BIOSYNTHESIS OF BRANCHED CHAIN AMINO ACIDS V.2

Biosynthesis of Branched Chain Amino Acids

Proceedings of the Workshop on the Biosynthesis of Branched Chain Amino Acids

Beer-sheva, Israel, November 1988

Edited by Z. Barak, D. M. Chipman, J.V. Schloss

Balaban Publishers

VCH

Distribution

VCH Verlagsgesellschaft, P.O. Box 101161, D-6940 Weinheim (Federal Republic of Germany)

Switzerland: VCH Verlags-AG, P.O. Box, CH-4020 Basel (Switzerland)

United Kingdom and Ireland: VCH Publishers (UK) Ltd., 8 Wellington Court, Wellington Street, Cambridge CB1 1HW (England)

USA and Canada: VCH Publishers, Suite 909, 220 East 23rd Street, New York, NY 10010-4606 (USA)

ISBN 3-527-28040-5 (VCH, Weinheim) ISBN 0-89573-961-5 (VCH, New York)

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Dihydroxyacid dehydratase — Isolation, characterization as Fe-S proteins, and sensitivity to inactivation by oxygen radicals

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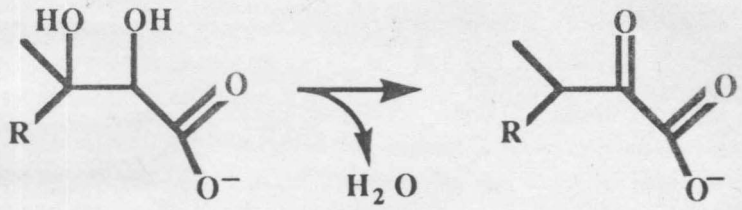
Abstract

Dihydroxyacid dehydratase has been purified from *Spinach oleracea* and *Escherichia coli*. In each case it has been found to be in its native state a dimer of identical subunits with molecular weights of 63,000 and 66,000 respectively. The enzyme from *S. oleracea* contains a [2Fe–2S] cluster and the enzyme from *E. coli* apparently contains a [4Fe–4S] cluster. The clusters appear to be involved in catalysis. The enzyme from *S. oleracea* is stable to O_2 and O_2^- whereas the enzyme from *E. coli* is inactivated by O_2 and inactivated rapidly by O_2^-.

Introduction

Dihydroxyacid dehydratase (2,3-dihydroxy-acid hydro-lyase, EC 4.2.1.9) catalyzes the third step in branched-chain amino acid biosynthetic pathway. This reaction is depicted in Scheme 1 and involves the conversion of two substrates differing only by a methyl group to their corresponding products. This enzyme is a critical component of the biological synthesis of branched-chain amino acids, coenzyme A, and the penicillin/cephalosporin class of antibiotics.

As a result of studies prompted by prior work on isoleucine and valine-requiring mutants of *Escherichia coli* and *Neurospora crassa*, in 1954 Myers and Adelberg published the first report on dihydroxyacid dehydratase (33). Their work showed the same enzyme was used by these organism to catalyze the conversion of both dihydroxyacid substrates to the corresponding keto products. The level of activity of dihydroxyacid dehydratase they found was adequate, but not excessive, for the flux through the branched-chain pathway.



R = Me or Et

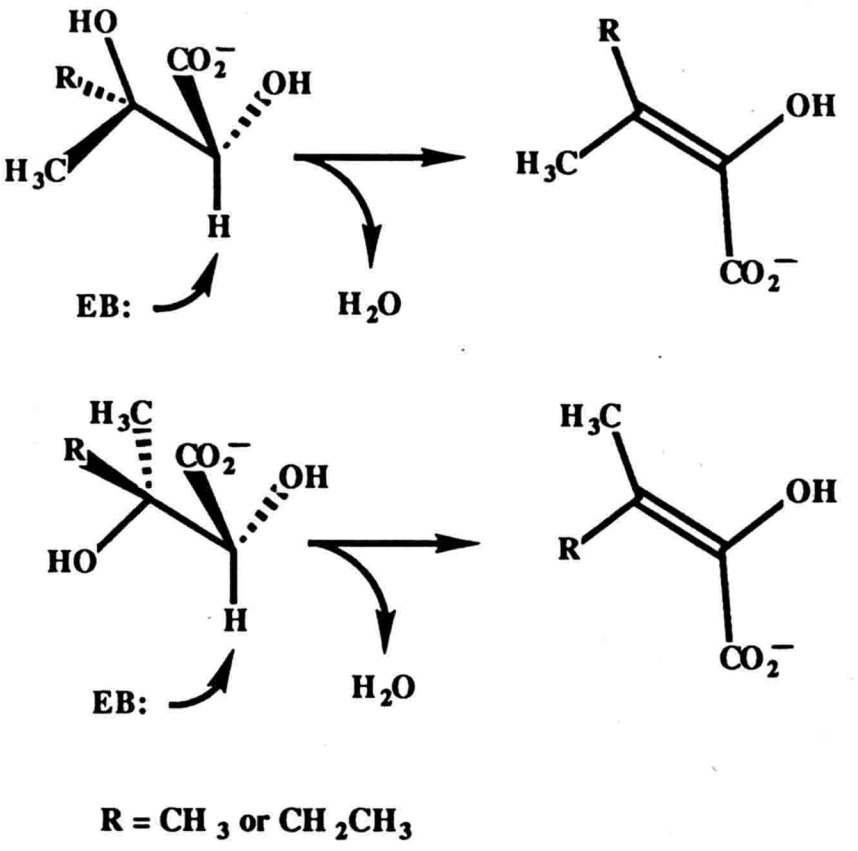
Scheme 1. The reaction catalyzed by dihydroxyacid dehydratase

