# Isocitrate dehydrogenase from extremophiles; Molecular adaptations to high temperatures

**Runar Stokke**


Thesis submitted in partial fulfilment of the requirements
for the degree philosophiae doctor (PhD)

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Department of Biology
University of Bergen

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## Contents

## Acknowledgements

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Bergen, 05-10-06

## Abbreviations

| | |
|---|---|
| 3D | 3-dimensional |
| *Af*IDH | *Archaeoglobus fulgidus* isocitrate dehydrogenase |
| *Ap*IDH | *Aeropyrum pernix* isocitrate dehydrogenase |
| ASA | Accessible surface area |
| DSC | Differential scanning calorimetry |
| HDH | Homoisocitrate dehydrogenase |
| IDH | Isocitrate dehydrogenase |
| IPMDH | Isopropylmalate dehydrogenase |
| *Mc*IDH | *Methylococcus capsulatus* isocitrate dehydrogenase |
| NAD+ | Nicotinamide adenine dinucleotid |
| NADP+ | Nicotinamide adenine dinucleotid phosphate |
| RMSD | Root Mean Square Deviation |
| *Ta*IDH | *Thermoplasma acidophilum* isocitrate dehydrogenase |
| TCA cycle | Tricarboxylic acid cycle |

## List of papers

The study is based on the following publications referred to by their roman numerals in the text:

**I** Mikael Karlström, Runar Stokke, Ida Helene Steen, Nils-Kåre Birkeland, and Rudolf Ladenstein. 2005.
Isocitrate Dehydrogenase from the Hyperthermophile *Aeropyrum pernix*: X-ray Structure Analysis of a Ternary Enzyme-Substrate Complex and Thermal Stability. Journal of Molecular Biology 345: 559-577.

**II** Runar Stokke, Mikael Karlström, Nannan Yang, Ingar Leiros, Rudolf Ladenstein, Nils-Kåre Birkeland, and Ida Helene Steen. 2006.
X-ray structure analysis of isocitrate dehydrogenase from the hyperthermophile *Archaeoglobus fulgidus*: thermal stability and domain swapping.
Submitted to Extremophiles 2006.

**III** Runar Stokke, Nils-Kåre Birkeland, and Ida Helene Steen.
Thermal stability and biochemical properties of isocitrate dehydrogenase from the thermoacidophilic archaeon *Thermoplasma acidophilum*
Submitted to Extremophiles 2006

**IV** Runar Stokke, Dominique Madern, Nils-Kåre Birkeland, and Ida Helene Steen.
Biochemcial characterization of isocitrate dehydrogenase from *Methylococcus capsulatus* reveals a unique $NAD^+$-dependent homotetrameric enzyme.
Submitted to Archives of Microbiology 2006.

## Summary

Life on earth has adapted to a wide variety of environmental conditions, many of which are extreme to us humans. Temperature is one of the most important factors limiting biological activity and survival, but so far, biological activity has been observed in a wide temperature range; from -20 to + 121 °C. The organisms living at different temperature ranges are classified according to their optimal growth temperature; psychrophiles below 15 °C, mesophiles 15-45 °C, thermophiles 45-80 °C and hyperthermophiles above 80 °C. The forces governing the high thermal stability of enzymes from (hyper)thermophiles are of great interest due to their possible applications in different industries. Although several comparative structural studies on homologous enzymes from mesophilic and (hyper)thermophilic organisms have been performed no universal feature responsible for the high thermal stability of (hyper)thermophilic enzymes have been observed. In order to understand more closely the molecular mechanisms underlaying protein stability, heat-adaptive features of isocitrate dehydrogenase (IDH) from two hyperthermophiles, *Aeropyrum pernix* (*Ap*IDH) and *Archaeoglobus fulgidus* (*Af*IDH), have been analysed based on their 3D-structures and comparisons to mesophilic homologs, in particular to IDH from *E. coli* (*Ec*IDH). Isocitrate dehydrogenase, a key enzyme in the tricarboxylic acid cycle (TCA), catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate using $NAD(P)^+$ as cofactor. A previous study revealed *Ap*IDH as a hyperstable IDH with an apparent melting temperature ($T_m$) of 109.9 °C, whereas *Af*IDH was less stable with a $T_m$ of 98.5 °C. In the present work, $T_m$s of the thermophilic *Thermoplasma acidophilum* IDH (*Ta*IDH), *Methylococcus capsulatus* IDH (*Mc*IDH) and the mesophilic *Ec*IDH were determined to 80.0, 70.3 and 52.6 °C, respectively. Each of the (hyper)thermophilic enzymes showed significantly higher $T_m$ than *Ec*IDH. To investigate the importance of certain structural traits for the thermal stability of the hyperthermophilic *Ap*IDH and *Af*IDH, a mutational analysis and a domain-swapping experiment was performed, respectively.

The hyperthermophilic IDHs showed additional stabilization of their N-terminus; *Ap*IDH by a disulfide bond, and *Af*IDH through an aromatic cluster. The size and positioning of ionic networks differed among the hyperthermophilic *Ap*IDH and *Af*IDH and the mesophilic *Ec*IDH. *Ap*IDH possessed a higher number of intra-and inter-subunit ion pairs and the ionic networks were larger than observed in *Af*IDH and *Ec*IDH. Mutational disruption of a 7-membered inter-domain network demonstrated the importance of this ionic network in the thermal stability of the former enzyme. The hyperthermophilic *Af*IDH was, however,

strikingly similar to *Ec*IDH and possessed almost the same number of ion pairs and ionic networks. However, a unique inter-subunit 4-membered ionic network between the clasp-domain and the small domain was found in the former enzyme. To investigate the contribution of the clasp-domain to the thermal stability of *Af*IDH, chimeras of the two enzymes were constructed by domain-swapping. An aromatic cluster which is believed to further strengthen the subunit interaction was also identified in *Ap*DIH and *Af*IDH. This cluster also seems to be conserved conserved in the moderately thermostable *Ta*IDH.

The biochemical characterization of *Mc*IDH identified the first homotetrameric $NAD^+$-dependent form of a bacterial IDH with a high sequence similarity to homoisocitrate dehydrogenase and isopropylmalate dehydrogenase.

In conclusion, the work presented in this thesis has increased our knowledge on how enzymes from (hyper)thermophiles can adapt to and resist thermal denaturation at high temperatures.

# 1. Introduction

The biosphere comprises a large variety of habitats having extraordinary variations in physical and chemical characteristics. In a human perspective many of these habitats seem "extreme" beeing a 37 °C species living on the surface of the planet exposed to an oxygen-rich atmosphere. A vide variety of organisms are adapted to these harsh conditions, termed extremophiles, and defined by the physical conditions at which they live, such as extreme temperatures ((hyper)thermophiles and psychrophiles), high salinity (halophiles), and extreme pH values (alkaliphiles and acidophiles). Most of the extremophiles belong to the archaeal lineage, however, extremophilic species are also found both amongst the Bacteria and Eukarya.

According to pylogenetic analyses based on 16S rRNA sequences, organisms adapted to high temperature occupy the shortest and deepest lineages close to the branching point between Eukarya, Bacteria and Archaea (Figure 1). Thus, heat-loving organisms are believed to be the early descendents of our last universal common ancestor (Stetter, 1996). Since temperature affects essentially all cellular content and processes, this has served as a major driving force in evolution. The enzymes from these heat-resistant organisms can therefore be used as model systems in order to understand enzyme evolution, the different mechanisms that confer protein stability, and also the temperature limits for enzyme function. The knowledge gained from such model systems can lead to efficient protein engineering studies with a wide range of biotechnological applications.

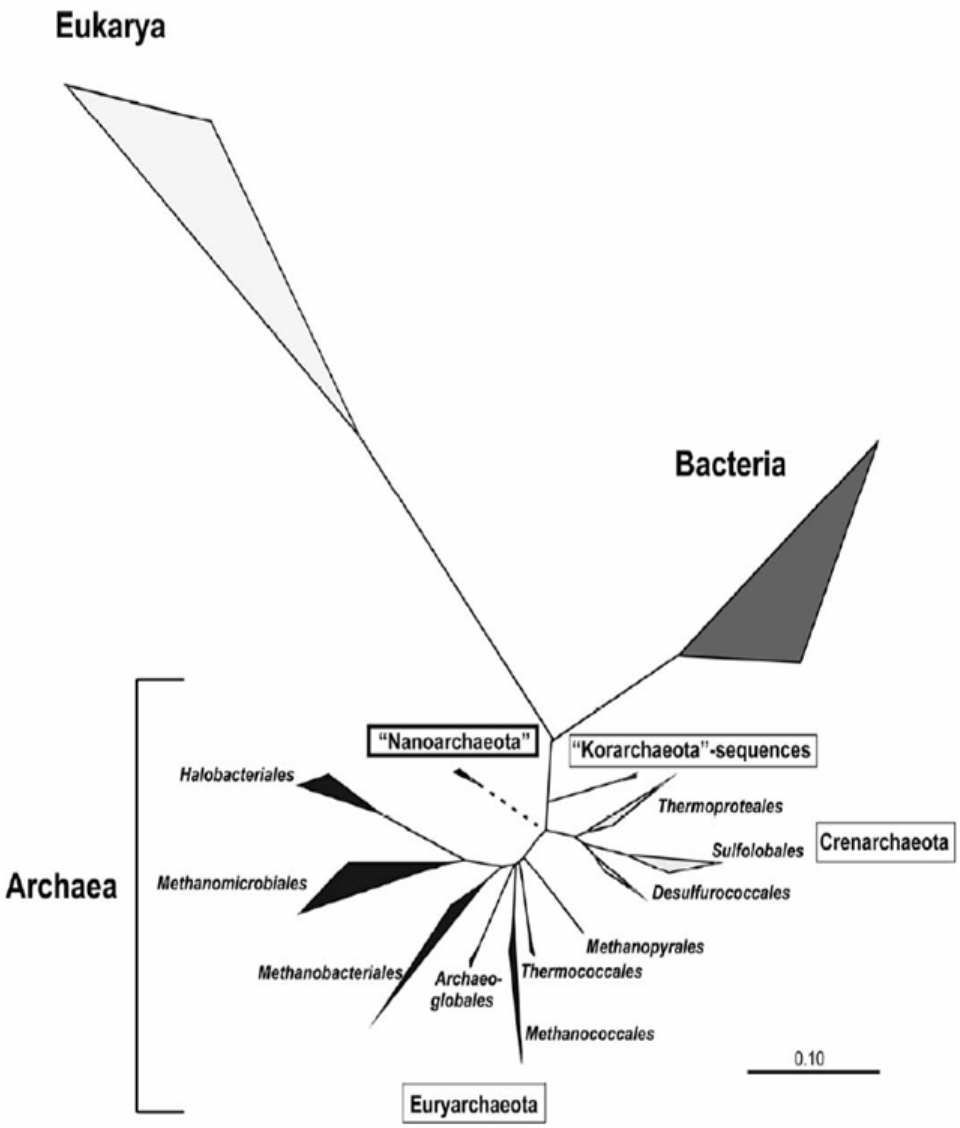


**Figure 1** Phylogenetic tree based on 16S rRNA sequence comparisons. The interrupted line of the "Nanoarchaeota" was set to outline the uncertain position of the branching point (Huber *et al.*, 2003).

