FAD-independent and Herbicide-resistant Mutants of Tobacco Acetohydroxy Acid Synthase

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Acetohydroxy acid synthase catalyzes the first common step in the biosynthesis of branched chain amino acids. AHAS plays two distinct metabolic roles, and is designated as anabolic AHAS and catabolic AHAS, depending on its function. Anabolic AHAS is FAD-dependent, while its catabolic counterpart is not. In this work, a conserved motif was identified in the β-domain of anabolic AHASs, but not in catabolic AHAS ($_{372}$RFDDR$_{376}$). In order to determine the functions of this motif, we replaced the motif with the corresponding sequence in FAD-independent AHAS, SPVEY. None of these three mutants (SPV, SPVE, and SPVEY) was detected with bound FAD. However, two of these mutants (SPVE and SPVEY) were active at a low level of specific activity. Although they exhibited pyruvate- and ThDP- dependent characteristics, the activity of the two active mutants appears to be FAD-independent. The SPVEY mutant was completely insensitive to the three tested herbicides, even at extremely high concentrations and is also somewhat more thermolabile than the wild type enzyme. The data provided in this work suggest that the RFDDR motif is a possible determinant of the FAD-dependent and herbicide-resistant properties of tobacco AHAS. The SPVEY mutant appears to exhibit catabolic AHAS-like activity.

Key Words : Acetohydroxy acid synthase, Sequence motif, Tobacco, Site-directed mutagenesis, Flavoenzyme

Introduction

Acetohydroxy acid synthase (AHAS, EC 2.2.1.6, also known as acetolactate synthase) catalyzes the first parallel step in the pathway of branched chain amino acid biosynthesis. These reactions involve the irreversible decarboxylation of pyruvate and the condensation of the acetaldehyde moiety with a second molecule of pyruvate to produce 2-acetolactate, or with a molecule of 2-oxobutanoate, yielding 2-aceto-2-hydroxybutyrate. This class of AHAS is called anabolic AHAS.[1] In certain microorganisms, AHAS performs another function in the fermentation pathway, which results in the formation of butanediol and related compounds.[2] This is called catabolic AHAS. These two classes of AHAS differ with regard to physiological activities, substrate specificities, and cofactor requirements.[1] Anabolic AHAS is FAD-dependent, while its catabolic counterpart is not.

Several sequence-structure analyses of FAD-containing enzyme have been carried out, and several conserved sequence motifs have been identified. Nevertheless, these studies have focused only on redox flavoenzymes.[3] Recently, the X-ray structures of anabolic and catabolic AHASs have been resolved by Duggleby's group.[4-6] The authors reported that, while the overall conformations of the FAD-dependent and FAD-independent AHASs were similar, we observed some salient differences in the β-domains of the two enzymes.[6]

In this work, a conserved motif was identified in the β-domain of anabolic AHAS from tobacco ($_{372}$RFDDR$_{376}$, tobacco numbering). Analysis of the structural model of tobacco AHAS revealed that this motif is closely associated with the FAD molecule. This motif, however, is not present in catabolic AHAS. The equivalent sequence of the catabolic AHAS is SPVEY, which is also conserved. From this evidence, we surmised that the RFDDR motif might be responsible for the difference in FAD requirements between the two classes of AHASs. In order to verify the function of this motif, we conducted three motif replacement mutations. Two of these are partial motif replacements, and one is a full motif replacement. The mutants were characterized with regard to their kinetic properties, cofactor requirements, and herbicide sensitivity.

Materials and Methods

Materials. Bacto-tryptone, yeast extract, and Bacto-agar were purchased from Difco Laboratories (Detroit, USA). Restriction enzymes were purchased from Takara Shuzo Co. (Shiga, Japan) and Boehringer-Mannheim (Mannheim, Germany). GSH, ThDP, FAD, α-naphthol, and creatine were obtained from the Sigma Chemical Co. (St. Louis, USA). Thrombin protease and epoxy-activated Sepharose 6B were obtained from Pharmacia Biotech (Uppsala, Sweden). *E. coli* XL1-blue cells harboring expression vector pGEX-AHAS were kindly provided by Dr. Soo-Ik Chang (Chungbuk National University, Cheongju, Korea). Oligonucleotides were obtained from Genotech (Daejon, Korea). Londax (a sulfonylurea herbicide) and Cadre (an imidazolinone herbicide)

Abbreviations used: AHAS, acetohydroxy acid synthase; GSH, glutathione; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactoside; PCR, polymerase chain reaction; TP, triazolopyrimidine sulfonamide; ThDP, thiamine diphosphate.