Subsequently, in an extensive series of papers Wixom and co-workers found dihydroxyacid dehydratase in yeast (51), in several species of fungi (49), in several species of bacteria (52, 46, 48), and in all the species of higher plants examined from 29 different genera representing 14 families of angiosperms (50). Dihydroxyacid dehydratase was not found in the 16 vertebrate species examined or in certain microorganisms that require valine for growth (52, 53). To date dihydroxyacid dehydratase has been found in all species examined which do not require valine.

Enzymatic dehydrations of vicinal diols can occur via an enol intermediate (31,5) or the co-enzyme B_{12} mediated 1,2 intramolecular shift of a hydride ion (1). Arfin has shown that dihydroxyacid dehydratase from *Salmonella typhimurium* acts via an enol intermediate with the loss of the proton from the α position to solvent (5). The enol mechanism presumably holds true for all bacterial enzymes and for plant enzymes as well since plants do not contain co-enzyme B_{12}. The dehydration required to reach the enol intermediate could be either *syn* or *anti* as depicted in Scheme 2. No experiments have been done that bear on this question in the case of dihydroxyacid dehydratase. The majority of known enzymatic dehydrations are *anti* (37).

After the enol intermediate is formed, a tautomerization is required to reach the keto acid product. In the case of the isoleucine precursor, tautomerization in solution would lead to a racemic product. It has been shown that only the (3*S*) isomer is formed in the reaction catalyzed by *E. coli* dihydroxyacid dehydratase (24). The tautomerization must therefore occur stereospecifically on the enzyme surface before the product is released into solution. This same situation must be the case with all dihydroxyacid dehydratases since only the (3*S*)-2-oxo-3-methylpentanoic acid product would lead to the correct natural stereoisomer of isoleucine. The stereospecific tautomerization in the case of dihydroxyacid dehydratase contrasts to 6-phosphogluconate dehydratase, another vicinal diol dehydratase, which releases the enol intermediate into the solution to tautomerize non-stereospecifically (31).

In an early investigation of the stereochemistry of the reaction catalyzed by dihydroxyacid dehydratase, Hill and Yan found the natural substrates are (2*R*)-2,3-dihydroxy-3-methylbutyric acid and (2*R*,3*R*)-2,3-dihydroxy-3-methylpentanoic acid (21).

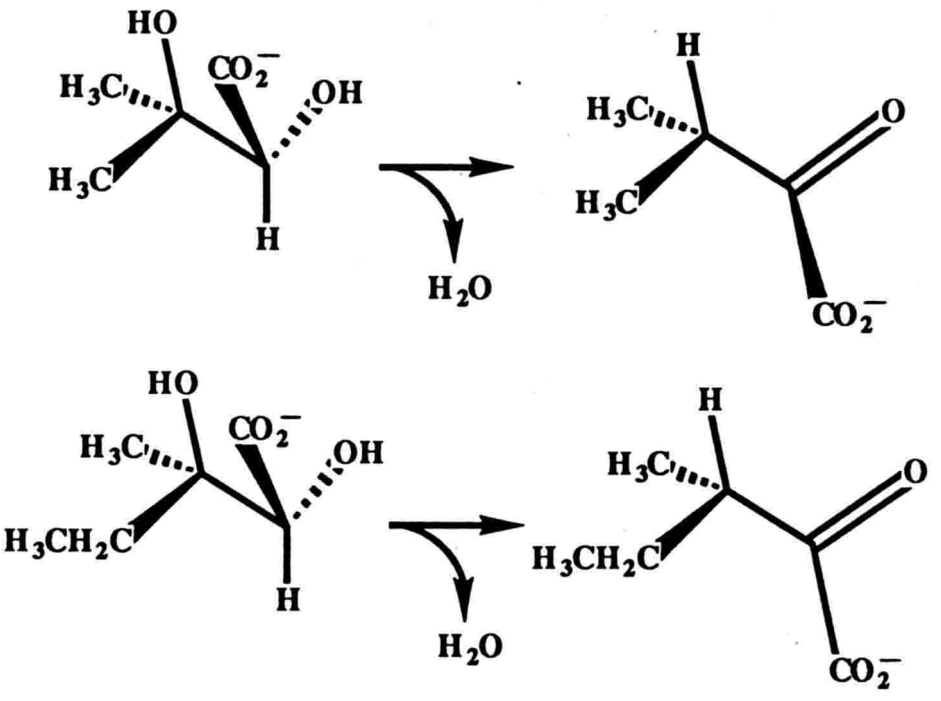


Scheme 2. Possibilities of *syn* and *anti* dehydration to the enol intermediate.