### 1.1 Life at high temperature

All organisms are adapted to a specific temperature growth range. Depending on their optimal growth temperatures ($T_{opt}$), microorganisms are classified into different groups; psychrophiles ($T_{opt}$< 15 °C), mesophiles ($T_{opt}$ 15-45 °C), thermophiles ($T_{opt}$ 45-80 °C) and hyperthermophiles ($T_{opt}$ > 80 °C) (Figure 2).

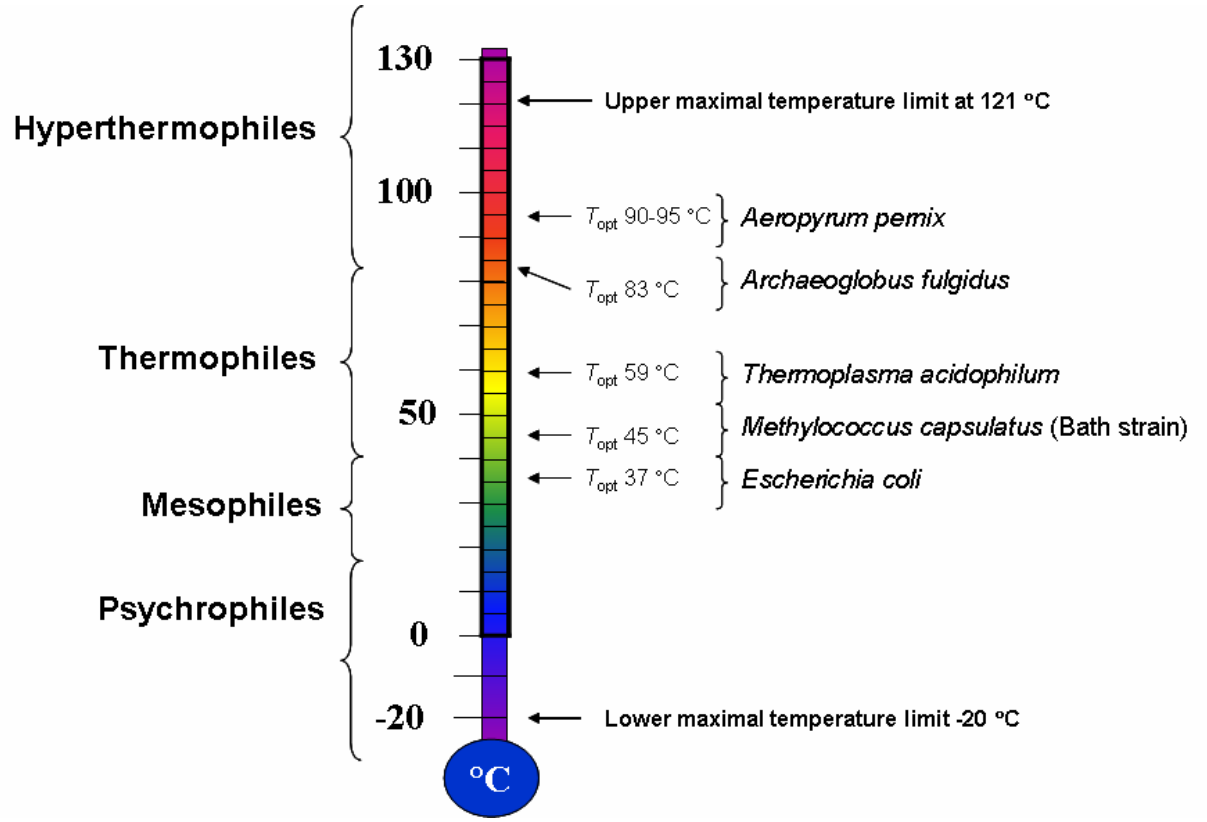


**Figure 2** Temperature scale showing the temperature limits for microbial growth. The optimal growth temperatures ($T_{opt}$) of microorganisms for which the enzymes studied in this thesis are indicated.

In the late 1960`s Thomas Brock and colleagues isolated the first (hyper)thermophile, *Sulfolobus acidocaldarius*, from an acidic hot spring in Yellowstone National Park, USA (Brock *et al.*, 1972). Since then (hyper)thermophiles have been isolated from diverse habitats including both terrestrial and marine hot environments in geothermally heated areas along tectonic fracture zones. The temperatures in active volcanoes are much too high to support life. However, fumaroles and hot springs associated with volcanic activity provide more suitable temperatures (Stetter, 2003). One of the most heat-resistant of all known hyperthermophiles is the facultatively aerobic crenarchaeon *Pyrolobus fumarii*, who thrives at 106 °C but grows up to 113 °C (Blöchl *et al.*, 1997). However, isolation of the archaeon "strain 121", a *Pyrodictium*- and *Pyrobaculum*-related strain, extended the upper temperature limit for life to 121 °C (Kashefi & Lovley, 2003). The lowest temperature limit reaches down to around -20 °C, which has been reported for bacteria living in permafrost soil and sea ice (D'Amico *et al.*, 2006).

All hyperthermophiles known so far are prokaryotes and most belong to the domain Archaea. However, hyperthermophilic bacterial species from the order of *Thermotogales* and *Aquificales* have been decribed (Deckert *et al.*, 1998; Huber *et al.*, 1986). Although no eukaryotic organisms have been described as hyperthermophilic, a few eukaryotes such as the Pompeii worm (*Alvinella pompejana*), have been found in the hot waters of deep-sea hydrothermal vents where they experience a thermal gradient of 60 °C or more over its body length (Campbell & Cary, 2001; Cary *et al.*, 1998).

Due to their significant scientific interest as model organisms for both basic research and as a source of biotechnologically important molecules, the genomes of hyperthermophilic microorganisms, i.e. *Methanocaldococcus jannaschii*, *Archaeoglobus fulgidus* and *Aquifex aeolicus* (Bult *et al.*, 1996; Deckert *et al.*, 1998; Klenk *et al.*, 1997), were among the first to be sequenced. There is now an increasing knowledge about how the cellular components such as the cellular membrane, DNA, RNA, and proteins of hyperthermophiles are adapted to function at high temperatures and how they withstand thermal denaturation at elevated temperatures.

The cytoplasmic membrane in Bacteria and Archaea functions as a barrier between the cytoplasm and the environment and thus, represents the first line of defence against external forces. The cytoplasmic membrane is further crucial for metabolic energy as generated through an electrochemical gradient across the membrane and subsequently transformed to other forms of energy (Albers *et al.*, 2000). The membrane composition of thermophilic and extremely acidophilic Archaea contains tetraether lipids (Albers *et al.*, 2000). These lipids have limited permeability for protons even at high temperatures which makes it possible for thermophilic Archaea to maintain a viable proton motive force under the extreme conditions. Furthermore, the Archaea, psychrophilic and mesophilic Bacteria have the ability to adjust the lipid composition of their membranes so that the proton permeability remains within a narrow range (Albers *et al.*, 2000). The changes in membrane composition is also needed to keep the membrane in a liquid crystalline state (Konings *et al.*, 2002).

Findings of unique elements in DNA-protection in hyperthermophiles have shed light into the increased molecular stability at elevated temperatures compared to mesophilic and thermophilic microorganisms. DNA transactions such as transcription, replication and recombination, require single-stranded DNA. In mesophiles, topoisomerase II is responsible for the negatively supercoiled (underwound) genome which favours the above transactions because it suffers local strand separation more frequently than relaxed DNA (Forterre *et al.*,

1996; Rodríguez & Stock, 2002). By contrast, hyperthermophiles contain a reverse gyrase (RG) consisting of a helicase-like domain and a 5`-type I DNA topoisomerase responsible for positively supercoiled DNA (Forterre *et al.*, 1985; Forterre *et al.*, 1996). RG is so far the only protein that is unique to hyperthermophiles (Forterre, 2002) and, hence, believed to play a crucial role in thermoadaptation at DNA level. A recent case study on the disruption of the RG gene from the hyperthermophilic archaeon, *Thermococcus kodakaraensis*, showed that disruption of the gene did not lead to a lethal phenotype at 90 °C (Atomi *et al.*, 2004). Furthermore, an apparent positive supercoiling activity that was observed in the host strain was not observed in the mutated strain. Hence, the authors concluded that RG is not a prerequisite for hyperthemophilic life at 90 °C. Remarks was however made to test this hypothesis also in hyperthermophilic strains growing above 90 °C. However, the case study also revealed a decreased growth rate in the RG mutant at elevated temperatures showing that disruption og the gene in fact influences hyperthermophilic growth (Atomi *et al.*, 2004).

An increasing G+C content of ribosomal and transfer RNA (rRNA and tRNA, respectively) have been found with elevated growth temperature of the organism (Galtier & Lobry, 1997; Wang & Hickey, 2002). Furthermore, the increase in G+C content in thermophiles contra mesophiles were located almost entirely within the double-stranded stem regions of the RNA molecule (Wang & Hickey, 2002). However, no correlation have been found between genomic G+C content and the $T_{opt}$ of the organisms (Galtier & Lobry, 1997). From the small subunit rRNA database of the Ribosomal Database Project the two organisms that contained the highest G+C content in their rRNAs, *Pyrolobus fumarii* and *Pyrodictium occultum* (68.9% and 68.8%, respectively), were in fact extreme hyperthermophiles. *Pyrolobus fumarii* can grow up to 113 °C and *P. occultum* grows between 85 – 105 °C (Wang & Hickey, 2002). Based on such observations it was suggested that structural RNAs could be identified as high G+C islands in a low G+C genomic background (Rivas & Eddy, 2000). Similar observations were also made for *Methanocaldococcus jannaschii*, *Mycoplasma genitalium* and *Borrelia burgdorferi*, all of which showed A+T rich genomes (Wang & Hickey, 2002).

Furthermore, posttranscriptional modifications in the tRNA of hyperthermophiles have been suggested as one of the leading mechanisms of structural stabilization. In *P. fumarii*, twenty-six modified nucleosides were detected when investigating posttranscriptional modifications in unfractionated tRNA (McCloskey *et al.*, 2000).

