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**New insights into catalyzing ethanol production at high temperatures by
thermostable iron-containing/activating alcohol dehydrogenases**

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Abstract

Alcohol dehydrogenases (ADHs) are important enzymes that catalyze alcohol oxidation and/or aldehyde reduction. As one of the NAD^+ -dependent ADH types, iron-containing/activating ADHs (Fe-ADHs) are ubiquitous in Bacteria, Archaea, and Eukaryotes, possessing similar “tunnel-like” structures that are composed of domain A in their N-terminus and domain B in their C-terminus. The conserved “GGGS” sequence in the domain A of Fe-ADHs associates with NAD(P)^+ , and one conserved Asp residue and three conserved His residues in the domain B are the catalytic sites of Fe-ADHs by surrounding with Fe atom, suggesting that they might employ similar catalytic mechanisms. Notably, all the biochemically characterized Fe-ADHs from hyperthermophiles possess two unique characteristics: thermophilicity and thermostability, thereby demonstrating that they are capable of oxidizing alcohol and reducing aldehyde at high temperatures. Considering these two characteristics, Fe-ADHs from hyperthermophiles are potential in alcohol biotransformation at high temperatures. Herein, we reviewed structural and biochemical characteristics of Fe-ADHs from hyperthermophiles, focusing on similarities and differences between Fe-ADHs from different hyperthermophiles and between Fe-ADHs from hyperthermophilic Archaea and their bacterial homologues. Additionally, we proposed future directions of thermostable Fe-ADHs from hyperthermophiles.

Keywords: Alcohol dehydrogenase; Hyperthermophiles; Thermophilicity and thermostability

Introduction

As the members of the oxidoreductase family, alcohol dehydrogenases (ADHs) are responsible for catalyzing the biotransformation of alcohols to be the corresponding aldehydes or ketones, thus playing important roles in primary and secondary alcohol metabolism. Based on their cofactor requirements, ADHs have been currently grouped into four types: pyrroloquinoline quinone-dependent ADH; flavine adenine dinucleotide-dependent ADH; heme or cofactor F420-dependent ADH; nicotinamide adenine dinucleotide (NAD⁺)-dependent ADH [1]. Furthermore, NAD⁺-dependent ADHs are further subdivided into three types: short-chain ADHs (type I ADH); medium-chain or zinc ADHs (type II or Zn-ADH); long-chain or iron ADHs (type III or Fe-ADH) [2-3]. Fe-ADHs are further classified into two groups: Fe-activated ADHs and Fe-containing ADHs. After purification, the Fe-ADHs do not harbor a Fe, but Fe must be required for their activities, thus designating these enzymes to be Fe-activated ADHs, such as iron-activated alcohol dehydrogenase from *Zymomonas mobilis* [4-6]. On other hand, Fe is present in Fe-ADHs after purification, thus terming these enzymes as Fe-containing ADHs, such as the ADH from the hyperthermophilic archaeon *Thermococcus litoralis* [7].

The first Fe-ADH was reported from *Z. mobilis* by Christopher Wills and co-workers [8], and then described as an iron-activated ADH [6]. Later, Tyrrell Conway et al. cloned and sequenced the gene encoding ADH II (*adhB*) from *Z. mobilis*, demonstrating no homology with all previously reported ADHs [9]. Besides, an ADH4 gene in *Saccharomyces cerevisiae* was reported by Williamson and Paquin [10], displaying sequence similarity with the Fe-activated ADH II from *Z. mobilis*. In addition,

Tyrrell Conway and Lonnie O. Ingram identified the third ADH homologue protein (1, 2-propanediol oxidoreductase), which is encoded by the *fucO* gene in *E. coli*, thus leading to the proposal that a novel ADH family of enzymes comprising these unusual ADHs [11]. Currently, Fe-ADH homologs are found in more organisms, including Archaea.

Hyperthermophiles are organisms that grow optimally at temperatures above 80°C, comprising Archaea and Bacteria [12-13]. Notably, hyperthermophiles that live in harsh high-temperature environments have become the valuable sources of novel enzymes that are potentially used for molecular biology and biotechnology [14-15]. Compared with the corresponding homologues from other organisms, the enzymes from hyperthermophiles possess two unique characteristics: thermophilicity and thermostability, thus making them particularly suitable industrial biocatalysts used for the high temperature reactions [16]. The Fe-ADH gene is encoded in the genomes of some of sequenced hyperthermophiles, especially hyperthermophilic Euryarchaea and Bacteria. As expected, Fe-ADHs from hyperthermophiles possess both alcohol oxidation and aldehyde reduction activities at high temperatures by using NAD^+ and NADH as the cofactors (Fig. 1), respectively.

Radianingtyas and Wright summarized research progress of Zn-dependent ADHs, short-chain ADHs, and Fe-ADHs from thermophilic and hyperthermophilic Archaea and Bacteria [17]. Gaona-Lopez revealed diversity and evolutionary relationship of Fe-ADHs in Eukaryotes [18], demonstrating that animal Fe-ADHs are only found in the hydroxyacid-oxoacid transhydrogenase (HOT) subfamily [19], while fungi Fe-ADHs are mainly present in HOT and maleylacetate reductase (MAR) and plant Fe-ADHs occur only in chlorophyta and are distributed in three different protein subfamilies. Different

Fe-ADHs from various organisms have been recently reviewed by Shanbhag et al. [20], targeting their potential applications. Although they display high sequence diversity, Fe-ADHs possess a similar “tunnel-like” structure containing a cofactor binding site and a Fe-atom binding site with particular functions. Recently, Asokumar et al. have reviewed the ADHs from hyperthermophiles, capable of catalyzing the production of ethanol at high temperatures [21], including Fe-ADHs. In this review, we focused on the recent progresses of Fe-ADHs from hyperthermophiles, highlighting similarities and differences of these proteins between hyperthermophilic species and between these proteins and homologues from other organisms to gain a better understanding of structural and functional relationship of these thermostable Fe-ADHs. Besides, we also proposed future directions of thermostable Fe-ADHs.