In a subsequent collaborative effort, the laboratories of Armstrong and Crout (2, 3, 4) investigated in greater detail the stereochemistry of the dihydroxyacid dehydratase reaction with the enzyme from *S. typhimurium*. It was found that dihydroxyacid dehydratase from this organism is not catalytically active on the following unnatural isomers of the two substrates (2*S*)-2,3-dihydroxy-3-methylbutyric acid, (2*S*,3*S*)-2,3-dihydroxy-3-methylpentanoic acid, or (2*S*,3*R*)-2,3-

dihydroxy-3-methylpentanoic acid. As expected it is catalytically active on the natural substrate isomers (2*R*)-2,3-dihydroxy-3-methylpentanoic acid; and (2*R*,3*R*)-2,3-dihydroxy-3-methylpentanoic acid; and in addition, it is catalytically active on one unnatural isomer (2*R*,3*S*)-2,3-dihydroxy-3-methylpentanoic acid. It is interesting that *S. typhimurium* dihydroxyacid dehydratase is active only on the stereoisomers with the natural (*R*) isomer at the 2-carbon; but it is active on both the natural (*R*) isomer and the unnatural (*S*) isomer at the 3-carbon. It is not known if this is the case with the dehydratase from other organisms.

The products of the action of *S. typhimurium* dihydroxyacid dehydratase on (2*R*,3*R*)-2,3-dihydroxy-3-methylpentanoic acid and (2*R*,3*S*)-2,3-dihydroxy-3-methylpentanoic acid are respectively (3*S*)-2-oxo-3-methylpentanoic acid and (3*R*)-2-oxo-3-methylpentanoic acid. Thus in the case of *S. typhimurium* dihydroxyacid dehydratase, the proton that replaces the 3-hydroxyl group of the substrate occupies the same stereochemical position of the original 3-hydroxyl group as shown in Scheme 3. This must be true for dihydroxyacid dehydratase from all organisms since



Scheme 3. Stereochemistry of the reactions in the branched-chain amino acid biosynthetic pathway catalyzed by *S. typhimurium* dihydroxyacid dehydratase

the same stereoisomer of isoleucine is produced. It also suggests the group that activates the hydroxyl group for elimination to form the enol intermediate, and the group that donates the proton to the enol intermediate to form the keto product are close together on the enzyme surface.

Nine different substrate analogs with varied alkyl groups and stereochemistry at the β-carbon as well as the two natural substrates were investigated for their relative activity as substrates for *S. typhimurium* dihydroxyacid dehydratase (3). These substrates and analogs are shown below in Fischer projections in rank order of the most to the least active as substrates in Figure 1.

In all organisms examined, the valine precursor 2,3-dihydroxy-3-methyl-butanoic acid is turned over under V_{max} conditions at a rate 2 to 6 fold faster than the isoleucine precursor 2,3-dihydroxy-3-methylpentanoic acid (32, 47)

Wixom and co-workers have shown that the following substrate analogs are not active as substrates for dihydroxyacid dehydratase from various organisms: DL-serine, DL-threonine, DL-β-hydroxyvaline, L-cysteine, DL-homocysteine, DL-dihydroxy-nonanoic acid, DL-dihydroxy-β-phenylpropanoic acid, D-, L-, and *meso*-tartaric acid, citric acid, D-glucuronic acid, D-galacturonic acid, D-ribonic acid, and D-mucic acid (2, 47).

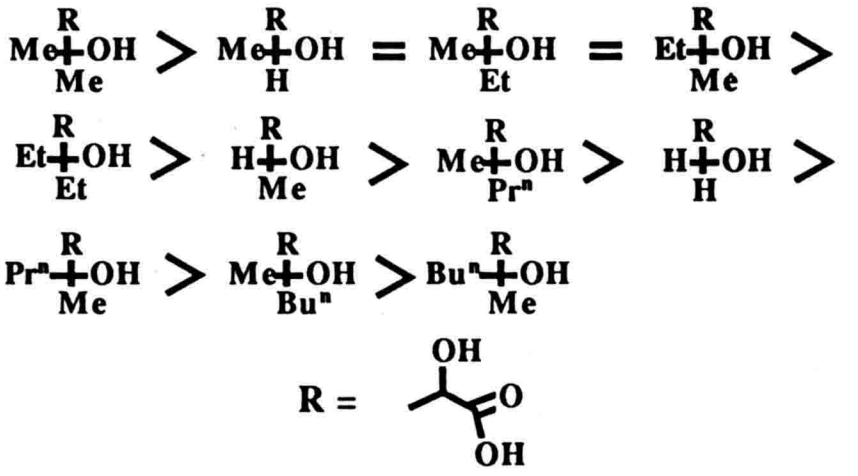


Figure 1: Substrate preference for *S. typhimurium* dihydroxyacid dehydratase.