## 1.2 Enzyme stability at elevated temperatures

Enzymes of hyperthermophiles are intrinsically stable and catalytically active at high temperature and have gained much interest in the context of both basic and applied protein science. Much effort has been done since the middle of 1990s to reveal how hyperthermophilic enzymes remain stable at elevated temperatures. The lessons learned from these investigations have subsequently been applied to enhance protein stability of less stable proteins, either by rational or evolutionary design. Numerous naturally occurring hyperthermophilic enzymes have been purified and characterized, and X-ray structures of hyperthermophilic enzymes have been solved and studied with regard to heat adaptive traits (Chang *et al.*, 1999; Dalhus *et al.*, 2002; Jaenicke & Bohm, 1998; Vieille & Zeikus, 2001; Wallon *et al.*, 1997). X-ray data of hyperthermophilic enzymes demonstrated that the 3D-structures of hypertherophilic enzymes are highly similar to their mesophilic homologs and that thermal stability is achieved by small but highly relevant changes at different locations throughout the structure (Vieille & Zeikus, 2001). So far, glutamate dehydrogenase (GDH) and citrate synthase (CS) are the best characterized enzyme families with regard to heat adaptitive traits (Arnott *et al.*, 2000; Bell *et al.*, 2002; Bhuiya *et al.*, 2005; Britton *et al.*, 1999; Knapp *et al.*, 1997; Lebbink *et al.*, 2002; Russell *et al.*, 1994; Russell *et al.*, 1997; Russell *et al.*, 1998; Yip *et al.*, 1995). Structural comparisons of *Pyrococcus furiosus* GDH (*Pf*GDH) with that of the mesophilic *Clostridium symbiosum* GDH revealed an increased number of ion pairs together with an organization of ionic amino acid residues in large networks in *Pf*GDH, implying an important role of this trait for its high thermal stability (Yip *et al.*, 1995). In the 3D-structure of GDH from *Thermotoga maritima* (Knapp *et al.*, 1997) these features were less obvious and actually absent in GDH from *Pyrobaculum islandicum* (Bhuiya *et al.*, 2005). A systematic study on CS from different organisms with optimal growth temperatues between 10 and 100 ºC has demonstrated that the increased stability at elevated temperatures may not be solely due to the actual number of ionic interactions but rather the position in structurally crucial areas (Bell *et al.*, 2002). These data illustrate an important point when analyzing heat-adaptive traits in hyperthermophilic enzymes, i.e. it may be difficult to define general rules for enhanced thermal stability from comparative work on X-ray structures. So far, no single structural feature has been found to be responsible for the high intrinsic thermotolerance of (hyper)thermophilic proteins. Table 1 lists the features found for protein stability at high temperature with the most common being ionic and electrostatic interactions. This is discussed in more detail in section 3.2.

**Table 1** Factors contributing to thermal stability of proteins

| | |
|---|---|
| • Ionic networks | (Alsop *et al.*, 2003; Karshikoff & Ladenstein, 2001; Kumar & Nussinov, 2002; Kumar & Nussinov, 2004; Littlechild *et al.*, 2004; Makhatadze *et al.*, 2003; Vetriani *et al.*, 1998; Yip *et al.*, 1995) |
| • Electrostatic interactions / salt bridges | (Alsop *et al.*, 2003; Elcock, 1998; Elcock & Thomas, 2004; Kumar & Nussinov, 2004; Makhatadze *et al.*, 2003; Mozo-Villarias *et al.*, 2003; Xiao & Honig, 1999) |
| • Helix stabilization | (Olson *et al.*, 2001; Vieille & Zeikus, 2001) |
| • Loop deletions (surface) | (Russell *et al.*, 1997) |
| • Hydrophobic packing | (Bell *et al.*, 2002; Elcock & McCammon, 2001; Jaenicke & Bohm, 1998; Schumann *et al.*, 1993; Tanner *et al.*, 1996; Vieille & Zeikus, 2001) |
| • Aromatic-aromatic interactions | (Anderson *et al.*, 1993; Massant *et al.*, 2003) |
| • Decrease in hydrophobic accessible surface area (ASA) | (Bell *et al.*, 2002) |
| • Increased no. of hydrogen bonds | (Vogt *et al.*, 1997) |
| • More secondary structure | (Irimia *et al.*, 2004) |
| • Disulphide bonds | (Littlechild *et al.*, 2004) |

Enzymes from hyperthermophiles offer major biotechnological advantages as compared to mesophilic or psychrophilic enzymes: (i) when expressed in mesophilic hosts, (hyper)thermophilic enzymes are easier to purify by heat treatment, (ii) their thermostability is associated with higher resistance to chemical denaturants, and (iii) enzymatic reactions at higher temperature allows higher substrate concentration, lower viscosity, less microbial contaminations, and often higher reaction rates (Vieille & Zeikus, 2001). Furthermore, they are often expressed and purified more easily from a mesophilic host. The requirement for thermostable enzymes in various industries is large where the major fields are detergents, food industry, starch, textile, leather, pulp and paper production and pharmaceuticals (Gomes & Steiner, 2004). However, more investigation is necessary to fully understand how enzymes from (hyper)thermophiles endure the physical constraints of extreme heat in order to engineer highly applicable enzymes for specific industrial purposes.

### 1.3 Isocitrate dehydrogenase

Isocitrate dehydrogenase (IDH) belongs to the family of $\beta$-decarboxylating dehydrogenases, a family of enzymes that catalyses the metal ($Mg^{2+}$ or $Mn^{2+}$) and $NAD(P)^{+}$-dependent

dehydrogenation at C2 followed by their metal-dependent decarboxylation at C3 of (2R, 3S) 2-hydroxy acids (Figure 3). $\beta$-decarboxylating dehydrogenases are classified as orthologs, i.e., the genes coding for the enzymes from different species have evolved from a common ancestral gene by speciation and normally retained the same function in the course of evolution. The classification of these enzymes as orthologs was established based on the observations that substitutions of only a few amino acid residues was sufficient to exchange substrate and coenzyme specificities amongst the enzymes (Chen & Jeong, 2000). Four different orthologs of $\beta$-decarboxylating dehydrogenases have so far been identified: $NAD^+$-dependent IDH (NAD-IDH; EC 1.1.1.41), $NADP^+$-dependent IDH (NADP-IDH; EC 1.1.1.42), $NAD^+$-dependent isopropylmalate dehydrogenase (NAD-IPMDH; EC 1.1.1.85) and $NAD^+$-dependent homoisocitrate dehydrogenase (NAD-HDH; EC 1.1.1.115). In addition, tartrate dehydrogenase (TDH) has been suggested as a member of this enzyme family (Chen & Jeong, 2000).

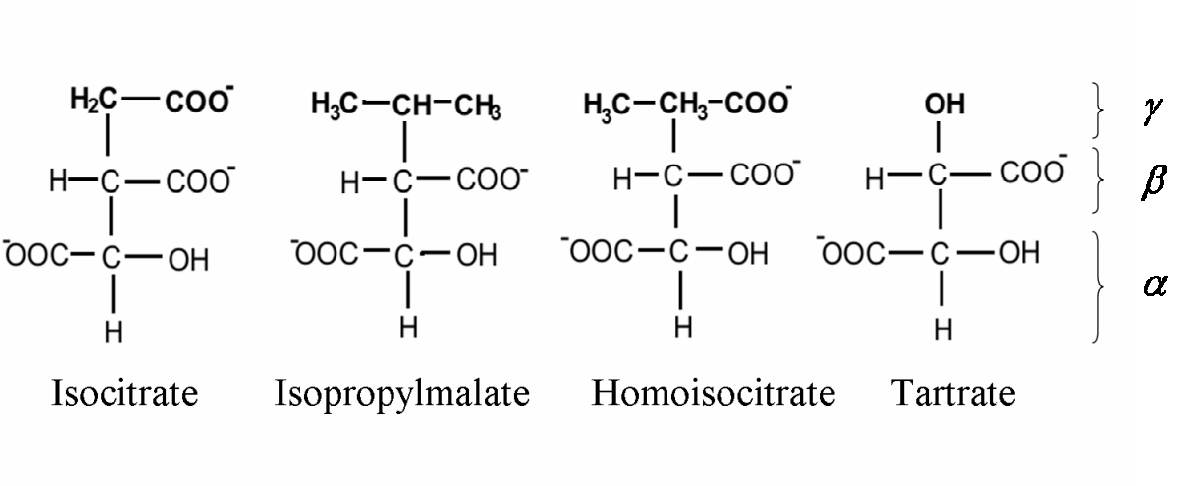


**Figure 3** The different structures of substrates for the family of $\beta$-decarboxylating dehydrogenases. $\alpha$ and $\beta$ refers to the $\alpha$- and $\beta$-carboxyl group, respectively. The unique $\gamma$-moieties recognized by the respective enzymes, IDH, IPMDH, HDH and TDH, are in bold. The figure was edited from Chen & Jong (2000).

The $\beta$-decarboxylating dehydrogenases share a common fold that lacks the $\beta\alpha\beta\alpha\beta$ motif characteristic of the nucleotide binding Rossmann fold of dehydrogenases (Rossmann *et al.*, 1974). Due to the high sequence identity and structural resemblance, they are believed to be evolutionary related and diverged from a common ancestral enzyme.

The dimeric conformation of the bacterial and archaeal enzymes consist of two identical subunits connected through a conserved 4-helix bundle and inter-locked in a clasp-like

domain (Figure 4). Tetramerization has been observed as dimer-dimer association in the clasp-like domain of *Thermus thermophilus* HDH (Miyazaki *et al.*, 2005). To date, no structure of TDH is available.

