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Glutamate dehydrogenase from hyperthermophilic Bacteria and Archaea: determinants of thermostability and catalysis at extremely high temperatures

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Abstract

Insight in the molecular mechanisms determining the extreme intrinsic thermostability of enzymes isolated from hyperthermophilic Archaea and Bacteria, is increasing rapidly as more comparative studies on their amino acid sequences, biochemical characteristics and three-dimensional structures are reported. In order to test the hypotheses arising from these data, protein engineering strategies have been applied to mesophilic and thermostable glutamate dehydrogenases (GDH) from different prokaryotic sources, followed by biochemical and structural characterization of the engineered enzymes. This review aims to provide an overview of (i) the state of the art on biochemical and structural characterization of thermostable GDHs, (ii) the construction and properties of hybrid GDHs obtained by domain swapping between GDHs from the mesophilic bacterium *Clostridium difficile* and the hyperthermophilic archaeon *Pyrococcus furiosus*, and (iii) the elucidation of the role of large ion-pair networks in conferring stability to GDHs from hyperthermophiles by the introduction of ion-pair networks into GDH from *Thermotoga maritima*.

Keywords: Glutamate dehydrogenase; Thermostability; Hybrid enzymes; Ion-pair networks

1. Introduction

The remarkable ability of hyperthermophilic Bacteria and Archaea to grow optimally at temperatures around the boiling point of water, has raised questions on how these microorganisms have stabilized their macromolecules, and notably, their enzymes. Comparative studies of amino acid sequence data, biochemical features and an increasing number of three-dimensional structures from homologous enzymes from mesophiles and (hyper)thermophiles, have revealed a large variety of molecular adaptations that might contribute to an elevated intrinsic stability. Proposed stabilizing features range from a decreased amount of thermolabile amino acid residues, improved hydrogen bonding patterns and hydrophobic packing, lowering the surface-to-volume ratio and multimerization, to an increased number of ion-pairs and the presence of large ion-pair networks (reviewed by

Abbreviations: GDH, glutamate dehydrogenase; GdmCl, guanidinium chloride

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Ref. [1]). To test the validity of these adaptation mechanism on the one hand, and generate novel enzymes with altered properties on the other hand, protein engineering approaches have been applied, followed by biochemical and structural characterization of the wild-type and engineered enzymes.

Among the enzymes used as model for studying adaptation mechanisms, glutamate dehydrogenase (GDH) is a suitable candidate because it is well studied, catalyzes an important reaction, and is present in all three domains of life [2,3]. A large number of primary sequences and several three-dimensional structures from GDHs obtained from organisms with a wide range of optimal growth temperatures are available (Table 1). Furthermore, the *gdh* genes from several organisms have been functionally overexpressed in *Escherichia coli*, facilitating the analysis of their expression products and allowing for a variety of mutagenesis approaches [4–6]. GDH couples carbon and nitrogen metabolism in the cell by the oxidative deamination of L-glutamate to α-ketoglutarate and ammonia, accompanied by the reduction of the cofactor NAD^+ or $NADP^+$. Phylogenetic analysis of GDH primary sequences revealed that GDHs can be organised in two enzyme families (Fig. 1) [5,7]. In family I, exclusively sequences from bacterial GDHs are present, in family II, GDH sequences from Bacteria as well as Archaea and Eukarya are found. In general, GDH is a multimeric enzyme consisting of six identical subunits (Fig. 2) [8]. The hexamer is composed of two trimers that are stacked upside down on top of each other. Each subunit contains an N-terminal, substrate-binding domain which forms almost all of the intersubunit interactions, and a C-terminal domain binding the cofactor $NAD(P)^+$. During catalysis the cleft in between the two domains, in which the active site is located, is opened and closed by a rotation of the cofactor binding domain with respect to the substrate binding domain [9]. This movement is thought to be mediated by residues and interactions in the so-called hinge region connecting the two domains. These interactions are therefore assumed to play an important role in determining activity and stability of the enzyme [10,11].

This overview describes biochemical and structural characteristics of available thermostable GDHs (summarized in Table 1), and

Table 1
Comparison of GDHs from mesophilic and hyperthermophilic Bacteria and Archaea

Organism	T_{growth} (°C)	% aa identity	Cofactor specificity	T_{opt} (°C)	$t_{1/2}$ (h; °C)	T_m (°C)	T_s (M)	3D	References
P. furiosus	100	100	NADP > NAD	> 90	12 (100)	113	6.1	2.2 Å	[4,10,16,19,21]
P. endeavori	98	96	NADP	nd	10.5 (100)	113			[22]
P. sp. KOD1	95	83	NADP > NAD	80	4 (100)				[24,52]
P. islandicum	100	nd	NAD	90	> 2 (100)				[23,51]
A. fulgidus	83	nd*	NADP	> 95	2.3 (100)				[27,53]
T. litoralis	88	87	NADP	> 95	2 (98)				[25,45]
T. profundus	80	nd	NADP	85	1 (90)				[26,54]
S. solfataricus	80	43	NADP > NAD	70	15 (80)		1.8		[17,18,55]
T. maritima	80	55	NADP < NAD	75	1.8 (85)	93	3.5	3.0 Å	[5,11,50]
C. difficile	37	52	NAD	60	5.1 (65)		1.5		[6,15]
C. symbiosum	37	37	NAD	nd	0.3 (52)	55		1.9 Å	[8,9,11,56,57]

