The biosynthesis of ansamycin antibiotics, like rifamycin B, involves formation of 3-amino-5-hydroxybenzoic acid (AHBA) by a novel variant of the shikimate pathway. AHBA then serves as the starter unit for the assembly of a polypeptide which eventually links back to the amino group of AHBA to form the macrolactam ring. The terminal enzyme of AHBA formation, which catalyzes the aromatization of 5-deoxy-5-amino-3-dehydroshikimic acid, has been purified to homogeneity from Amycolatopsis mediterranei, the encoding gene has been cloned, sequenced, and overexpressed in Escherichia coli. The recombinant enzyme, a (His)$_6$ fusion protein, as well as the native one, are dimers containing one molecule of pyridoxal phosphate per subunit. Mechanistic studies showed that the enzyme-bound pyridoxal phosphate forms a Schiff’s base with the amino group of AHBA. This enzyme catalyzes the aromatization of 5-deoxy-5-amino-3-dehydroshikimic acid and catalyzes both an $\alpha,\beta$-dehydration and a stereospecific 1,4-enolization of the substrate. Inactivation of the gene encoding AHBA synthase in the A. mediterranei genome results in loss of rifamycin formation; production of the antibiotic is restored when the mutant is supplemented with AHBA.

The clinically important ansamycin antibiotic, rifamycin B (Scheme I), contains a biosynthetically unique structural element called a mC$_7$N unit (shown in bold in the rifamycin B structure) (1, 2). This mC$_7$N unit is derived from 3-amino-5-hydroxybenzoic acid (AHBA) (3–7), which serves as the starter unit for the assembly of a linear polypeptide by addition of acetate and proionate units. The C terminus of the assembled polypeptide eventually forms an amide linkage to the amino group of the AHBA moiety to close the macrolactam ring.

AHBA, in turn, is generated by a newly discovered biosynthetic reaction sequence, the aminoshikimate pathway, which parallels the first three steps of the shikimate pathway, but is modified by the introduction of nitrogen in the first step (Scheme I) (8,9) to give 3,4-dideoxy-4-amino-d-arabino-heptulosonic acid 7-phosphate (aminoDAHP) instead of the normal shikimate pathway intermediate, 3-deoxy-d-arabino-heptulosonic acid 7-phosphate (DAHP). Cyclization and dehydration leads to the 5-amino analog of 3-dehydroshikimic acid, 5-deoxy-5-amino-3-dehydroshikimic acid (aminoDHS), which is then aromatized by the enzyme, AHBA synthase. In this article, we report on the purification and preliminary mechanistic analysis of this enzyme, which has no parallel in the normal shikimate pathway; on the cloning, sequence analysis, and expression of the gene encoding it; and on the effect of deletion of this gene on rifamycin production.
AHBA Synthase Assay—AminoDHS solution (500 μl, 0.6 mm) and buffer B were mixed with a portion of the protein solution (50–300 μl) to give a total volume of 1 ml. After 1 h of incubation at 28 °C, the reaction was stopped by the addition of 200 μl of 15% trichloroacetic acid solution. After centrifugation, the production of AHBA was assessed by measuring the increase in A₄₆₅ relative to a blank. Protein concentration was determined with Bio-Rad protein assay solution, using bovine serum albumin as a standard.

To determine the kinetic parameters of the native or recombinant enzyme, initial velocities were measured by incubating 50 or 100 μl of enzyme solution at 33 °C with 0.1–1.1 mM aminoDHS in buffer C in a total volume of 1 ml and following the absorbance at 310 nm. The enzyme solution from the Phenyl-Sepharose column (bed volume of 1 ml) at 0.5 ml/min and then eluted with a linear gradient of 50–250 mM KCl in buffer C for 5 min at 0.5 ml/min and then eluted with a linear gradient of 50–250 mM KCl in buffer C for 5 min at 1 ml/min. Fractions were collected every 2 min. Step gradients were continued for 10 min with 250 mM KCl and for 15 min with 1 M KCl at a flow rate of 0.5 ml/min. The fractions containing high AHBA synthase activity, eluting just before 250 mM KCl, were pooled.

