Yeast Acetohydroxyacid Synthase. Crystal Structure of an Enzyme Containing Non-Catalytic FAD

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Introduction

Acetohydroxyacid synthase (AHAS; E.C. 4.1.3.18) (1, 2) is involved in the first reaction that is common to the biosynthesis of branched-chain amino acids. The enzyme catalyzes the decarboxylation of pyruvate and condensation with either a second molecule of pyruvate to give acetolactate, or a molecule of 2-ketobutyrate to yield acetohydroxybutyrate. The former product is then converted in several more steps to valine and leucine, and the latter to isoleucine. AHAS is also the target site of several classes of potent and widely used herbicides including sulfonylureas (3, 4) and imidazolinones (5).

AHAS requires three cofactors; thiamin diphosphate (ThDP), a divalent metal and has an unexpected requirement for FAD since the reaction catalyzed involves no oxidation. The presence of FAD is believed to play a solely structural role and is an evolutionary remnant from a pyruvate oxidase (POX)-like ancestral enzyme (6). Schoss *et al.* (7) have reported that the flavin cofactor of a bacterial AHAS can be replaced by $FADH_2$ and various FAD analogues (5-deaza FAD and 8-chloro FAD) with little or no effect on the enzymatic activity. This rules out any hidden redox function in AHAS.

AHAS contains both catalytic and regulatory subunits. The catalytic subunit contains all the enzymatic machinery while the regulatory subunit confers upon the enzyme allosteric regulation by end-product feedback inhibition (8). Here we report the first crystal structure of any AHAS catalytic subunit. Examination of the active site and FAD binding site of the enzyme may provide us with some clues to why the flavin cofactor is required.

Materials and Methods

The AHAS catalytic subunit of *Saccharomyces cerevisae* was over-expressed as a hexa-histidine fusion protein in *Escherichia coli* stain BL21(DE3) (9). The recombinant protein was isolated by immobilized-metal affinity chromatography, and further purified by size-exclusion chromatography. The enzyme was crystallized by

hanging-drop vapour diffusion in the presence of ThDP, $MgCl_2$ and FAD (10). X-ray data were collected from cryoprotected crystals at 100 K on Beam Line 14C at the Advance Photon Source in the Argonne National Laboratory, Chicago, USA. The structure was solved by molecular replacement using the program AMoRe (11). The probe structure is the partial monomer structure of benzoylformate decarboxylase (BFDC) (12). Model rebuilding and refinement were carried using O (13) and CNS (14). The final structure of AHAS has R_{factor} and R_{free} values of 0.188 and 0.219, respectively (15). The figures were generated using INSIGHT2000.1 (MSI, San Diego, CA).

Results and Discussion

Overall Structure

The crystal structure of AHAS was refined at a resolution of 2.6 Å. AHAS is a dimeric enzyme (Fig. 1b). Each monomer is folded into three domains, designated as α, β and γ (Fig. 1a). The domains are of similar size (about 180 amino acids) and have an α-β architecture consisting of a central six-stranded parallel β-sheet surrounded by several helices. In the dimer, the subunits are associated mainly through the α and γ domains, with the β domains on either side of the protein (Fig. 1b).

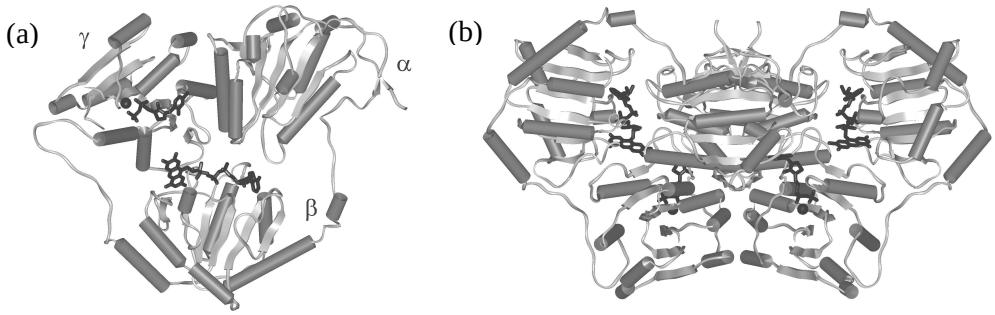


Figure 1: The three-dimensional structures of yeast AHAS. (a) The AHAS monomer is folded into three domains designated α, β and γ. (b) In the AHAS dimer, the α and γ domains of each subunit associate with each other to form the central core of the enzyme with the β domains on either side. Cofactors ThDP and FAD are depicted as stick model and Mg^{2+} as CPK sphere.

The overall fold of the AHAS monomer is similar to that observed in other ThDP-containing enzymes such as BFDC and POX. The former structure (12) was used as the probe model in the solving of the AHAS structure by molecular replacement. Unlike AHAS, BFDC contains no FAD. POX and AHAS are believed to be

evolutionarily related based on the analysis of their DNA and amino acid sequences (6). These similarities lead to the suggestion that the flavin requirement in AHAS is a vestigial remnant from a POX-like ancestral enzyme that has been retained for structural reasons. The phylogeny is further supported when the three-dimensional structures of POX (16) and AHAS are compared. Both enzymes have very similar domain organization and secondary structure topology (Fig. 2a).