Several reports of the effects of metal ions on dihydroxyacid dehydratase have been made. In *E. coli* Fe^{++}, Mg^{++}, and Mn^{++} activated the enzyme with Fe^{++} giving 3 fold more activity than the other two (32). In *Phaseolus radiatus* these same divalent metals along with several others activated the enzyme, but Mn^{++} and

Mg^{++} gave the greatest activation (38). In *N. crassa* and *Spinach oleracea* (hereafter referred to as spinach) Mg^{++} was required during purification to maintain enzyme activity and activated the enzyme as well, but besides Mg^{++} several other divalent metal ions would activate the enzyme (26, 23). In all of these cases the enzyme activity was 20–50% maximal without the addition of any divalent metal ion. Because of the impure state of these preparations, it is difficult to tell if the enzyme activity in the absence of added divalent metal ions was due to contamination with divalent metal ions in the preparation, or if dihydroxyacid dehydratase retains some activity in the absence of any divalent metal ions.

Dihydroxyacid dehydratase from *E. coli* is known to be an unstable enzyme (45, 20, 29). Presumably because of this instability early attempts to purify this enzyme from bacteria failed. In addition to the instability *in vitro*, the intriguing investigations of Brown and co-workers have shown that *E. coli* become starved for branched-chain amino acids when the cells are grown in minimal media in the presence of hyperbaric O_2 (7). This starvation develops because dihydroxyacid dehydratase is rapidly inactivated *in vivo* under these conditions (8). The observation that dihydroxyacid dehydratase was similarly inactivated when *E. coli* were grown aerobically in the presence of paraquat suggested the inactivating species was an oxygen radical produced in excess in the presence of hyperbaric O_2 and paraquat (17). One obvious candidate for the oxygen radical was O_2^-. This lead to a more recent study by Fridovich and co-workers in which they obtained indirect evidence that dihydroxyacid dehydratase from *E. coli* was sensitive to O_2^- (29). However, no rationale for the unusual sensitivity of this enzyme to oxygen radicals has previously been proposed.

Purification and characterization of spinach dihydroxyacid dehydratase

Dihydroxyacid dehydratase has been purified to homogeneity from spinach according to the protocol in Table 1 (19). The spinach enzyme is quite stable *in vitro* at pH 8 but loses activity rapidly if the pH is much below 8.

As shown in Figure 2, purified spinach dihydroxyacid dehydratase ran on SDS gels as a single band with a molecular weight compared to standard proteins of 63,500 and on native gels as a single band with a molecular weight compared to standard proteins of 110,000. The elution volume of dihydroxyacid dehydratase on a calibrated Superose 6B column was characteristic of a protein of molecular weight 105,000 (data not shown). These results indicate that dihydroxyacid dehydratase in its native state is a dimer.