Hydrophobic Interaction FPLC—Ammonium sulfate was added to the AHBA synthase pool from the Mono Q FPLC to give 30% saturation. The solution (1.6 mg of protein) was applied to a Phenyl-Superose FPLC column (bed volume of 1 ml) at 0.5 ml/min. The column was flushed with buffer C containing 50% saturated ammonium sulfate for 3 min at 50% KCl, followed by elution at a linear gradient of 30–0% saturated ammonium sulfate in buffer C over 30 min. Fractions were collected every 2 min, and the fractions containing high AHBA synthase activity, eluting at 20–22 min, were pooled. Protein from this step was subjected to SDS-PAGE, and the band corresponding to AHBA synthase was cut out and transferred electrophoretically to Immobilon for gas phase microsequencing at the protein analysis facility of the Department of Biochemistry, University of Washington.

DNA Sequencing and Sequence Analysis—DNA sequencing was performed using the Sequenase kit (U.S. Biochemical Corp.) and (α-35S) dCTP (NEN Life Science Products) according to the manufacturer’s protocol. Sequencing reactions were analyzed on polyacrylamide gels (8% (v/v) acrylamide, 5% bisacrylamide, 8 M urea, 45 mM Tris borate, pH 8.0, and 1 mM EDTA). SK, KS, Tφ, or T primers (Stratagene) for dDNA sequencing reactions and M13 primer (Stratagene) for ssDNA sequencing reactions were used. ssDNA was prepared using M13K07 (Promega) following the manufacturer’s protocol. DNA and protein sequences were analyzed using the University of Wisconsin Genetics Computer Group (UWGCG) program version 7.2.

Inactivation of AHBA Synthase Gene—A primary disruption of the AHBA synthase gene was engineered using a marker-replacement suicide vector, pSK/AHBA2. In this plasmid, the hygromycin resistance gene (hyg) of pJB963 (11), which resides on a 1.75-kb BglII fragment, had been inserted into the 2.3-kb Xhol fragment of pSK/AHBA1 (Fig. 24) at the only BglII site, which lies at the N-terminal part of the AHBA synthase gene, in an orientation such that transcription of the hyg gene would occur in the same direction as that of the AHBA synthase gene, leaving 1.2 and 1 kb of homologous DNA flanking this insertion to the left and right, respectively. Through electroporation (12), approximately 20 transformants of A. mediterranei S699 that were resistant to hygromycin were obtained per microgram of heat-treated denatured pSK/AHBA2. Southern hybridization of the transformants (data not shown) demonstrated that they arose by the expected single crossover, either upstream, named HGF001, or downstream, named HGF002, of the AHBA synthase gene, in approximately equal numbers. HGF001 produced normal rifamycin B yields; however, all HGF002 recombinants showed delayed production and reduced yield of rifamycin B (approximately half the yield of wild-type A. mediterranei S699). One of the HGF001 strains for maintenance agar medium (13) lacking hygromycin. After propagation through three subsequent generations, about 0.5–1% of the colonies showed sporulation 1–2 days earlier and lost the ability to produce rifamycin B. Southern hybridization with six random choices of these rifamycin B nonproducing colonies (HGF003–1 to –6 (data not shown)) confirmed that pSK–/AHBA2 had integrated into the chromosome in HGF001, and that all
six HGF003 strains had undergone the second crossover event to replace the endogenous AHBA synthase gene with one that was truncated with the insertion of the hygromycin resistance gene marker (Fig. 2B).

Expression of the AHBA Synthase Gene—pSK-AHBA1, which contained the 2.9-kb XhoI fragment of A. mediterranei DNA carrying the AHBA synthase gene, was digested with EcoRI. The resulting 1.8-kb EcoRI fragment was resolved on 0.8% agarose gel and ligated into pRSET digested with EcoRI using T4 DNA ligase. The ligated productions were transformed into BL21(DE3)/pLysS. Several colonies of E. coli BL21(DE3)/pLysS/pRSET(AHBA) grown on LB agar plates containing carbenicillin (50 μg/ml) and chloramphenicol (34 μg/ml) were inoculated with 10 ml of LB medium containing the same antibiotics at a 5–6 h of growth at 37 °C with shaking (280 rpm). 1 ml of these cultures was used to inoculate 100 ml of LB medium containing 1 M sorbitol, 2.5 mM betaine, carbenicillin, and chloramphenicol (500 mM KCl). The protein solution was loaded onto the column at a flow rate of 1 ml/min, which was then washed with 10 volumes of the same buffer overnight. The dialyzed protein solution was loaded onto a nickel resin (Novagen) following the manufacturer’s protocol. The fractions containing AHBA synthase were collected and resolved by preparative layer chromatography (silica gel, n-butanol/water/acetic acid 2:1:1, RF = 0.26). The resulting N-phosphopyridoxyl-AHBA was then dephosphorylated by incubation with alkaline phosphatase and the N-pyridoxyl-AHBA purified by preparative layer chromatography in the same system. N-Pyridoxyl-aminOA was prepared analogously from aminoSA and PLP.

