Common Ancestry of *Escherichia coli* Pyruvate Oxidase and the Acetohydroxy Acid Synthases of the Branched-Chain Amino Acid Biosynthetic Pathway

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A number of enzymes require flavin for their catalytic activity, although the reaction catalyzed involves no redox reaction. The best studied of these enigmatic nonredox flavoproteins are the acetohydroxy acid synthases (AHAS), which catalyze early steps in the synthesis of branched-chain amino acids in bacteria, yeasts, and plants. Previously, work from our laboratory showed strong amino acid sequence homology between these enzymes and *Escherichia coli* pyruvate oxidase, a classical flavoprotein dehydrogenase that catalyzes the decarboxylation of pyruvate to acetate. We have now shown this homology (i) to also be present in the DNA sequences and (ii) to represent functional homology in that pyruvate oxidase has AHAS activity and a protein consisting of the amino-terminal half of pyruvate oxidase and the carboxy-terminal half of *E. coli* AHAS I allows native *E. coli* AHAS I to function without added flavin. The hybrid protein contains tightly bound flavin, which is essential for the flavin substitution activity. These data, together with the sequence homologies and identical cofactors and substrates, led us to propose that the AHAS enzymes are descended from pyruvate oxidase (or a similar protein) and, thus, that the flavin requirement of the AHAS enzymes is a vestigial remnant, which may have been conserved to play a structural rather than a chemical function.

Virtually all flavoproteins are involved in oxidation-reduction reactions. However, a few anomalous enzymatic reactions are known (4, 10, 44; J. V. Schloss and D. E. Van Dyk, *Methods Enzymol.*, in press) which have an obligate requirement for flavin but for which the reaction catalyzed is not a redox reaction. In these enzymes, flavin adenine dinucleotide (FAD) can be replaced with FADH₂ or a variety of synthetic flavins without loss of enzyme activity (4; Schloss and Van Dyk, in press). The best-studied of these anomalous flavoproteins are the acetohydroxy acid synthases (AHAS) (Fig. 1), which catalyze early steps in the synthesis of branched-chain amino acids in bacteria (49), yeasts (13), and plants (35, 37). These enzymes have received much recent attention, since they are the targets for several widely used herbicides (12, 24, 25, 35, 37).

A possible clue to the enigmatic role of flavin in the AHAS enzymes comes from our finding of strong amino acid sequence homologies between the large subunits of the three AHAS isozymes of *Escherichia coli* and *E. coli* pyruvate oxidase (18). Pyruvate oxidase (pyruvate:ubiquinone-8 oxidoreductase; EC 1.2.2.2) of *E. coli* is a classic two-electron flavin dehydrogenase that catalyzes the oxidation of pyruvate to acetate and CO₂ (Fig. 1) (15). The oxidase is a peripheral membrane protein containing tightly bound FAD. Oxidase activity is greatly stimulated by phospholipid and several synthetic detergents (e.g., sodium dodecyl sulfate [SDS]) (15, 32). Phospholipid activation can be mimicked in vitro by a limited proteolytic digestion done in the presence of pyruvate and thiamine PP_i (39, 40, 41).

Pyruvate oxidase and the AHAS large subunit proteins are related by other criteria in addition to the amino acid sequence homologies. All four enzymes require FAD, thiamine PP_i, and Mg²⁺ for activity, utilize pyruvate, produce CO₂ from the pyruvate carboxyl group, and have similar monomeric molecular weights. We have now explored this

relationship in greater detail; in this paper we report evidence indicating that pyruvate oxidase is evolutionarily related to the AHAS isozymes.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains used were derivatives of *E. coli* K-12 (Table 1). Strain MH4 was constructed by M. Henry of our laboratory and has the Kan^r gene of Tn5 inserted into the *SalI* site in the middle of the *poxB* gene. Strain YYC365 is a derivative of strain RK4988. It was constructed by first transducing strain MI262 to Tet^r with a bacteriophage P1 lysate of strain YYC186 and then transducing the $\Delta aceEF$, *ilvI614*, *ilvH612*, and *leuB6* loci in a single P1 transduction into strain RK4988 by selecting for the *zac::Tn10* insertion of the donor. The resulting strain was then made Tet^s by selection on fusaric acid plates (29). The *poxB::Kan^r* allele was subsequently introduced by P1 transduction from strain MH4 with selection for Kan^r. The *recA* lesion of strain YYC365 was introduced by P1 transduction from strain JC10289 with selection for Tet^r.

Strain YYC395 was constructed by transduction of strain CU1126 with a P1 phage lysate grown on strain RK5596 followed by selection of Tet^r transductants, which were screened for $\Delta(ilvB-uhpA)$ lesion by lack of growth on fructose 6-phosphate (22). The $\Delta aceEF$ lesion was introduced by P1 transduction from strain YYC186. The other markers of strain YYC395 were introduced as in strain YYC365. Strain YYC396 was constructed by first transducing strain FD1050 with a P1 lysate grown on strain YYC186 and then introducing the $\Delta aceEF$ and $\Delta(ara-leu-ilvIH)863$ loci into strain CU1126 by a single phage P1 transduction with selection for the *zac::Tn10* insertion as above.

