No differences in secondary and tertiary structure between the partially and fully active conformations could be detected in UV absorbance difference spectra, fluorescence emission and far-UV CD spectra (results not shown). These results indicate that *P.furiosus* GDH produced in *E.coli* is identical to that produced in *P.furiosus*, allowing for its use in protein engineering studies.

Construction and expression of hybrid *gdh* genes

Hybrid *gdh* genes were constructed containing either the substrate binding domain I or the cofactor binding domain II of *P.furiosus* GDH and the complementary domain of *C.difficile* GDH. The latter GDH was chosen because the family II *C.difficile* and *P.furiosus* GDHs share 52% identical amino acid residues (Lyerly *et al.*, 1991; Eggen *et al.*, 1993). In addition, there are considerable differences in the thermal stability and kinetic properties of the two enzymes (see below). To maximize the possibility of obtaining hybrid enzymes with catalytic activity, the fusion was located in between two fully conserved glycine residues, that in the known GDH structure of *C.symbiosum* are located in the loop between β -sheet f and α -helix 9 connecting the two domains (corresponding to residues Gly200 and Gly201 in *C.symbiosum*; Baker *et al.*, 1992).

Based on two pUC19 derivatives, pLUW401 and pLUW420, carrying the *P.furiosus* and *C.difficile* *gdh* genes, respectively,

Table II. Kinetic parameters for *P.furiosus* GDH isolated from *P.furiosus* and *E.coli*, *C.difficile* GDH and hybrid GDH obtained at 25°C by varying the concentration of the respective substrate while keeping all other parameters fixed

Substrate	<i>P.furiosus</i> GDH		<i>P.furiosus</i> GDH (<i>E.coli</i>)		<i>C.difficile</i> GDH		Hybrid GDH	
	K_m (mM)	Catalytic efficiency ^a (min ⁻¹ mg ⁻¹)	K_m (mM)	Catalytic efficiency ^a (min ⁻¹ mg ⁻¹)	K_m (mM)	Catalytic efficiency ^a (min ⁻¹ mg ⁻¹)	K_m (mM)	Catalytic efficiency ^a (min ⁻¹ mg ⁻¹)
L-Glutamate	1.0	5.5	–	–	2.7	28	18.1	0.69
α -Ketoglutarate	0.039	405	0.029	600	0.16	5906	0.74	225
Ammonia	4.6 ^b	3.4	5.1 ^b	1.9	121 ^d	7.9	228 ^d	0.74
	24.0 ^c	1.1	30.0 ^c	0.6				
NAD	–	–	–	–	0.65	145	1.08	12
NADH	–	–	–	–	0.043	27 621	0.047	3932
NADPH	0.0030	6533	0.0050	4600	ND	ND	ND	ND

ND, not detectable; –, not determined.

^a V_{max}/K_m .

^b1.25–20 mM ammonia.

^c30–150 mM ammonia.

^d8–900 mM ammonia.

two reciprocal hybrid genes were constructed following a procedure described previously (Tomic *et al.*, 1990). The essential reactions included PCR amplification with appropriate primers that created a *Bsp*MI site (Table I) followed by digestion with *Bsp*MI and ligation into linearized pUC19 (Figure 1). The complete DNA sequence of each hybrid gene was determined and found to be identical to that expected from the *P.furiosus* and *C.difficile* *gdh* gene sequences (Lyerly *et al.*, 1991; Eggen *et al.*, 1993). The resulting plasmids pLUW440 and pLUW450 (Figure 1) were each introduced into *E.coli* strain TG1. Cell-free extracts were assayed for GDH activity and compared with those obtained with the parental plasmids carrying the wild-type genes.

The activity of the hybrid GDH specified by pLUW440, containing the substrate binding domain I of *P.furiosus* and the cofactor binding domain II of *C.difficile*, could be detected using NAD(H) as cofactor. No activity could be detected with NADP(H). This *P.furiosus*–*C.difficile* hybrid enzyme amounted to up to 1% of total cell protein according to SDS–PAGE, possessed a subunit molecular mass of 46 kDa like the *P.furiosus* GDH, and showed a weak cross-reaction with antibodies raised against pyrococcal GDH (data not shown).