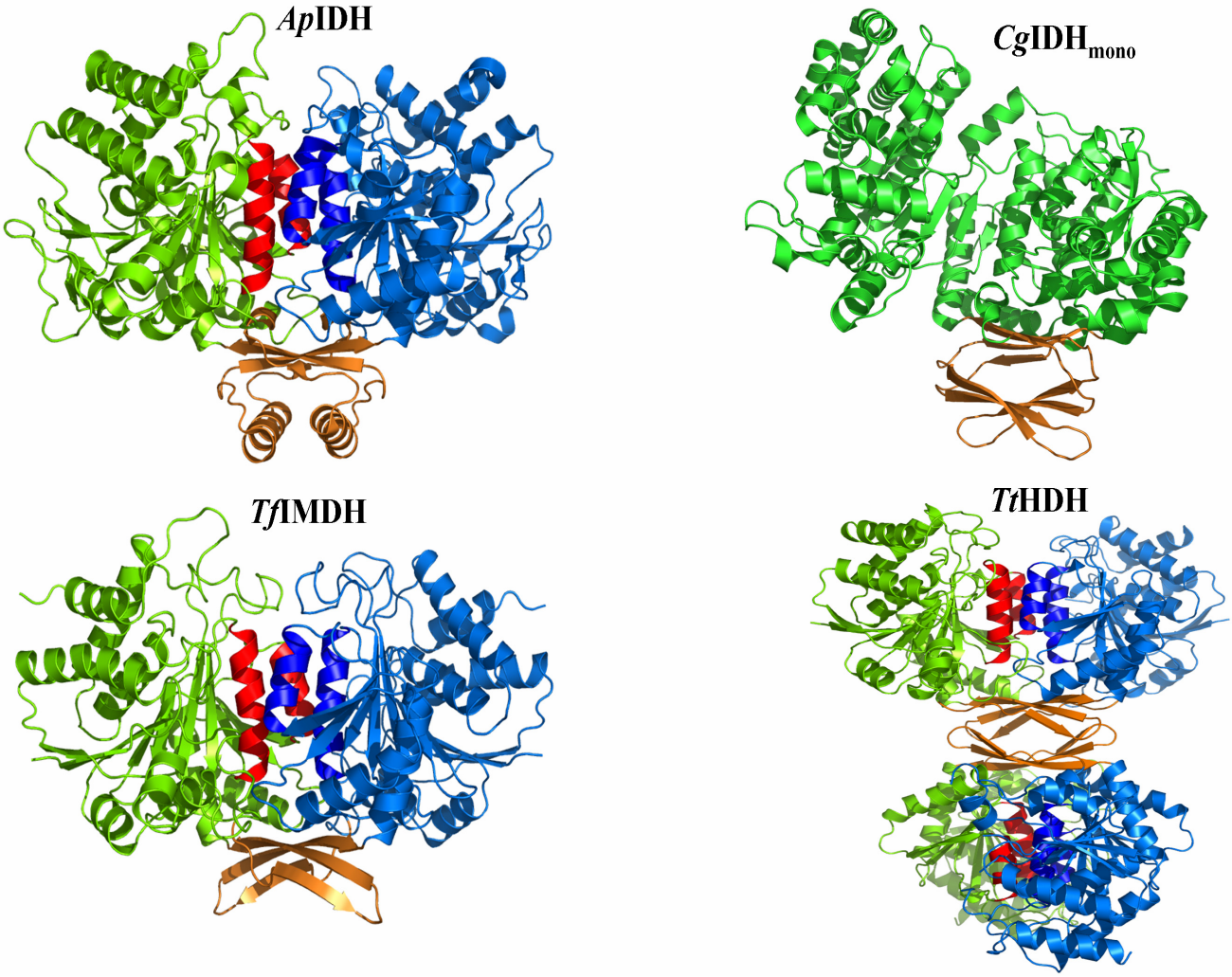


**Figure 4** Resolved structures of $\beta$-decarboxylating dehydrogenases: dimeric *Ap*IDH; *Aeropyrum pernix* IDH (1TYO), monomeric *Cg*IDH; *Corynebacterium glutamicum* IDH (2B0T), dimeric *Tf*IPMDH; *Thiobacillus ferrooxidans* IPMDH (1A05) and homotetrameric *Tt*HDH; *Thermus thermophilus* HDH (1X0L). Conserved 4-helix bundle (between subunits) in the interface between the subunits is coloured red and dark blue. Clasp-domain is coloured orange.

More specific, IDH is a key enzyme in central metabolism important for controlling the metabolic flux between the tricarboxyl acid cycle (TCA cycle) and the glyoxylate shunt. The complete TCA cycle serves as the final step in the oxidation of all major nutrients in

eukaryotes and bacteria grown under aerobic conditions. However, several variations of the TCA cycle have been found reflecting adaptation to different environments such as anaerobic conditions. The TCA cycle provides essential metabolites for energy generation and biosynthetic reactions and generates precursors for lipids, amino acids and heme. During energy conservation in the TCA cycle, IDH catalyses the dehydrogenation and decarboxylation of *D*-isocitrate to α-ketoglutarate using $NAD^+$ or $NADP^+$ as cofactor (Hurley *et al.*, 1991) (Figure 5). A distinguishing feature for the organisms without a complete TCA cycle is their lack of α-ketoglutarate dehydrogenase activity.

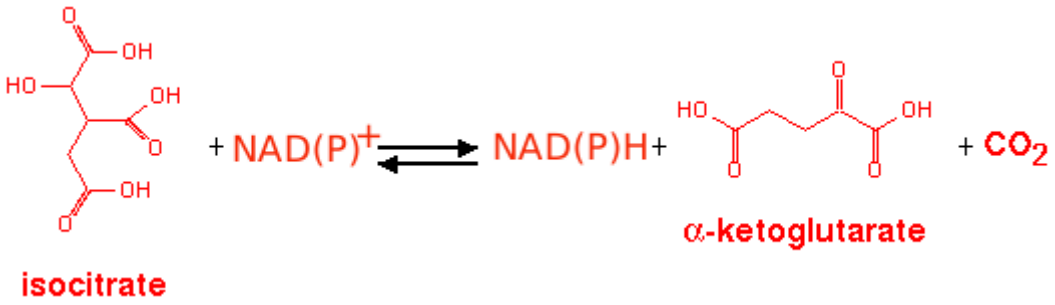


**Figure 5** Conversion of isocitrate to α-ketoglutarate by IDH in the TCA cycle.

## 2. Aims of the study

The work included in this thesis is part of the strategic university programme "Biological adaptations to extreme temperature" supported by the Norwegian Research Council, where the main goal was to "Contribute to the understanding of the molecular basis for protein stability and thermoactivity using enzymes from extremophiles as model systems".

Initially, IDH was chosen as a model enzyme because it is a well-characterized enzyme family with biochemical as well as structural data available. In addition, IDH is a highly conserved and universal group of enzymes present in Archaea, Bacteria and Eukarya. Finally, IDH catalyses a reaction in the central metabolism and has therefore a ubiquitous distribution in nature and is present in organisms living in the entire temperature range compatible with life. Hyperthermophilic IDH from *Aeropyrum pernix* (*Ap*IDH), *Archaeoglobus fulgidus* (*Af*IDH), *Pyrococcus furiosus* (*Pf*IDH) and *Thermotoga maritima* (*Tm*IDH) has previously been cloned, and expressed in *E.coli* and apparent melting temperatures were determined to 109.9, 98.5, 103.7 and 98.3 °C, respectively (Steen *et al.*, 2001). In order to investigate comparatively the structural basis for the thermal stability of these hyperthermophilic IDHs, efforts were made to obtain their 3D-structures.

In order to test the hypothesis of structural features of IDH from *A. pernix* and *A. fulgidus* that may be responsible for their high thermal stability, mutational analyses aiming at the removal of stabilizing interactions were performed. To gain information on the importance of structural domains for thermal stability, hybrid enzymes (domain swapping) carrying parts of a highly thermostable enzyme and parts of a less stable enzyme was to be constructed.

Isocitrate dehydrogenase from *Methylococcus capsulatus* and *Thermplasma acidophilum* were chosen to obtain biochemical data on a sub-set of IDHs from microorganisms growing in the mid-range temperatures between mesophilic and hyperthermophilic species.

## 3. Results and Discussion

Results from papers I-IV are summarized and discussed in the following sections.

### 3.1 Phylogeny, oligomeric states and available structures of IDH

A previous study on the phylogenetic relationship amongst IDHs from Eukarya, Bacteria and Archaea, suggested a three-family division of IDH, whilst IPMDH, HDH and TDH branched in a separate cluster (Steen *et al.*, 2001) (Figure 6).

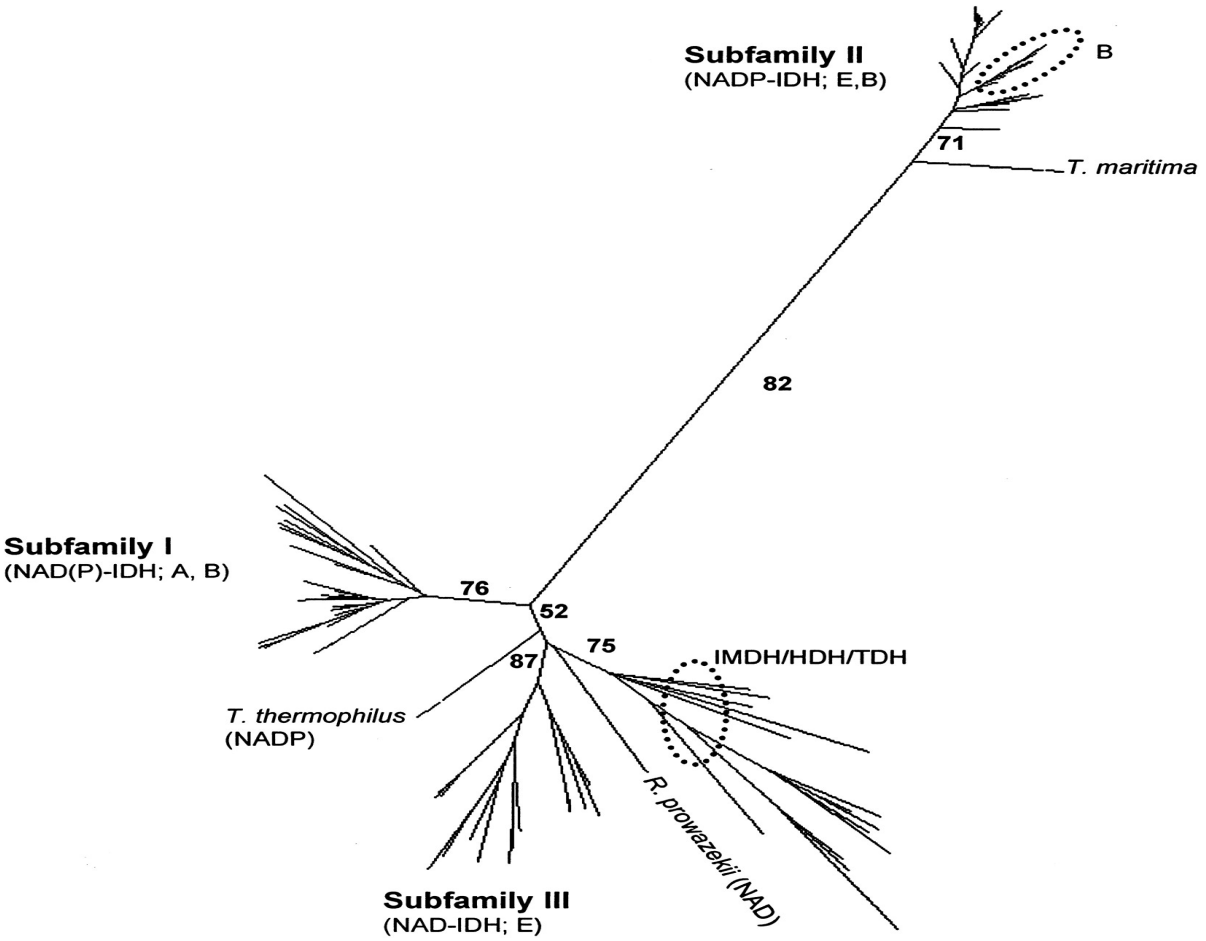


**Figure 6** A phylogenetic study which included 101 primary sequences of IDH, IPMDH, HDH and TDH have divided the IDHs into three subfamilies (Steen *et al.*, 2001).