Plasmid pYYC35 (6 kilobase pairs [kbp]), which carries the *ilvBN* loci, was made by subcloning a 3.4-kbp *HindIII* fragment (49) of pDSE5 (45) into the *HindIII* site of plasmid pUC19 (52). Plasmid pYYC36 (4.8 kbp), which carries only

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FIG. 1. Reactions catalyzed by acetohydroxy acid synthase (AHAS) isozymes leading to valine, isoleucine, and leucine biosynthesis, and the reaction catalyzed by pyruvate oxidase.

the *ilvB* gene, was constructed by deleting a 1.2-kbp *SacI* fragment carrying the *ilvN* gene from pYYC35. Plasmid pYYC37 (5.2 kbp) was made from pYYC35 by complete digestion with *SmaI* and partial digestion with *EcoRV* followed by isolation of the 5.2-kbp *SmaI-EcoRV* DNA fragment from an agarose gel and ligation. This removed an *EcoRV* site, thus simplifying further constructions. Plasmid pYYC39 was made from pYYC37 and pYYC26 (see Fig. 3). Plasmid pYYC26 is a derivative of pYYC1 (3) in which a 1-kbp chromosomal DNA fragment downstream from the *poxB* gene was removed and two restriction sites, *KpnI* and *HindIII*, were introduced into the *poxB* gene at 527 and 1,348 bp from the initiation codon. These sites were introduced by in vitro oligonucleotide-directed mutagenesis without altering the amino acid sequence of pyruvate oxidase (Y. Y. Chang, and J. E. Cronan, Jr., unpublished results). In addition, the *HindIII* site on vector pBR322 of plasmid

pYYC26 was removed, but the vector remained Tet^r owing to restoration of a functional promoter sequence. Plasmid pYYC38 (4.8 kbp) was made in a manner analogous to pYYC39, except that pYYC36 was used in place of pYYC37.

Plasmid pYYC51 (4.8 kbp) was constructed from plasmids pYYC36 and pYYC40. Plasmid pYYC40 (4.8 kbp), which carries the *poxB* gene, was made by subcloning a 2.2-kbp *PstI-XhoI* fragment of pYYC26 into the *PstI* and *SalI* sites of vector pUC19. Plasmid pYYC40 was first digested completely with *EcoRI* and then digested partially with *EcoRV*. (There are two *EcoRV* sites 57 bp apart near the middle of the *poxB* gene. The *EcoRV* site near the N terminus of the oxidase is the site of fusion used in making the hybrid protein-coding genes.) The larger *EcoRI-EcoRV* fragment (ca. 0.8 kbp) of pYYC40, which encodes the C-terminal half of pyruvate oxidase, was ligated with the *EcoRI-EcoRV* fragment (4.0 kbp) of pYYC36 to form plasmid pYYC51. Plasmid pYYC52 (5.2 kbp) was made from plasmids pYYC36 and pYYC41. Plasmid pYYC41 (5.2 kbp) was formed by subcloning the 2.6-kbp *PstI-StuI* fragment of plasmid pCG20 (19), which encodes a pyruvate oxidase lacking the C-terminal last 24 amino acids between the *PstI* and *HindIII* sites of vector pUC19. Plasmid pYYC52 was made similarly to pYYC51 by ligating the 1.2-kbp *EcoRI-EcoRV* fragment of pYYC41 and the 4.0-kbp *EcoRI-EcoRV* fragment of pYYC36. Plasmids pYYC51 and pYYC52 were checked by restriction mapping, which confirmed the presence of two *EcoRV* sites.

Recombinant-DNA methods. The alkaline lysis plasmid preparation method, transformation, and gel electrophoresis and other DNA manipulation methods were those of Maniatis et al. (30). Restriction fragments were recovered from agarose gels by the method of Dretzen et al. (8) with a DEAE membrane (NA-45; Schleicher & Schuell, Inc., Keene, N.H.).

Preparation of extracts, enzyme assays, and other experimental conditions. Pyruvate oxidase was purified by P. Porter in the laboratory of R. Gennis (36) from strain CG6, an oxidase-overproducing strain constructed by C. Grabau of our laboratory. The enzyme preparations were about 95% homogeneous as assayed on SDS-polyacrylamide gels and by absorption spectra and had a specific activity of 75×10^3 U/mg of protein when the enzyme was activated with α -chymotrypsin. The oxidase (30 μ g) was activated with α -chymotrypsin by preincubating the enzyme in the presence of 0.1 M sodium pyruvate, 0.1 mM thiamine PP_i, 10 mM MgCl₂, and 20 μ g of α -chymotrypsin per ml in a final volume of 15 μ l for 30 min at room temperature. Unactivated enzyme consisted of the enzyme without treatment with α -chymotrypsin or SDS. Chymotrypsin-inactivated oxidase was prepared by preincubating the enzyme with α -chymotrypsin in the absence of pyruvate, thiamine PP_i, and MgCl₂. When the enzyme was activated with SDS, the detergent (20 μ M) was added to the assay mixture (see below) and incubated for 10 min at room temperature. Acetolactate synthase (AHAS) activity of the purified pyruvate oxidase was assayed by using a mixture (1 ml) consisting of 0.1 M sodium phosphate buffer (pH 6.0), 0.2 M sodium pyruvate, 0.1 mM sodium thiamine PP_i, 0.01 M MgCl₂, 25 μ M FAD, and α -chymotrypsin-activated purified oxidase (30 μ g). The mixture was incubated for 30 min at 37°C. The acetolactate formed was converted to acetoin by H₂SO₄ treatment, and acetoin was determined by the method of Westerfeld (51) as modified by LaRossa and Schloss (24). When the reaction was stopped by alkali, 0.1 ml of 2.5 N NaOH was added instead of H₂SO₄.

