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Distinct metal dependence for catalytic and structural functions in the L-arabinose isomerases from the mesophilic *Bacillus halodurans* and the thermophilic *Geobacillus stearothermophilus*

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Abstract

L-Arabinose isomerase (AI) catalyzes the isomerization of L-arabinose to L-ribulose. It can also convert D-galactose to D-tagatose at elevated temperatures in the presence of divalent metal ions. The *araA* genes, encoding AI, from the mesophilic bacterium *Bacillus halodurans* and the thermophilic *Geobacillus stearothermophilus* were cloned and overexpressed in *Escherichia coli*, and the recombinant enzymes were purified to homogeneity. The purified enzymes are homotetramers with a molecular mass of 232 kDa and close amino acid sequence identity (67%). However, they exhibit quite different temperature dependence and metal requirements. *B. halodurans* AI has maximal activity at 50 °C under the assay conditions used and is not dependent on divalent metal ions. Its apparent K_m values are 36 mM for L-arabinose and 167 mM for D-galactose, and the catalytic efficiencies (k_{cat}/K_m) of the enzyme were 51.4 $mM^{-1}\,min^{-1}$ (L-arabinose) and 0.4 $mM^{-1}\,min^{-1}$ (D-galactose). Unlike *B. halodurans* AI, *G. stearothermophilus* AI has maximal activity at 65–70 °C, and is strongly activated by Mn^{2+}. It also has a much higher catalytic efficiency of 4.3 $mM^{-1}\,min^{-1}$ for D-galactose and 32.5 $mM^{-1}\,min^{-1}$for L-arabinose, with apparent K_m values of 117 and 63 mM, respectively. Irreversible thermal denaturation experiments using circular dichroism (CD) spectroscopy showed that the apparent melting temperature of *B. halodurans* AI ($T_m = 65$–67 °C) was unaffected by the presence of metal ions, whereas EDTA-treated *G. stearothermophilus* AI had a lower T_m (72 °C) than the holoenzyme (78 °C). CD studies of both enzymes demonstrated that metal-mediated significant conformational changes were found in holo *G. stearothermophilus* AI, and there is an active tertiary structure for *G. stearothermophilus* AI at elevated temperatures for its catalytic activity. This is in marked contrast to the mesophilic *B. halodurans* AI where cofactor coordination is not necessary for proper protein folding. The metal dependence of *G. stearothermophilus* AI seems to be correlated with their catalytic and structural functions. We therefore propose that the metal ion requirement of the thermophilic *G. stearothermophilus* AI reflects the need to adopt the correct substrate-binding conformation and the structural stability at elevated temperatures.

Keywords: L-Arabinose isomerase; Metal dependence; D-Tagatose; *Bacillus halodurans*; *Geobacillus stearothermophilus*

Many bacteria can use L-arabinose as carbon source, converting it into D-xylulose 5-phosphate, an intermediate in the pentose phosphate pathway [1–4]. D-Xylulose 5-phosphate is formed from arabinose by the sequential action of arabinose isomerase, ribulokinase, and ribulose 5-phosphate epimerase. These enzymes are encoded by the *araA*, *araB*, and *araD* genes, respectively, of the L-arabinose operon [5,6]. In *Escherichia coli*, the L-arabinose operon also encodes the regulatory C protein (the

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araC gene), while a further three genes (*araE*, *araF*, and *araG*) specify products involved in the transport of arabinose into the cell [4].

L-Arabinose isomerase (AI, EC 5.3.1.4)[1] converts the aldo-pentose, L-arabinose to the ketose, L-ribulose, and this enzymatic isomerization has industrial application in the production of a novel sweetener, D-tagatose, from D-galactose [7–10]. However, as D-galactose is a poor substrate for AI, and the isomerization requires high temperatures [8,10] mesophilic AIs are of limited use in this application. Indeed the mesophilic AIs from *E. coli* [11], *Lactobacillus gayonii* [12], and *Salmonella typhimurium* [13] all fail to carry out this isomerization. It has been reported that *E. coli* AI is hexameric and that Mn^{2+} is the only metal that confers catalytic activity [14,15]. However, the catalytic activities of purified *E. coli* and *L. gayonii* AIs are not markedly affected by Mn^{2+} [11,12].