T_{growth}: optimal temperature for growth of the organism. T_{opt}: temperature at which optimal enzymatic activity occurs. $t_{1/2}$: time in which 50% enzymatic activity is retained after incubation at given temperature. T_m: melting temperature as determined by differential scanning microcalorimetry or thermal denaturation monitored by circular dichroism. T_s: concentration of guanidinium chloride in M in which 50% of enzyme has retained native conformation. 3D: resolution of the available crystal structure. nd = not determined. *GDH has been isolated from *A. fulgidus* strain 7324 (DSM 8774) [27], no GDH is present in *A. fulgidus* strain VC-16 (DSM 4304) of which the complete genome sequence has been determined [59].

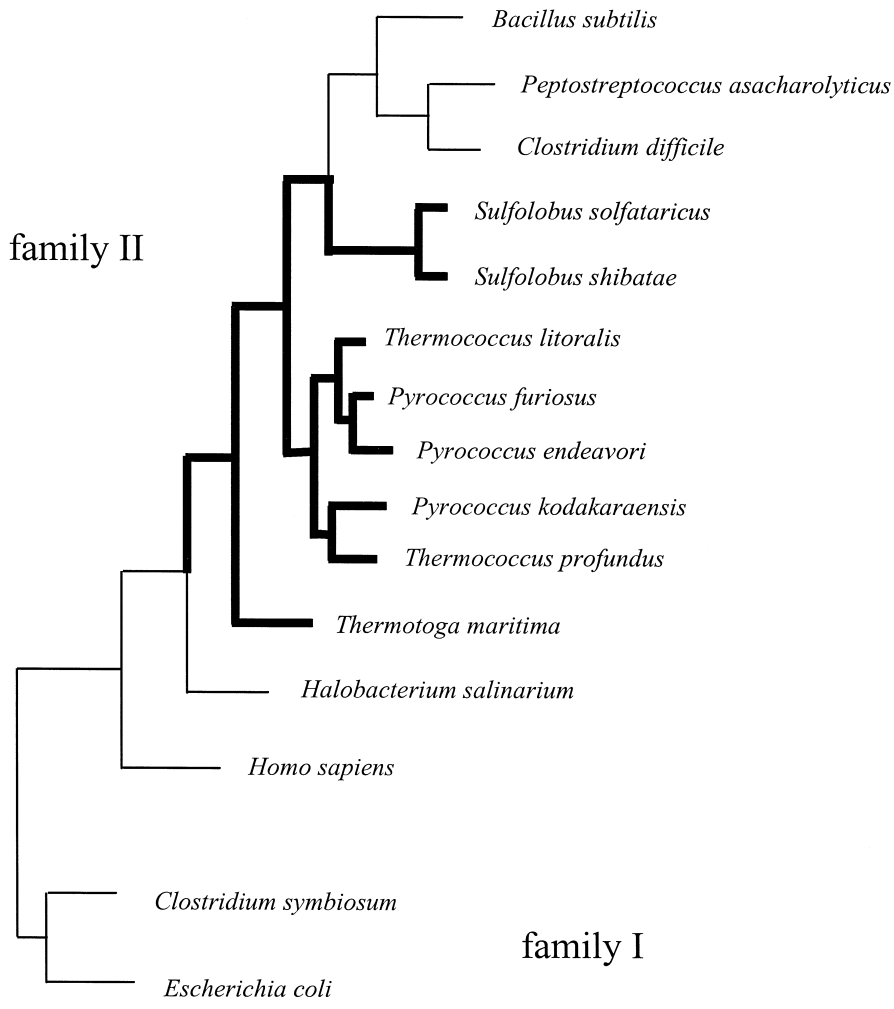

Fig. 1. Schematic phylogenetic tree for GDH amino acid sequences, redrawn and modified from Ref. [5] with additions taken from Refs. [7,24] showing the division of GDHs into two families. Branchings corresponding to GDHs from hyperthermophilic organisms are drawn in bold. For accession numbers see Refs. [5,7,24].

the characterization of mutant enzymes that have been constructed in order to study the mechanisms that are responsible for the thermoactivity and -stability of this well-studied class of enzymes (summarized in Table 2).

2. Thermostable GDHs

One of the most extensively studied GDHs is the enzyme from the mesophilic bacterium *Clostridium symbiosum*, that grows optimally at 37°C (Table 1). *C. symbiosum* GDH clusters among other bacterial GDH sequences in family I (Fig. 1). Its crystal structure was determined at 1.9 Å resolution and the complexes with the cofactor NAD and glutamate have been solved [8,9,12]. These different structures showed a rotation of the cofactor-binding domain with respect to the substrate-binding domain upon ligand binding, indicating hinge bending during catalysis. As a consequence, the active site which is located in the cleft between the two domains will close upon substrate and/or cofac-