The complementary hybrid GDH specified by pLUW450, containing domain I of *C.difficile* GDH and domain II of *P.furiosus* GDH, also amounted to up to 1% of total cell protein according to SDS–PAGE, had a molecular mass of 45 kDa like *C.difficile* GDH, and showed a strong cross-reaction with the pyrococcal GDH antibodies (results not shown). However, despite its efficient production, this hybrid GDH showed no activity above the *E.coli* background GDH activity. In contrast, active *C.difficile* GDH was highly over-produced in *E.coli* TG1 harboring pLUW420, the parental plasmid that only differs from pLUW450 by the absence of *P.furiosus* DNA encoding domain II (results not shown; Lyerly *et al.*, 1991).

The active *P.furiosus*–*C.difficile* hybrid GDH specified by pLUW440 and the *C.difficile* GDH were subsequently purified to homogeneity, as judged by SDS–PAGE, and used for further characterization.

Kinetic parameters and substrate specificity

Pyrococcus furiosus GDH was purified from either *P.furiosus* or *E.coli* and used to determine the kinetic parameters as well as the substrate and cofactor specificities that were compared

with each other and those obtained with purified *C.difficile* or *P.furiosus*–*C.difficile* hybrid GDH (Table II).

Similar K_m values for α -ketoglutarate, ammonia and NADPH were obtained with *P.furiosus* GDH isolated from *P.furiosus* and *E.coli*. Even the Lineweaver–Burk plots that were obtained with the *P.furiosus* GDH from either source showed similar curvature for ammonia, indicating that at low concentrations the affinity for ammonia was higher than at high concentrations. The affinity for ammonia of the *P.furiosus* GDH from both sources at 25°C was the same as that reported previously at 60°C (Consalvi *et al.*, 1991). However, the K_m values of the *P.furiosus* GDH for NADPH and notably α -ketoglutarate appeared to be temperature dependent because those found at 25°C were four and nine times lower, respectively, than those reported previously at 60°C (Consalvi *et al.*, 1991). The calculated V_{max}/K_m values of the *P.furiosus* GDH isolated from either *P.furiosus* or *E.coli* were similar, indicating that this GDH showed a host-independent specific activity and efficiency.

The purified *C.difficile* GDH followed Michaelis–Menten kinetics for NAD, NADH and ammonia. No activity was found with phosphorylated cofactors. For L-glutamate and α -ketoglutarate, substrate inhibition was observed above 50 and 4 mM, respectively. *Clostridium difficile* GDH was unable to convert any of the tested substrate analogs.

The hybrid *P.furiosus*–*C.difficile* GDH also followed Michaelis–Menten kinetics only for NAD and NADH. For L-glutamate and α -ketoglutarate, substrate inhibition was observed for this enzyme above 60 and 6 mM, respectively. No saturation was observed with the hybrid GDH for ammonia up to 900 mM. V_{max}/K_m values indicated that the hybrid GDH was much less efficient than both parental enzymes. Substrate binding appeared to be affected in the hybrid because K_m values for glutamate, α -ketoglutarate and ammonia were much higher than for both parental enzymes (Table II). In addition, the hybrid was found to be more specific than GDH isolated from *P.furiosus* because it was not able to convert L-norvaline or 2-oxovalerate, while the *P.furiosus* GDH was able to utilize any of these substrate analogs (Consalvi *et al.*, 1991). Comparable K_m values for NAD and NADH were found with the hybrid and *C.difficile* GDH, while neither showed activity with the phosphorylated cofactor. The identical cofactor affinity and specificity of the *P.furiosus*–*C.difficile* hybrid and the *C.difficile* GDH indicate that these properties are solely determined by the cofactor binding domain.

Optimum temperature

Pyrococcus furiosus GDH isolated from *E.coli* showed an identical temperature optimum of 100°C as the enzyme isolated

Fig. 3. Temperature dependence of *C.difficile* GDH (●) and hybrid GDH (■) for glutamate breakdown.

Fig. 4. GdmCl-induced denaturation of *C.difficile* GDH (●), hybrid GDH (■) and *P.furiosus* GDH (▲) followed by fluorescence emission at 342 nm. The fraction of GDH present in the native conformation (f_N) is plotted against the GdmCl concentration.

from *P.furiosus* (Consalvi *et al.*, 1991; results not shown). This temperature optimum was much higher than that observed with the *P.furiosus*–*C.difficile* hybrid GDH that showed a similar optimum to the *C.difficile* GDH for glutamate formation (56–63°C). For glutamate breakdown, the optimum temperature of the hybrid GDH was slightly higher than that of *C.difficile* GDH (61–64 and 57–61°C, respectively; Figure 3). This indicates that exchange of the *P.furiosus* cofactor binding domain for that from *C.difficile* lowers the optimum temperature of the resulting hybrid enzyme to the level obtained with the GDH from the mesophilic parent.