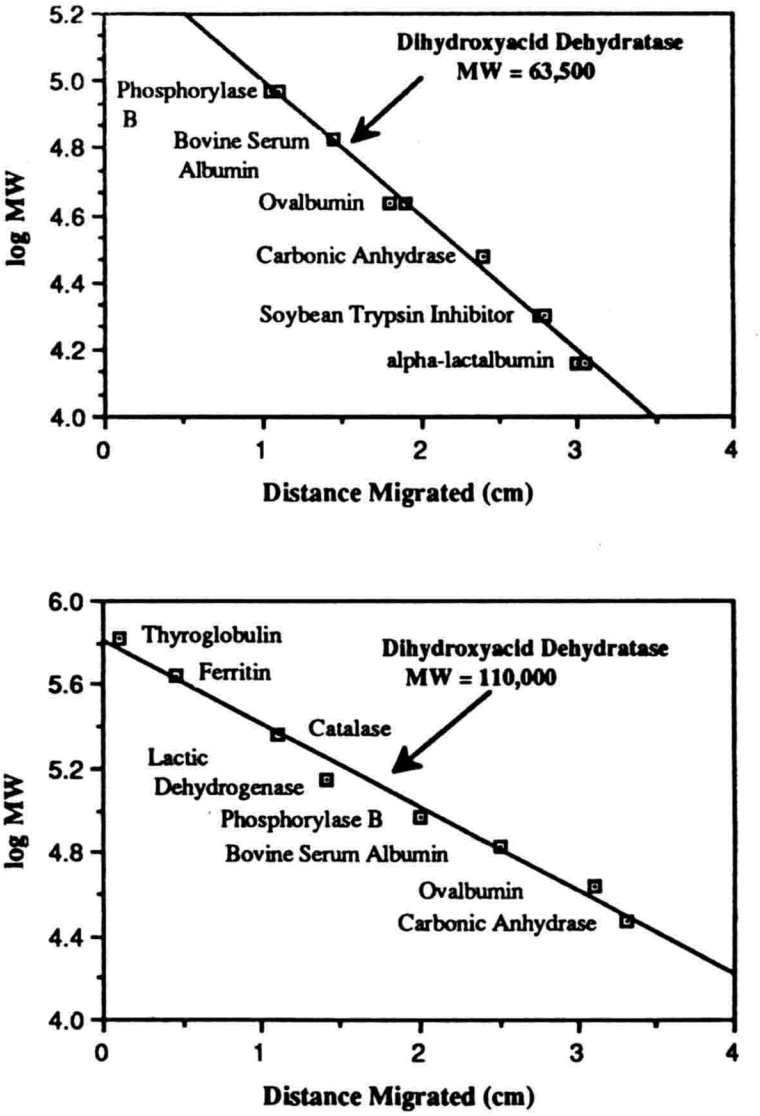


Figure 2: Molecular weight determination of SDS denatured and native spinach dihydroxyacid dehydratase by SDS and native polyacrylamide gel electrophoresis. The distance the standard proteins migrated is plotted against the log of the molecular weight. The distance dihydroxyacid dehydratase migrated in each case is indicated by an arrow. The molecular weight (MW) corresponding to these migration distances given.

Table 1: Purification of spinach dihydroxyacid dehydratase

	Volume *ml*	Protein *mg*	Activity *units*	Specific Activity *units/ mg protein*	Yield	Purif. Factor
Acetone Powder	solid	27400	4900	0.09	100	1
Acetone Precipitate	solid	9120	3920	0.43	80	5
DEAE-Sephacel	572	480	3890	8.1	79	95
Phenyl-Sepharose	225	90	2400	26.0	49	293
Matrix Green Gel A	52	10.5	1660	158	34	1780
Superose 6B	25	3.4	1650	486	34	5470

Kanamori and Wixom determined the value for K_m on impure spinach enzyme as 6.3 mM (23). Values determined by several groups for crude enzyme from other organisms vary from 0.17 mM to 2.4 mM (27).

The values we have determined for the K_m of the pure spinach enzyme using the racemic substrate 2,3-dihydroxy-3-methyl butyrate at pH 8.0 is 1.5 mM. We have also found under these same conditions at 37.5°C $k_{cat} = 25\ s^{-1}$ (see Figure 3).

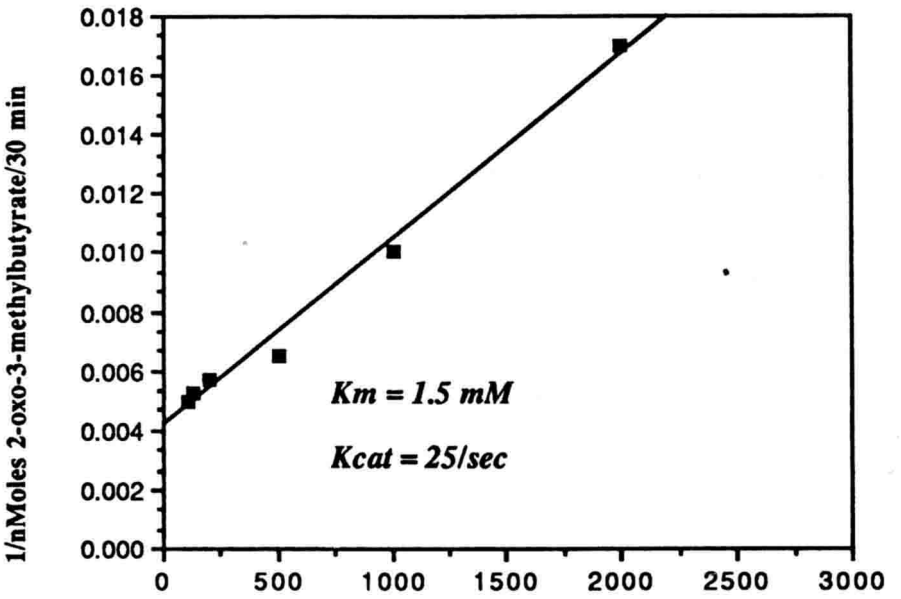


Figure 3: K_m and k_{cat} determination for spinach dihydroxyacid dehydratase.