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were kindly provided by Dr. Dae-Whang Kim (Korea Research Institute of Chemical Technology, Daejon, Korea). TP, a triazolopyrimidine derivative, was obtained from Dr. Sung-Keon Namgoong (Seoul Women's University, Seoul, Korea).

Site-directed mutagenesis. Site-directed mutagenesis procedures for the construction of tobacco AHAS mutants were performed as described earlier,[7] using the following mutagenic oligonucleotides:

SPV, 5'-GGTGAGcccgGtTGATAGAG-3'

SPVE, 5'-GGTGAGcccgGtTGAaAGAGTTAC-3'

SPVEY, 5'-GGTGAGcccgGtTGAatacGTTACTG-3'

The lower-case bases were mismatched to introduce the mutation, and the underlined bases encode for the desired residues. The PCR products were doubly digested with *Nco*I and *Bgl*II, and cloned into the expression vector, which was prepared from the *Nco*I/*Bgl*II-excised pGEX-wAHAS. The resulting pGEX-mAHAS was used to transform the *E. coli* strain XL1-Blue cells, using standard $CaCl_2$ transformation instructions.[8]

DNA sequence analysis. DNA sequencing was carried out by the dideoxy chain-termination procedure[9] at Macrogen, Inc. (Seoul, Korea), in order to confirm the correct mutations.

Expression and purification of tobacco wAHAS and mAHAS. Bacterial strains of *E. coli* BL21-DE3 cells harboring the expression vector pGEX-AHAS were grown at 37 °C in Luria-Bertani (LB) medium containing 50 mg/ml ampicillin, to an OD_{600} of 0.7-0.8. pGEX-AHAS gene expression was induced via the addition of 0.1-0.3 mM isopropyl-D-thiogalactoside (IPTG). Cells were grown for an additional 4 hours at 30 °C, and were harvested by centrifugation at 5000 g for 30 minutes. wAHAS and mAHAS were purified as described previously.[10] The cell pellets were suspended in standard buffer (50 mM Tris-HCl, pH 7.5, 1 mM pyruvate, 10% (v/v) ethylene glycol, 10 mM $MgCl_2$) containing protease inhibitors. The cell suspension was then lysed by sonication at 4 °C. The homogenate was centrifuged for 20 minutes at 20,000 g twice, and the supernatant was applied to a GSH-coupled Sepharose 6B column. Unbound proteins were removed by washing with standard buffer. The GST-AHAS fusion protein was recovered from the column with elution buffer (50 mM Tris-HCl, pH 7.5, 15 mM GSH, and 10% (v/v) ethylene glycol). In order to obtain the cleaved AHAS, the purified GST-AHAS was incubated overnight at 4 °C with thrombin (10 U/mg protein). The AHAS was then purified by an additional step of GSH-affinity chromatography. The isolated protein was identified *via* SDS-PAGE analysis,[11] and the protein concentration was determined by the method of Bradford.[12]

Enzyme assay. The enzyme activities of the purified wAHAS and mAHAS were assessed according to the method of Westerfeld,[13] with some modifications, as described previously.[14] The V_{max}, and K_m values for the substrate were determined by fitting the data into Equation (1), while the activation constant (K_c) values were obtained by fitting the data into equation (2).

$$v = V_{max} / (1 + Km/[S]) \quad (1)$$

$$v = V_0 + V_{max} / (1 + K_c/[C]) \quad (2)$$

In these equations, v is the reaction velocity, V_{max} is the maximum velocity, V_0 is the activity due to the trace of cofactors present in the apo-enzyme, K_m is the Michaelis Menten constant, K_c is the activation constant, [S] is the substrate concentration, and [C] is the added cofactor concentration. The K_i^{app} values were determined by fitting the data into Equation (3).

$$v_i = v_0 / (1 + [I] / K_i^{app}) \quad (3)$$

In this equation, v_i and v_0 represent the rates in either the presence or absence of the inhibitor, respectively, and [I] is the concentration of the inhibitor. The K_i^{app} is the apparent K_i, or the concentration of the inhibitor necessary to induce 50% inhibition under standard assay conditions, also known as the IC_{50}. All fittings and data analysis were performed with the program Sigma Plot 8.0 (Systat Software Inc., California, USA).