Three conserved motifs in Fe-ADHs from hyperthermophiles

Currently, the Fe-ADH homologues from hyperthermophiles have been structurally or biochemically reported from *Thermococcus* species: *Thermococcus litoralis* [12], *Thermococcus* sp. AN1 [22-23], *Thermococcus hydrothermalis* [24], *Thermococcus* sp. ES1 [25-26], *Thermococcus thio还原ens* [27], and *Thermococcus barophilus* Ch5 [28-29], *Pyrococcus* species: *Pyrococcus horikoshii* OT3 [30], and *Thermotoga* species: *Thermotoga maritima* [31], *Thermotoga hypogea* [32-33], and *Thermotoga neapolitana* [34-35], possessing the oxidation and reduction activities at high temperatures. Sequence alignment demonstrates that Fe-ADHs possess three highly conserved motifs, among which Motif I contains “GGGSxxDxxK”, Motif II harbors “DxxxHxxE”, and Motif III possesses three discontinued histidine residues (HxxxHxxxxxxxxxxH) (Fig. 2), where x represents any amino acid residue. These three conserved Motifs suggest that Fe-ADHs

might employ similar catalytic mechanism for alcohol oxidation and acetaldehyde reduction. In addition, sequence alignment also shows that 19 amino acids (MxWESxxxINQxFxxxxxT) in the N-terminal are conserved in Fe-ADHs from *T. barophilus* Ch5 (Tba ADH₆₄₁), *T. paralvinellae*, *Thermococcus* sp. and *T. hydrothermalis*, but not in Fe-ADHs from other *Thermococcus* species, which could be used as a unique molecular marker for distinguishing these Fe-ADHs from *Thermococcus* species.

Interestingly, the hyperthermophilic and piezophilic euryarchaeon *T. barophilus* Ch5 possesses two Fe-ADHs: Tba ADH₅₄₇ (GenBank: ALM74514) and Tba ADH₆₄₁ (GenBank: ALM74601), which are encoded by the gene TBCH5v1_0547 and the gene TBCH5v1_0641 [36], respectively. Tba ADH₅₄₇ displays 71% ~ 79% similarities to the Fe-ADHs from *T. litoralis*, *Thermococcus* sp. AN1, *T. thio还原ens* and Tko ADH1. On the other hand, 80% ~ 87% similarities are observed between Tba ADH₆₄₁ and the Fe-ADH homologues from *Thermococcus paralvinellae*, *Thermococcus* sp., *T. hydrothermalis*, Tko ADH2, and *P. furiosus*. Notably, Tba ADH₅₄₇ exhibits 26% similarities to Tba ADH₆₄₁. Thus, Fe-ADHs from *Thermococcus* species are divided into two types, which might be exemplified by Tba ADH₅₄₇ and Tba ADH₆₄₁, respectively. However, Tko ADH2 is an exception since this ADH displays 33% and 25% amino acid similarities to Tba ADH₆₄₁ and Tba ADH₅₄₇, respectively, which suggests that Tko ADH2 is a novel Fe-ADH with novel biochemical function distinct from other reported Fe-ADHs.

Phylogenetic relationship of Fe-ADHs from hyperthermophiles

As shown in Fig. 3, Tba ADH₅₄₇ displays a closely evolutionary relationship with the Fe-ADHs from *T. litoralis*, *T. zilligii*, *T. thio还原ens* and Tko ADH1, while Tba

ADH₆₄₁ is clustered with the Fe-ADHs from *T. paralvinellae*, *Thermococcus* sp. and *T. hydrothermalis*, which is consistent with the observation of their high amino acid similarity. Currently, only one Fe-ADH has been biochemically characterized from *Pyrococcus* species: *Pyrococcus horikoshii* OT3. The Fe-ADH from *P. horikoshii* OT3 is clustered with Tba ADH₅₄₇ rather than Tba ADH₆₄₁. Besides, 65% and 26% similarities are observed between *P. horikoshii* OT3 Fe-ADH and Tba ADH₅₄₇ and between *P. horikoshii* OT3 Fe-ADH and Tba ADH₆₄₁, respectively. Thus, these observations suggest that the Fe-ADH from *P. horikoshii* OT3 resembles Tba ADH₅₄₇. Additionally, the Fe-ADHs from other Archaea, such as *Thermoplasma volcanium*, *Methanothermobacter thermautotrophicus* and *Archaeoglobus fulgidus* display a closer evolutionary relationship with Tba ADH₅₄₇ than with Tba ADH₆₄₁. Likewise, the Fe-ADH from the hyperthermophilic crenarchaeon *Aeropyrum pernix* K shows a closer relationship with Tba-ADH₅₄₇ than Tba-ADH₆₄₁, although it has not been characterized to date.

The Fe-ADHs from hyperthermophilic *Thermotoga* species (*Thermotoga maritima*, *Thermotoga neapolitana* and *Thermotoga hypogea*) have been biochemically or structurally characterized. *T. maritima* and *T. hypogea* possess one Fe-ADH, while *T. neapolitana* harbors three Fe-ADHs. Intriguingly, *T. hypogea* Fe-ADH displays a far evolutionarily relationship with *T. maritima* Fe-ADH, but a closer evolutionarily relationship with Tba ADH₆₄₁ and *T. neapolitana* Fe-ADH1. By comparison, a closer evolutionarily relationship is found between *T. maritima* Fe-ADH and *T. neapolitana* Fe-ADH2. Additionally, *T. neapolitana* Fe-ADH3 (Fe-AAdh) is clustered with *P. furiosus* Fe-ADH.