TABLE 1. Bacterial strains used in this work

Strain	Relevant markers	Source or reference
CY265	HfrC $\Delta aceEF$	1
CU1126	<i>ilvBN2102 ilvIH2202</i>	H. E. Umbarger
MH4	Kan ^r of Tn5 insert into <i>poxB</i>	This work ^a
JC10289	$\Delta(srl-recA) srl::Tn10$	CGSC ^b
MI262	<i>leuB6 ilvI614 ilvH612</i>	CGSC 5769
RK4988	MC4100 $\Delta(ilvB-uhpA)2089$	R. Kadner
RK5596	<i>zib-646::Tn10</i> $\Delta(ilvB-uhpA)$	R. Kadner
FD1050	$\Delta(ara-leu-ilvIH)863$	R. Lawther (9)
YYC88	<i>zac::Tn10</i> $\Delta aceEF$	1
YYC186	<i>zac::Tn10</i> $\Delta aceEF$ of CY265	CY265 $\times$ P1 (YYC88)
YYC329	<i>srl::Tn10</i> $\Delta(srl-recA)$ of CU1126	CU1126 $\times$ P1 (JC10289)
YYC365	$\Delta aceEF leuB6 ilvI614 ilvH612$ <i>poxB::Kan^r srl::Tn10</i> $\Delta(srl-recA)$ derivative of RK4988	This work ^a
YYC395	$\Delta aceEF \Delta(ilvB-uhpA) poxB::Kanr$ <i>srl::Tn10</i> $\Delta(srl-recA)$ derivative of CU1126	This work ^a
YYC396	$\Delta aceEF \Delta(ara-leu-ilvIH)863$ <i>srl::Tn10</i> , $\Delta(srl-recA)$ derivative of CU1126	This work ^a

^a For details of strain construction, see Materials and Methods.

^b CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

The condensation product formed by purified pyruvate oxidase was identified by using [3-¹⁴C]pyruvate (25 μ Ci; specific activity, 9.3 μ Ci/ μ mol) as substrate in a reaction analogous to that described above except on a larger scale. The radioactive product (ca. 1.4×10^6 cpm) was first decarboxylated with H₂SO₄ and then mixed with 2.36 g of acetoin, 4 g of semicarbazide hydrochloride, 6 g of sodium acetate, and water (final volume, 20 ml) and crystallized at 0°C. The acetoin semicarbazone crystals were recovered and washed with ice water, dried, and weighed. The crystals were dissolved in hot water, a sample was taken for radioactive counting, and the remainder was recrystallized. Recrystallization was repeated five times, and a constant radioactivity per gram (dry weight) was obtained for the last four crystallizations.

Pyruvate oxidase activity of the purified oxidase or crude extracts (1) was assayed by using Na₂Fe(CN)₆ as an artificial electron acceptor as described previously (1). One unit of pyruvate oxidase activity was defined as 1 nmol of pyruvate decarboxylated per min.

Crude extracts prepared from strains carrying plasmids were from log-phase cells grown on minimal glucose medium supplemented with sodium acetate (10 mM) (if required), L-isoleucine, L-leucine, and L-valine (0.01% each), and ampicillin (100 μ g/ml). Cells were harvested and washed once with 0.05 M potassium phosphate buffer (pH 7.0) containing 0.2 mM dithiothreitol, sonicated in the same buffer (0.5 g [wet weight] per ml), and centrifuged at $25,000 \times g$ for 30 min. The crude extract was then treated twice with activated charcoal (Norite; Fisher Scientific Co., Fair Lawn, N.J.) at 10 mg of charcoal per ml of extract. The assay of AHAS I activity in crude extracts of plasmid-carrying strains was similar to that described above, except that 0.1 M potassium phosphate buffer (pH 7.0) and 0.05 M sodium pyruvate were used and acetolactate was determined by the method of Umbarger (28). The FAD substitution activity of hybrid proteins was assayed by adding a fixed amount of the crude extract of strain YYC365 carrying plasmid pYYC37, and incubation was done in the absence of FAD. One unit of AHAS activity was defined as 1 nmol of acetolactate formed per min.

The experiments on the removal and reconstitution of the FAD moiety of the hybrid protein (see Table 4) were done with cells obtained from a 25-liter fermentor. The medium and the preparation of crude extracts were the same as described above, except that the buffer used throughout the experiment consisted of 20 mM potassium phosphate (pH 7.0), 0.5 mM dithiothreitol, 1 mM MgCl₂, and 0.1 mM thiamine PP_i (standard buffer) plus 20% glycerol. The 30 to 60% (NH₄)₂SO₄ fraction was dialyzed in standard buffer plus 20% glycerol for 1 h. Bound FAD was dissociated from proteins with KBr-acidic ammonium sulfate by the method of Gupta and Vennesland (21). The protein precipitate was redissolved in standard buffer plus 20% glycerol with or without FAD (25 μ M). After the protein was completely dissolved, Norite was added to remove free FAD. Protein concentrations were determined by a biuret procedure (27).