Influence of GdmCl on spectral properties

The effect of GdmCl on fluorescence emission intensity and ellipticity of the *P.furiosus*, *C.difficile* and hybrid *P.furiosus*–*C.difficile* GDHs was studied. Identical denaturation curves were obtained with both spectroscopic techniques. Transition midpoints were found at 6.6, 3.2 and 1.5 M GdmCl for *P.furiosus* GDH, the hybrid and *C.difficile* GDH, respectively (Figure 4). The loss of secondary structure was accompanied by a loss of ability to regain catalytic activity after dilution of the denaturant because transition midpoints found by fluorescence and CD were similar to those found in reactivation experiments in which GdmCl was removed after 24 h of incubation by an at least 50-fold dilution (data not shown).

Thermostability

The thermostability of *P.furiosus* GDH purified from *P.furiosus* or *E.coli* was studied at 90 and 105°C (Table III). The 260 kDa gel permeation fraction containing *P.furiosus* GDH that was purified from *E.coli* without heat treatment lost its activity almost completely within several minutes of incubation at 105°C (data not shown). However, *Pyrococcus furiosus* GDH that was purified from *E.coli* using heat incubation showed an initial fast inactivation (loss of >50% activity within several minutes) and a second much slower inactivation at 105°C. The half-life of this second inactivation process was similar to that of GDH purified from *P.furiosus* (~1 h; Table III). At 90°C, there was only a small initial loss of activity of the heat-treated GDH purified from *E.coli*. *Pyrococcus furiosus* GDH isolated from *E.coli* showed a similar half-life at this temperature to that isolated from *P.furiosus* (~6 h; Table III).

The thermostability of *C.difficile* GDH and the *P.furiosus*–*C.difficile* hybrid GDH was determined at 63, 65, 67 and 69°C. At all these incubation temperatures the hybrid *P.furiosus*–*C.difficile* GDH showed a 2-fold shorter half-life than the *C.difficile* GDH, irrespective of the protein concentration (data not shown). At 65°C, this difference in stability amounted to up to 1.89 kJ mol⁻¹ (Table III). Both *P.furiosus* and *C.difficile* GDH remained soluble after thermal inactivation, whereas centrifugation of the thermally inactivated hybrid resulted in the

Table III. Half-life and $\Delta G^\ddagger$ values for the thermal inactivation of *P.furiosus* GDH isolated from *P.furiosus* and *E.coli* at 90 and 105°C, and of *C.difficile* GDH and hybrid GDH at 65°C

	65°C		90°C		105°C	
	$t_{1/2}$ (h)	$\Delta G^\ddagger$ (kJ mol ⁻¹)	$t_{1/2}$ (h)	$\Delta G^\ddagger$ (kJ mol ⁻¹)	$t_{1/2}$ (h)	$\Delta G^\ddagger$ (kJ mol ⁻¹)
<i>C.difficile</i> GDH	5.1	111.70	–	–	–	–
Hybrid GDH	2.6	109.81	–	–	–	–
<i>P.furiosus</i> GDH	–	–	5.5	120.41	1.20	120.72
<i>P.furiosus</i> GDH (<i>E.coli</i>)	–	–	6.4	120.87	0.86	119.68

$\Delta G^\ddagger$ values were calculated according to Segel (1975). –, not determined.

complete precipitation of the aggregated enzyme presumably because of the exposure of buried hydrophobic residues.

Discussion

We have reported previously the functional expression of the *P.furiosus* *gdh* gene in *E.coli* under the control of the λ P_L promoter (Eggen *et al.*, 1994). After further optimization described here, up to 20% of the total *E.coli* cell protein could be identified as *P.furiosus* GDH. After heat treatment, the enzyme was fully active, suggesting that it needs a temperature closer to the *in vivo* growth optimum of *P.furiosus* to be able to adopt its correct conformation. *Pyrococcus furiosus* GDHs isolated from *P.furiosus* and *E.coli* have similar apparent molecular weights, N-terminal sequences, subunit compositions, specific activities, thermoactivities, kinetic parameters and both chemical and thermal stabilities. This confirms that the overproduced enzyme can be used to study molecular adaptations to high temperatures.