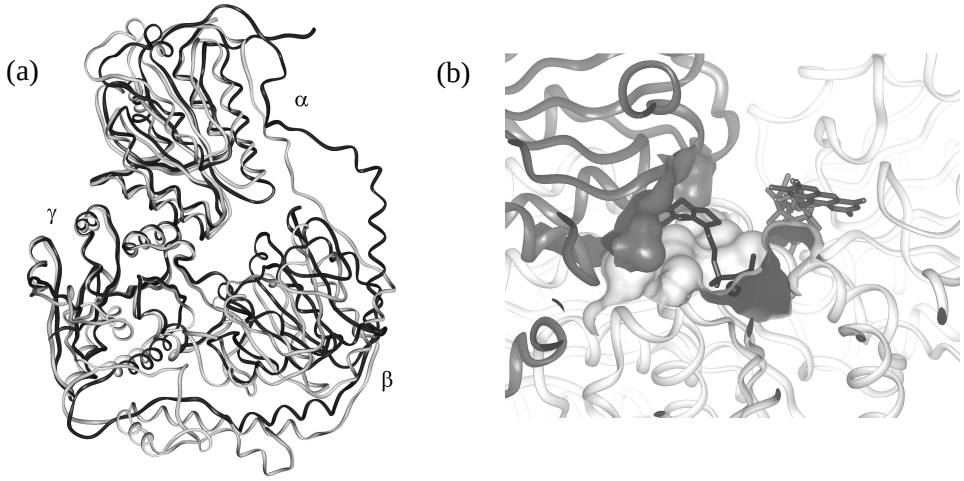


Figure 2: (a) Superimposition of the Cα traces of AHAS (black ribbon) and POX (grey ribbon), giving an rmsd value of 3.1 Å. (b) The active site of AHAS is located at the dimer interface. The polypeptide is represented in ribbon with residues within 4 Å from ThDP modeled as solid Connolly surfaces. Residues from different subunits are shown in different shades of grey. ThDP and FAD are depicted as stick models and Mg^{2+} as CPK sphere.

Active Site

AHAS has two active sites that are located at the dimer interface (Figs. 1b and 2b). The binding sites for ThDP, Mg^{2+} and FAD are located in or near the active site. ThDP is positioned centrally in the active site. During catalysis, the C2 atom of ThDP forms covalent bond with the reaction intermediates. ThDP forms contacts with residues from both subunits in the dimeric structure and binds to AHAS in a V-conformation (Fig. 2b). Similar cofactor interactions and conformations have also been described in the structures of other ThDP-dependent enzymes (12, 16-18). With ThDP in the V-conformation, a close approach, 3.1 Å, is made between the 4'-amino nitrogen and C2 (active site) atoms, which is critical for cofactor activation. The divalent metal Mg^{2+} is not involved directly in catalysis but functions to secure ThDP to the protein by coordinating the diphosphate group of ThDP and amino acid side-chains.

FAD Binding Site: Comparison of AHAS and POX

In AHAS, the cofactor FAD is bound to a double Rossmann fold in an extended conformation (Fig. 1a), similar to that observed in POX (16). The cofactor interacts almost exclusively with the β domain and forms numerous contacts with the protein. Although the FAD binding site is close to the dimer interface (Fig. 2b), the cofactor has only one interaction across subunits. Thus, FAD does not appear to play a direct role in stabilizing the dimer interface.

AHAS and POX are similar in many aspects, including sequence homology, substrate and cofactor requirements and three-dimensional structure. One obvious difference between the two enzymes is that POX uses FAD in a redox reaction while the cofactor in AHAS is not involved in catalysis. Examining the active sites of both enzymes show that the distance between the N5 atom of the flavin ring and the active site (C2 atom of ThDP) is more than 10 Å. In POX, the route by which electrons are transferred from the reaction intermediates to FAD is unclear. Based on the distance, direct electron transfer seems unlikely and several potential indirect routes have been proposed (16). Unlike POX, the flavin ring of AHAS is orientated such that N5 nitrogen is pointing away from the active site (Fig. 3a), which may render it inefficient for electron transfer. The other factor that may allow FAD to be redox active in POX but not in AHAS is that it is planar in AHAS (Fig. 3b) but bent by 15 ° across the N5-N10 axis in POX. This bend favors the reduced form and has been described in several flavin-dependent enzymes, although it does not appear to be an absolute requirement for redox function (19).

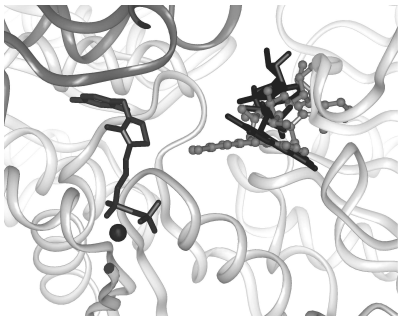
(a)  (b) 

Figure 3: Two different views of the orientation and conformation of FAD in AHAS (black, stick model) and POX (grey, ball and stick model), after the superimposition of the active sites (ThDP). (a) The isoalloxazine ring of AHAS is pointing away from the active site. (b) The isoalloxazine ring of POX is bent while it is planar in AHAS.

Conclusion

The first crystal structure of any AHAS catalytic subunit has been described. AHAS is a dimeric enzyme and the active site is located at the dimer interface. The crystal structure also reveals the conformation of FAD and its position in the active site. Comparison with the three-dimensional structure of the FAD-dependent POX shows that both enzymes have very similar overall structures and hence share a common ancestry. Examination of the active site of both enzymes, particularly the orientation and conformation of the isoalloxazine ring of FAD, suggest that it is unlikely that FAD plays a direct role in AHAS catalysis.

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