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Notes

Characterization of Acetohydroxyacid Synthase Cofactors from *Haemophilus influenza*

Vinayakumar Gedi, Bon-Sung Koo,† Dong-Eun Kim,‡ and Moon-Young Yoon*

*Department of Chemistry, College of Natural Sciences, and Research Institute of Natural Sciences, Hanyang University, Seoul 133-791, Korea. *E-mail: myyoon@hanyang.ac.kr*
†Functional Biomaterial Division, National Academy of Agricultural Science, RDA, Suwon 441-857, Korea
‡Department of Bioscience and Biotechnology, WCU Program, Konkuk University, Seoul 143-701, Korea
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Haemophilus influenzae (Hin) is a Gram-negative, rod-shaped bacterium usually involved in community-acquired respiratory tract infections, acute otitis media, acute sinusitis, exacerbation of chronic bronchitis, and childhood meningitis.[1] Major respiratory pathogens are rapidly developing antibiotic resistance, creating both a serious problem of effectively managing respiratory tract infections and the need for new antibiotics. Previous studies of leucine and isoleucine-valine-leucine auxotrophs of *M. tuberculosis*^{H37Rv},[2] leucine auxotrophs of *Mycobacterium bovis* BCG,[3] and the AHAS mutant of *Burkholderia pseudomallei*[4] have shown that enzymes in branched chain amino acid (BCAA) synthesis pathways could be suitable targets for antimicrobial agents. In contrast, plants, algae, fungi, bacteria, and archaea synthesize these BCAAs, but animals do not.

The anabolic enzyme, acetohydroxyacid synthase (AHAS; EC 2.2.1.6) catalyzes the first step in the biosynthesis of the BCAAs valine, leucine, and isoleucine. AHAS, in common with several other enzymes that catalyze the decarboxylation of 2-ketoacids, uses thiamine diphosphate (ThDP) as a cofactor and also requires a divalent metal ion to anchor ThDP in the active site.[5] However, AHAS does not exhibit a high level of metal ion specificity for catalytic activity. *Salmonella typhimurium* AHASII was active in the presence of Mn^{2+}, Mg^{2+}, Co^{2+}, Ca^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}, or Ba^{2+}. The three-dimensional structure of yeast AHAS reveals the location of several active-site features, including the position and conformation of the cofactors ThDP, Mg^{2+}, and FAD.[7] The reported crystal structures of AHAS assume that the metal ion observed in the structure is Mg^{2+}, since it was added in high concentration (> 5 mM) to the crystallization buffer and to the protein during purification.[7-8]

In this study, metal ion activation of AHAS was performed with Hin-AHAS. Previously, we had purified recombinant Hin-AHAS and identified potent inhibitors.[9] Cofactors and metal ion-free Hin-AHAS (apo-AHAS), prepared by exhaustive dialysis, showed a complete loss of residual activity. Subsequent addition of 1 mM ThDP and 10 mM Mg^{2+} (final concentrations) resulted in full reconstitution of activity (100%). Besides Mg^{2+}, the metal ions Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, Cu^{2+}, and Zn^{2+} were tested as potential activators of apo-AHAS in the presence of 1 mM ThDP, 10 μM FAD and 100 mM pyruvate. Only Ca^{2+}, Mn^{2+}, and Co^{2+} were activators, providing 103%, 110%, and 27% residual activity, respectively, whereas Ni^{2+}, Cd^{2+}, Zn^{2+}, and Ba^{2+} with Hin-AHAS showed no activity. These results were not completely identical with *S. typhimurium* AHAS activation,[6] in which significant activity was found with Ni^{2+}, Cd^{2+}, Zn^{2+}, or Ba^{2+} activated AHAS. The k_{cat} values of Mg^{2+}, Mn^{2+}, and Ca^{2+}activated Hin-AHAS were in a similar range (4.48 - 6.68 s^{-1}), whereas, the K_c value for Mn^{2+} (0.37 mM) was significantly lower than the K_c values for Mg^{2+} and Ca^{2+} (Fig. 3 and Table 1). The K_c value for ThDP activation was about 9.1 μM (Table 1), as determined by measuring the catalytic activity of the 10 mM Mg^{2+}-activated Hin-AHAS in the presence of 100 mM pyruvate and 10 μM FAD.

AHAS has an essential requirement for FAD, which is unexpected, because the catalytic reaction does not involve oxidation or reduction. However, it was reported that the enzyme-bound FAD in AHAS is reduced in the course of catalysis in a side reaction.[10] In this study, we found experimental evidence for the presence of FAD and its reduction in Hin-AHAS. Purified Hin-AHAS has a bright yellow appearance. Even with no additional FAD, maximum enzyme activity was observed, indicating that the purified Hin-AHAS contained sufficient endogenous FAD and a nearly saturated FAD binding site (data not shown). The UV-visible absorbance spectrum of Hin-AHAS showed two peaks at 378 and 451 nm, which correspond to FAD (Figure 1). The molar concentration of bound FAD in 30.2 μM

Table 1. Metal ion and ThDP dependence of steady-state kinetic constants.