Pyrococcus furiosus GDH produced by *E.coli* appears to be partially active in untreated cell-free extract. Native PAGE and subsequent immunodetection showed that at least 90% of the GDH formed in *E.coli* is present in a hexameric conformation. Furthermore, the hexameric GDH fraction obtained by gel filtration showed a clear increase in specific activity after heat treatment. These results strongly suggest the presence of partially active, hexameric GDH in the *E.coli* cell-free extract, which upon heat treatment arranges into a fully active structure. Furthermore, the enzyme isolated from *E.coli* without heat treatment is not fully thermostable because it showed an almost complete loss of activity upon incubation at 105°C, while the heat-incubated enzyme showed a considerably longer half-life (almost 1 h) at this temperature. This implies that either the *P.furiosus* GDH is present in *E.coli* in two conformations, one fully thermostable and one less thermostable, or that the enzyme always assumes a partially active conformation which is lost during its purification from *P.furiosus* but maintained in the purification method applied to *E.coli*. At present, we cannot discriminate between these two possibilities. It was reported recently that the expression of the *P.furiosus* *gdh* gene as a transcriptional and translational fusion using the T7 system in *E.coli* resulted in ~15% GDH of total cell protein. However, this overproduced *P.furiosus* GDH consisted of 50% inactive monomeric enzyme that assembled into hexamers upon heat incubation (Diruggiero and Robb, 1995). The correct hexameric subunit assembly in *E.coli*, which is reported here when the *P.furiosus* *gdh* gene is expressed under the control of the λ P_L promoter, might be caused by the activity of *E.coli* heat-shock proteins that are induced upon incubation at 42°C. However, more trivial explanations may also account for the previously observed incorrect folding in *E.coli* because the *P.furiosus* *gdh* gene used in those studies was PCR amplified and may contain unforeseen mutations (Diruggiero and Robb, 1995).

Exchanging parts of the polypeptide chain between homologous enzymes with different characteristics can be very useful in studying the role and localization of residues involved in achieving thermostability. The cloning and successful expression of the *P.furiosus* *gdh* gene allowed us to exchange domains between this GDH and GDH from any other organism to study the effects on its functionality and stability. The phylogenetically very distant mesophilic bacterium *C.difficile* was chosen to be the partner in the domain exchange. The gene fusion was located between two conserved glycine residues in the loop connecting the two domains, so as to

disturb as little as possible the folding of the resulting hybrids in comparison with the parental enzymes.

The activity of the *C.difficile*-*P.furiosus* hybrid GDH, containing the substrate binding domain I of *C.difficile* GDH and the cofactor binding domain II of *P.furiosus* GDH, could not be detected above the *E.coli* background, although it was clearly present in *E.coli* cell-free extract as judged by SDS-PAGE and immunoblotting. Apparently, the correct folding and/or assembly into an active hexameric structure was impaired in this mutant. In contrast, the complementary *P.furiosus*-*C.difficile* hybrid, with the substrate binding domain I from *P.furiosus* GDH and the cofactor binding domain II of *C.difficile* GDH, showed significant GDH activity when produced in *E.coli*. According to gel permeation chromatography, it had the same apparent molecular weight as *P.furiosus* GDH and *C.difficile* GDH, indicating a hexameric assembly similar to that of the parental enzymes. The *P.furiosus*-*C.difficile* hybrid enzyme is less efficient than both parental GDHs, as indicated by its kinetic parameters. Similar affinities for NAD and NADH for the hybrid and *C.difficile* GDH, and the inability to use the phosphorylated cofactor, indicate that binding of the cofactor is not altered in the hybrid that contains domain II from *C.difficile* GDH. This result agrees with the structures of the binary and tertiary complexes of *C.symbiosum* GDH, which show that the cofactor is almost exclusively bound by domain II (Baker *et al.*, 1992; Stillman *et al.*, 1993). Moreover, this result indicates that the cofactor binding domain II of GDH is structurally independent of domain I with respect to the binding of the cofactor. A similar independent activity was observed with the cofactor binding domain of the glyceraldehyde-3-phosphate dehydrogenase from *T.maritima* which, after overproduction in *E.coli*, retained its capacity to bind NAD and NADH (Jecht *et al.*, 1994). Both dehydrogenases show a similar domain arrangement, with the main difference being the orientation of the cofactor binding domain that is C-terminal in GDH and N-terminal in glyceraldehyde-3-phosphate dehydrogenase.