Spectroscopic measurement. Absorption spectra were recorded on a Beckman DU-600 Spectrophotometer. The protein solution was dispensed with 1 mL black-walled quartz cuvettes, and the spectrum of each sample was scanned over a range of 250-550 nm. Fluorescence emission spectra were recorded using a Hitachi F-3000 fluorescence spectrophotometer. The fluorescence spectra of FAD bound to wAHAS and mAHAS were scanned over a range of 450-650 nm *via* excitation at 370 nm.

Analysis of structural models. Analysis of the homology structure of tobacco AHAS was performed with DeepView, and the structural illustration was created using the same program,[15] coupled with Molecular Image software (http://www.molimage.com).

Results

From the multiple sequence alignment, we discovered that the RFDDR motif was conserved in the FAD-dependent AHASs. In the meantime, the corresponding sequence in FAD-independent AHASs is SPVEY, which is also conserved (Fig. 1). Analysis of the tobacco AHAS homology model constructed earlier[7] revealed that the motif forms part of the pocket which accommodates the FAD molecule (Fig. 2).

A pattern search using the tool developed by the Supercomputer Lab, at the Institute for Chemical Research, Kyoto University (http://motif.genome.jp) revealed 59 proteins which contained the predicted motif in the Swiss-prot database (*accessed date Oct 23, 2004*). In this search, 29 proteins were found to be FAD-dependent AHASs. Apart from the AHASs, some of the non-AHAS proteins which contained the motif were reported to be FAD-dependent or ATP-dependent enzymes.[16-18]

In order to determine the function of the motif, we performed three motif replacement mutations *via* site-direct-

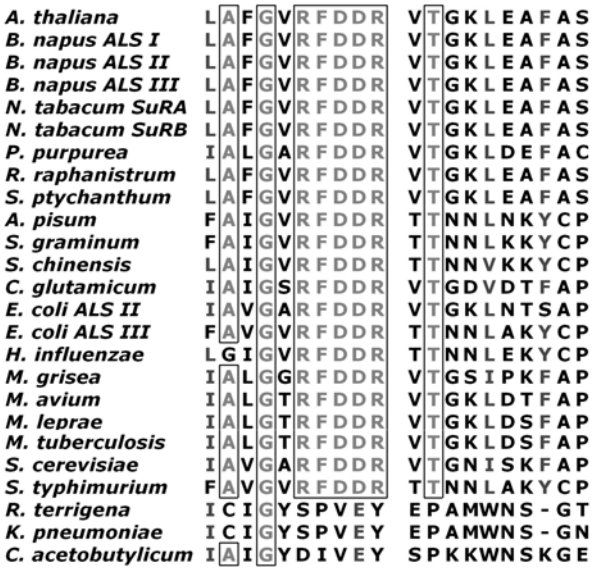

Figure 1. Multiple sequence alignment of 25 AHASs showing the conserved sequence motif in this study. The bottom three sequences are catabolic AHASs, and the other are anabolic AHASs. Alignment was carried out using ClustalW[19] integrated with the BIOEDIT program.[20]

ed mutagenesis. Three mutants were obtained, designated SPV (where $_{372}$RFD$_{374}$ is replaced by SPV), SPVE ($_{372}$RFDD$_{375}$ is replaced by SVPE), and SPVEY ($_{372}$RFDDR$_{376}$ is replaced by SPVEY) mutants. Three mutants were expressed and

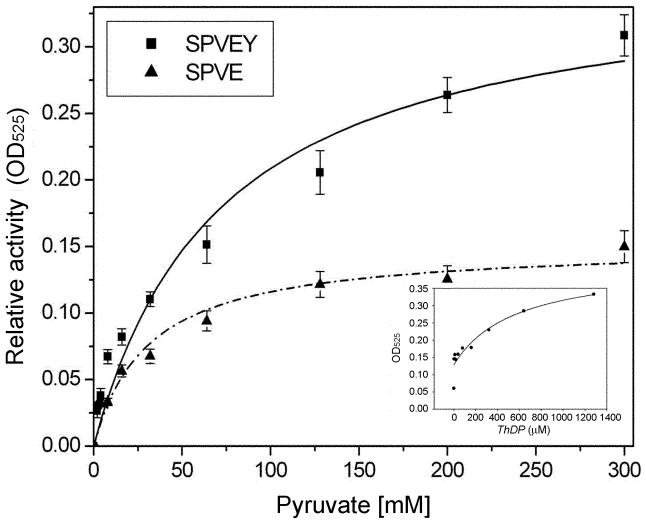

Figure 3. Pyruvate-dependent activity of the motif-replacement mutants (SPVEY and SPVE). Each reaction tube contains 37 μg of SPVEY protein in a 200 μL reaction volume. Activity was expressed arbitrarily as optical density at a 525-nm wavelength. Inset, ThDP-dependent activity of SPVEY mutant.