Subfamily I comprises dimeric IDHs from Archaea and Bacteria, including the model enzymes *Ap*IDH, *Af*IDH, *Ta*IDH and *Ec*IDH. Among previously characterized IDHs, *Mc*IDH showed highest identity to the eukaryal allosterically regulated $NAD^+$-dependent IDH (**Paper IV**). Interestingly, the primary sequence of *Mc*IDH revealed higher sequence identity to *Tt*HDH. However, as presented in **Paper IV**, *Mc*IDH showed highest sequence identity to a

new group of putative bacterial IDHs not included in the previous phylogentic analysis (Steen *et al.*, 2001). The high similarity to HDH and IPMDH suggest that they represent an ancient form of IDH and the presence of $NADP^+$-signatures among these enzymes indicates that $NADP^+$-specificity first evolved among these IDHs. Subfamily II comprises dimeric IDHs from Eukarya and Bacteria (although *Tm*IDH have been observed as both dimeric and tetrameric in solution (Karlström *et al.*, 2006)), and Subfamily III of multimeric IDHs from Eukarya.

Isocitrate dehydrogenase represents a diverse enzyme family with regard to cofactor specificity and oligomeric states and includes hetero-oligomeric $NAD^+$-IDHs, homodimeric $NAD^+$-IDHs, monomeric $NADP^+$-IDHs, homodimeric $NADP^+$-IDHs, and homotetrameric $NADP^+$-IDH (Karlström *et al.*, 2006; Steen *et al.*, 2001). Several 3D-structures are resolved of mesophilic $NADP^+$-dependent IDHs and *Ec*IDH is crystallized as apo-enzyme and in complex with its substrates (Table 2). So far, three structures of hyperthermophilic IDHs have been resolved including, *Ap*IDH (**Paper I**), *Af*IDH (**Paper II**) and *Tm*IDH (Karlström *et al.*, 2006). Structural analysis of *Ap*IDH and *Af*IDH revealed homodimeric enzymes consisting of two symmetrical subunits with high structural similarity to mesophilic *Ec*IDH (Paper I and II; Jeong et al. 2004). The secondary structure elements were conserved and the hyperthermophilic IDHs had a similar folding topology of the subunits as mesophilic *Ec*IDH (**Paper** I and **II**, Figure 7). Hyperthermophilic *Ap*IDH and *Af*IDH are grouped together with *Ec*IDH within subfamily I of IDH and the high structural similarity within subfamily I IDHs, make these good model enzymes for comparative analysis of temperature adaptations. It should be noted that *Ta*IDH (**Paper III**) has a sequence identity of 55.8% with *Af*IDH which implies a similar folding topology.

The 3D-structures of IDH from subfamily II, *Tm*IDH and porcineIDH (Karlström *et al.*, 2006), showed low RMSD values within the subfamily. However, there are distincts structural characteristics of IDH from the two subfamiles with a characteristic clasp-domain which is formed by two anti parallel α-helices beneath a single four stranded anti-parallell β-sheet in subfamily I and by two stacked four stranded anti-parallell β-sheets in subfamily II (Hurley *et al.*, 1989; Karlström *et al.*, 2006). Another major difference is that the N-and C-termini are separated by only approximately 4Å in subfamily II IDHs and by more than 40Å in subfamily I IDHs. This difference may be reflected in the way these termini are stabilized in order to resist thermal denaturation (see section 3.2).

**Table 2** Available IDH structures in the PDB database (http://www.rcsb.org/pdb/).

| PDB-codes | Source organism | Describtion | References |
|---|---|---|---|
| 3ICD | *Escherichia coli* | $NADP^+$-dependent homodimeric, apo-form | (Hurley *et al.*, 1989) |
| 9ICD | | Complex with substrates | (Hurley *et al.*, 1991) |
| 1SJS | | Apo-form | (FinerMoore *et al.*, 1997) |
| 1LWD | Porcine | $NADP^+$-dependent homodimeric, complex with Mn2+ and isocitrate | (Ceccarelli *et al.*, 2002) |
| 1T09<br>1T0L | Human cytosolic | $NADP^+$-dependent homodimeric, complex with $NADP^+$, isocitrate, and $Ca^{2+}$ | (Xu *et al.*, 2004) |
| 1TYO<br>1XKD<br>1XGV | *Aeropyrum pernix* | $NADP^+$-dependent homodimeric, complex with etheno-$NADP^+$ (1TYO), complex with isocitrate, $NADP^+$ and $Ca^{2+}$(1XKD), and apo-form (1XGV) | **Paper I** |
| 2IVO | *Archaeoglobus fulgidus* | $NADP^+$-dependent homodimeric, apo-form | **Paper II** |
| 1V94 | *Aeropyrum pernix* | $NADP^+$-dependent homodimeric, apo-form | (Jeong *et al.*, 2004) |
| 1ZOR | *Thermotoga maritima* | $NADP^+$-dependent homodimeric, apo-form | (Karlström *et al.*, 2006) |
| 1HQS | *Bacillus subtilis* | $NADP^+$-dependent homodimeric | (Singh *et al.*, 2001) |
| 1ITW | *Azotobacter vinelandii* | $NADP^+$-dependent monomeric, complex with isocitrate and $Mn^{2+}$ | (Yasutake *et al.*, 2002) |
| 1J1W | | Complex with $NADP^+$ | (Yasutake *et al.*, 2003) |
| 2B0T | *Corynebacterium glutamicum* | $NADP^+$-dependent monomeric | (Imabayashi *et al.*, 2006) |

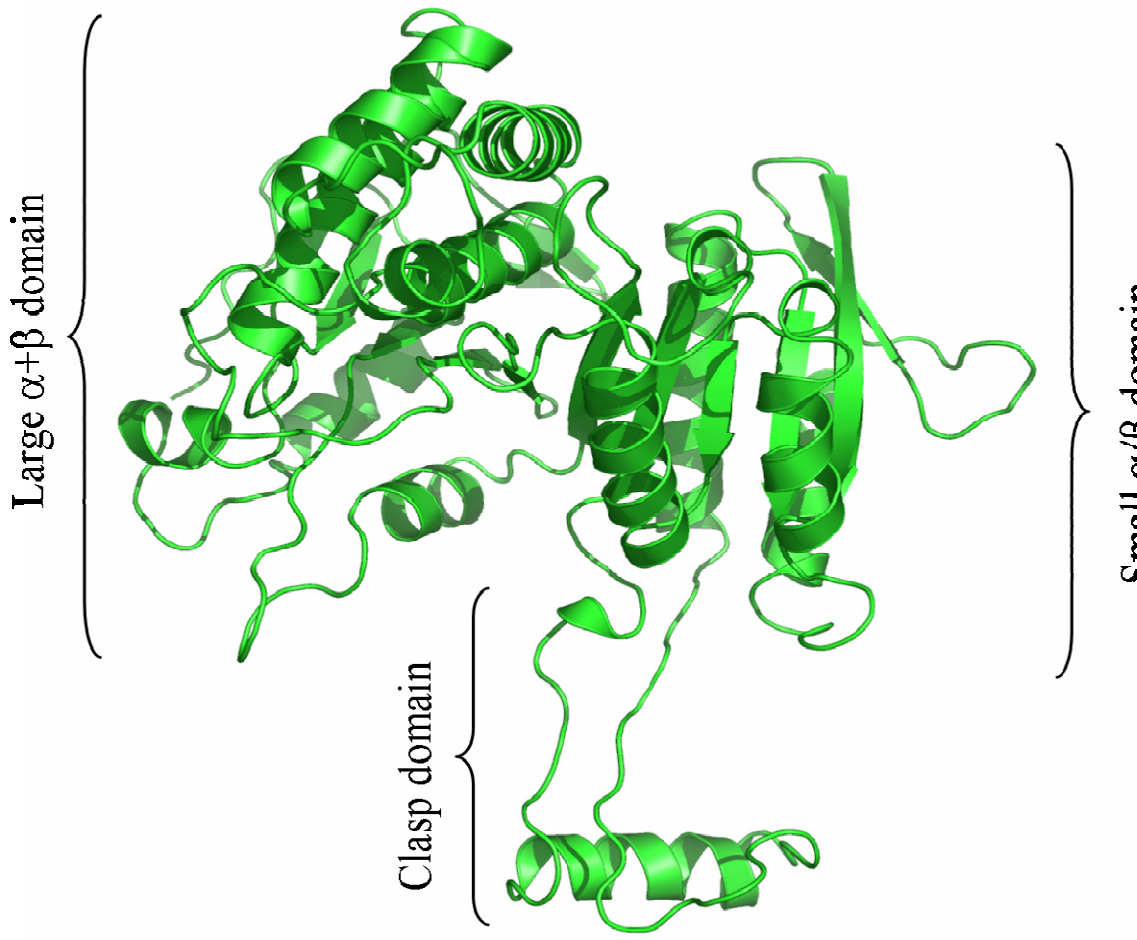


**Figure 7** Topology of the monomeric subunit of Subfamily I IDHs. Each monomer contains three domains; a large $\alpha + \beta$ domain, a small $\alpha/\beta$ domain, and an $\alpha/\beta$ clasp-like domain involving both subunits.

Characterization of the $NAD^+$-dependent *Mc*IDH in **Paper IV** revealed a homotetrameric enzyme, a unique oligomeric state for $NAD^+$-dependent bacterial IDHs. *Tt*HDH is so far the only structure among $\beta$-decarboxylating dehydrogenases that is resolved in a homotetrameric form (Miyazaki *et al.*, 2005a). From this structure it appeared that the arm-like protrusion of the clasp-domain was involved in tetramer formation via hydrophobic interactions. Val135 contributed to a hydrophobic environment with Tyr125 and Val141 from another subunit of the same dimer and Tyr132 and Leu133 from the second dimer. A sequence alignment of *Mc*IDH with *Tt*HDH based on the structural assigments of the latter enzyme, and a subunit model of *Mc*IDH with the *Tt*HDH structure as template, indicated that it is very likely that

Val132, Tyr123 and Val144 (Val135, Tyr125 and Val141 in *Tt*HDH) contributes to a hydrophobic environment with Phe129 and Ile130 (corresponding to Tyr132 and Leu133 in *Tt*HDH). This suggests that tetramer formation in *Mc*IDH is achieved through hydrophobic interactions between the two clasp-like domains. Mutational analysis is in progress to test this hypothesis

### 3.2 Thermostability

One important aspect of protein stability is the mode in which the proteins unfold. The most ideal situation would be to have a set of proteins from closely related organisms, with different growth optimum temperatures which unfold reversible, thereby allowing determination of thermodynamic data. As a consequence, an increase or a decrease in the stability of a protein upon mutation could be related directly to the introduced element. Enzyme thermostability encompasses thermodynamic and kinetic stabilities, in which the thermodynamic stability of a protein is defined by two factors; the protein`s free energy of stabilization ($\Delta G_{stab}$), and by its $T_m$ (Vieille & Zeikus, 2001). The $\Delta G_{stab}$ directly reflects the thermodynamic stability of the folded protein (Eq. 1), however, the $\Delta G_{stab}$ difference between (hyper)thermophilic and mesophilic enzymes is small, usually in the range of 5 to 20 kcal/mol (Vieille & Zeikus, 2001).