Structural comparison of Fe-ADHs from hyperthermophiles

Currently, the crystal structures of only two Fe-ADHs from hyperthermophiles have been solved, one which is from the hyperthermophilic bacterium *T. maritima* (PDB: 1O2D) (Fig. 4A) and the other is from the hyperthermophilic euryarchaeon *T. thioreducens* (PDB: 6C75) (Fig. 4B). The two solved structures of Fe-ADHs from hyperthermophiles are similar to the structures of the Fe-ADHs from *Z. mobilis* and FucO protein (Figs. 4C and 4D) [37-38], possessing the conserved domain A and domain B.

***Thermotoga maritima* Fe-ADH homologue**

T. maritima is a hyperthermophilic and anaerobic organism, which was isolated from the sediment of a marine geothermal area near Vulcano, Italy [39]. This archaeon possesses the ability to ferment many different types of carbohydrates to produce hydrogen (clean energy) by employing Fe-hydrogenases [39]. Interestingly, five alcohol dehydrogenase homologues were identified in the *T. maritima* proteome (TM0920, TM0111, TM0285, TM0423, and TM0820), among which TM0920 is a putative iron-containing 1,3-propanediol dehydrogenase that catalyzes the oxidation of propane-1,3-diol to 3-hydroxypropanal with the simultaneous reduction of NADP⁺ to NADPH. TM0920 is a homologue of Fe-ADH since the enzyme possesses three conserved motifs present in Fe-ADH superfamily.

The crystal structure of TM0920 was solved, possessing 19 α helices, and 8 β -strands (Fig. 4A). The α/β N-terminal domain A (residues 1 to 177) consists of 8 β -strands while the C-terminal domain B (residues 178 to 359) comprises helices H10 to H19 organized in two helical bundles [31]. The active site is composed of Asp189, His193, His256, and His270, which are located in domain B (Fig. 5A). Interestingly, the Fe²⁺ ion is deeply positioned into the catalytic cleft, while the NAD binding site is

situated between domains A and B. The interaction between the conserved “GGGGS” and with NAD is depicted in Fig. 5A. A structural similarity comparison suggests that TM0920 is also structurally similar to a glycerol dehydrogenase from *Bacillus stearothermophilus* (PDB: 1JQA) [40] in addition to the Fe-ADH from *Z. mobilis* and FucO protein (Figs. 5C and 5D). Thus, the solved TM0920 structure, which represents an iron-containing 1,3-propanediol dehydrogenase in complex with NADP, has provided valuable insights into substrate specificity, catalysis and thermostability of Fe-ADHs.

***T. thioreducens* Fe-ADH**

T. thioreducens is a sulfur-reducing, hyperthermophilic, organo-heterotrophic archaeon, which was isolated from 'black smoker' chimney from the Rainbow hydrothermal vent site on the Mid-Atlantic Ridge [41]. This archaeon is strictly anaerobic, capable of growing optimally at 83-85°C and also is obligately dependent upon elemental sulfur as an electron acceptor. The genome of *T. thioreducens* encodes a Fe-ADH. The crystal structure of the *T. thioreducens* Fe-ADH was solved, which is the first solved Fe-ADH from hyperthermophilic Archaea. The structure of Fe-ADH from *T. thioreducens* is a dimer under both high-salt and low-salt conditions [27].

The Fe-ADH from *T. thioreducens* possesses a Fe-atom binding site, where the Fe atom is associated with D193, H260, H197 and H272 (Fig. 5B). The NADP contacts with the Fe atom in van der Waals, which is presumably in the ferric state. Like the *T. maritima* enzyme TM0920 [31] and other almost all dehydrogenases [37-38], *T. thioreducens* Fe-ADH comprises two domains: domain A and domain B (Fig. 4B). Domain A has amino acids 1–182 and domain B has amino acids 183–378. A NADP-binding site is located in domain A, while the Fe²⁺ ion-binding site is situated in

domain B. Specially, the conserved residues D193 and H197 interact with Fe atom plus residues H260 and H272 located in the conserved motif III. The Fe atom is bound to the second domain by the side chains of His197, His260, His272 and Asp193, which occupy four of the five positions of a trigonal bipyramid around the Fe atom.

Biochemical comparison of Fe-ADHs from hyperthermophiles

Thermophilicity and thermostability are two unique common characteristics of Fe-ADHs from hyperthermophiles [14-15]. However, these unusual Fe-ADHs display distinct biochemical characteristics, including the optimal temperature and pH, thermostability, divalent metal ion requirements, substrate specificity and kinetic characteristics (Table 1).

***T. barophilus* Fe-ADH (Tba ADH₅₄₇)**

T. barophilus Ch5 is a thermo-piezophilic euryarchaeon that can grow optimally at 88°C and at 40 MPa of hydrostatic pressure [42]. This strain possesses two Fe-ADHs: Tba ADH₅₄₇ and Tba ADH₆₄₁ [36], which were biochemically characterized. The recombinant Tba ADH₅₄₇ possesses two activities for oxidize ethanol and reduce acetaldehyde, demonstrating the same optimal temperature (75°C) for its two activities [28]. Moreover, similar thermostability is observed for oxidization and reduction reactions catalyzed by the enzyme. On the other hand, the optimal pH values for oxidation and reduction of Tba ADH₅₄₇ are 8.5 and 7.0, respectively. Interestingly, a divalent ion is required for Tba ADH₅₄₇ to oxidize alcohol and Mn²⁺ is an optimal one. However, Fe²⁺ is optimal for reduction activity of Tba ADH₅₄₇. Additionally, the substrate specificity of the enzyme shows that 1-butanol and 1-hexanol are its preferable substrates over ethanol and other alcohols, and butylaldehyde is its better substrate than

acetaldehyde. Kinetic data demonstrate that Tba ADH₅₄₇ possesses greater catalytic activity for ethanol synthesis than that for ethanol oxidation, suggesting that the recovered NAD⁺ might be used for Entner-Doudoroff glycolytic pathway under anaerobic conditions.