Glycerol gradients (10 to 30%, vol/vol) (see Fig. 4) were prepared in standard buffer–0.1 M pyruvate–10 μ M FAD (when FAD was present). Samples were loaded in the same buffer containing 5% glycerol. Centrifugation was done at 40,000 rpm at 4°C for 24 h in an SW41 rotor (Beckman Instruments, Inc., Fullerton, Calif.). At the end of centrifugation, a hole was punctured at the bottom of the tube and 0.3-ml fractions were collected.

Materials. Sulfometuron methyl (99% pure) was kindly

provided by E. I. du Pont de Nemours & Co., Inc., Wilmington, Del. Acetoin (acetylmethyl carbinol) and semicarbazide were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. FAD, thiamine PP_i, and other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. [3-¹⁴C]pyruvate was purchased from Du Pont, NEN Research Products, Boston, Mass. The media used were the rich or minimal media described previously (1). The dot-matrix analysis program was written and provided by C. Marck (31) and run on an Apple IIe computer.

RESULTS

Homology between pyruvate oxidase and the AHAS large subunits. Grabau and Cronan (18) previously reported that pyruvate oxidase has a similar extent (29 to 30%) of homology with the large subunits of each of the three AHAS isozymes by amino acid sequence alignment. (All three AHAS enzymes are composed of two types of subunits encoded by neighboring genes [*ilvBN*, *ilvGM*, and *ilvIH* for AHAS I, II, and III, respectively; 14, 26, 46, 50]. The large subunits are believed to carry the catalytic sites, whereas the small subunits seem to play regulatory roles [11, 23].) Since such direct amino acid sequence alignments can give misleading results (16), we have confirmed this amino acid sequence homology by a dot-matrix analysis of the amino acid sequence of pyruvate oxidase versus each of the three AHAS isozyme amino acid sequences. This analysis (Fig. 2A) confirmed that the protein sequence of pyruvate oxidase has a strong homology to all three large subunit sequences. Regions of homology were found at sites throughout the amino acid sequences, except at the extreme carboxy-terminal segment of pyruvate oxidase, a region that plays a key role in lipid activation of the oxidase (18, 19). Doolittle (7) argues that proteins with 20% or more primary sequence homology have a genuine relationship of common ancestry.

We also compared the DNA sequences of pyruvate oxidase with the sequences encoding the large subunits of the three AHAS isozymes. A dot-matrix plot of the nucleotide sequences of pyruvate oxidase gene (*poxB*) versus each of the three AHAS large-subunit genes (Fig. 2B) indicated that the AHAS I large-subunit gene (*ilvB*) was more homologous to the *poxB* gene than are the other two synthase large-subunit genes (*ilvG* and *ilvI*). Wek et al. (50) previously postulated from the protein sequences of the three isozymes that *ilvB*, *ilvG*, and *ilvI* came from a common ancestral gene. Our work strongly suggests that *poxB* is evolutionarily related to the AHAS isozymes and, in particular, that *poxB* is more closely related to the *ilvB* gene than to either of the *ilvI* or *ilvG* genes.

Pyruvate oxidase catalyzes the AHAS reaction. Since the discovery of the sequence homology between pyruvate oxidase and the AHAS isozymes, it has seemed possible that pyruvate oxidase catalyzes the AHAS reaction. We found that the purified pyruvate oxidase catalyzed the synthesis of α -acetolactate at a rate of 9.2×10^{-3} nmol/min per μ g of enzyme. The product of the reaction was identified by converting the product to acetoin with [3-¹⁴C]pyruvate as described in Materials and Methods. On the other hand, the enzyme catalyzed the decarboxylation of pyruvate in the presence of an artificial electron acceptor at a rate of 75 nmol/min per μ g of enzyme. Although the AHAS activity was low compared with the oxidase activity (ca. 10^{-4}), the reaction was a true property of pyruvate oxidase and could not be attributed to contamination of the 95% pure oxidase with AHAS isozymes. The data which support this conten-

FIG. 2. (A) Dot-matrix plots of the protein sequence of pyruvate oxidase versus each of the AHAS isozymes. The horizontal axis is pyruvate oxidase, and the vertical axis is AHAS isozyme I, II or III (in order from left to right). Each dot represents a minimum of 7 amino acids matched out of 21 amino acids compared. (B) Dot-matrix plots of the DNA sequence of pyruvate oxidase versus each of the AHAS isozymes. The horizontal axis is pyruvate oxidase (*poxB*), and the vertical axis is AHAS isozyme I (*ilvB*), II (*ilvG*), or III (*ilvI*) (in order from left to right). Each dot represents a minimum of 15 nucleotides matched out of 25 nucleotides compared.

tion (Table 2) are as follows: (i) AHAS activity was stimulated by activation of the oxidase with either SDS or α -chymotrypsin; (ii) addition of FAD to the reaction mixture failed to stimulate the reaction (AHAS isozymes I and II require added FAD for activity [6, 10, 44, 48]); (iii) aceto-

TABLE 2. α -Acetolactate synthase activity of *E. coli* pyruvate oxidase

Conditions ^a		Amt of acetolactate formed (nmol)
Activation	Assay mixture	
With α -chymotrypsin	Complete	8.3
None	Complete	2.0
Inactivated with α -chymotrypsin	Complete	1.7
With SDS	Complete	8.7
With α -chymotrypsin	Without FAD	8.3
With α -chymotrypsin	Without TPP ₁ ^b	3.1
With α -chymotrypsin	Without MgCl ₂ ^b	6.1
With α -chymotrypsin	Plus 1.7 mM L-valine	8.3
With α -chymotrypsin	Plus 1 mM sulfometuron methyl	8.6
With α -chymotrypsin	Complete but stopped by alkali	0.7

^a The conditions of activation and assay are described in Materials and Methods. Controls in which the enzyme was omitted from the assay mixture or in which the reaction was stopped at zero time had similar values and were subtracted from the values given.