Eq. 1 $$\Delta G_{stab} = \Delta H_{stab} - T\Delta S_{stab}$$

The kinetic stability of an enzyme depends on the energy barrier for that enzyme upon unfolding, i.e. the activation energy of the unfolding, and often expressed as its half-life ($t_{1\backslash 2}$) at defined temperatures (Vieille & Zeikus, 2001).

Unfortunately, only a limited number of thermophilic proteins undergo fully reversible unfolding. The majority of these proteins are small monomers and consists of a single structural domain, e.g. the small DNA-binding protein from *Sulfolobus solfataricus* (Sso7d) (Knapp *et al.*, 1996). For the enzymes that unfolds irreversibly only $T_m$ can be determined, i.e., the transition state temperature of thermal denaturation (temperature at which 50 % of the protein is in its unfolded state).

In **Paper I**, **III** and **IV** the thermal stability of *Ec*IDH, *Ta*IDH and *Mc*IDH, respectively, were investigated using DSC. As previously noted for the hyperthermophilic IDHs (Steen *et al.*,

2001), they unfolded irreversibly upon heating. Due to this irreversible unfolding, their stability in the following discussion is refered to as thermal stability. As expected from the thermophilic nature of the source organism *Ta*IDH had a $T_m$ midway between *Ec*IDH and the hyperthermophilic IDHs (Table 3). *Mc*IDH had however, a higher thermal stability than expected from the growth optimum of the source organism which may be explained by the higher level of oligomeric state observed for this enzyme (**Paper IV**).

**Table 3** Thermal stabilities of wild-type IDHs and mutants as determined by DSC.

| | Subfamily | $T_m$ (°C) | Δ $T_m$ between wild-type and respective mutatants (°C) | Δ $T_m$ as compared to *Ec*IDH (°C) | Reference |
|---|---|---|---|---|---|
| *Ap*IDH | I | 109,9 | - | 57.3 | (Steen *et al.*, 2001) |
| E188/Q | | 108,5 | - 1.4 | | |
| E188/A | | 107,6 | - 2.3 | | |
| D130/N | | 106,4 | - 3.5 | | **Paper I** |
| D334/N | | 104,4 | - 5.5 | | |
| R211/Q | | 101,2 | - 8.7 | | |
| R211/M | | 98,6 | - 11.3 | | |
| C87S | | 100.3 | - 9.6 | | |
| *Pf*IDH | I | 103.7 | | 51.1 | (Steen *et al.*, 2001) |
| *Af*IDH | I | 98.5 | - | 45.9 | (Steen *et al.*, 2001) |
| *Af*IDH/*Ec*IDH clasp | | 80.0 | - 18.5 | | **Paper II** |
| *Tm*IDH | II | 98.3 | | 45.7 | (Steen *et al.*, 2001) |
| *Ta*IDH | I | 80.0 | | 27.4 | **Paper III** |
| *Mc*IDH | | 70.3 | | 17.7 | **Paper IV** |
| *Ec*IDH | I | 52.6 | - | - | **Paper I** |
| *Ec*IDH/*Af*IDH clasp | | 56.4 | + 3.8 | | **Paper II** |

The large difference in apparent $T_m$ of *Ap*IDH and *Af*IDH to that of the mesophilic *Ec*IDH ($\Delta T_m$ of 57.3 and 45.4 °C, respectively) was used to relate structural features to the increased thermotolerance of the two former enzymes (**Paper I** and **II**). In order to relate the differences in $T_m$s to structural features, the molecular structures were compared with regard to amino acid composition, ASA, interface, ionic interaction and ionic networks (quantitative and qualitative analyses), aromatic interaction, and presence of disulfide bonds. Mutational studies were performed to confirm the relevance of certain structural traits to increased stability. These data are also presented in Table 3.

*Disulfide bond formation*

Previously, 100 °C was believed to be the upper limit for the thermal stability of proteins containing disulfides, due to the susceptibility of disulfides and cystein residues to destruction at high temperature (Vieille & Zeikus, 2001). However, recent computational genomics and structural studies supports the formation of disulfide bonds in intracellular enzymes from (hyper)thermophiles (Beeby *et al.*, 2005; Mallick *et al.*, 2002). The predicted disulfide abundance, expressed as a proximity score for cysteine-cystein pairs, was shown to be the greatest in *Aeropyrum pernix*. In **Paper I** we have shown that a disulfide bond, between Cys9-Cys87, was present in the structure of the hyperthermophilic *Ap*IDH. Disruption of the disulfide bond by site-directed mutagenesis resulted in a mutated enzyme with a reduced overall stability ($\Delta T_m$ -11 °C) compared to the wild-type enzyme (Table 3). In the tetrameric triosephosphate isomerase from *T. maritima*, a disulfide bond was found as the major inter-dimeric contact (Maes *et al.*, 1999). Engineered disulfides have also shown to stabilize enzymes in the higher temperature range. In malate dehydrogenase from the moderately thermophilic bacterium *Chloroflexus aurantiacus*, an engineered disulfide in the dimer-dimer interface of the tetrameric enzyme resulted in an increase of 15 °C in apparent $T_m$ compared to the wild-type enzyme (Bjørk *et al.*, 2003). However, an engineered disulfide bond in holo azurin have been shown to increase the stability of the wild-type by only 3.7 °C (Tigerström *et al.*, 2004). The different impact on stability by introducing a disulfide bond, from 3-15 °C increase in $T_m$, might suggest the structural positioning of these disulfide bonds as important for stability.

Furthermore, a specific protein known as protein disulfide oxidoreductase (PDO) has been recognized as a potential key contributer in the intracellular disulfide-shuffling in hyperthermophiles (Beeby *et al.*, 2005; Ladenstein & Ren, 2006). Recently, a novel member of this enzyme family was found in *A. pernix* and, the functional and structural study of this PDO revelead the ability to catalyze the reduction, oxidation and isomerisation of disulfide bonds (D'Ambrosio *et al.*, 2006). Hence, supporting the presence of the *Ap*IDH disulfide bond under *in situ* conditions. The observed disulfide bond in the hyperthermophilic *Ap*IDH is so far unique to the IDH family.

*Accessible surface area*

The distribution of charged, polar and hydrophobic content of the ASA and interface area of *Ap*IDH and *Af*IDH compared the mesophilic *Ec*IDH was first analyzed residue-wise (**Paper I**

and **II**). However, it would be more accurate to perform this analysis atom-wise since even a charged residue possesses hydrophobic entities. An atom-wise distribution of the ASA and interface is presented in Figure 8. The atom-wise distribution showed the same trend as the residue-wise distribution of the total ASA as presented in **Paper I and II**, whereby a slightly decrease in charged residues and a significant increase in the polar content was observed in *Ap*IDH and *Af*IDH as compared to *Ec*IDH.

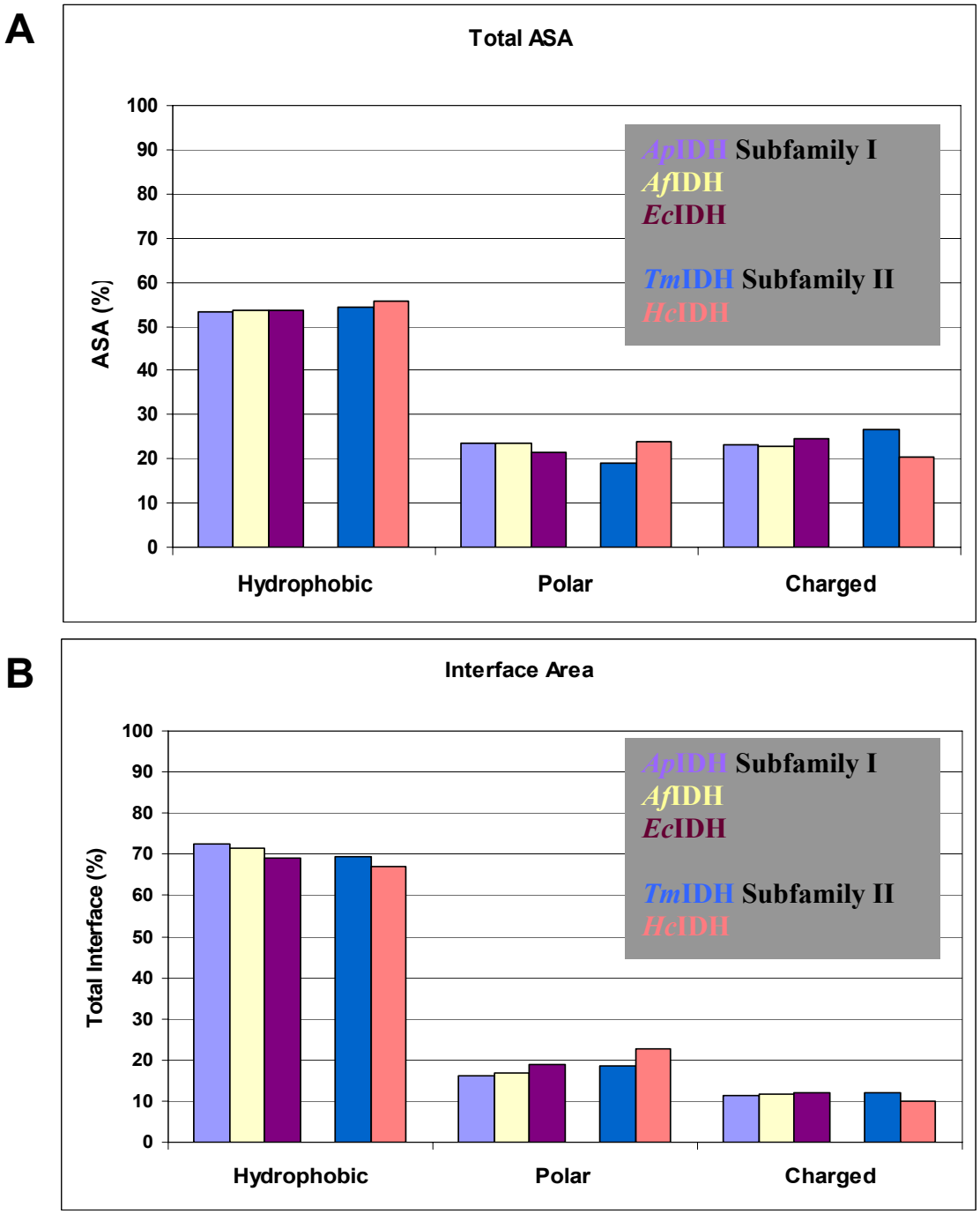


**Figure 8** Distribution of hydrophobic, polar and charged residues in the total ASA (A) and the interface area (B) of subfamily I and II IDHs.