Mutational data show that a combination of residues H262, H266 and H274 in Tba ADH₅₄₇, which are three conserved histidine residues in the motif III, is essential for catalysis. It is the first reported Fe-ADH from *Thermococcus* with the function the three highly conserved histidine residues in the motif III to be clarified. Besides, residues D195 and H199 in Tba ADH₅₄₇, which is the highly conserved Asp and His in the “DxxxH” of motif II, are essential for catalysis and maintaining their structural roles. It is also the first reported roles of “DxxxH” in motif II of ADHs from *Thermococcus*, thus providing biochemical support for structural mechanistic of key role of the conserved motif II in the solved crystal structures of *T. thio-reducens* Fe-ADH (Fig. 5B) and other Fe-ADHs.

***T. barophilus* Fe-ADH (Tba-ADH₆₄₁)**

The low similarity is observed between Tba ADH₅₄₇ and Tba ADH₆₄₁ and, Tba-ADH₆₄₁ displays a far evolutionarily relationship with Tba ADH₅₄₇ (Fig. 3), which leads us to propose these two typical Fe-ADHs as the representatives from hyperthermophiles. Distinct biochemical characteristics, including optimal reaction temperature, thermostability, divalent ion requirement, and substrate specificity, are observed for Tba ADH₆₄₁ and Tba ADH₅₄₇ [29]. Firstly, the optimal temperature of Tba ADH₆₄₁ is 10 ~ 20°C degrees lower for alcohol oxidation than Tba ADH₅₄₇, suggesting that the former is less thermophilic than the latter. Additionally, Tba ADH₆₄₁ appears to be less thermostable than Tba ADH₅₄₇. Besides, Tba ADH₆₄₁ differs from Tba ADH₅₄₇ in

the aspect of the optimal divalent metal ion requirements: Fe^{2+} for ethanol oxidation of Tba ADH₆₄₁ but Mn^{2+} for ethanol oxidation of Tba ADH₅₄₇. Moreover, ethanol is a preferable substrate for Tba ADH₆₄₁ over 1-butanol while 1-butanol is a better substrate for Tba ADH₅₄₇ than ethanol.

As discussed above, the conserved “GGGSxxD” motif I is a typical motif present in Fe-ADHs. Like other Fe-ADHs, Tba ADH₆₄₁ harbors the conserved motif I. Our lab revealed for the first time that residue D115 in the conserved “GGGSxxD” in Tba ADH₆₄₁ is essential for its activity. Besides, residues K118, E159, D190, and E215 in Tba ADH₆₄₁, which are conserved in other Fe-ADHs, are involved in ethanol oxidation and acetaldehyde reduction, thus providing insight into roles of the conserved residues in Fe-ADH.

***T. litoralis* Fe-ADH**

Thermococcus litoralis is a strictly anaerobic and hyperthermophilic archaeon [43]. The native Fe-ADH was purified from the *T. litoralis* cells and characterized biochemically [11]. The enzyme displays the optimal pH for ethanol oxidation of 8.8 and the optimal temperature of 80°C. Heating at 85°C and 96°C for about 2 h and 15 min leads to the 50% loss of activity of the enzyme, suggesting that the enzyme is thermostable. No metal ion is contained in the native enzyme. The enzyme can utilize NADP as the cofactor rather than NAD^+ . Additionally, the enzyme can utilize a very broad of primary alcohols but not secondary alcohols, thus suggesting that the enzyme is a primary ADH. Furthermore, the enzyme displays the low affinity for ethanol, demonstrating that ethanol is not the physiological substrate, which is confirmed by the observation that the *T. litoralis* cells have no ability to metabolize alcohols. On the other

hand, the enzyme can utilize NADPH as a cofactor to reduce acetaldehyde, and display much lower affinity for ethanol than that for acetaldehyde, suggesting that the enzyme might play a psychological role in acetaldehyde reduction rather than ethanol oxidation.

***Thermococcus* sp. AN1 Fe-ADH**

Thermococcus sp. AN1, the proposed name as *Thermococcus zilligii*, is an anaerobic hyperthermophilic, sulfur-metabolizing and thermophilic euryarchaeon that can be routinely grown under anaerobic conditions at 80°C [44]. The native Fe-ADH from the *Thermococcus* sp. AN1 was purified from its host cells [22-23]. The enzyme is a homotetramer, capable of oxidizing a series of primary linear alcohols but not including methanol, isopropanol, or glycerol, and also reducing acetaldehyde. Besides, the enzyme displays the optimal temperature and pH for ethanol oxidation at 85°C and 6.8 ~ 7.0. Kinetic parameters of the enzyme shows that the K_m values for butanol, ethanol, and propanol were 1.4 mM, 10 mM, and 12 mM, respectively, suggesting that butanol is a preferred substrate for the enzyme. Besides, the K_m values for NADP and NAD^+ were 80 μ M and 1.5 mM, respectively, demonstrating that NADP is a preferred NADP as the cofactor for the enzyme. However, the enzyme is able to reduce acetaldehyde with a preferred cofactor of NADPH. Additionally, *Thermococcus* sp. AN1 ADH is less thermostable than other thermophilic Fe-ADHs since the enzyme has a half-life of only 16 min at 80°C.