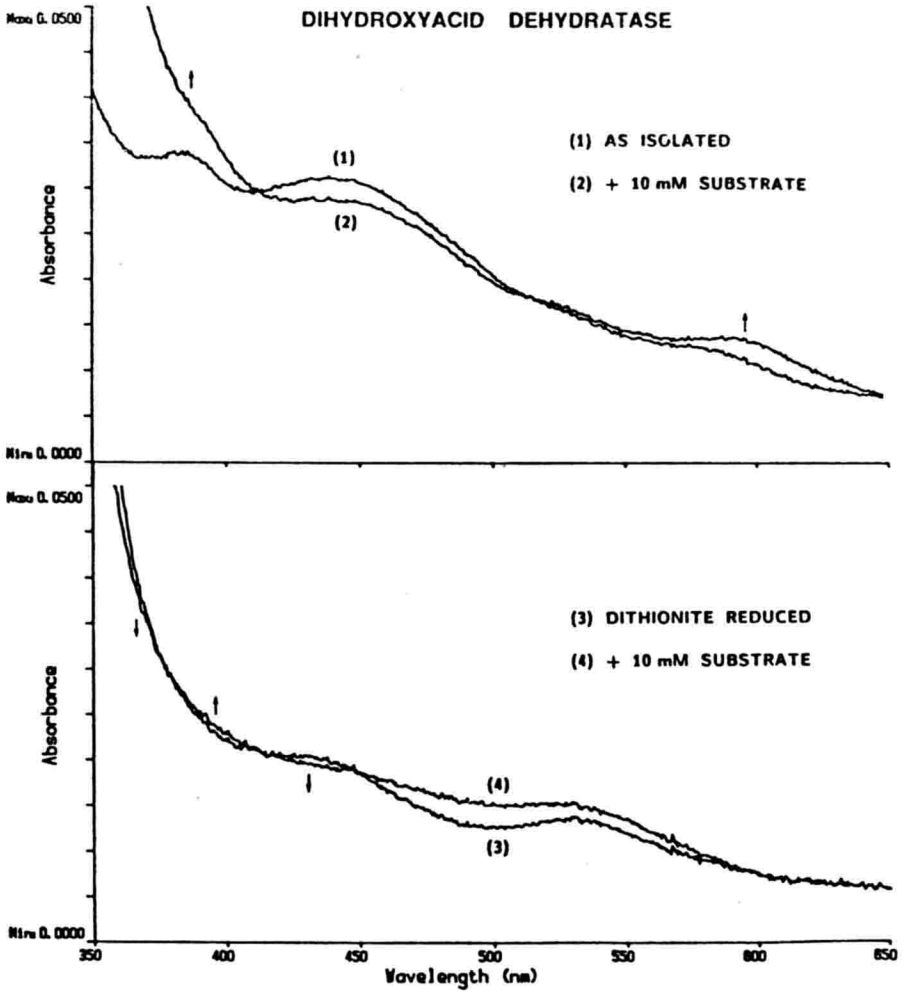


Figure 4: Absorbance spectrum of native and reduced spinach dihydroxyacid dehydratase.

Dihydroxyacid dehydratase isolated from spinach has a distinct brown color. The UV-visible absorbance spectrum is shown in Figure 4. The brown color arises from absorbance in the visible region that is characteristic of proteins with Fe-S clusters. The absorbance of dihydroxyacid dehydratase is bleached upon reduction by dithionite, which is another characteristic of proteins with Fe-S clusters. These results suggest that spinach dihydroxyacid dehydratase contains a Fe-S cluster. While the absorbance spectrum of dihydroxyacid dehydratase is not exactly like any other known Fe-S protein, it more closely resembles that of proteins with [2Fe-2S] clusters (34) particularly the Rieske Fe-S proteins (36, 15). Also of interest is the

effect the substrate has on the absorbance spectrum in both the reduced and oxidized state as shown in Figure 4. Since the absorbance of Fe-S proteins in the visible region is due to charge transfer bands of the cluster, the effect the substrate has on this region of the absorbance spectrum of dihydroxyacid dehydratase suggests the electronic nature of the cluster is perturbed upon substrate binding.

Spinach dihydroxyacid dehydratase was analyzed for the presence of Fe and S. The results are shown in Table 2. Approximately 2 mole of Fe and 2 mole of S was found per mole of protein monomer indicating the presence of a single [2Fe-2S] cluster per monomer.