The interface area of subfamily I IDHs showed an increase in the hydrophobic content with increasing stability. In addition, the interface of *Ap*IDH and *Af*IDH was compensated with a significant lower degree of buried charges as compared to the mesophilic *Ec*IDH (**Paper I and II**). The same tendency for increased hydrophobic content was also observed for subfamily II IDHs (Karlström *et al.*, 2006). A major driving force in protein folding and stability is considered to be the hydrophobic effect, i.e. the aversion of water from non-polar residues (Dill, 1990) and, may explain the trend of a significant increase in the number of buried hydrophobic residues in hyperthemophilic proteins. Such feature has been observed for GDH where there is an increase of hydrophobic content in the hyperthermophilic *Tm*GDH compared to the mesophilic *Clostridium symbiosum* GDH. However, the most stable GDH, *P. furiosus* GDH, showed a significant decrease of hydrophobic residues (discussed in detail in a later section) most likely due to large compensated ionic networks (Knapp *et al.*, 1997; Yip *et al.*, 1995). Similar observations were made for members of the citrate synthases. With a $T_{opt}$ of 100 °C, the hyperthermophilic *P. furiosus* CS showed a greater degree of hydrophobicity in the interface region as compared to the psychrophilic and mesophilic enzymes, but lower than other (hyper)thermophilic CS enzymes. As for GDH, this is believed to be compensated by the more extensive ionic interactions in the hyperthermophilic *Pf*CS enzyme (Bell *et al.*, 2002).

*Charged residues and ionic interactions*

Amino acid exchanges such as Lys to Arg, Ser to Ala, Gly to Ala, Ser to Thr and Val to Ile found in thermophilic proteins, have been considered as a possible strategy in thermal stabilization (Scandurra *et al.*, 2000). However, statistical analysis using a larger number of proteins have revealed numerous exemptions (Scandurra *et al.*, 2000). Furthermore, Asn, Gln, Met and Cys have been classified as thermolabile due to their tendency to undergo deamination or oxidation at high temperatures. As previously shown for *Af*IDH, *Pf*IDH and *Ap*IDH (Steen *et al.*, 2001), only a decrease in the cystein content was significant when mesophilic IDHs where compared to (hyper)thermophilic homologs.

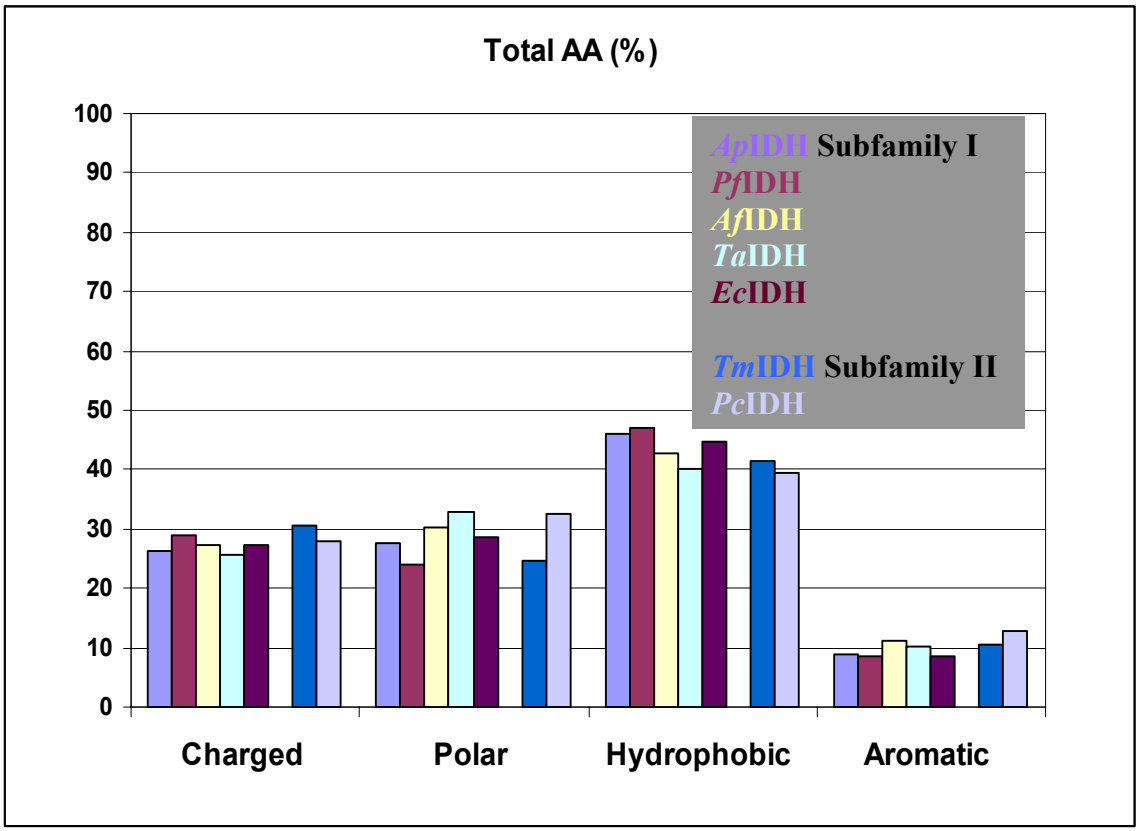


**Figure 9** Distribution of charged, polar, hydrophobic and aromatic residues from subfamily I and II IDHs.

The total number of charged residues per dimer was basically the same in the *Ap*IDH and *Af*IDH compared to *Ec*IDH, 228 (26.3%), 224 (27.2%) and 226 (27.2%), respectively (**Paper I and II**; Figure 9). In general, more charged residues (mostly at the expence of polar residues) and slightly more hydrophobic residues are found in hyperthermophilic proteins compared to mesophilic proteins. However, data from genome sequencing have shown that the *A. pernix* protein pool actually contained fewer charged residues, fewer large hydrophobic residues and fewer aromatic residues than mesophiles (Vieille & Zeikus, 2001). Thus, the observed bias in the amino acid composition of hyperstable enzymes have been suggested to often have evolutionary reasons, rather than being an indication of thermal adaptation (Vieille & Zeikus, 2001). Instead, distribution of residues and their interactions in the protein are probably more relevant for thermal stability (Vieille & Zeikus, 2001).

The contribution of ionic bonds to thermal stability has been a subject of great discussion. The contribution of desolvation of charges to the free energy of folding associated with bringing oppositely charged residues together is large and unfavourable, thus, suggesting that ion pairs are destabilizing in proteins. However, a continuum solvation model have shown that ion pairs have stabilizing effects at higher temperature although being destabilizing at room temperature (Elcock, 1998), the reason being that the unfavourable desolvation penalty decreases at high temperatures as a consequence of a decrease in the water dielectric constant. As a result, water is less ordered and further away from charged residues at high temperature, and in turn, leads to to conformational rearrangement of charged residues to improve their direct electrostatic interaction among each other (Elcock, 1998). In addition, it has been claimed that the dielectric constant of thermophilic proteins is increased, reducing the dielectric difference between the protein and the solvent, and as a consequence the desolvation penalty is reduced even more (Brian N. Dominy, 2004).

As a trend, an increase in the net number of ion pairs in (hyper)thermophilic proteins compared to mesophilic homologs have been observed (Karshikoff & Ladenstein, 2001).

In *Ap*IDH, the charged residues formed about the same number of ion pairs compared to *Ec*IDH when a cutoff distance of 4 Å was used (Table 4). However, an extension of the cutoff to 4.2 Å resulted in a dramatic increase of ion pairs in *Ap*IDH: 73 ion pairs in *Ap*IDH compared to 58 in *Ec*IDH (**Paper I**). A series of unforced molecular dynamics (MD) simulations by Elcock and Thomas between two salt-bridge-forming amino acids have shown that increasing the temperature makes the free energy of charge-charge association more favorable at all separation distances, thus, not only at the distances at which the salt-bridge atoms are in direct contact (Elcock & Thomas, 2004). Surprisingly, increasing the cutoff distance had little impact on the ion pair content in the hyperthermophilic *Af*IDH compared to the mesophilic *Ec*IDH. In fact, the ion pair content in *Ec*IDH was higher compared to *Af*IDH at cutoff distances of 4, 6 and 8 Å (**Paper II**). In the subfamily II IDH structures there was a more significant difference in the ion pair content (Table 4) (Karlström *et al.*, 2006).

**Table 4** Total number of ion pairs and ionic networks in the open structures of subfamily I and II IDHs.

| | Subfamily I | | |
|---|---|---|---|
| (4 Å cutoff distance) | *Ap*IDH$_{open}$ | *Af*IDH$_{open}$ | *Ec*IDH$_{open}$ |
| Ion pairs per dimer | 62 | 53 | 58 |
| Large ionic networks | 1 six member<br>1 seven member<br>(3 seven member at 4.2 Å) | 2 four member | 2 four member |
| | Subfamily II[A] | | |
| | *Tm*IDH$_{open}$ | *Hc*IDH$_{open}$ | |
| Ion pairs per dimer | 70 | 49 | |
| Large ionic networks | 2 five member<br>4 four member | 2 four member | |

[A] Karlström et al. 2006

*Ionic networks*

The structure of the hyperthermophilic *Pf*GDH gave support to a possible correlation between the extent and character of ion pair networks with the relative stabilities within this enzyme family (Yip *et al.*, 1995; Yip *et al.*, 1998). Overall, both total number of ion pairs and ionic networks appared to decrease with a decrease in stability. The apparent abcense of large intricate ion pair networks in enzymes operating at temperatures below 100 °C suggested that multiple ion pair interactions are critical only in the proteins of those organisms that function at the highest temperatures (Yip *et al.*, 1998). Although not a clear trend in the total number of ion pars in *Ap*IDH and *Af*IDH, the large ion pair networks was only observed in the structure of *Ap*IDH (**Paper I and II**), with the largest being a 7-membered and a 6-membered network at 4.0 Å cutoff. However, with at 4.2 Å cutoff two additional 7-membered networks were identified in *Ap*IDH. A muational analysis confirmed the significance of one of the 7-membered ionic network for the high stability in *Ap*IDH (Table 3). Extending the cutoff distances for ionic interactions resulted in a dramatic extension in the size of the ionic networks in the *Ap*IDH enzyme. In contrast, no dramatic effect was observed in the number of ionic networks in the hyperthermophilic *Af*IDH and the mesophilic *Ec*IDH when increasing the cutoff distance. These results suggested that the cooperation of weaker ionic interactions in large networks might also be important for the thermal stability of the hyperthermophilic *Ap*IDH as previously observed for other enzymes (Karshikoff & Ladenstein, 2001). Similar

observations were also made in the subfamily II IDHs (Table 4). Previous attempts to experimentally increase the thermal stability of the bacterial *Tm*GDH by enlarging ionic networks to the size of the ionic networks in the archaeal *Pf*GDH, demonstrated that an exchange of structural features between archaeal and bacterial enzymes with moderate sequence identity, although high structural homology, is not straightforward (Lebbink *et al.*, 1999).