**RESULTS**

Enzyme Purification—AHBA synthase was purified from 54-h-old mycelia of A. mediterranei strain S699 as summarized in Table 1. The enzyme activity was assayed by measuring the amount of AHBA formed after 1 h incubation of the protein with 0.3 mM aminoDHSA at 28 °C, followed by addition of trichloroacetic acid. In the early stages of purification, AHBA was quantitated by an inverse isotope dilution GC-MS assay (9), in the later stages by measuring the increase in A290, the absorption maximum of AHBA at an acidic pH. The six-step 180-fold purification (Table I) gave AHBA synthase of a specific activity of 72 units/mg protein in 5% overall yield. The protein at this stage was judged by SDS-PAGE to be homogeneous (Fig. 1).

Properties—The native molecular mass of AHBA synthase was estimated by gel filtration as 74 kDa and by nondenaturing PAGE as slightly higher than that of bovine serum albumin (66,700 Da). Elution of the enzymatically active band from the DE 52 column step, assaying activity by following the change in A310 during the initial 10-min linear phase of the reaction. The enzyme is most active at 33 °C and has a pH optimum of 7.5. AHBA synthase retained its activity over a broad range of temperature and pH. Over 84% of the maximum activity of AHBA synthase was maintained over a temperature range from 28 to 50 °C and a pH range from 7.0 to 9.0. The Km value for aminoDHSA was determined from Lineweaver-Burk plots to be 0.164 mM. When the substrate aminoDHSA was added to a final concentration of 0.1 mM, AHBA synthase reached 1.0. Isopropyl-1-thio-β-D-galactopyranoside was then added to a final concentration of 0.5 mM, and the expression level during further incubation was checked by SDS-PAGE and assay of AHBA synthase activity.

### Table I

| Step | Volume | Activity | Recovery |
|------|--------|----------|----------|
|      | ml     | Units/mg | %        |
| Crude extract | 110 | 453 | 0.4 |
| 50–70% (NH₄)₂SO₄ | 16 | 325 | 1 |
| DE 52 | 30 | 317 | 3.2 |
| Phenyl-Sepharose CL-4B | 15 | 220 | 6 |
| Sephadex G-200 | 15 | 130 | 17 |
| Mono Q (FPLC) | 2 | 88 | 55 |
| Phenyl-Superose (FPLC) | 2 | 22 | 72 |

*a* Nanomoles of AHBA produced per minute.

*b* Obtained from 25 g wet cells.

*c* Cumulative yield is given in parentheses.
3,4-dideoxy-4-amino-3-arabino-heptulosonic acid 7-phosphate (aminoDAHP, 1 mM), aminoSA (1 mM) or 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP, 1 mM), none of the compounds except DAHP affected the enzyme activity, with DAHP showing 40 to 50% activation of the enzyme. It had been shown by others (16) that DAHP synthase from A. mediterranei is inhibited by rifamycin. Activation of AHBA synthase by DAHP provides another piece of evidence that the AHBA biosynthetic pathway is related to the shikimate pathway, and suggests some type of cross-regulation of the two pathways.

Partial Amino Acid Sequence Analysis—To obtain partial amino acid sequence information for the construction of oligodeoxynucleotide primers for the cloning of the AHBA synthase gene, the protein from the Phenyl-Superose FPLC step was further purified by SDS-PAGE. The specific region containing the AHBA synthase band was cut out from the gel after visualization with Coomassie Blue and electrophoretically transferred to PVDF membrane (17, 18). Gas-phase microsequencing of the intact protein revealed a 10-amino acid N-terminal sequence H$_2$N-N-A-R-K-A-P-E-F-P-A (sequence 1). Internal amino acid sequences were determined after in situ CNBr cleavage (19, 20) of the enzyme on the PVDF membrane. The cleavage products were separated by reverse-phase HPLC (21) on a C$_4$ column, and two peptides were chosen for amino acid sequence analysis. They yielded sequences of 26 and 16 amino acids, R-L-N-E-F-S-A-S-V-L-R-A-Q-L-A-R-L-D-E-Q-I-A-V-R-L-E (sequence 2) and G-V-G-P-I-G-T-E-V-I-V-P-A-T-E-L-S (sequence 3).