purified to homogeneity (data not shown). The SPV mutant, however, was found to be inactive under a variety of assay conditions (Table 1). The SPVE and SPVEY mutants were active, and their V_{max} values were quite low, but reliably detected. Their V_{max} values were 0.15% and 0.33% of that the corresponding values of the wild type enzyme,

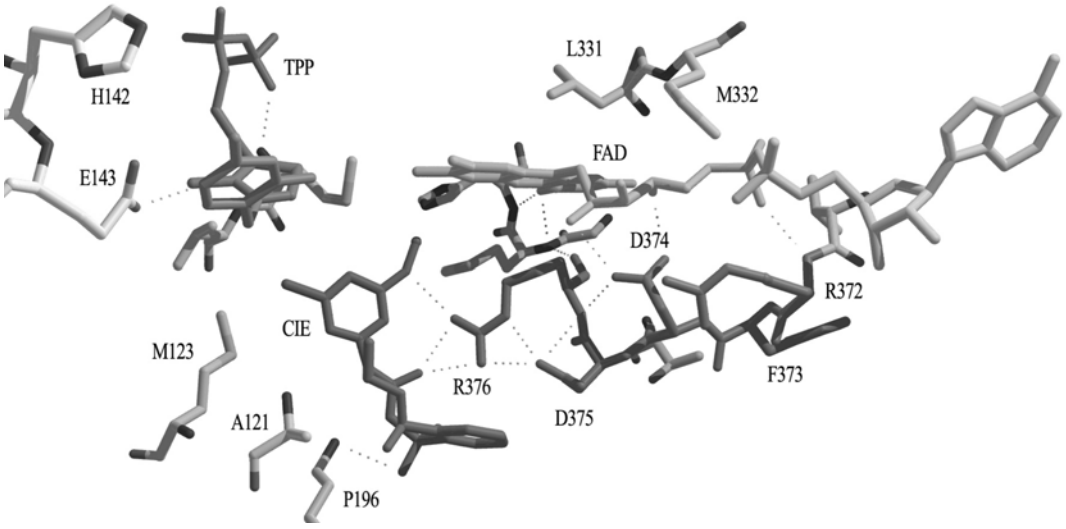

Figure 2. The structural model of the active site, herbicide-binding site, and cofactor-binding site of tobacco AHAS. CIE is the chlorimuron ethyl herbicide (a sulfonylurea). Dotted lines represent hydrogen bonds. This figure was created using Molw 4.0.

Table 1. Kinetic parameters of the wild type and motif-replacement mutants

Enzymes	V_{max} (U.mg^{-1})	K_m (pyruvate, mM)	V_{max}/K_m	Affinity for FAD		Affinity for ThDP	
				V_0	$(V_{max}-V_0)/K_{FAD}$	V_0	$(V_{max}-V_0)/K_{ThDP}$
wAHAS	1.55 ± 0.05	11.7 ± 1.9	132.47×10^{-3}	0.96	28.5×10^{-3}	0.86	23.6×10^{-3}
SPV	*No enzymatic activity*						
SPVE	0.002 ± 0.0002	28.9 ± 7.6	0.08×10^{-3}	0.002	∞	–	–
SPVEY	0.005 ± 0.0005	75.65 ± 18.1	0.07×10^{-3}	0.005	∞	0.0022	0.005×10^{-3}