^b TPP₁ and MgCl₂ are present owing to their presence in the activation incubation.

lactate synthesis was not inhibited by either valine or sulfo-meturon methyl (all known AHAS isozymes are inhibited by at least one of these two compounds [5, 24, 25]); (iv) the pH optimum of acetolactate synthesis (pH 6.0) is the optimum of the oxidase reaction, whereas the known AHAS activities are more active at higher pH values (6); and (v) the AHAS isozymes are unusually labile enzymes (10, 44; Schloss and Van Dyk, in press), which would perish under the conditions used to purify the oxidase. The first of these criteria is the most compelling, since the agents used to activate the oxidase (SDS and α -chymotrypsin) are generally potent inhibitors of enzyme activities. Indeed, if incubated with α -chymotrypsin in the absence of pyruvate and thiamine PP₁-Mg²⁺, the oxidase was irreversibly inactivated in its catalysis of both the oxidation reaction (data not shown) (39) and acetolactate synthesis (Table 2).

We also have tested whether a 19-fold overproduction of AHAS I activity (encoded by plasmid pDSE5 [45]) in vivo could substitute for the lack of pyruvate oxidase in a *poxB* strain (strain CG3) (2, 17). These AHAS I-overproducing strains showed no evidence of pyruvate oxidase activity either in vitro (<0.15% of the AHAS activity) or as assayed in vivo (2, 17). Thus, we conclude that although pyruvate oxidase has AHAS activity, AHAS I has no oxidase activity. Schloss et al. (43) have reached the same conclusion by using homogeneous AHAS isozyme preparations.

Formation and properties of a *poxB-ilvBN* hybrid protein. The homology data and the AHAS activity of pyruvate oxidase noted above suggested that substitution of *ilvB*

FIG. 3. Construction of plasmid pYYC39 carrying the hybrid *poxB-ilvB* gene. Restriction enzyme sites are *Eco*RI (RI), *Sac*I (Sc), *Kpn*I (K), *Eco*RV (RV), *Sma*I (Sm), *Hind*III (D), *Hind*II (H), *Pst*I (P), *Sph*I (Sp), and *Xho*I (X). Restriction sites (RI, Sc, K, Sm, P, Sp, and D) that bracket the *ilvBN* DNA fragments of pYYC37 come from the vector pUC19. The arrows show the coding sequences, and *Ap^R* and *Tet^R* show the ampicillin and tetracycline resistance genes, respectively. For details, see Materials and Methods.

nucleotide sequences with the corresponding sequences of the *poxB* gene might result in a hybrid protein with AHAS activity. Functionality of a hybrid protein would be strong evidence that the sequence homology denoted functional homology.

A gene encoding a hybrid protein was constructed by using the *Eco*RV site present in the conserved sequence found in both the *poxB* and *ilvB* genes (14, 18, 50) (Fig. 2). The conserved *Eco*RV site is centrally located in both genes. (Note that the *poxB* gene contains two *Eco*RV sites separated by 57 bp at 874 and 931 bp from the initiation codon. It is the first of these sites that corresponds to the *Eco*RV site of *ilvB* on alignment by homology.) The construction scheme is shown in Fig. 3, and the structure of the plasmids carrying the hybrid gene was confirmed by restriction mapping. Two plasmids encoding the hybrid protein were made; one also carried the *ilvN* sequence encoding the small subunit of AHAS I (pYYC39), whereas the other (pYYC38) lacked this sequence.

To test the functionality of the protein encoded by the hybrid gene, we transformed pYYC39 into a strain, YYC329, carrying mutations in the *ilvBN* and *ilvIH* genes. Strain YYC329 required both isoleucine and valine for growth on minimal medium, but we found that transformants of the strain with pYYC39 grew (after a prolonged lag) in the absence of valine, although an isoleucine requirement was still evident. Plasmid curing and reconstitution experiments showed that both the plasmid and the host strain of those transformants retained their original characteristics, and

thus the hybrid protein was able to complement (or bypass) the chromosomal mutations and allow valine synthesis. However, we subsequently constructed strain YYC365, carrying a deletion of the *ilvBN* genes and a different *ilvIH* allele, and found that transformants of this strain were unable to grow without both valine and isoleucine. Construction of strains carrying the various *ilvBN* and *ilvIH* mutant alleles (YYC395 and YYC396; Table 1) showed that a combination of the *ilvBN* lesion of strain YYC329 and the pYYC39 plasmid were needed for valine-independent growth.

Neither strain YYC329 nor pYYC39 transformants of strain YYC365 contained detectable AHAS activity, whereas strain YYC329 carrying pYYC39 grown in the absence of valine had a high level of AHAS activity (17 U/mg of protein). The activity was sensitive to valine inhibition and insensitive to sulfometuron methyl (properties characteristic of AHAS I activity).