Table 2: Iron, sulfur, and spin analysis for spinach dihydroxyacid dehydratase

Fe analysis by atomic absorption (With the assistance of Barton Holmquist, Harvard University)	**nM DHAD** (monomer)	**nM Fe**
Sample 1	1.3	2.58
Sample 2	0.95	1.94

colorimetric Fe and S= analysis	**mM DHAD** *(monomer)*	**mM Fe**	**mM S=**
Batch 12	110	160	180

EPR spin concentration for reduced enzyme	**mM DHAD** (monomer)	**mM Spins**	**mM Fe** (@ 2Fe/Spin)
Batch 13	34	35	70

The EPR spectra of dithionite reduced dihydroxyacid dehydratase at 30 K is shown in Figure 5. The upper spectrum is of the reduced enzyme in Tris buffer Double integration of the derivative spectrum gives a value of 1 spin/1.9 irons using Cu^{++}-EDTA as a spin standard. It is composed of two rhombic species with roughly equivalent intensities, with g-values at 1.99, 1.91 and 1.82 for component and 2.04, 1.89 and 1.82 for component II. While the ratio of the two components is not significantly affected by changes in the concentration of $MgCl_2$ (0–100 mM), it is sensitive to the type of buffer. In 50 mM EPPS, pH 8, component I becomes the

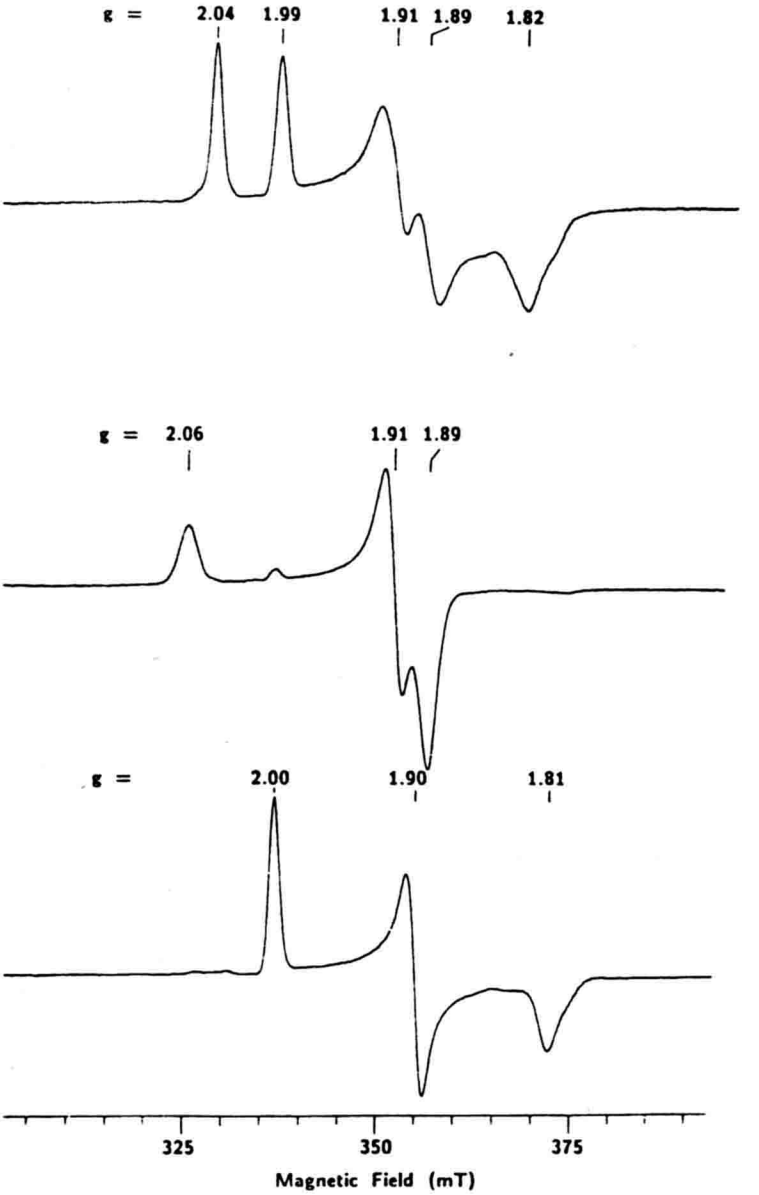


Figure 5: **EPR spectra in order from top to bottom of reduced dihydroxyacid dehydratase, reduced dihydroxyacid dehydratase with substrate present, and reduced dihydroxyacid dehydratase with product present.**

dominant species, representing 80–90% of the total spins. We conclude the two species are not due to protein impurities because 1) both native and SDS gels show single bands, 2) addition of either substrate or product produce a single EPR species, and 3) changing buffers affects the ratio of two species. The EPR spectra of the reduced Fe-S protein aconitase also exhibit buffer effects (12). The g-values of component I are very similar to those seen for reduced Rieske Fe-S proteins (36, 15). The EPR spectrum of component I continues to show sharp spectral features at temperatures as high as 80 K (data not shown) which is also indicative of proteins with [2Fe-2S] clusters. However, the spectrum of component II has broadened to the point of being invisible at 80 K, indicative of fast spin-lattice relaxation of component II at this temperature.