Cloning of AHBA Synthase Gene—Based on the above partial amino acid sequences of AHBA synthase, three degenerate oligodeoxynucleotides were designed (Table II), taking into account the preferred codon usage (22) of genes from organisms with GC-rich DNA, like A. mediterranei or Streptomyces (>90% G or C in the third base). These oligonucleotides were used as PCR primers to amplify a 500-bp (combination primers 1 and 2) and a 250-bp (combination primers 1 and 3) region of genomic DNA from A. mediterranei S699. Both PCR products hybridized strongly to the same bands of restriction-digested genomic DNA, establishing them to originate from the same region. Sequencing of the two PCR products revealed that the deduced amino acid sequence of the 257-bp PCR product contained the last two amino acids of the N-terminal peptide sequence and the first six amino acids of the internal peptide sequence 3, neither of which had been used in the construction of the primers. On the other hand, the deduced amino acid sequence of the 505-bp PCR product contained the first 13 amino acids of the internal peptide sequence 2, which had not been encoded by the primer, but did not have the N-terminal amino acid sequence corresponding to oligonucleotide 1 at the 5' end of the PCR product. Instead, the 505-bp PCR product also had the same sequence as the oligonucleotide 2 at the 5' end, suggesting that oligonucleotide 1 might bind nonspecifically within a region where the AHBA synthase gene is located. Furthermore, none of the deduced amino acid sequence corresponding to the peptide 3 was found in the 505-bp PCR product.

Two nondegenerate primers (primers 4 and 5, Table II) were then synthesized, representing base sequences near the 3' and 5' ends, respectively, of the 505- and 257-bp PCR products. PCR with these primers gave a ~700-bp DNA fragment; sequencing revealed a 717-bp base sequence, which included the coding region of internal peptide 3. This 717-bp PCR product was then used to isolate the complete AHBA synthase gene from a cosmid library of A. mediterranei S699 genomic DNA. The library was constructed from partially Sau3A-digested A. mediterranei S699 genomic DNA, which was cloned into cosmid vector pOJ446 (23) restriction digested with BamHI and HpaI. The titer of the library was 2 × 10$^{10}$ colonies/µg of DNA and the average insert size was 30–40 kb. Screening with the 717-bp PCR probe led to the isolation of 6 colonies from 2000 colonies screened, which contained DNA hybridizing strongly to the probe. Southern hybridization analysis of restriction digests of the inserts from the isolated six colonies utilizing the 717-bp PCR product identified a 2.3-kb XhoI fragment carrying the AHBA synthase gene.

Sequencing and Sequence Analysis of the AHBA Synthase Gene—The 2.3-kb XhoI fragment was subcloned into XhoI-digested pSK–. Two white colonies were selected, in which the 2.3-kb XhoI fragment was present in two different orientations. A detailed restriction map was obtained (Fig. 2A) using several restriction enzymes, including EcoRI, PstI, SmaI, SacI, and SalI. XhoIEcoRI (~800 bp), EcoRI/SacI (~1 kb), and SmaIXhoI (~700 bp) digests of the 2.3-kb fragment were subcloned into pSK– or M13mp18 and sequenced using pSK–, M13mp18, or sequence-specific primers. The nucleotide sequence of the AHBA synthase gene was determined in both directions, and the restriction sites used for subcloning were verified by determination as part of an overlapping sequence.