The low catalytic efficiency of the *P.furiosus*-*C.difficile* hybrid enzyme is very likely caused by less efficient substrate binding, as indicated by the higher K_m values for L-glutamate and α -ketoglutarate as well as ammonia compared with both parental enzymes. Interactions in the pocket on the surface of domain I, where the substrate side chain is bound (Stillman *et al.*, 1993), are not expected to be changed significantly because they are solely made by residues from domain I and the hybrid is even more specific towards substrate analogs than *P.furiosus* GDH. It is possible that the interdomain interactions involved in positioning the two domains in the correct orientation with respect to each other (and thus the positioning of substrate and cofactor) are less efficient in the hybrid, although preliminary modeling studies indicated that these interactions are considerably conserved in *C.difficile* and *P.furiosus* GDH.

The *C.difficile*-*P.furiosus* hybrid GDH shows a thermoactivity that is only slightly higher than that of the mesophilic parental enzyme. Apparently, the presence of a domain from a hyperthermostable enzyme does not significantly enhance the temperature at which the enzyme becomes susceptible towards thermal inactivation during catalysis, and thermoactivity might be determined by the weakest or more flexible part of the protein. It may be envisaged that the mesophilic domain reaches its maximum flexibility at the temperature optimum and starts unfolding, while the thermostable domain

is still too rigid to be able to bind and release substrates efficiently, explaining the low catalytic efficiency of the hybrid. Although the thermoactivity of the hybrid is relatively low, it is more stable towards chemical denaturation than its mesophilic parental enzyme, most likely because of the presence of the *P.furiosus* glutamate binding domain. The apparent two-state cooperative transition profile, obtained by fluorescence emission and ellipticity measurements of GdmCl-denatured hybrid GDH, is the same as for *C.difficile* GDH. Although kinetic parameters suggest that both domains are structurally independent and may be able to fold independently, the hybrid unfolds without any detectable preliminary loss of secondary structure of the *C.difficile* cofactor binding domain.

The effect of domain exchange on thermostability is dramatic. Instead of obtaining a hybrid displaying a thermostability in between those of the parental enzymes, as found in the case of chemical stability, the hybrid is even less thermostable than *C.difficile* GDH. Enzymes from (hyper)-thermophilic organisms are usually characterized by a high stability towards physical and chemical denaturation (Fontana, 1991). In the case of the hybrid, the increased stability towards GdmCl with respect to *C.difficile* GDH is not accompanied by a corresponding increase in thermostability. It should be noted that domain I of the hybrid does not consist completely of *P.furiosus* residues; the C-terminal α -helix 17 (numbering as in *C.symbiosum* GDH; Baker *et al.*, 1992) crosses over from domain II and is composed of *C.difficile* residues. Preliminary modeling studies indicate that the efficient packing of this α -helix against the *P.furiosus* domain is impaired because the possibility of forming hydrogen bridges is reduced and several residues will cause steric hindrance. The importance of a C-terminal α -helix for thermostability has been shown previously with the glyceraldehyde-3-phosphate dehydrogenase because its removal led to a dramatic decrease in the thermostability of the *M.fervidus* enzyme (Biro *et al.*, 1990). Furthermore, the packing of this C-terminal helix in GDH may also have effects on thermoactivity and catalytic efficiency because it is involved in orientating the two domains with respect to each other. The construction of hybrid GDHs with an additional exchange of this α -helix is in progress to allow us to study the possibility of obtaining more efficient and thermostable enzymes.

In conclusion, we have demonstrated that it is possible to successfully exchange parts of homologous proteins from micro-organisms that are phylogenetically very distant and belong to the archaea and bacteria. Furthermore, it has been shown that thermoactivity and thermostability are not necessarily linked to chemical stability and may be engineered independently, offering the possibility of constructing enzymes that are optimally active at low temperatures while retaining an extreme chemical stability.

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