***T. hydrothermalis* Fe-ADH**

T. hydrothermalis is a strictly anaerobic and hyperthermophilic archaeon, capable of grows optimally at temperature of 85°C and at pH of 6 [45]. This archaeon possesses a Fe-ADH [45]. The native and recombinant Fe-ADHs from *T. hydrothermalis* were

characterized, demonstrating that they have similar catalytic properties, including thermophilicity and thermostability [24]. This ADH is NADP-dependent Fe-ADH. Notably, the enzyme displays the optimal pH 10.5 for alcohol oxidation, and is a 197 kDa tetramer (subunit of 45 kDa) at this pH. However, the enzyme has the optimal pH 7.5 for aldehyde reduction, but the enzyme is an 80.5 kDa dimer at pH 7.5. Additionally, the kinetic data of the enzyme demonstrate that the enzyme exhibits the greater affinity for the aldehyde substrate and NADPH cofactor than the alcohol substrate and NADP, respectively. The substrate specificity of *T. hydrothermalis* Fe-ADH shows that a series of primary aliphatic and aromatic alcohols are its preferential substrates. Besides, *T. hydrothermalis* Fe-ADH can oxidize methanol and glycerol and stereospecific for monoterpenes. Overall, *T. hydrothermalis* Fe-ADH is the first cloned and overproduced *Thermococcale* ADH in *E. coli*, and identical characteristics occur in the recombinant and the native forms.

***Thermococcus* sp. ES-1 Fe-ADH**

Thermococcus sp. ES1 is an anaerobic archaeon that grows optimally at 82°C [46]. The genome of this archaeon encodes one Fe-ADH. The native and recombinant enzymes display indistinguishable biochemical characteristics. *In vivo*, the native enzyme exhibits much higher activity when growing the cells under S^0 -limited growth conditions [47], thus confirming its physiological role. The native and recombinant enzymes are homotetramers. Additionally, the 1.0 ± 0.1 g-atom iron per subunit was found in the recombinant ADH [25-26]. Both enzymes display a half-life of about 4 min upon exposure to air, suggesting that they were sensitive to oxygen. Distinct optimal pH-values of both enzymes are observed for ethanol oxidation (pH 10.4) and acetaldehyde reduction

(pH 7.0). Additionally, both enzymes also have similar temperature-dependent activities. The substrate specificity of both enzymes shows that they can oxidize primary alcohols rather than methanol and secondary alcohols. Besides, the enzyme displays lower apparent K_m -values for acetaldehyde and NADPH than those for ethanol and NADP.

***P. horikoshii* OT3 Fe-ADH**

P. horikoshii OT3 is an anaerobic and hyperthermophilic archaeon, which was isolated from a hydrothermal vent at a depth of 1395 m in the Okinawa Trough in the Pacific Ocean. This strain can thrive at a broad temperature range from 85°C to 105°C and optimally at 98°C. The genome of *P. horikoshii* OT3 encodes a typical Fe-ADH [48]. The enzyme was heterologously expressed in *E. coli* in a culture medium containing nickel (Ni-PhADH). The recombinant Ni-PhADH displayed the strong activity while the recombinant enzyme without adding nickel showed the weak activity, suggesting that binding a nickel ion to PhADH is helpful for avoiding inactivation [30]. Besides, binding a nickel ion to PhADH allows the enzyme to be stable in oxygen, which provides a new strategy for oxygen-sensitive Fe-ADHs to catalyze acetaldehyde reduction and ethanol oxidation.

In contrast with other thermophilic group III ADHs, Ni-PhADH displayed an affinity preference for NAD(H) over NADP(H). The optimal pH for the reduction activity of Ni-PhADH was 5.5, while the optimal pH for its oxidation activity was 9.0. Additionally, the optimal temperature for enzyme activity exceeds 95°C. Intriguingly, the E_a values were estimated to be 21.3 kJ mol⁻¹ (<80°C) and 36.3 kJ mol⁻¹ (>80°C) by the Arrhenius plot of the kinetic data. In addition, Ni-PhADH can oxidize a broad range of primary alcohols (C2–C8) and diols by utilizing NAD⁺ as the cofactor, displaying the

highest activity for 1-butanol oxidation. In contrast, the enzyme has no activity toward methanol or secondary and tertiary butanol oxidation. On the other hand, a range of linear aldehydes can be reduced by Ni-PhADH with NADH as the electron donor, demonstrating the highest activity for the reduction of propanal. Furthermore, the Ni-PhADH displays the order of magnitude higher aldehyde-reducing activities than alcohol-oxidizing activities. Furthermore, an unusually high K_{cat} value of the enzyme was observed toward aldehyde reduction rather than ethanol oxidation. Additionally, the Ni-PhADH has a half-life of approximately 6 h at 95°C.

***Thermotoga hypogea* Fe-ADH**

T. hypogea is an anaerobic and extremely thermophilic bacterium that grows optimally at 90°C [49]. This bacterium possesses one Fe-ADH (*ThFe-ADH*). The native enzyme was characterized, capable of oxidizing alcohols and reducing aldehydes at high temperature [32]. The fully active enzyme is a homodimer, harboring iron atom. Interestingly, the enzyme appears to be inactive after exposure to oxygen, but the enzyme activity can be recovered with the addition of dithiothreitol and Fe^{2+} , confirming that the enzyme is oxygen sensitive. Additionally, the enzyme displays the increased activity along with the rise of temperature up to 95°C, suggesting that the enzyme is extremely thermophilic. Moreover, the enzyme has a half-life of about 10 h at 70°C, demonstrating that the enzyme is thermostable. The enzyme exhibits the distinct optimal pH values: 8.0 for aldehyde reduction and 11.0 for alcohol oxidation. Besides, the enzyme can utilize primary alcohols and aldehydes as substrates, demonstrating its broad substrate specificity. In addition, the kinetic parameters of the enzyme shows that apparent K_m values for acetaldehyde and butyraldehyde are much lower than those for ethanol and

1-butanol, suggesting that the enzyme might catalyze the reduction of aldehydes to alcohols *in vivo*.