Cofactor	K_c (mM)	k_{cat} (s^{-1})
Mg^{2+}	0.74 ± 0.02	4.48
Mn^{2+}	0.37 ± 0.01	6.68
Ca^{2+}	1.06 ± 0.04	5.36
ThDP	0.0091 ± 0.01	4.5

The metal ion dependence of the steady-state kinetic constants for AHAS catalysis was measured in the presence of 100 mM pyruvate, 10 μM FAD, and 1 mM ThDP at pH 7.5. The ThDP-dependent catalysis was performed with 10 mM Mg^{2+}-activated AHAS in the presence of 100 mM pyruvate and 10 μM FAD with varied concentrations of ThDP.

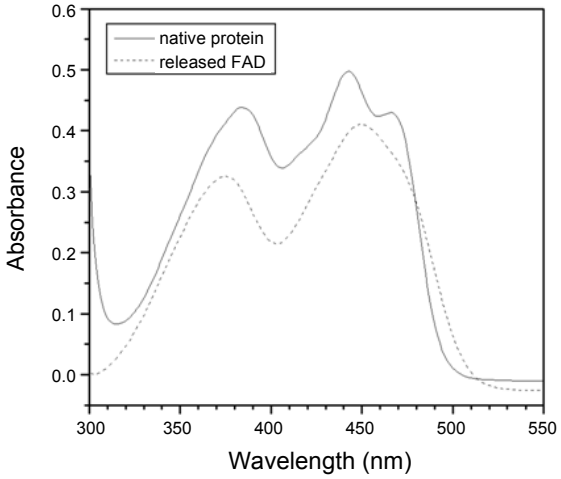

Figure 1. UV-vis absorbance spectra of native Hin-AHAS and released FAD. The released FAD was obtained by heat treatment, as described in the Methods section.

of Hin-AHAS was determined to be 36.3 μM, based on the released FAD spectrum, indicating a 1:1 stoichiometry (Figure 1). Furthermore, the reduction of enzyme-bound FAD in Hin-AHAS in the course of catalysis was analyzed. The UV-visible absorbance spectrum of Hin-AHAS in 100 mM potassium phosphate at pH 7.5 and 1 mM ThDP was recorded after addition of pyruvate at varied time intervals (0 - 20 min). As shown (Fig. 2), a time-dependent reduction of FAD was found with the addition of pyruvate to the reaction mixture. This result provides further evidence that the FAD bound in AHAS was reduced completely within 20 min.

By sequence alignment of Hin-AHAS with well-characterized AHASs from yeast, *A. thaliana* and *E. coli*, several key amino acid residues located at the active site or important for catalytic activity, respectively, were identified. The common step for all ThDP-dependent enzymes is the activation of the nucleophilic C2-atom, which occurs *via* deprotonation. A conserved glutamate residue (E139 in Yeast AHAS) donates a hy-

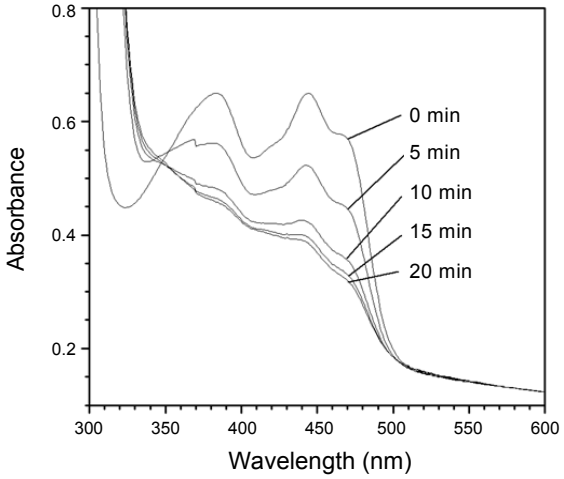

Figure 2. Time-dependent absorbance spectra of Hin-AHAS-bound FAD in the course of anaerobic reduction. The reaction was performed, as described in the Methods section. The spectra were recorded at 0, 5, 10, and 20 min after adding pyruvate.

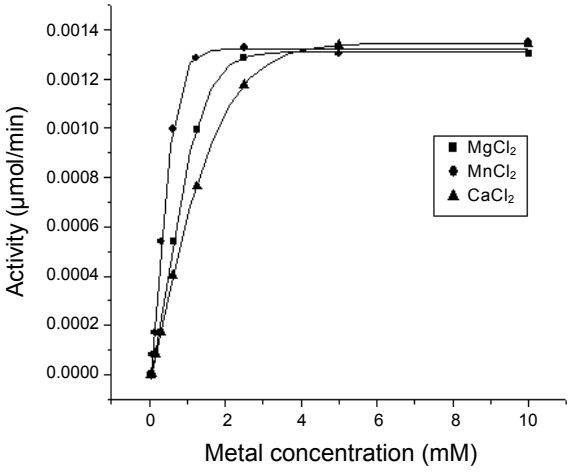

Figure 3. The activation of apo-AHAS in the presence of divalent metal ions, Mg^{2+}, Mn^{2+}, and Ca^{2+}. The preparation of apo-AHAS and activation experiments were conducted, as described in the Methods section. The data were fit to a Hill equation, and values are reported in Table 1.