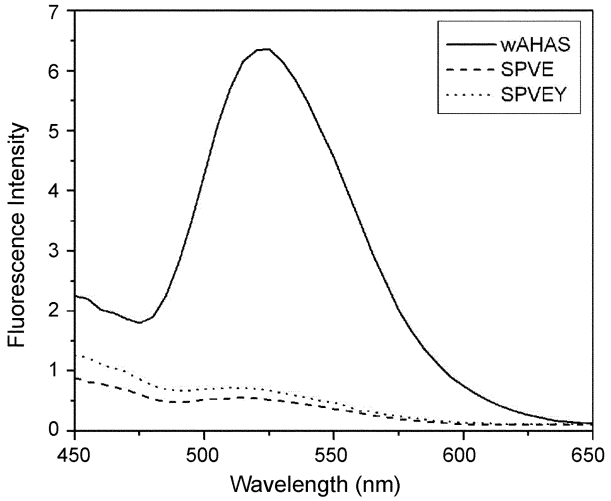

Figure 4. Fluorescence spectra of wAHAS and motif replacement mutants. The wild type and mutant enzymes were at a protein concentration of 0.4 mg/mL.

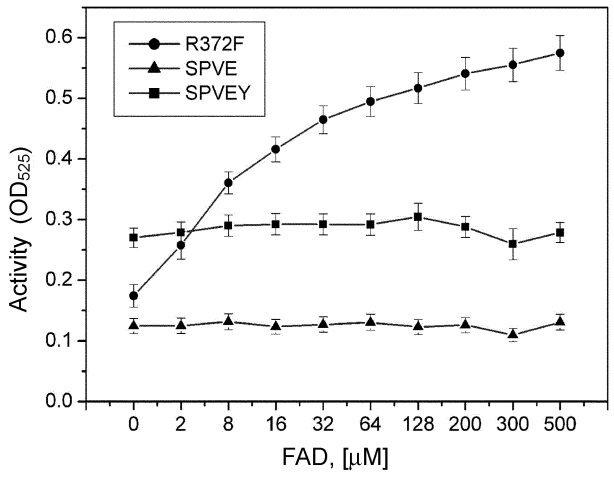

Figure 5. The activity of R372F, SPVE, and SPVEY mutants as a function of FAD concentration. No activation was observed with motif replacement mutants. The R372F mutant was reported previously to have reduced FAD-binding affinity. However, the R372F mutant was still activated by FAD.

respectively (Table 1). The pyruvate saturation curves of the mutants were hyperbolic, as was that of the wild type enzyme (Fig. 3).

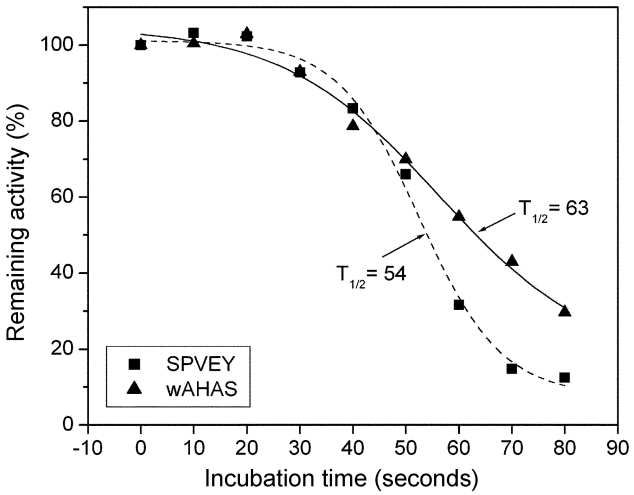

Figure 6. Thermostability of wAHAS and SPVEY mutants. Enzymes were incubated for different periods of time (as indicated in the graph), and then chilled on ice for 5 minutes before undergoing activity assays.

We also determined the absorption and fluorescence spectra of FAD bound to the three mutants. The data in Figure 4 (and data not shown) revealed that none of the mutants were able to bind with FAD. The FAD-dependent activities of the two active mutants were determined and compared to a single-point R372F mutant, which exhibited very week FAD-binding affinity (unpublished data). As shown in Figure 5, neither of the motif replacement mutants could be activated by the addition of FAD, even at high concentrations. Meanwhile, the R372F mutant was activated by the addition of FAD. In addition, the SPVEY mutant was completely insensitive to the three tested herbicides (Table 2). We also determined the thermo-stability of the SPVEY mutant. The data in Figure 6 indicate that the SPVEY mutant was slightly more thermolabile than was the wild type enzyme.