These data strongly suggest that the *ilvBN* lesion of strain YYC329 is a missense lesion that encodes an inactive protein that is somehow converted to an active species by interaction with the hybrid protein encoded by pYYC39. However, we have not studied this interaction further, since we found that the hybrid protein has a related but more readily studied property, the ability to activate AHAS I in the absence of its required FAD moiety.

Flavin substitution activity of the hybrid protein. AHAS I has an absolute requirement for added flavin for activity in vitro, presumably because loosely bound endogenous flavin

TABLE 3. FAD substitution activity of the hybrid protein^a

Sample	Amt of protein added	FAD added	Amt of acetolactate formed (nmol)
YYC365(pYYC37)	3 μ g	—	<0.02
YYC365(pYYC37)	3 μ g	+	65
YYC365(pUC19)	1.3 mg	—	4.3
YYC365(pUC19) plus YYC365(pYYC37)	1.3 mg 3 μ g	—	8.5
YYC365(pYCC39)	1.4 mg	—	5.2
YYC365(pYCC39) plus YYC365(pYYC37)	1.4 mg 3 μ g	—	46

^a The preparation of crude extracts and the assay of AHAS activity were described in Materials and Methods. The incubation was done at 37°C for 30 min.

is lost during the dilution accompanying cell disruption. By serendipity, we found that addition of extracts of AHAS-defective strains carrying pYYC39 (but not the parental plasmid pUC19) to AHAS I-containing extracts gave activity without addition of flavin (Table 3). These results could not be attributed to the presence of free flavin in these extracts, since activated-charcoal treatment (which quantitatively removed FAD) had no effect on the efficiency of the substitution. Moreover, strains carrying the vector pUC19 or plasmid pYYC38, which carries the hybrid *poxB-ilvB* gene but lacks the small-subunit gene (*ilvN*), were inactive. The IlvN protein presumably stabilizes the hybrid protein in vivo, as it does the IlvB protein (11).

The flavin substitution activity was linear with respect to both the AHAS I-containing extract and the pYYC39 extract (data not shown); however, a 100-fold excess of the pYYC39 extract over the AHAS I containing extract was required for substitution activity. This could be attributed to an inherently inefficient protein-protein interaction between the hybrid protein and AHAS I, a low level of the hybrid protein (presumed to be due to instability in vivo), or both. The major effect seemed to be low levels of the hybrid protein. This was shown in an experiment in which we mixed the crude extract from a strain carrying plasmid pYYC39 labeled with [³⁵S]methionine with the crude extract from a strain carrying plasmid pUC19 labeled with [³H]methionine, centrifuged the mixture in a 10 to 30% glycerol gradient, and counted both isotopes. There was no significant change in the ³⁵S-to-³H ratio throughout the gradient (data not shown). However, there was a definite peak in the ³⁵S-to-³H ratio in a parallel experiment when the crude extract from a strain carrying the AHAS I plasmid pYYC37 mixed with the crude extract from a strain carrying the vector only. This result indicated that the concentration of the hybrid protein encoded by plasmid pYYC39 was much lower than that of AHAS I encoded by plasmid pYYC37 under the same conditions. We roughly estimate that the concentration of hybrid protein was <0.1 that of AHAS I.

We also found that the hybrid protein became physically associated with the subunit of AHAS I when the two proteins were mixed (Fig. 4). When the crude extract of a strain carrying plasmid pYYC37 was centrifuged on a 10 to 30% glycerol gradient containing the substrate (pyruvate) and cofactors (thiamine PP_i-MgCl₂) in either the absence or the presence of FAD, the AHAS I activity was found in the

FIG. 4. Profiles of various samples after centrifugation on velocity glycerol gradients. The broad peak in panel B is due to the presence in the gradient of all of the components required for AHAS activity. The enzyme synthesizes α -acetolactate during centrifugation and leaves this product at the top of the gradient. The position of the enzyme is thus at the lower edge of the peak (20). The experimental conditions are described in Materials and Methods. Panels A and B show results for crude extracts prepared from strain YYC365 carrying plasmid pYYC37 (encoding AHAS I) in the absence (panel A) or in the presence (panel B) of FAD. The acetolactate synthase activity in each fraction (0.1 ml) was detected by assay in the presence of FAD. Panels C and D show results for crude extracts prepared from strain YYC365 carrying plasmid pYYC39 (hybrid protein) in the absence (panel C) or presence (panel D) of FAD. For panel C, the FAD substitution activity of the hybrid protein of each fraction (0.3 ml) was assayed by adding exogenous AHAS I prepared from strain YYC365 carrying plasmid pYYC37 (3 μ g of protein) in the absence of FAD, whereas for panel D, the background value of AHAS I activity (due to the presence of FAD in the gradient) was subtracted. For panel E the crude extract from strain YYC365 carrying plasmid pYYC37 was mixed with the crude extract prepared from strain YYC365 carrying vector pUC19. The gradient contained no FAD, and the AHAS activity of each fraction (0.25 ml) was assayed in the presence of FAD. Panel E was essentially the same as panel A, except that it had fivefold less AHAS I. Panel F was similar to panel E, except that the crude extract from strain YYC365 carried plasmid pYYC39 rather than the vector pUC19. The gradient contained no FAD, and the AHAS activity of each fraction was assayed in the absence of FAD. The sedimentation behavior of pyruvate oxidase (POX) under the same conditions is shown. The amounts of crude extract centrifuged corresponded to the cells from 13 ml of culture for strains carrying the hybrid gene or pUC19, whereas for the *ilvB* plasmid-carrying strain, 1 ml of culture (panels A and B) or 0.2 ml of culture (panels E and F) was used. Δ OD, Optical density.