The nucleotide and deduced amino acid sequences of the 2.3-kb XhoI fragment, shown in Fig. 3, revealed that AHBA synthase is encoded by an open reading frame (ORF) of 1164 bp, corresponding to a protein of 388 amino acids with a calculated molecular mass of 42,281.37 Da. The ORF was detected by CODON PREFERENCE (24) analysis, which also revealed the presence of parts of two additional ORFs to either side of the AHBA synthase gene. The TGA codon at bp 1383 was assigned as the stop codon of the AHBA synthase gene because it is the only stop codon in the coding region giving a molecular mass of the protein close to the value estimated by SDS-PAGE (39 kDa). The sequence of the ORF shows a typical Streptomyces codon preference (G+C content in the first position 72%, second position 48%, and third position 97%; the G+C content for the entire fragment was 72%). The apparent Shine-Dalgarno sequence (GGAG), which is complementary to the 3' end of the 16 S rRNA of Streptomyces lividus (25), lies 11 nucleotides upstream of the initiation codon. TERMINATOR (26) and STEMLOOP (27) analysis did not reveal any significant secondary structures in the regions upstream and down-
Table II

| Primers | Degenerated oligonucleotides | Peptide templates |
|---------|-----------------------------|-------------------|
| Primer 1 | 5'-AAG GGC GGG CAG TGT-3' | Asn-Ala-Arg-Lys-Ala-Pro-Glu-Phe |
| Primer 2 | 5'-GAT CTT TCT YTC GCG YGC YAG-3' | Leu-Ala-Arg-Leu-Asp-Glu-Gln-Ile |
| Primer 3 | 5'-GGT GAA YGC YGC YAC GAT YAC TGC-3' | Glu-Val-Ile-Val-Pro-Ala-Phe-Thr |
| Primer 4 | 5'-GCT GCG CCC CAG CAT GGC CCC-3' | Pro-Ala-Arg-Pro-Gln-Tyr-Asp-Asp |
| Primer 5 | 5'-CTG GCC GCC CAG CGC GGC CCC-3' | Ala-Ser-Val-Leu-Arg-Ala-Gln |

Fig. 2. Genomic maps of the AHBA synthase gene. A, restriction map of the region of the A. mediterranei S699 chromosome and of pSK-AHBA1, encompassing the AHBA synthase gene. The orientation and extent of the AHBA synthase gene were deduced from the nucleotide sequence (see Fig. 3). B, A. mediterranei HGF003 carries a mutated AHBA synthase gene into which has been inserted a 1.7-kb BglII fragment carrying the hygromycin resistance gene (hyg) through in vitro and in vivo gene replacement (see text). C, the expression of the His6-AHBA synthase under the control of the T7 promoter in pRSET/T/AHBA. An EcoRI DNA fragment, from pSK-AHBA1, which carried the functional AHBA synthase gene was ligated into EcoRI-digested pRSET B (Invitrogen). The T7 promoter (pT7), ribosome binding sites (RBS; underlined) and indicated N-terminal amino residues (in one-letter code) of the recombinant AHBA synthase were inherited from the vector pRSET B.

Screening of protein sequence data bases with the deduced amino acid sequence of AHBA synthase using the FASTA and BLAST (28) programs showed homology to the products of a series of genes implicated (29) primarily in transamination (Fig. 4) or dehydration/deoxygenation (Fig. 5) reactions involved in deoxyhexose biosynthesis (cf. Refs. 42-45). Of the products of these genes, at the time of our work, only the AscC protein had been studied in detail. AscC encodes an enzyme, E1, containing a pyridoxamine phosphate (PMP) cofactor and an iron-sulfur cluster, which catalyzes the 3-deoxygenation of CDP-4-keto-6-deoxy-d-glucose in the biosynthesis of ascosarylate in Yersinia pseudotuberculosis (46-48). Ascarylate is one of the antibiotic determinants in the cell wall polysaccharides of Y. pseudotuberculosis (43). However, the sequence homology between AscC and the AHBA synthase gene does not include the iron-sulfur cluster motif in AscC. In view of this partial homology to a known PMP-containing enzyme, the amino acid sequence of AHBA synthase was compared with those of representative other pyridoxal-phosphate (PLP) and PMP enzymes (49-51). The alignments revealed that AHBA synthase contains a typical PLP binding motif with a conserved aspartate (Asp-159) and the active site lysine (Lys-188), which presumably binds the cofactor as a Schiff's base (Fig. 4). In the PMP-containing AscC and the deduced sequences of related gene products, this lysine is replaced by a histidine (Fig. 5). This led to the expectation that AHBA synthase is a PLP enzyme, although catalysis via a PMP cofactor would be mechanistically equally plausible.