Discussion

Anabolic AHAS catalyzes the first common step in the biosynthesis pathway of valine, leucine, and isoleucine, in both plants and microorganisms. The enzyme requires ThDP, FAD, and Mg^{2+} as cofactors. Anabolic AHAS is

Table 2. Effects of three herbicides on activity of SPVEY and D375E mutants

Herbicide concentrations (mM)	Activity and IC_{50} values*					
	Londax		Cadre		TP	
	SPVEY	D375E	SPVEY	D375E	SPVEY	D375E
0	100	100	100	100	100	100
0.32	107	14.5	109.1	6.8	105.7	72.2
0.64	95.9	8.7	109.8	4.3	115.3	55.2
1.28	108.1	8.5	132.7	4	134.2	25.9
	N.D.*	90.7 ± 1.7 μM*	N.D.*	38.9 ± 7.4 μM*	N.D.*	8.71 ± 0.02 mM*

Activity was expressed as a percentage of the control (without herbicides) N.D, not detectable within the concentration range of 0-1.28 mM. D375E mutant was strongly resistant to three tested herbicides in comparison with that of the wild type enzyme (unpublished data).

capable of forming acetolactate and acetohydroxy-butyrate. It is comprised of two types of subunits, designated catalytic and regulatory subunits, and its activity is inhibited by the end products of the pathway. Catabolic AHAS can be distinguished from its anabolic counterpart by several characteristics: it possesses only one subunit, it is capable of forming only acetolactate, it is FAD-independent, it exhibits a low optimum pH, and it is insensitive to branch chain amino acids.[1] Recently, Han *et al.* has reported that catabolic AHAS from *Serratia marcescens* was insensitive to sulfonylurea herbicide.[21,22] More recently, chemical mechanism and catalytic properties of anabonic AHAS from tobacco have been reported.[23,24]

Multiple sequence alignments of anabolic and catabolic AHASs were performed, and we identified a unique anabolic AHAS sequence motif. This motif is identical in anabolic AHAS, but can not be found in catabolic AHAS (Fig. 1). This motif is located in the β-domain of AHAS, which was reported to be the principal difference between anabolic and catabolic AHASs.[6] This motif was also determined to form part of the pocket which accommodates cofactor FAD in the tobacco AHAS homology model (Fig. 2). The protein sequence motifs of the flavoenzymes have been the focus of special attention by a number of researchers. Several efforts have been made to analyze the protein structure-sequence motif of the FAD-dependent enzymes.[3] However, no report has yet provided and analysis of the structure-sequences of the non-redox flavoenzymes.

As an initial attempt to determine the structural motifs of the non-redox flavoenzymes, we assessed the functions of the identified motifs of anabolic AHAS, by replacing, either partially or fully, the motifs in the corresponding sequences in catabolic AHAS. Three of these constructed mutants were discovered to have no FAD bound to the enzyme (Fig. 4 and data not shown). The two active mutants, SPVE and SPVEY, exhibited FAD-independent AHAS activity (Fig. 5), although their activity was still dependent on the concentrations of ThDP and pyruvate (Fig. 3). Thus, it appears quite likely that the replacement of the motif by its catabolic counterpart resulted in a FAD-independent AHAS.

Since the herbicide-binding site is also located proximally to the FAD-binding site, we carried out herbicide inhibition tests using the sulfonylurea Londax, the imidazolinone Cadre, and the triazolopyrimidine TP. The results shown in Table 2 indicated that the SPVEY mutant was completely insensitive to the three tested herbicides. This motif appears also to be involved in the formation of the herbicide-binding site, and hence, removal of the motif deletes the herbicide binding site(s). The structural model of tobacco AHAS[7] also supports this hypothesis.

As the mutants lost their binding affinity for FAD, we are interested in ascertaining to what degree this could affect the thermostability of the enzyme. The SPVEY mutant and wAHAS were kept in a 60 ^{o}C water bath for different periods of time, and then evaluated for remaining activity. Our data indicated that the SPVEY mutant was slightly less stable than was the wild type enzyme. This may be attributable to the mutants' loss of FAD. The data also imply that this activity is, indeed, a true example of biochemical catalysis (which is thermolabile).

Overall data suggested that this motif is a possible determinant for the FAD-dependent characteristics of the anabolic AHASs. The replacement of this motif by an equivalent sequence in catabolic AHAS resulted in a mutant with catabolic AHAS-like properties, including FAD-independence and herbicide insensitivity. This motif may serve as a preliminary marker in the classification of acetohydroxy acid synthases into anabolic or catabolic groups, based on their amino acid sequences.

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