Recently, Hao et al. has reported that the recombinant *ThFe*-ADH displays similar biochemical characteristics to the native enzyme [33]. For instance, the optimal pH values of the recombinant *ThFe*-ADH were measured to be 11.0 and 8.0 for alcohol oxidation and aldehyde reduction, respectively, which are observed for the native enzyme. By comparison, a half-life of the recombinant *ThFe*-ADH was 5 h at 70 °C, which is shorter than that of the native enzyme. Besides, the homologous structure model was constructed by using the *T. maritima* ADH as a template, demonstrating that this enzyme harbors an α/β -dinucleotide-binding motif in its N-terminal domain and an α -helix-rich region containing the iron-binding motif in its C-terminal domain. As observed in other Fe-ADHs, the modeled structure of the recombinant *ThFe*-ADH possesses the highly conserved Asp195His199His268His282 and Gly39Gly40Gly41Ser42 in typical iron- and NADP-binding motifs, respectively.

***Thermotoga neapolitana* Fe-ADH**

T. neapolitana, which was isolated from a submarine thermal vent at Lucrino near Naples, Italy, is a hyperthermophilic bacterium that can grow as high as 90°C, possessing with a complete lignocellulosic hydrolase system [50-51]. The sequenced genome of *T. neapolitana* encodes three Fe-ADHs (Fe-AAdh, encoded by CTN_0580; Fe-Adh1, encoded by CTN_1655; Fe-Adh2, encoded by CTN_1756), one Zn-ADH encoded by CTN_0257 and one Aldh encoded by CTN_1548, which can catalyze from ac-CoA to acetaldehyde.

Currently, these three Fe-ADHs from *T. neapolitana* have been biochemically characterized. Fe-AAadh is a bifunctional iron-acetaldehyde/alcohol dehydrogenase, capable of catalyzing the acetyl-CoA (Ac-CoA) reduction to produce ethanol via an acetaldehyde intermediate [34]. Biochemical data show that maximum activity of Fe-AAadh for the reduction of ac-CoA to produce Ac-ald and the reduction of Ac-ald to produce ethanol was observed at pH 7.54. However, different optimal pH values were observed for Fe-AAadh to catalyze the oxidation reactions: 9.32 for Ac-ald \rightarrow Ac-CoA and 8.82 for ethanol \rightarrow Ac-ald. On the other hand, Fe-AAadh displays the same optimal temperatures for both Ac-CoA \rightarrow Ac-ald and Ac-ald \rightarrow Ac-CoA, but the different optimal temperatures: 80°C for Ac-ald \rightarrow ethanol and 95°C for ethanol \rightarrow Ac-ald. Additionally, Fe-AAadh can use NADPH instead of NADP as a cofactor to catalyze from Ac-CoA to Ac-ald, but can utilize both NADPH and NADH to catalyze from Ac-ald to ethanol. However, both NADP and NAD can be utilized as a cofactor for Fe-AAadh to oxidize from Ac-ald \rightarrow ethanol and from Ac-CoA \rightarrow Ac-ald. Furthermore, the enzyme possesses a half-life of 1-h at about 92°C.

Biochemical data have shown that both Fe-Adh1 and Fe-Adh2 displayed the highest activity of ethanol oxidation at pH 9.0 [35]. By contrast, Fe-Adh1 has the optimal pH value of 8.5 for acetaldehyde reduction, which is higher than that of Fe-Adh2 (pH 7.0). Furthermore, Fe-Adh1 has the same optimal temperatures (95°C) for ethanol oxidation and acetaldehyde reduction. However, Fe-Adh2 has the optimal temperatures of 100°C for ethanol oxidation and of 85°C for acetaldehyde reduction. Interestingly, both Fe-Adh1 and Fe-Adh2 can utilize NADPH as a cofactor rather than NADP to catalyze from acetaldehyde to ethanol. On the other hand, both NADP and NAD can be used as a

cofactor for both Fe-Adh1 and Fe-Adh2 to catalyze ethanol oxidation and acetaldehyde reduction. Overall, Fe-Adh2 displays the highest activity of catalyzing the reduction of acetaldehyde to produce ethanol, which resembled the optimal growth conditions of the protein host. Thus, Fe-Adh2 might play an important role in catalyzing ethanol formation in the hyperthermophilic bacterium *T. neapolitana*.

Ethanol production pathway in hyperthermophilic anaerobes

The discovery of a bifunctional aldehyde/alcohol dehydrogenase in *T. neapolitana* provides a complete pathway example for producing ethanol in hyperthermophilic anaerobes. Surprisingly, the Fe-AAadh gene of *T. neapolitana* is present in other *Thermotoga* species: *T. maritima* MSB8 (GenBank: TM_0111), *Thermotoga* sp. RQ7 (GenBank: TRQ7_04415), *Thermotoga petrophila* (GenBank: Tpet_0813), *T. kodakarensis* (GenBank: TK_RS07830) and *P. furiosus* (GenBank: PF_0075). Besides, some hyperthermophiles possess the CoA-dependent Aldh that can catalyze from ac-CoA to acetaldehyde.

Based on the recent progresses of Fe-ADHs, we proposed an ethanol fermentation pathway mediated by iron-containing ADHs and CoA-dependent Aldh in hyperthermophilic anaerobes (Fig. 6). The glucose would be transformed to be pyruvate and acetaldehyde by glycolysis and ED pathway under anaerobic conditions, respectively. The acetaldehyde can be reduced by Fe-ADH to be ethanol using NADH as a cofactor. By contrast, ethanol is oxidized by Fe-ADH to be acetaldehyde by using NAD⁺ as a cofactor. On the other hand, the pyruvate created by glucose glycolysis is further transformed to be Acetyl-CoA by POR (pyruvate ferredoxin oxidoreductase). Besides, acetyl-CoA can be produced by oxidation of acetaldehyde by Fe-AADH. In the opposite

direction, Fe-AADH can reduce Acetyl-CoA to be acetaldehyde. Additionally, acetyl-CoA can be phosphorylated by phosphate acetyltransferase (PTA) to produce Acetyl-P, and then Acetyl-P is transformed to be acetic acid by acetate kinase (ACK). In addition, acetaldehyde can be formed to acetic acid by aldehyde dehydrogenase. In another pathway, acetaldehyde can be formed from pyruvate by pyruvate decarboxylase (PDC).