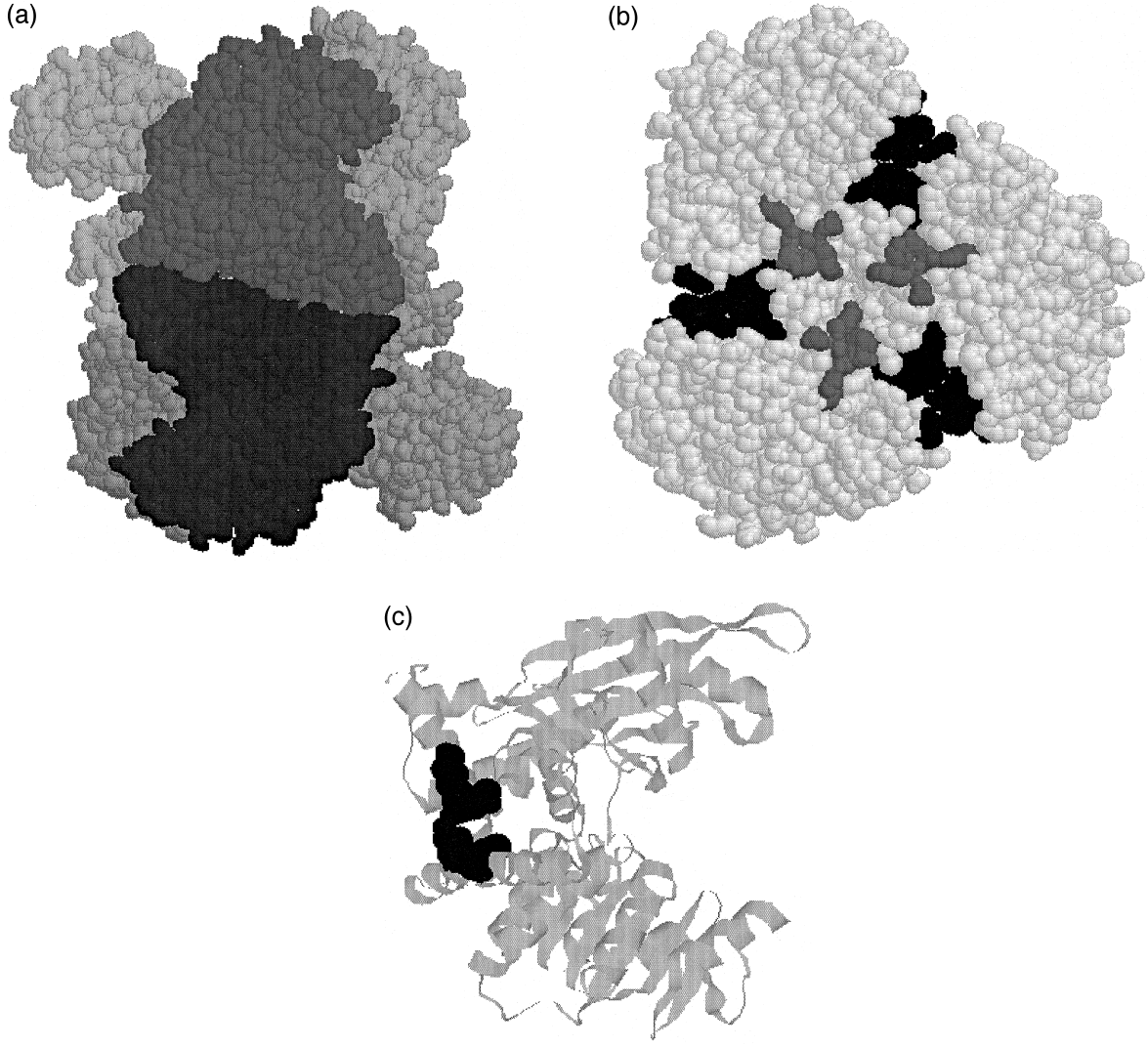

Fig. 2. Hexameric structure of *P. furiosus* GDH (a) and a view of the trimer interface (b). The 18-residue ion-pair networks are highlighted in black, the pairs of six-residue networks in grey. (c) The composition of each subunit in two separate domains. The upper, N-terminal domain binds the substrate in the cleft in between the two domains, the lower C-terminal domain binds the cofactor and is separated by a flexible hinge region from the first domain, allowing rotation of this domain resulting in opening and closure of the active site during catalysis. The five-residue hinge ion-pair network is highlighted in black. The figure was generated using coordinates of the *P. furiosus* GDH [10] and the programme RASMOL [58].

tor binding, thereby creating an adequate hydrophobic environment for hydride transfer during catalysis [9]. Construction of a dimeric form of *C. symbiosum* GDH by substitution of a phenylalanine residue for an aspartate at the dimer-interface resulted in an inactive enzyme, indicating that catalytic activity requires subunit interaction along the 3-fold symmetry axis [13]. Mutation of the general base of the catalytic reaction, aspartate 165 to histidine in the glutamate binding site, almost completely inactivates the enzyme. Moreover, this substitution results in the formation of a dimeric conformation instead of a hexamer [14]. This again indicates

Table 2
Overview of mutations and their effect on stability and activity of different enzymes that have been described in the text