Figure 4. Sequence alignment of Hin-AHAS, as compared with the sequences of *A. thaliana*, *E. coli*, and yeast AHASs. Conserved AHAS amino acid residues, which were catalytically important, are shown in boxes with symbols. Each symbol represents a group of amino acids responsible for cofactor and substrate binding, respectively (■, ThDP; ▲, Mg^{2+}; ▼, FAD and ◆, pyruvate binding).

drogen bond to the N1'-atom of the pyrimidine ring of ThDP, resulting in the activation of the C4'-amino group, which is the acceptor of the attached C2-ThDP-proton.[7] In the Hin-AHAS sequence, a strictly conserved glutamate residue, E51 (Hin-AHAS numbering), was identified and was predicted to be necessary for ThDP activation (Fig. 4). From several 3-D structures of ThDP-dependent enzymes, it was apparent that a divalent metal ion functions to anchor the ThDP molecule by coordinating two of the diphosphate oxygen atoms to the side chain oxygen atoms of the aspartate and the second asparagine residue of the ThDP-binding motif (D550 and N577 in Yeast AHAS).[7] These two residues (D447 and N474, Hin-AHAS numbering) were also found to be conserved in Hin-AHAS (Fig. 4). A recent study of the pyruvate mechanism in *E. coli* AHAS indicated that a valine and a phenylalanine residue (V375 and F109 in *E. coli* AHAS) hydrophobically interact with the methyl substituent of pyruvate,[11] and the alignment analysis also suggests conservation of these residues in all of the AHAS sequences (Fig. 4).

In conclusion, the present study characterizes the unique properties of AHAS cofactors. One molecule of FAD was found to be present in one molecule of Hin-AHAS, and bound FAD was reduced in a time-dependent manner with the addition of pyruvate. Further, Hin-AHAS was found be active in the presence of the varied divalent metal ions, Mg^{2+}, Mn^{2+} and Ca^{2+}. In the absence of a Hin-AHAS 3-D structure, we have compared various AHAS sequences by sequence alignment for which 3-D structures are available, and several conserved residues were identified in the Hin-AHAS sequence that are located at the active site or are important for catalytic activity.

Experimental Section

The molar ratio of FAD/Hin-AHAS. A reaction mixture containing 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 5 mM β-mercaptoethanol, 10% glycerol, and 30.2 μM Hin-AHAS (final concentration) was prepared, and the absorbance spectrum between 300 - 550 nm was recorded on a Lambda25 UV-vis Spectrophotometer, PerkinElmer, USA. After this scan, the Hin-AHAS was denatured by boiling for 5 min, cooled in an ice bath, and the protein precipitate was removed by centrifugation at 23,000 × g for 30 min. The supernatant containing released FAD was collected, and the absorbance spectrum between 300 - 550 nm was recorded. The concentration of released FAD was determined from the absorbance at 450 nm (A_{450} = 0.41), using the extinction coefficient for free FAD (ε_{450} = 11,300 M^{-1} cm^{-1}).

FAD reduction during Hin-AHAS catalysis. Reduction of FAD was detected by recording the UV-visible spectra of Hin-AHAS between 300 - 600 nm. Hin-AHAS (final concentration, 26.6 μM) was pre-incubated with 100 mM potassium phosphate, pH 7.5, and 1 mM ThDP at 37 °C for 10 min in a quartz cuvette with a stopcock. The reaction was initiated by the addition of 100 mM pyruvate, followed by purging the cuvette with argon gas. UV-visible spectra between 300 - 600 nm were obtained at 37 °C until FAD reduction was complete.

Metal ion and ThDP activation. Cofactor-free AHAS (apo-AHAS) was prepared by dialysis against a buffer containing 100 mM HEPES, 20 mM EDTA, and 1 mM DTT at pH 7.5, followed by exhaustive dialysis against a buffer containing 100 mM HEPES and 1 mM DTT at pH 7.5 in order to remove EDTA. Metal ion activation assays were performed in 200 μL reaction mixtures containing 100 mM pyruvate, 1 mM ThDP, and 10 μM FAD, and varying concentrations of divalent metal ions in a buffer containing 100 mM HEPES at pH 7.5. The reaction was initiated by the addition of 1 μg of apo-AHAS. The enzyme assay was performed as described previously.[12] The ThDP activation of apo-AHAS was performed, as described previously, with Mg^{2+} as the metal ion.[13] All data points represent the average of at least two independent experiments.

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