Concluding remarks

ADHs are the important enzymes that catalyze oxidation or reduction reactions by using electron transfer cofactors such as NAD(P)⁺/NAD(P), which offers an sustainable and environmentally friendly alternative to traditional chemical synthesis in terms of green chemistry [3, 52-53]. As the members of ADHs, Fe-ADHs are widespread in Bacteria, Archaea and Eukaryotes, possessing the conserved motifs and similar structures, thereby suggesting that they employ similar mechanism for catalyzing ethanol oxidation and acetaldehyde reduction. Generally, the Fe-ADHs from hyperthermophilic Euryarchaea and Bacteria possess two common characteristics: thermostability and thermophilicity, which demonstrates that they can withstand high-temperature and catalyze their oxidation and reduction at high temperatures. Thus, these two unique properties of Fe-ADHs from hyperthermophiles might allow them to be the ideal catalysts for creating industrial products at high temperatures.

Recently, Chen et al. has revealed that an NAD⁺-dependent Fe-ADH from *Acinetobacter venetianus* RAG-1 can effectively oxidize a wide range of substrates including alkyl alcohols (C1-C32) and some isomeric alcohols, and reduce the alkyl aldehyde (C1-C12) as well [54]. Since the *A. venetianus* RAG-1 Fe-ADH possesses the

conserved motifs present in other Fe-ADH homologs, it will be interesting to investigate whether the Fe-ADHs from hyperthermophiles can act on long-chain alkane degradation at high temperatures.

Hyperthermophilic Crenarchaea is an important branch of hyperthermophilic Archaea. Most of the structurally or biochemically characterized Fe-ADHs have derived from hyperthermophilic Euryarchaea, but no Fe-ADH has been characterized from hyperthermophilic Crenarchaea to date. For example, the hyperthermophilic crenarchaeon *Aeropyrum pernix* K1 encodes a putative Fe-ADH (Genbank: BAA80973.2). Although the structural and functional relationship of the oxidoreduction reactions catalyzed by Fe-ADHs from hyperthermophiles has been illustrated, there remain a number of gaps in our understanding regarding the biochemical characteristics and mechanism of Fe-ADH from hyperthermophilic Crenarchaea. Thus, it is worthwhile to investigate structure and function of Fe-ADHs from hyperthermophilic Crenarchaea by using a combination of biochemical, structural and molecular biological methods.

Structural and biochemical data of Fe-ADHs from hyperthermophiles have provided important insights into understanding structural and functional relationship of Fe-ADHs. However, no Fe-ADH from hypothermophiles has been reported for industrial applications to date. It will be great interest to generate novel Fe-ADHs with better properties suitable for practical application as industrially advantageous biocatalysts by tailoring thermostable Fe-ADHs from hyperthermophiles by using a combination of biochemistry, computational aid design and protein engineering. Therefore, further probing structural and functional relationship of Fe-ADHs from hyperthermophiles will

be helpful for developing Fe-ADHs from hyperthermophiles with superior properties, which in turn will expand their potential applications in the future.

Author Contributions

YL and YY made the figures and table and wrote the manuscript. LZ and XZ wrote and revised the manuscript.

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Conflict of Interests

All authors declare that we have no conflict of interest.

Ethical Statement

This article does not contain any studies with human participants or animals performed by any of the authors

Figure legends

Figure 1. Redox reactions by Fe-ADH.

Figure 2. Sequence alignment of Fe-ADHs from Bacteria, Archaea and Eukaryotes.

The similar and same residues are boxed and colored with red. Fe-ADHs possess three conserved motifs. “GGGS”, “DxxxHxxE” and three highly conserved histidine residues are located in Motif I, Motif II and Motif III, respectively. The roles of amino acid residues that have been clarified are marked by an asterisk. Afu: *Archaeoglobus fulgidus* (GenBank: WP_048064618); Tvo: *Thermoplasma volcanium* (GenBank: WP_010916654); Pab: *Pyrococcus abyssi* (GenBank: WP_010868426); Pho: *Pyrococcus horikoshii* (GenBank: WP_010884837); Tli: *Thermococcus litoralis* (GenBank:

AAB63011); Tba: *Thermococcus barophilus* (GenBank: ALM74514 for ADH₅₄₇ and ALM74601 for ADH₆₄₁); Tth: *Thermococcus thio-reducens* (GenBank: ASJ12775); Tzi: *Thermococcus* sp. AN1 (GenBank: WP_010477684); Tko: *Thermococcus kodakarensis* (GenBank: WP_011249959 for ADH1 and WP_083755808 for ADH2); Zmo: *Zymomonas mobilis* (GenBank: TWE26216); Sce: *Saccharomyces cerevisiae* (GenBank: GAX68104); Tet: *Thermoanaerobacter ethanolicus* (GenBank: EGD50333); Mth: *Methanothermobacter thermautotrophicus* (GenBank: WBF07343); Pfu: *Pyrococcus furiosus* (GenBank: WP_011011187); Aae: *Aquifex aeolicus* (GenBank: WP_010880720); Thy1: *Thermococcus hydrothermalis* (GenBank: CAA74334); Tsp: *Thermococcus* sp. (GenBank: AAB63011); Tpa: *Thermococcus paralvinellae* (GenBank: AHF80371); Eco: *Escherichia coli* (GenBank: UKM87074); Thy2: *Thermotoga hypogea* (GenBank: WP_031504348); Tma: *Thermotoga maritima* (GenBank: WP_227738386); Ape: *Aeropyrum pernix* K1 (GenBank: BAA80973); Tne: *Thermotoga neapolitana* (GenBank: ACM23932 for ADH1, ACM23831 for ADH2 and ACM22756 for ADH3).