upper third of the gradient, as reported by Grimminger and Umbarger (20) and Eoyang and Silverman (10) in similar experiments. (The activities shown in Fig. 4 vary with the assay used, the amount of extract loaded, and possibly differential lability during centrifugation and thus are useful mainly for determining the location of the activities in the gradient.) In the presence of pyruvate, thiamine PP_i-MgCl₂, and FAD, AHAS I had a higher sedimentation coefficient than that found in the absence of FAD (presumably dimers)

(Fig. 4A and B). When a similar experiment was performed with a crude extract of a strain carrying plasmid pYYC39, the hybrid protein in the gradient was found near the top of the gradient either in the presence or in the absence of FAD (Fig. 4C and D). This suggested that the hybrid protein had a lower molecular weight (presumably monomers) under these conditions. When a crude extract of a strain carrying plasmid pYYC39 was mixed with a crude extract of a strain carrying pYYC37 and was centrifuged on a gradient in the absence of FAD (Fig. 4F), the reconstituted AHAS I activity was detected (in the absence of FAD) at a position below that of the hybrid protein. This result indicated that the hybrid protein was associated with AHAS I (perhaps as heterodimers) and that this association allowed AHAS I to fold and function in the absence of FAD. This association was specific, because under the same conditions the crude extract of the same host strain carrying vector pUC19 lacked FAD substitution activity (Fig. 4E). One could argue the possibility that the FAD moiety of the hybrid protein is somehow transferred to the AHAS I subunit during AHAS I-hybrid protein association. This transfer would have to occur during a protein-protein interaction, since if the flavin were released into solution, the concentration would be too low to activate AHAS I. Also, interprotein transfer seems most unlikely on thermodynamic grounds, since transfer would be from a high-affinity site to a low-affinity site. The hybrid protein binds its FAD moiety tightly, so that treatment with an acidic and chaotropic solution is necessary to effect its removal from the protein (see below), whereas AHAS I spontaneously loses its FAD moiety.

We also found that valine could completely inhibit the AHAS I activity resulting from addition of the hybrid protein (data not shown). The valine sensitivity of AHAS I is determined by interaction of the IlvN protein with the IlvB protein (11), and thus the valine sensitivity of the flavin substitution activity suggests that the IlvB-hybrid protein complex also binds the small (IlvN) protein and that the observed activity is due to the IlvB component of the complex. It should be noted that the hybrid protein did not have oxidase activity.

The *poxB-ilvBN* hybrid protein contains tightly bound FAD. We suspected that the reason that the hybrid protein was able to satisfy the FAD requirement of AHAS I was that the hybrid protein contained bound FAD and hence was properly folded. We were able to dissociate the bound FAD from the hybrid protein by the standard KBr-acidic ammonium sulfate treatment used to remove bound FAD moieties (21). We partially purified the hybrid protein encoded by plasmid pYYC39 and treated this preparation with KBr-acidic ammonium sulfate. An extract of the strain carrying the vector pUC19 and treated in parallel was used as a control. After FAD removal by this treatment, the hybrid protein lost most of its FAD substitution activity (Table 4). The FAD substitution activity could be restored by adding FAD and then removing free FAD by activated-charcoal adsorption (Table 4). This result clearly demonstrated that the hybrid protein consisting of the N-terminal half of pyruvate oxidase and the C-terminal half of AHAS I contained tightly bound FAD. Since pyruvate oxidase binds FAD tightly, whereas AHAS I does not, we conclude that the FAD-binding site of the hybrid protein is probably that of pyruvate oxidase.

Properties of *ilvB-poxB* hybrid proteins. We also constructed genes encoding two other hybrid proteins. One, encoded by plasmid pYYC51, had the N-terminal half of AHAS I and the C-terminal half of pyruvate oxidase. Another hybrid protein, encoded by plasmid pYYC52, was

TABLE 4. FAD substitution activity of the hybrid protein under various conditions

Sample treatment	FAD substitution activity ^a for:	
	Control	Hybrid protein
None (crude extract)	3.3	13
30 to 60% (NH ₄) ₂ SO ₄ fraction	3.2	34
KBr-acidic (NH ₄) ₂ SO ₄ treatment	2.0	7.4
FAD addition with free-FAD removal	4.6	37

^a The control and the hybrid protein extracts were prepared from strain YYC365 carrying vector pUC19 or plasmid pYYC39, respectively. Sample preparation and other experimental conditions were as described in Materials and Methods. Activity is expressed in nanomoles of acetolactate formed per milligram of protein.

identical to the one encoded by pYYC51, except that the C-terminal 24 amino acids of pyruvate oxidase were deleted. The fusion site in these constructions is the same *EcoRV* site described for the *poxB-ilvBN* hybrid protein. The formation of plasmids pYYC51 and pYYC52 is described in detail in Materials and Methods. Neither of these two hybrid proteins had AHAS activity (in the presence or absence of FAD) or pyruvate oxidase activity. They also lacked FAD substitution activity for AHAS I. This is consistent with the notion that the FAD-binding site of pyruvate oxidase is at the N-terminal half of pyruvate oxidase and that the FAD substitution activity is a special attribute of the *poxB-ilvB* hybrid protein.