Taken together the data from the UV-visible spectra, the Fe and S analysis, and the EPR studies provide three fold evidence that spinach dihydroxyacid dehydratase has a [2Fe-2S] cluster. First, the Fe and labile $S^{=}$ analysis results show 2 atoms of Fe and $S^{=}$ per mole of enzyme monomer. Second, the double integration of the EPR spectrum of the reduced enzyme yields a value of 1 spin per 1.9 irons. Third, the g-values and the temperature dependence of the EPR spectra are characteristic of proteins with [2Fe-2S] clusters.

There is a growing number of enzymes in the hydro-lyase class (EC 4.2.1.x.) which have been shown to be Fe-S proteins, namely, aconitase (EC 4.2.1.3.) (25), phosphogluconate dehydratase (EC 4.2.1.12.) (40), maleate hydratase (EC 4.2.1.31.) (10), lactoyl-CoA dehydratase (EC 4.2.1.54.) (28), tartrate dehydratase (EC 4.2.1.32.) (43), and isopropylmalate isomerase (EC 4.2.1.33.) (11). However, other than spinach dihydroxyacid dehydratase only aconitase has been studied in enough detail to determine cluster type with certainty and has been found to have a [4Fe-4S] cluster. Spinach dihydroxyacid dehydratase is therefore the first example of a member of the hydro-lyase class with a [2Fe-2S] cluster. One wonders if it is possible that there are subtile functional differences in some aspect of the reactions carried out by these hydro-lyases that favor a [2Fe-2S] verses a [4Fe-4S] cluster. Two of the enzymes mentioned above, phosphogluconate dehydratase and tartrate dehydratase, convert vicinal diol carboxylic acids to the corresponding 2-keto acids as is the case with dihydroxyacid dehydratase. It will be interesting to see if they contain [2Fe-2S] clusters when they have been characterized well enough to identify the cluster type.

The spectroscopic studies of the [2Fe-2S] cluster of dihydroxyacid dehydratase demonstrate that it is novel compared to other known proteins with [2Fe-2S] clusters. It has some features which resemble the Rieske Fe-S proteins and other features which resemble 2Fe ferredoxins. Like the EPR spectra of the Rieske proteins, components I and II in the spectrum of reduced dihydroxyacid dehydratase have low g_{avg} values of 1.91 and 1.92, respectively.

Because the g_{avg} value is a reflection of the electronic properties of Fe-S clusters (6), the similarities in between the g_{avg} of dihydroxyacid dehydratase and the Rieske protein suggests some similarity in the clusters of these proteins . The cluster of the Rieske proteins has been studied in considerable detail (44, 16), and it may be of some value as a model of the cluster of dihydroxyacid dehydratase.

The unusual low g_{avg} of the Rieske proteins in comparison to 2Fe ferredoxins (g_{avg} = 1.91 vs 1.96, respectively) is thought to arise from an asymmetrical ligand environment at one Fe of the [2Fe-2S] cluster. In the case of the Rieske proteins this asymmetry arises from the replacement of two thiol ligands (cysteine) by nitrogen ligands (probably histidine), as demonstrated by ^{14}N electron nuclear double resonance spectroscopy (9, 43). In addition, the replacement of two negatively charged ligands by neutral ligands can explain the higher redox potential observed for Rieske proteins [+150 to +330 mV (16)] as compared to the 2Fe ferredoxins [–420 to –240 mV (35)].

The similarity of the g_{avg} value of the EPR spectra of dihydroxyacid dehydratase and the Rieske proteins led us to expect that the ligands and redox potential of [2Fe-2S] cluster of dihydroxyacid dehydratase also would be similar. However, the measured value for the redox potential of dihydroxyacid dehydratase is –470 mV, about 600 mV lower than the Rieske protein and in the lower end of the range of 2Fe ferredoxins. The low redox potential implies that the charge of the ligands in the [2Fe-2S] cluster of dihydroxyacid dehydratase is the same as in the 2Fe ferredoxins and is different than the ligands of the [2Fe-2S] cluster of the Rieske proteins. This result makes it unlikely that the [2Fe-2S] cluster of dihydroxyacid dehydratase will have nitrogen ligands like the Rieske proteins. If this is true it will leave sulfur and oxygen as possible ligands.

Two possible models for a cluster with negatively charged non-bridging ligands and a Rieske-like low g_{avg} are suggested by the work of Bertrand's group (6). The first is a cluster whose non-bridging ligands are all sulfur but whose geometry is distorted by compression of the tetrahedron of ligands around the Fe(II) ion. The second is a cluster whose two non-bridging ligands on one side of the cluster are oxygen (from phenolate or carboxylate). We are pursuing studies that would allow us to decide between these possibilities for the case for dihydroxyacid dehydratase.

Function of the Fe-S Cluster.

The presence of the [2Fe-2S] cluster in dihydroxyacid dehydratase raises a question about the role it may play in the catalytic events of the enzyme. In connection with this question, the EPR spectrum in the middle panel of Figure 5 shows the addition of substrate to the reduced spinach enzyme produces a transient