Involvement of AHBA Synthase Gene in Rifamycin Biosynthesis—The isolation and characterization of the gene encoding AHBA synthase reported above sets the stage for the cloning of the rifamycin biosynthetic gene cluster based on the paradigm (52) that in Actinomycetes the genes encoding the biosynthesis of a given antibiotic are clustered in the genome of the producing organism. Thus, analysis of the DNA surrounding the AHBA synthase gene should reveal other rifamycin biosynthesis genes, provided that the AHBA synthase gene is indeed essential for rifamycin formation. To verify this point, we carried out a gene disruption experiment. An inactivated version of the AHBA synthase gene was constructed by insertion of a 1.7-kb BglII fragment carrying DNA fragment from plJ963 (11) into the unique BglII site in pSK-AHBA1 (Fig. 2A). To reduce the restriction limitation and increase the integration efficiency, this suicide vector was denatured and then introduced into A. mediterranei S699 by electroporation. Successive selection first for single-crossover mutants (Hyg') and then for a second crossover gave a double-crossover mutant (Hyg') (Fig. 2B) in which the functional AHBA synthase gene had been replaced by the inactivated version. This mutant was unable to produce rifamycin B, the normal metabolite of the wild-type, but production was restored to wild-type levels by supplementation of the culture with AHBA. When the mutant culture was supplemented with [7-13C]AHBA (89% 13C), the resulting rifamycin B contained at least 81% 13C, confirming the absence of endogenous AHBA synthase in the mutant. Thus, the cloned AHBA synthase gene is indeed essential for rifamycin formation, justifying the expectation that it is part of the rifamycin biosynthesis gene cluster of A. mediterranei.

Expression of the AHBA Synthase Gene in E. coli—To establish whether AHBA synthase is indeed a PLP enzyme and to generate larger amounts of enzyme for mechanistic studies, the AHBA synthase gene was overexpressed in E. coli. An EcoRI restriction digest of pSK-AHBA1 containing the 2.3-kb XhoI restriction fragment produced a 1.6-kb EcoRI fragment in which 23 nucleotides from the start of the coding region of the AHBA synthase gene had been deleted. The 1.6-kb EcoRI fragment was cloned into the expression vector pRSET for expres-
sion as a (His)$_2$ fusion protein under the control of the T$_7$

promoter. The cloned vector pRSET(AHBA) (Fig. 2C) was transfor-
mated into the host E. coli BL21(DE3)/pLysS. Growth of the cells at 37 °C in LB medium containing chlor-
amphenicol and carbenicillin and induction with 0.5 mM iso-
propyl-1-thio-

b-D-galactopyranoside produced high levels of a

protein of the expected molecular mass (43 kDa), but all in

insoluble form. No AHBA synthase activity was detected in the

soluble fractions. Attempts to refold the insoluble protein into

the active enzyme were unsuccessful. However, modification of

the culture and induction conditions led to the formation of

some of the protein in soluble, enzymatically active form. Low-

ering the temperature to 30 °C and the isopropyl-1-thio-

b-D-galactopyranoside concentration to 0.1 mM allowed detection of

AHBA synthase activity in the soluble fractions. The addition

of sorbitol (1M) and betaine (2.5 mM) to the medium led to a

retardation of cell growth but the AHBA synthase activity of

the soluble fractions increased 2–3-fold. The addition of PLP or

FIG.3. Nucleotide sequence of the 2.3-kb XhoI fragment from A. mediterranei S699 carrying the AHBA synthase gene. The deduced

gene products are indicated in the one-letter code under the DNA sequence. The possible ribosome binding sites (rbs), and the

XhoI, EcoRI, and BglII restriction sites are

underlined. The start (except ORF1) and direction of each of the ORFs are indicated by

arrows and named accordingly. A stop codon is identified by an asterisk. The amino acid sequences determined by Edman degradation are

underlined. Numbers to the left of the sequences show the positions of nucleotides and amino acid residues for the AHBA synthase gene product, respectively.

\[ \text{3-Amino-5-hydroxybenzoic Acid Synthase} \]

\[ 6035 \]
PMP to the fermentation broth, however, did not lead to an increase in enzyme activity. The final AHBA synthase activity was 13 times higher than in cell-free extracts of A. mediterranei S699. The recombinant enzyme was purified 38-fold by ammonium sulfate precipitation, DE 52 chromatography and adsorption on a nickel column as summarized in Table III to give nearly homogeneous protein (Fig. 6) in 46% yield.