Surprisingly, no significant differences in the amount and sizes of ion pair networks were observed in the structure of the hyperthermophilic *Af*IDH compared to the mesophilic *Ec*IDH (**Paper II**). However, as seen from the comparative structural analysis, the positioning of the ion pair networks in the hyperthermophilic *Af*IDH compared to the mesophilic *Ec*IDH is most likely crucial for the thermal stability of the former enzyme, e.g. loop-loop and inter-subunit stabilization. A unique 4-membered ionic network was observed in *Af*IDH, connecting the clasp-domain in one subunit to the small domain of the adjacent subunit, i.e. representing both an inter-subunit and an inter-domain ionic network.

Little is known about the contribution of the clasp-domain to the thermal stability of these enzymes. Construction of chimeras between the hyperthermophilic *Af*IDH and the mesophilic *Ec*IDH was therefore performed (**Paper II**). The clasp-domain from *Af*IDH was swapped with the clasp-domain from *Ec*IDH (*Af*IDH/*Ec*IDH$_{clasp}$) and vice versa (*Ec*IDH/*Af*IDH$_{clasp}$). Both chimeras were functionally active and their enzymatic properties resembled those of the wild-type enzymes indicating that no conformational changes had occurred. The most dramatic effect concerning stability was observed for *Af*IDH/*Ec*IDH$_{clasp}$ where the thermal stability decreased by 18 °C compared to the wild-type enzyme (Table 3). This large effect was mainly assigned to the disruption of the unique 4-membered ionic network and an aromatic cluster in the clasp-domain as observed from the structure of *Af*IDH (**Paper II**). The reproduction of these specific interactions in the chimer *Af*IDH/*Ec*IDH$_{clasp}$ was not observed due to none-conserved residues in the *Ec*IDH clasp-domain sequence. Replacement of the clasp-domain of *Ec*IDH with the clasp-domain from *Af*IDH in the chimer *Ec*IDH/*Af*IDH$_{clasp}$ resulted in a chimeric enzyme with an increased global stability of 4 °C compared to the wild-type enzyme (Table 3). Although it was difficult to assign the changes in stability to specific interactions in the clasp-domain without structural data of this chimer, we explained the increased stability with the possible formation of an aromatic cluster in the *Af*IDH clasp as observed from the structure of the wild-type enzyme. Disruption of an aromatic cluster in the

hyperthermophilic *Tm*IDH by one amino acid have recently been shown to reduce the thermal stability of the enzyme by 3.5 °C (Karlström *et al.*, 2006).

As a conclusion, large ionic networks were only present in *Ap*IDH and extending the cutoff distances for ionic interactions revealed a dramatic increase in large networks in this enzyme compared to other hyperthermophilic and mesophilic subfamily I homologs. Hence, the size and positioning of ionic networks differed in mesophilic and hyperthermophilic IDHs. However, this does not exclude the possibility for electrostatic compensation of the hyperthermophilic *Af*IDH compared to the less stable *Ec*IDH. In a study by Xiao and Honig (1999), that electrostatic interactions were found more favourable in (hyper)thermophilic proteins than mesophilic homologs. However, the electrostatic free energy was found not to be correlated with the number of ionizable residues, ion pairs or ionic networks, but rather the specific location of these features within the protein structure (Xiao & Honig, 1999)

*Aromatic interactions*

Aromtic interactions have previously been recognized as important factors in structural stabilization of proteins (Anderson *et al.*, 1993; Burley & Petsko, 1985; Kannan & Vishveshwara, 2000). One pair of aromatic amino acids involved in aromatic interactions with each other contributes between -0.6 and -1.3 kcal/mol to the protein stability (Burley & Petsko, 1985). In **Paper I** and **II**, an aromatic cluster was observed in the clasp-region of *Ap*IDH and *Af*IDH, strengthening the dimer interface, and hence, the stability of the enzymes. The residues involved in this cluster were also conserved in the primary structure of *Ta*IDH (**Paper III**) and believed to have similar impact on the thermal stability of this enzyme. The cluster was not conserved in the mesophilic *Ec*IDH. Furthermore, a non-conserved aromatic cluster was observed in the N-terminus of *Af*IDH, suggesting an aromatic stabilization of this region (**Paper II**). In *Tm*IDH, a cluster of aromatic residues was observed in the small domain. Disruption of this cluster resulted in a decrease in $T_m$ of 3.5 °C (Karlström *et al.*, 2006).

*Protection of N- and C-termini*

The N- and C-termini are usually regions with high thermal factors in a protein crystal structure and likely to unfold first during thermal denaturation (Vieille & Zeikus, 2001). In contrast to earlier beliefes that these regions were not important for protein stability, the availability on structural data from hyperthermophilic proteins have revealed structural

features in these regions that could be of crucial importance to protein stabilization (Vieille & Zeikus, 2001). As described above, *Ap*IDH contained a dilsulfide bond in the N-terminus region, anchoring the region to the large domain (**Paper I**). As shown for *Af*IDH, this enzyme contained a large amount of aromatic residues at the N-terminus compared to *Ap*IDH and *Ec*IDH (**Paper II**). An aromatic cluster containing three residues, most likely protect this region in *Af*IDH from thermal unfolding. In addition, an ionic interaction was observed in the N-terminus of *Af*IDH between Lys14 and Glu90. However, this interaction was also conserved in *Ec*IDH (**Paper II**). The length of the N-terminus was shown to vary within the subfamily I IDHs with the longest one belonging to the hyperthemophilic *Ap*IDH. A structure-based sequence alignment showed that the N-terminus of *Ta*IDH was considerable shorter than both the mesophilic *Ec*IDH and the hyperthermophilic *Ap*IDH, *Af*IDH and *Pf*IDH (**Paper III**). Hence, a substantial shortening in this area could constitute to protection of the N-terminus and aid in the protection of *Ta*IDH from thermal degradation.

The side chains of the C- and N-termini residues in IDH from *Tm*IDH are only separated by 4.2 Å (Karlström *et al.*, 2006). The termini are also brought close together in the subfamily II structures from porcine IDH (*Pc*IDH) and human cytosolic IDH (*Hc*IDH) (Ceccarelli *et al.*, 2002; Xu *et al.*, 2004). By contrast, the termini are widely separated on the large domain (> 40 Å) in *Af*IDH, *Ap*IDH and *Ec*IDH from subfamily I (**Paper I and II**). In *Tm*IDH from subfamily II, an ionic interaction between Asp389 in the C-terminus and Lys29 close to the N-terminus is involved in protection of both termini from thermal unfolding. Site-directed mutagenesis at Asp389 resulted in a considerable decrease in thermal stability of almost 22 °C compared to the wild-type enzyme. Although conserved in the mesophilic *Hc*IDH and *Pc*IDH, this ion pair is extended to a 4-membered ionic network in *Tm*IDH at 6.0 Å cutoff distance, which is not observed in the former enzymes (Karlström *et al.*, 2006). This indicates that the stabilizing effect of this ion pair is dependent on the local environment of Asp389. Furthermore, electrostatic compensation was shown to be crucial for preventing thermal unfolding of the N-terminus of *Tm*IDH (Karlström *et al.*, 2006).

As a conclusion, each of the (hyper)thermostable IDHs from both subfamily I and II showed additional stabilization of the N-terminus; *Ap*IDH by a disulfide bond (**Paper I**), *Af*IDH by an aromatic cluster (**Paper II**), *Ta*IDH by shortening of the N-terminus (**Paper III**) and *Tm*IDH by long range electrostatic interactions (Karlström *et al.*, 2006).

*Oligomeric states*

At the start of this work the dimeric form of IDH represented the highest oligomeric state of prokaryotic NAD-IDHs. The biochemical analysis of *Mc*IDH revealed a novel $NAD^+$-dependent homotetrameric enzyme closely related to HDH and IPMDH (**Paper IV**).

Site-directed mutagenesis altering the homotetrameric *Tt*HDH to a homodimeric enzyme was shown to affect the stability of the HDH indicating that tetramer formation was involved in the thermal stabilization of this enzyme (Miyazaki *et al.*, 2005). A higher oligomeric state has previously been found in other (hyper)thermophilic enzymes (Vieille & Zeikus, 2001). The homotetrameric state of *Mc*IDH may thus partly explain the relatively higher $T_m$ than that observed for *Ec*IDH (**Paper IV**). However, the effect of a higher oligomeric state in the *Mc*IDH enzyme has to be investigated by a mutational approach.

## 4. Concluding remarks

IDH represent an enzyme family where the hyperthermophlic members show both unique as well as conserved molecular mechanisms responsible for their high thermal stability. This result is in line with previous investigations of intra-family members of hyperthermophilic enzymes. An unexpected result was however, that the extreme thermal stability of *Ap*IDH appears to derive, at least in part, from a N-terminal disulfide bond. Disulfide bonds were previously considered rare as stabilizing features in intracellular hyperthermophilic proteins due to the susceptibility of disulfides and cystein residues to destruction at high temperature. However, recent work has revealed a frequent precence of stabilizing disulfide bonds in intracellular enzymes in hyperthermophilic crenarchaeota, supported by the observations in *Ap*IDH. The most frequent molecular stabilizing mechanisms in hyperthermophilic enzymes are increased number of ion pairs and large ionic networks. However, the work included in this thesis, as well as the recent resolved 3D-structure of *Tm*IDH, has not revealed a clear trend of such interactions in the IDH enzyme family. These findings do not however, exclude that each of the hyperthermophilic IDH are electrostatic compensated. The combination of three molecular structures of hyperthemophilic IDHs and the high number of mutations aimed at disrupting ionic interactions in these model enzymes, offer a unique dataset to explore the contribution of electrostatics comparatively within one enzyme family.

It is well known that the IDH family encompasses a diverse enzyme family with regard to cofactor specificity and oligomeric states. Despite that $NAD^+$-specificity is suggested to have developed before $NADP^+$-specificity, the $NADP^+$-dependent IDHs are predominant.

However, NAD-IDHs similar to *Mc*IDH are widespread within the domain of Bacteria and, the homotetrameric form of $NAD^+$-dependent *Mc*IDH extends the diversity in cofactor specificity and oligomeric states in the IDH enzyme family.

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