Enzyme	Mutation	Description of mutation and effect	References
C. symbiosum GDH	Phe187Asp	Mutation at subunit interface results in inactive, dimeric GDH. Activity requires communication along 3-fold axis.	[13]
	Asp165His	Mutation of general base inactivates and results in dimer: Communication between active sites and subunit interfaces.	[14]
P. furiosus GDH *C. difficile* GDH	Hybrid GDHs containing subunit/cofactor binding domains from *P. furiosus*/*C. difficile* GDH.	Thermoactivity and stability towards thermal inactivation are determined by weakest or most flexible part of protein. Unlinking of stability towards temperature and chemical denaturants.	[15]
T4 lysozyme Barnase Arc repressor	Introduction of ion-pairs at protein surface or buried in interior	Surface exposed salt-bridges contribute only marginally to protein stability due to entropic cost of side-chain localization. Cooperativity in network: sharing of entropic costs. Buried salt-bridges may contribute considerably to stability because entropic cost has already been provided for during protein folding. Buried salt-bridges may be replaced by more stabilizing hydrophobic clusters.	[40–43]
T. litoralis GDH	Thr138Glu Thr138Glu/Asp157Thr	Addition of glutamate to charge cluster at subunit interface is destabilizing; second-site mutation results in mutant enzyme more stable than wild-type GDH.	[47]
T. maritima GAPDH	Removal of charges from surface-exposed or buried charge clusters.	Removal of peripheral ion-pairs has no effect. Disruption of four-residue ion-pair network leads to accelerated thermal denaturation.	[48,49]
T. maritima GDH	Asn97Asp Gly376Lys Asn97Asp/Gly376Lys	Introduction of six-residue ion-pair network into flexible hinge region does not stabilize GDH but has significant effects on thermoactivity and substrate affinity.	[50]

communication between the active site and the subunit interfaces. *C. symbiosum* GDH is not a thermostable enzyme and has a melting temperature of only 55°C [11]. Therefore, *C. symbiosum* GDH serves as mesophilic model enzyme in many studies dealing with GDH thermostability.

The gene encoding GDH from the mesophilic bacterium *C. difficile* has been cloned, sequenced and overexpressed in *E. coli* [6]. Interestingly, its expression product shows a remarkable thermostability with a half-life of activity at 65°C of 5.1 h and is optimally active around 60°C [15]. Amino acid sequence homology comparison showed that *C. difficile* GDH does not belong to family I with the other bacterial GDHs, but clusters with the eukaryal and archaeal sequences in family II (Fig. 1).

The gene coding for GDH from the hyperthermophilic bacterium *Thermotoga maritima* (optimum temperature for growth 80°C) was cloned and overexpressed in *E. coli* and the recombinant enzyme was characterized [5]. The enzyme showed optimal activity at 75°C [5] and a melting temperature of 93°C [11]. Whereas several archaeal GDHs prefer NADP over NAD, the *T. maritima* enzyme uses NAD in favor of NADP; while other bacterial GDHs only use the non-phosphorylated cofactor. Interestingly, in the phylogenetic tree *T. maritima* GDH does not

cluster with the other bacterial GDHs but is found in between that of halophilic and hyperthermophilic Archaea (Fig. 1) [5].

GDH from the hyperthermoacidophile *Sulfolobus solfataricus* (optimal temperature for growth 80°C) displays a half-life of activity of 15 h at 80°C (0.2 mg/ml protein) [16]. This stability was shown to decrease at lower protein concentrations and increased in the presence of guanidinium chloride. This suggests that the inactivation is probably caused by the exposure to the solvent of hydrophobic regions that leads to inactive aggregates, whose formation is efficiently prevented by GdmCl [17].

GDH from the hyperthermophilic archaeon *Pyrococcus furiosus* is the most stable GDH described up to now with a half-life of activity at 100°C of 12 h [18] and a melting point of 113°C [19]. In contrast to the thermostability of the *S. solfataricus* enzyme, that increased upon higher protein concentrations, the stability of the *P. furiosus* GDH appears to be independent of protein concentration, suggesting differences in the mechanism of stabilization between the two enzymes. The *P. furiosus gdh* gene was cloned, characterized and its expression was studied [3,4]. Efficient overexpression was achieved under control of the phage lambda P_L promoter that requires expression under heat-shock conditions. This resulted in identification of GDH hexamers that have a 10-fold lower specific activity than GDH isolated from *P. furiosus* [15]. A heat-incubation of the cell-extract resulted in the formation of fully active hexamers, indicating that enzymes from hyperthermophiles may need a temperature close to the in vivo growth optimum of the organism in order to fold correctly. A translational fusion of the PCR amplified *P. furiosus gdh* gene with the phage T7 system in *E. coli* resulted in the production of GDH comprising up to 15% of total cell protein [20]. Fifty percent of this GDH was formed in an inactive monomeric conformation and could also be fully activated by heat-treatment. In vitro refolding of *P. furiosus* GDH occurs via structured monomers to the formation of higher association states with a tertiary structure different from that of the native enzyme [21]. Again heat-treatment at 70°C is required for the hexamer to acquire activity.