DISCUSSION

Our evidence that pyruvate oxidase is closely related to the AHAS isozymes can be summarized as follows. First, the homology seen on amino acid alignment is strong. Second, the enzymes have common coenzymes (thiamine PP_i and FAD), the same substrate (pyruvate), and a common product (CO₂). Third, pyruvate oxidase catalyzes the AHAS reaction. The fourth line of evidence, the ability of the hybrid *poxB-ilvB* protein to replace FAD in the oligomerization of AHAS I, indicates that the amino acid sequence homology has functional significance. This last finding indicates that the curious requirement of the AHAS enzymes for FAD is due to structural rather than chemical considerations. This had been suggested for the AHAS enzymes (42; Schloss and Van Dyk, in press) and another nonredox flavoprotein (4), but only from negative results: the inability to find a chemical role for the flavin. The FAD replacement activity requires the presence of FAD in the hybrid protein, and since the flavin is tightly bound, we assume the FAD-binding site resides in the PoxB portion of the hybrid protein. Thus, it seems that the pyruvate oxidase domain of the hybrid protein somehow allows AHAS to fold into an active conformation in the absence of FAD. Hence, it also seems that the AHAS retains another attribute of pyruvate oxidase, the requirement of FAD for proper subunit association (38). A final piece of evidence comes from the work of Schloss et al., (43) who have studied the inhibition of the enterobacterial AHAS isozymes by various herbicides. Following our report of sequence homology between pyruvate oxidase and the AHAS enzymes, they sought and found a vestigial ubiquinone-binding site on all three AHAS isozymes that defines the site of herbicide binding. Ubiquinone binding has no mechanistic role in nonredox proteins such as the AHAS isozymes, but plays a key role in the pyruvate

oxidase reaction. We therefore believe that the combination of these lines of evidence virtually precludes the possibility that the AHAS isozymes and pyruvate oxidase are not evolutionary kin.

We believe that pyruvate oxidase is an ancestor of the AHAS enzyme rather than the reverse situation (or parallel evolution) for the following reasons. Early environments are thought to have been rich in amino acids, and thus amino acid biosynthetic enzymes should be a late development. Our results argue that the flavin required for AHAS activity has only a structural role; that of facilitating protein-protein interactions. The rule of parsimony argues that flavins would not have first evolved to perform a structural role in protein folding, since this role can be (and is, in the vast majority of proteins and even another acetolactate-forming enzyme [47]) satisfied by amino acid side chain interactions. In this context the requirement for flavin binding and the ubiquinone-binding site of the AHAS isozymes are simply vestigial remains that presumably will be lost given further time. It should be noted that Schloss et al. (43) propose that the AHAS flavin requirement may have been retained to shield the hydroxyethylthiamine PP_i reaction intermediate from the solvent, whereas we propose a purely structural role owing to the flavin substitution activity of the *poxB-ilvB* hybrid protein. In our view, the FAD-binding site of the AHAS enzymes has been retained, since crystallographic studies of other flavoproteins indicate a large number of contacts between FAD and amino acid side chains. Thus, elimination of the flavin would require numerous amino acid alterations.

A plausible and straightforward scenario for the evolution of these enzymes is as follows. Investigators performing experiments to attempt to recreate the primordial conditions have invariably reported (33) the synthesis of large amounts of amino acids (including branched-chain amino acids), and thus amino acid biosynthetic pathways would be a relatively late development, selected only when the environment became more dilute. It seems logical that the first cells to synthesize amino acids would conscript and alter previously encoded proteins for this task. Pyruvate oxidase has AHAS I activity and thus is a logical candidate for conscription. The first task would be to eliminate oxidase activity, since decarboxylation of pyruvate to acetate would preclude condensation of two molecules of pyruvate (the AHAS reaction). Grabau and Cronan (19) have shown that deletion of 24 amino acids from the carboxy terminus of pyruvate oxidase results in loss of enzyme activity *in vivo*. A similar truncated protein is active in the AHAS reaction *in vitro*, and thus a reasonable first step in the evolution of the oxidase into an AHAS enzyme would be truncation of the protein by introduction of a termination codon or deletion of the carboxy-terminal gene segment. Indeed, when aligned by homology, the AHAS isozymes are about 20 amino acids shorter than the oxidase protein and the carboxy-terminal regions of the oxidase and AHAS enzymes have little or no homology. Therefore, the AHAS and oxidase protein differ most in the region where the novel and essential property of the oxidase, lipid binding, is located.

Following alteration of the carboxyl terminus, natural selection could account for conversion of pyruvate oxidase into a highly active AHAS enzyme. This would require about a 1,000-fold increase in the intrinsic AHAS activity of the oxidase, but this is not unreasonable. There are several examples of laboratory selection of cells containing enzymes of greatly increased (several hundredfold) activity by making such changes essential to cell growth (34). We therefore expect that by altering the gene and perhaps the level of gene

expression, we should be able to produce an altered oxidase that functionally replaces AHAS I in valine biosynthesis.

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