Figure 3. Phylogenetic analyses of Fe-ADHs from hyperthermophiles, bacteria and eukaryotes.

Figure 4. Structural comparison of Fe-ADHs. The domain A and domain B in Fe-ADHs are colored with magenta and yellow, respectively. The GGGs sequence in the domain A is colored with cyan. Fe atom is shown with a green ball. NAD, the conserved Asp and three conserved His residues are labeled with sticks with different colors. A. The crystal structure of Tma-Fe-ADH. B. The crystal structure of Tth-Fe-ADH. C. The crystal structure of *Z. mobilis* Fe-ADH. D. The crystal structure of FucO protein.

Figure 5. Active site and cofactor binding site of Fe-ADHs. Fe-ADHs possess the conserved active sites and cofactor binding site. The active site of Fe-ADH is composed of one conserved Asp and three conserved His residues, which surround with Fe atom. The cofactor binding site comprises the GGGS sequence that associates with NAD. A. The active site and cofactor site of Tma-Fe-ADH. B. The active site and cofactor site of Tth-Fe-ADH. C. The active site and cofactor site of *Z. mobilis* Fe-ADH. D. The active site and cofactor site of FucO protein.

Figure 6. Ethanol fermentation pathways mediated Fe-ADHs in hyperthermophilic anaerobes. The details are seen in the text. The pathways ① and ② are catalyzed by Fe-ADH, while the pathways ③ and ④ are catalyzed by Fe-AAdh, which is a bifunctional iron-acetaldehyde/alcohol dehydrogenase. ACK: acetate kinase; Aldh: aldehyde dehydrogenase; ED: Entner-Doudoroff; PDC: pyruvate decarboxylase; POR: pyruvate ferredoxin oxidoreductase; PTA: phosphate acetyltransferase.

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Table 1 Comparison of biochemical characteristics of iron-containing ADHs from hyperthermophiles

Kingdom	Hydrothermophile	Oxidation or Reduction	Cofactor	T _{opt} (oC)	Thermostability	pH _{opt}	K _m (mM)	K _{cat} (s ⁻¹)	K _{cat} /K _m (s ⁻¹ M ⁻¹)	Oxygen-sensitive	Reference
Archaea	<i>Thermococcus barophilus</i> ^a	Oxidation	NAD/NADP	75	Retaining 20% activity after heating at 100°C for 30 min	8.5	92	0.27	2.9	Not	28
		Reduction	NADH/NADPH	75	Retaining 20% activity after heating at 100°C for 30 min	7.0	34.5	69	2.0 × 10 ³		
	<i>Thermococcus barophilus</i> ^b	Oxidation	NAD/NADP	50 ~ 65	Retaining 5.6% activity after heating at 90°C for 20 min	8.0	52	7.2	0.14	Not	29
		Reduction	NADH/NADPH	75 ~ 80	Retaining 21% activity after heating at 100°C for 20 min	8.0	1.5	40	27		
	<i>Thermococcus litoralis</i>	Oxidation	NADP	85	The 50% loss of activity after heating at 85°C and 96°C for about 2 h and 15 min	8.8	11	N.D.	N.D.	Yes	11
		Reduction	NADPH			N.D.	0.4	N.D.	N.D.		
	<i>Thermococcus</i> sp. AN1	Oxidation	NAD/NADP	85	A half-life of only 16 min at 80°C	6.8 ~ 7.0	10	N.D.	N.D.	Not	22-23
		Reduction	NADPH	N.D.		7.0	0.12 - 0.13	N.D.	N.D.		
	<i>Thermococcus hydrothermalis</i>	Oxidation	NADP	80	A half-life of 15 min at 80°C	10.5	2.0	22.6	11.3	Yes	24
		Reduction	NADPH			7.5	0.01	1.7	170		
	<i>Thermococcus</i> sp. ES-1	Oxidation	NADP	>95	A half-life of about 4 h at 95°C	10.4	9.7	81	1.5 × 10 ⁵	Yes	25-26
		Reduction	NADPH	>95		7.1	0.84	22.9	2.7 × 10 ⁴		
<i>Pyrococcus horikoshii</i> OT3	Oxidation	NAD/NADP	>95	A half-life of 6 h at 95°C	9.0	0.52	0.48	0.92	Yes	30	
	Reduction	NADH/NADPH	>95		5.5	0.25	6.96	28.05			
Bacteria	<i>Thermotoga hypogea</i>	Oxidation	NADP	>95	A half-life of 10 h at 70°C and 2 h at 90°C	11.0	9.7	14	1.5 × 10 ³	Yes	32-33
		Reduction	NADPH	>95		8.0	3.1	15	5.0 × 10 ³		
	<i>Thermotoga neapolitana</i> Fe-Adh1 ^c	Oxidation	NAD/NADP	95	N.D.	9.0	N.D.	N.D.	N.D.	Yes	35
		Reduction	NADPH	95		8.5	N.D.	N.D.	N.D.		
	<i>Thermotoga neapolitana</i> Fe-Adh2 ^d	Oxidation	NAD/NADP	>100	N.D.	9.0	N.D.	N.D.	N.D.	Yes	35
		Reduction	NADPH	85		7.0	N.D.	N.D.	N.D.		
	<i>Thermotoga neapolitana</i> Fe-AAadh3 ^e	Oxidation	NAD/NADP	95	A half-life of 1-h at about 92°C	8.82	N.D.	N.D.	N.D.	Yes	34
		Reduction	NADH/NADPH	80		7.54	N.D.	N.D.	N.D.		

a: ADH547;

b: ADH641;

c: Fe-Adh1 is encoded by CTN_1655;

d: Fe-Adh2 is encoded by CTN_1756;

e: Fe-AAadh is encoded by CTN_0580;

N.D.: Not determined.