Thermostable GDHs have furthermore been isolated from *P. endeavori* (formerly known as ES4, Ref. [22]), *Pyrobaculum islandicum* [23], *Pyrococcus* sp. KOD1 [24], *Thermococcus litoralis* [25], *T. profundus* [26] and *Archaeoglobus fulgidus* [27]. The GDH from *P. endeavori* is highly homologous to *P. furiosus* GDH (96%, Table 1) and has a similar half-life of activity and an identical melting point. *P. endeavori*, *T. litoralis*, *T. profundus* and *A. fulgidus* GDH, interestingly, all use exclusively NADP as cofactor, in contrast to the hyperthermostable GDHs mentioned before. *P. islandicum*, however, only is able to use NAD. All enzymes of which the amino acid sequence is known, cluster with the other hyperthermophilic sequences in family II (Fig. 1).

3. Hybrid GDH

The construction of hybrid enzymes by the exchange of large parts of the polypeptide chain between homologous proteins, has been an established approach to study the contribution of large substructures in determining stability and activity [28]. This approach was taken in order to investigate the role of the substrate and the cofactor domains of GDH [15]. Hybrid enzymes were constructed between the GDHs from the hyperthermophilic archaeon *P. furiosus* and the mesophilic bacterium *C. difficile*. Although the two microorganisms are phylogenetically very distant, their GDHs share 52% amino acid identity and both belong to family II (Fig. 1). On the other hand, there are considerable differences in thermostability and kinetic properties between the enzymes. Two hybrid genes were constructed, containing gene fragments coding for either the substrate or the cofactor binding domain of *P. furiosus* or *C. difficile* GDH (Fig. 3). The gene fusion was located in between two

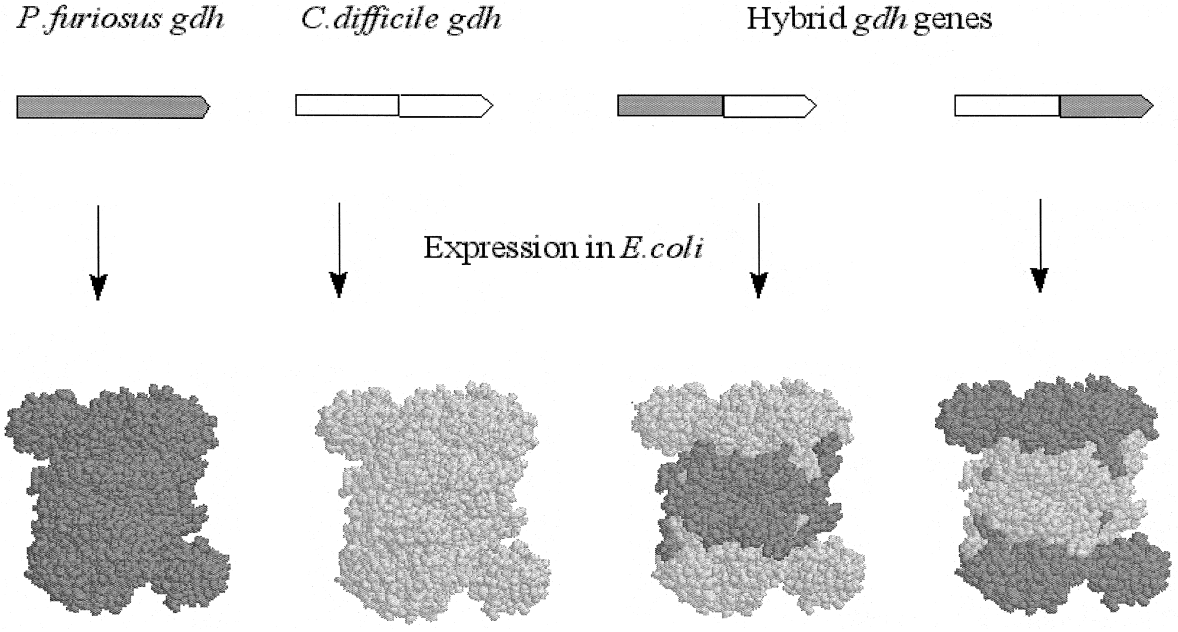

Fig. 3. Schematic drawing of wild-type and hybrid *gdh* genes and the expression in *E. coli* of their corresponding gene products.

conserved glycine residues in the loop connecting the two domains, in order to disturb as little as possible the folding of the resulting hybrids in comparison with the parental enzymes. Expression of the genes in *E. coli* resulted in formation of hybrid proteins as shown by SDS-PAGE and immunoblotting using antibodies raised against pyrococcal GDH. Activity of the hybrid containing the substrate binding domain of *C. difficile* and the cofactor binding domain of *P. furiosus* GDH could not be demonstrated, indicating that correct folding and/or assembly into an active hexameric structure is impaired in this mutant. In contrast, the complementary hybrid GDH with the substrate binding domain from *P. furiosus* and the cofactor domain from *C. difficile* GDH, was produced in a hexameric, active conformation. This *P. furiosus–C. difficile* hybrid maintained efficient cofactor binding as indicated by similar affinities for NAD and NADH as found for parental *C. difficile* GDH and the inability to use the phosphorylated cofactor. However, its overall catalytic activity was low, probably caused by less efficient substrate binding. This might be due to changed interdomain interactions that are involved in positioning the two domains in the right orientation with respect to each other. The temperature optimum of the *P. furiosus–C. difficile* hybrid is, apparently, determined by the weakest or more flexible part of the protein, because the optimum of the hybrid is only a few degrees higher than that of the *C. difficile* parental enzyme (65°C instead of 60°C). 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. In contrast, the presence of the pyrococcal substrate binding domain is increasing the transition point in guanidinium chloride induced denaturation by as much as 2-fold from 1.5 M GdmCl for *C. difficile* GDH to 3.0 M for the hybrid; *P. furiosus* GDH has a transition point at 6.1 M. However, the effect of domain exchange on thermostability is the most dramatic: Instead of obtaining a hybrid displaying a thermostability in between that of the parental en-

zymes, as found in the case of chemical stability, the *P. furiosus–C. difficile* hybrid is less thermostable than *C. difficile* GDH. These results indicate that, while in hyperthermostable enzymes properties like thermoactivity, thermostability and stability towards denaturants are very well optimized, they are not necessarily linked.