We recently cloned and expressed hyperthermophilic AIs from *Thermotoga neapolitana* [8] and *Thermotoga maritima* [10]. The enzymes were activated and temperature stabilized by Mn^{2+} and Co^{2+}, and had no catalytic activity in the absence of divalent metal ions. Homologous AIs from a number of sources, with widely differing thermal characteristics, are very similar in sequence, subunit composition, and enzymatic properties. The AIs from a mesophile (e.g., *E. coli*, $T_{max} = 45\,^{\circ}C$), a moderate thermophile (*Geobacillus stearothermophilus*, $T_{max} = 70\,^{\circ}C$), and a hyperthermophile (e.g., *T. maritima*, $T_{max} = 90\,^{\circ}C$) showed high similarity [10]. For this reason, AIs from a variety of mesophilic and thermophilic species have been used to elucidate the basic mechanisms of thermophilic adaptation. Present evidence suggests that homologous proteins from mesophiles and thermophiles have evolved to possess similar degrees of conformational flexibility at their respective temperature optima [16,17]. However, this hypothesis has not been confirmed in the case of AIs, by comparing the conformational changes in view of the substrate–enzyme binding structure. Moreover, although we found that hyperthermophilic AIs have absolute metal dependence for their catalytic activity, not only a detailed comparative physicochemical description of a moderate thermophilic AI with a mesophilic counterpart is still lacking but also the exact role of metal ions on AIs is somewhat vague.

[1] *Abbreviations used:* AI, L-arabinose isomerase; LB, Luria–Bertani; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; IPTG, isopropyl-β-D-thiogalactopyranoside; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(4-butanesulfonic acid); TLC, thin-layer chromatography; HPIC, high-performance ionic chromatography; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propane-sulfonic acid); ICP, inductively coupled plasma; CD, circular dichroism; GI, galactose isomerase; TIM, triose phosphate isomerase; GPI, glucose 6-phosphate isomerase; RPI, ribose 5-phosphate isomerase; FI, fucose isomerase; XI, xylose isomerase; HFCS, high fructose corn syrup.

We are interested in the mechanism of aldose/ketose interconversion and in the structural basis of thermostability. Of particular interest are the conformations achieved during the isomerization of D-galactose to D-tagatose and L-arabinose to L-ribulose, and the metal requirements for isomerization that discriminate between homologous enzymes. As described above, *E. coli* AI is a hexamer [11,18] whereas thermophilic AIs are tetramers [10]. For this reason, we chose *Bacillus halodurans* AI as the mesophilic counterpart; its amino acid sequence and oligomeric state are very similar to those of *G. stearothermophilus* AI, e.g., 67% identity and 83% similarity. In the present study, we characterize the activity and conformation of these two enzymes in response to temperature, and describe the thermostability and the substrate-binding conformation of *B. halodurans* AI and *G. stearothermophilus* AI in the presence and absence of divalent metal ions. The difference in the conformations of the AIs between the metal-free and the metal-bound forms as a function of temperature provides insight into the divergent evolution of homologous AIs.

Materials and methods

Materials

Reagents were purchased from the following sources: restriction enzymes, Ex-tag DNA polymerase, dNTP, and chemicals for PCR from Takara Biomedicals; pGEM-T easy vector and T4 DNA ligase from Promega; and all columns for purification from Pharmacia; genomic-tip, plasmid miniprep kits from Qiagen, and the pET-22b(+) expression vector from Novagen; electrophoresis reagents from Bio-Rad; and all chemicals for assay and characterization of enzymes from Sigma. Oligonucleotides were synthesized by Cosmo. Plasmid pGEM-T easy vector was used for cloning and sequencing, and pET-22b(+) vector for expression.

Bacterial strains and culture conditions

Bacillus halodurans (DSM 497) and *G. stearothermophilus* (DSM 22) were obtained from Deutsche Sammlung von Mikroorganismen (DSM), Braunschweig, Germany. *B. halodurans* was grown in an alkaline nutrient medium (pH 9.7) containing 3 g of beef extract, 5 g peptone, 5 g L-arabinose, 4.2 g $NaHCO_3$, and 5.3 g Na_2CO_3 per liter at 37 °C. *G. stearothermophilus* was grown in a nutrient broth supplemented with 5 g L-arabinose at 55 °C. *E. coli* DH5α was used as host for the construction of expression vectors, and *E. coli* BL21 (DE3) as host for expression. Both strains were grown in Luria–Bertani (LB) medium with ampicillin (100 μg/ml) in a rotary shaker at 37 °C.