4. Ion-pairs and ion-pair networks in GDH

The recent elucidation of a large number of three-dimensional structures from enzymes from hyperthermophilic Bacteria and Archaea, comprise GDH, aldehyde ferredoxin oxidoreductase, ornithine carbamoyl transferase and citrate synthase from *P. furiosus* [10,29–31], Fe-superoxide dismutase from *Aquifex pyrophilus* [32], β-glycosidase and indole-3-glycerol phosphate synthase from *S. solfataricus* [33,34] and GDH, D-glyceraldehyde-3-phosphate dehydrogenase, phosphoribosyl anthranilate isomerase and signal transduction protein CheY from *T. maritima* [11,35–37]. In the majority of these structures, a high number of ion-pairs and large ion-pair networks were identified that are only partially or not at all present in homologous enzymes from mesophilic organisms. This finding strongly suggests an important role of these electrostatic interactions in determining enzyme hyperthermostability and/or thermoactivity.

The role of ion-pair interactions in stabilization of proteins has been a subject of debate for years. Some 20 years ago, Perutz and Raidt [38] and Perutz [39] already showed that ion-pairs may play a role in stabilization of proteins. Site-directed mutagenesis involving single, surface-located salt bridges in T4 lysozyme showed that their contribution to the stability of the protein is only marginal (0.1–0.25 kcal/mol) [40,41]. This is caused by the fact that the gain in free energy of folding is about equal to the entropic cost of dehydration and the reduction of the conformational freedom. In contrast, a buried salt-bridge in the same enzyme was shown to stabilize the native state by 3–5 kcal/mol as compared to the unfolded state [42]. However, a buried salt-bridge triad in the Arc repressor from bacterial phage P22 could be replaced by more stable hydrophobic interactions [43].

Theoretical considerations indicate that extensive networks may play an important role in maintaining enzyme stability or function at extreme temperatures. For each additional ion-pair that is added to a network, only a single residue needs to be desolvated and fixated. Indeed, multiple salt bridges in barnase show cooperativity; each single ion-pair in a three-residue network was found to enhance the strength of the other interaction by 0.8 kcal/mol [41]. In addition, part of the entropic cost of fixation is in many cases already provided during the folding of the protein since networks are often located in cavities and at interfaces. Furthermore, at high temperatures electrostatic interactions may become more important because hydration effects play a minor role and the dielectric constant decreases with temperature, resulting in an increased electrostatic energy upon formation of an ion-pair.

Ion-pair networks have been characterized most extensively in GDH. GDH from *P. furiosus* was found to contain more ion-pairs and larger ion-pair networks than GDHs from the mesophilic homologues from *E. coli*, *Neurospora crassa* and *C. symbiosum* (Table 3) [10,44]. The number of ion-pairs in *P. furiosus* GDH has been doubled compared to *C. symbiosum* and *E. coli* GDH. In the hyperthermostable enzyme, the residues involved in these ionic interactions, preferably pair with more than one partner. While in the *C. symbiosum* hexamer only about 10% of these residues form two and three ionic interactions, in the *P. furiosus* hexamer more than half of the residues form multiple ion-pair interactions. Consequently, extensive interacting networks are present in the *P. furiosus* GDH. In *C. symbiosum* and *E. coli* GDH, approximately 25% of the ion-pairs are arranged in networks of four residues, respec-

Table 3
Analysis of ion-pairs and ion-pair networks in GDH from *E. coli*, *C. symbiosum* and *P. furiosus* [44] and *T. maritima* [11]

	E. coli	*C. symbiosum*	*T. maritima*	*P. furiosus*
No. of ion-pairs per subunit	26	28	37	45
No. of residues per hexamer forming 1/2/3 ion-pairs	168/36/6	144/36/6	132/78/18	108/102/30
No. of residues forming 2/3/4 membered networks	84/24/12	72/24/12	66/33/18	54/24/12
No. of residues forming 5/6/7/18 membered networks	0/0/0/0	0/0/0/0	0/0/6/0	12/6/0/3
% of ion-pairs in ≥ four-residue networks	21	23	40	62
No. of intersubunit ion-pairs	nd	36	34	54

tively; in *P. furiosus* GDH this number amounts to 62%. While these four-residue clusters are the largest to be identified in the mesophilic enzymes, *P. furiosus* GDH contains networks comprising five, six and up to 18 residues.

The elucidation of the crystal structure of GDH from the hyperthermophilic bacterium *T. maritima* confirmed the ion-pair network hypothesis [11]. In line with the stability of this enzyme being intermediate between that of *C. symbiosum* and *P. furiosus* GDH, the total number of ion-pair interactions per monomer is in between that of the other enzymes (Table 3). In comparison to the *C. symbiosum* hexamer, the number of three- and four-residue networks in *T. maritima* GDH increased and six networks of seven residues are present. However, these extra ion-pairs are mainly located within each subunit, while in *P. furiosus* GDH many large networks are found at the subunit interface. In contrast, the subunit interface in *T. maritima* GDH seems to be optimized by hydrophobic interactions.

In order to obtain more support for the ion-pair network hypothesis, a structure-based homology modelling study was recently carried out using sequences from GDHs from 10 different sources, spanning a large temperature spectrum [45]. Specific attention was given to the 18-residue network that is three times present at the subunit interface of *P. furiosus* GDH, and the three pairs of six-residue networks, also present at the subunit interface in between the 18-residue networks (Fig. 2). For all GDHs that were studied, the networks decreased in size, became fragmented and finally completely disappeared with decreasing thermostability. The formation of large, intricate ion-pair networks seems to be specifically correlated with enzymatic operation close to or above 100°C.

The first experimental approach addressing the ion-pair network hypothesis in a hyperthermophile, is the acid-induced denaturation of *P. furiosus* GDH and biochemical characterization of the unfolded enzyme [46]. Lowering the pH into the acidic region results in protonation of negatively charged groups in the enzyme, and the shielding of dipoles by the added ions resulting in the disruption of electrostatic interactions. Therefore, any residual structure at low pH values should be a result of hydrophobic interactions. GdmCl-induced denaturation was found to be pH dependent, indicating that electrostatic interactions play indeed a major role in determining the stability of the enzyme. However, at pH 1 a monomeric protein was formed that could be further unfolded by GdmCl with a higher transition midpoint than found for other thermophilic and mesophilic GDHs [46]. This finding indicates that, at least within one subunit, electrostatic interactions cannot be the predominant force in *P. furiosus* GDH.

Recent results on mutagenesis of *T. litoralis* GDH indicate that the introduction of a charged residue at the subunit interface (T138E) in first instance results in a lower thermostability of the mutant *T. litoralis* GDH [47]. The introduction of a second, nearby mutation (D157T) that does not take part in the presumed ionic network, elevates the thermostability of the double mu-

tant above that of the wild-type enzyme. These results confirm that, assuming that the presumed ionic interactions are indeed formed in the mutant enzyme, ion-pair networks might indeed be a stabilizing feature in hyperthermostable GDH.

5. Introduction of ion-pair networks into *T. maritima* GDH

In order to experimentally verify the presumed stabilizing contribution of ion-pair networks to the thermostability of enzymes from hyperthermophiles, several strategies can be followed. One is to remove the interaction from the enzyme by replacement of the charged amino acid for an uncharged residue using site-directed mutagenesis. This approach was used to demonstrate the stabilizing effect of small ion-pair networks in glyceraldehyde-3-phosphate dehydrogenase from *T. maritima* [48,49]. A drawback of this approach, however, is the fact that always more than only the ionic interaction is affected. The hydrogen bonding potential will be changed and cavities or steric hindrance may be introduced. An alternative approach is to introduce charged residues that are present in a thermostable enzyme, into a less stable homologue. We employed this strategy to study the role of *P. furiosus* GDH ion-pair networks by introducing these into the less stable GDH from the hyperthermophilic bacterium *T. maritima* [50]. The GDHs from *P. furiosus* and *T. maritima* share 55% amino acid identity and are very homologous in secondary and tertiary structure as well [11]. However, the *P. furiosus* GDH has a higher melting point than the *T. maritima* GDH (113°C vs. 93°C) [19,11] and contains significantly larger ion-pair networks. The trimer interface in *P. furiosus* is almost completely charged because of the presence of three 18-residue networks which are separated by three pairs of six-residue networks (Fig. 2) [10]. The *P. furiosus* GDH 18-residue network is fragmented into two small ones of only four residues in the *T. maritima* GDH and from the pyrococcal six-residue network only one residue is conserved. The major difference between the hinge regions connecting the two domains, is the presence of a five-residue ion-pair network in the *P. furiosus* enzyme that is absent in the less stable *T. maritima* GDH. This five-residue ion-pair network links secondary structure elements from both domains, is surface exposed, and located opposite to the substrate binding site at a distance of more than 12 Å (Fig. 2). The five participating residues in *P. furiosus* GDH are positioned in such a way that four salt bridges are formed. *T. maritima* GDH contains, instead of aspartate and lysine, asparagine and glycine at positions 97 and 376, respectively, resulting in the loss of three of the four ionic interactions. An additional lysine in *T. maritima* GDH (which is an asparagine in the pyrococcal enzyme and does not interact at all with the network) brings the number of ion-pairs in this region to two [50].

Two single mutant *T. maritima* GDHs were generated and characterized, containing the substitutions asparagine to aspartate at position 97 and glycine to lysine at position 376, as well as double mutant N97D/G376K [50]. The three-dimensional structure of the double mutant was solved at 3.0 Å resolution and revealed that a six-residue ion-pair network is present in this mutant, this network being even larger than the one in the *P. furiosus* enzyme. Apparent melting temperatures of 91, 92 and 91°C were determined for N97D, G376K and the double mutant, respectively, not differing significantly from that of the wild-type GDH (93°C). In addition to this, identical transition midpoints in guanidinium chloride induced unfolding were found; 3.5 M for wild-type GDH and the single mutants, 3.6 M for the double mutant. In contrast to this, thermal inactivation at 85°C occurred more than 2-fold faster for all mutant enzymes than for the wild-type GDH. The effect of the two single mutations was not additive, suggesting a stabilizing feature between these two residues in the double mutant which

might be either or both the ionic interaction or the extra hydrogen bond.

In addition to the effect of the mutations on the stability of the enzyme, changes in the thermoactivity and kinetic parameters of the mutants in comparison with the wild-type GDH were studied [50]. At temperatures of 65°C and higher, the wild-type and the three mutant enzymes showed identical specific activities. However, at 58°C the specific activity of N97D/G376K and G376K was found to be significantly higher than that of the wild-type and N97D GDHs. Enzyme inactivation started at 58°C for G376K and N97D/G376K, at 62°C for the wild-type GDH and at 66°C for N97D. For the wild-type GDH, K_m values for α-ketoglutarate and NADH were found to decrease, and that for ammonia to increase, with increasing temperature. K_m values for all substrates changed differently for each mutant GDH and with temperature, indicating that the mutations have pronounced effects on catalysis and enzyme efficiency at different temperatures. The most pronounced effect was found for mutant N97D that has a 10-fold lower K_m value for NADH at 58°C than at 25°C and is at this temperature more efficient than the wild-type GDH on this substrate.

In conclusion, these results indicate that the engineered ion-pair interactions in the hinge region do not affect the stability towards temperature- or guanidinium chloride-induced denaturation but rather affect the specific activity and substrate affinity of the enzyme and the temperature at which it functions optimally.

In addition to studying the role of ion-pair networks in the flexible hinge region of GDH, we studied the contribution of the large intersubunit network that comprises 18 residues in *P. furiosus* GDH and is fragmented into two small networks of only four residues in *T. maritima* GDH (Fig. 2) (Lebbink et al., manuscript in preparation). In order to reconstruct the network into the less stable enzyme, two positive and two negative charges were introduced into *T. maritima* GDH as single substitutions, several double and triple combinations and the quadruple mutant. Preliminary analysis revealed different effects of each substitution on thermal inactivation and activity of the enzyme and also pointed out the need for balanced charges (Lebbink et al., manuscript in preparation).

6. Conclusion

A large amount of structural and biochemical data of wild-type and mutant GDHs has been generated during recent years, contributing greatly to our knowledge of the molecular adaptations that govern enzyme hyperthermostability and activity. This wealth of information and the fact that GDH is a large, multimeric enzyme containing a high number of complex ion-pair networks, makes it an excellent model system to study the role of these features in determining protein hyperthermostability, which is undoubtedly one of the main topics in protein chemistry at the present time. We have shown that the introduction of a six-residue ion-pair network in the flexible hinge region of *T. maritima* GDH is not affecting the melting temperature of the enzyme, leads to somewhat faster thermal inactivation, but has pronounced effects on thermoactivity and kinetic parameters [50]. The finding that the presence of the ion-pair network does not seem to increase the stability of the enzyme, may indicate that in this region of the enzyme, ion-pairs indeed do not contribute to stability. Alternatively, these results may reflect the considerable differences between the archaeal and the bacterial enzyme (amino acid identity 55%). Although the direct environment of the hinge network is highly conserved, small changes that are not visible in the three-dimensional structures, or unfavourable interactions with macroscopic parameters like the existing electrostatic field, may lead to non-optimal interactions in the mutant enzyme, resulting in a faster thermal inactivation. This consideration is supported by the fact that in *T. litoralis* GDH two mutations (one introducing a charge, the

second one being nearby but removing a charge) apparently are needed for stabilization of this enzyme [47]. The degree of homology between *P. furiosus* and *T. litoralis* GDH is much higher (87%) than that between *P. furiosus* and *T. maritima* (55%) and the results suggest that already in a highly homologous system, second-site mutations are required to obtain the desired stabilization. For a further interpretation of these data, it is essential to determine the three-dimensional structures of mutant *T. litoralis* GDH, and investigate whether the additional charge indeed participates in an ion-pair network and what is the basis for the stability-rescuing effect of the second-site mutation.

The fact that GDH is a large, multimeric enzyme is not only an advantage. Due to this complexity, GDH does not unfold reversibly during thermal incubation or guanidinium chloride induced denaturation and therefore no thermodynamic analysis of wild-type and mutant GDHs can be performed. This means that only data about the thermoactivity and the kinetic stability of GDH can be obtained. To circumvent this problem, smaller model systems like monomeric proteins or isolated domains could be used. However, only few monomeric thermostable enzymes have been reported to date and the enzymatic activity of isolated domains is difficult to assess. Furthermore, much smaller networks are to be expected in these systems, and intersubunit networks—the most prominent feature in the most stable GDH—cannot be studied in this way. Therefore, GDH will remain one of the challenging model systems for analyzing structure-stability relations in the near future.

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