Properties of Recombinant AHBA Synthase—Although the recombinant enzyme was obtained as a fusion protein and lacked the first six N-terminal amino acids, it showed very similar behavior to the enzyme isolated from A. mediterranei. The native molecular mass of the recombinant AHBA synthase was determined as 80 kDa and that of the denatured protein as 44 kDa by gel-filtration and SDS-PAGE. These values are somewhat low, compared with the molecular mass of 46,101 calculated from the DNA sequence. Nevertheless, the recombinant enzyme, as the native one, is most likely a dimer with subunits of identical molecular mass. The $K_m$ value of the recombinant enzyme for aminoDHS was 0.133 mM, compared with 0.164 mM for the native enzyme. The temperature and pH optima, 37 °C and 8.5, respectively, also differed only slightly or predictably from those of the native enzyme, 33 °C and 7.5.

The recombinant enzyme displayed an absorbance maximum at 418 nm, typical of a PLP-lysine Schiff’s base (53). Reduction with sodium borohydride abolished the enzymatic activity and led to a change in the absorbance maximum to 330 nm, characteristic of PMP derivatives. PLP forms an adduct with cysteine that absorbs at 330 nm, and this property was used to estimate the amount of PLP bound per mole of AHBA synthase holoenzyme (54). Solutions of AHBA synthase were mixed with 200 mM cysteine in 10 mM HCl, and the $A_{330}$ was measured. By comparison to standard solutions of PLP, the AHBA synthase was found to contain 0.6 $\pm$ 0.05 mol of PLP/mol of enzyme subunit. The protein concentration was measured by both the Bradford method and by UV, using the absorption coefficient calculated (55) from the deduced amino acid sequence of AHBA synthase. The low value compared with an expected one of 1 mol/mol of subunit left open the possibility that only one of the two subunits in the dimeric enzyme carries a PLP. This possibility was ruled out by ESI-MS analysis. ESI-MS was run on the NaBH$_4$-reduced and unreduced, then denatured protein and gave an average molecular mass of 46,075 $\pm$ 9 Da for the denatured apoenzyme and 46,297 $\pm$ 13 Da for the reduced, denatured holoenzyme. The difference, 222 Da, is close to the molecular mass of the phosphopyridoxyl moiety, 231 Da. Each sample gave only a single protein species, whereas in a spec-

Fig. 4. Amino acid sequence alignment of AHBA synthase (AHBA) with nine putative PLP-dependent aminotransferase-like or pleiotropic regulatory proteins. The data were generated using the PILEUP program in the UWGCG package. Amino acids are presented in the one-letter code. Numbers to the left of the sequence show the position of amino acids. Identical residues in all proteins are shown in bold letters. The two conserved PLP-binding residues, an aspartate and a lysine, which presumably hydrogen-bond to N1 and form a Schiff’s base with the PLP cofactor, respectively, are indicated by vertical arrows. Aligned proteins are from A. mediterranei (AHBA) (the present work), B. griseus (StrC: a L-glutamine-dependent scyllo-inosose aminotransferase; 32.0% identity; 38.1% similarity) (30), B. pertussis (BplF: 31.8% identity; 42.5% similarity) (31), B. stearothermophilus (DegT: 30.4% identity; 40.9% similarity) (32), S. erythraea (EryC1: 36.3% identity; 44.6% similarity) (33), S. fradiae (TylB: 30.9% identity; 38.5% similarity) (34), S. griseus (StrS: a putative aminotransferase; 30.7% identity; 37.1% similarity) (30), S. peucetius (DnrJ: 30.4% identity; 41.0% similarity) (35), S. alboniger (Pur4: a putative aminotransferase; 28.7% identity; 35.8% similarity) (36), S. griseus (StsA: a putative L-alanine dependent N-amidino-3-keto-scyllo-inosamine aminotransferase; 31.7% identity; 39.8% similarity) (30). The values of identity and similarity refer to comparisons of the different proteins with AHBA.

PMP to the fermentation broth, however, did not lead to an increase in enzyme activity. The final AHBA synthase activity was 13 times higher than in cell-free extracts of A. mediterranei S699. The recombinant enzyme was purified 38-fold by ammonium sulfate precipitation, DE 52 chromatography and adsorption on a nickel column as summarized in Table III to give nearly homogeneous protein (Fig. 6) in 46% yield.

Properties of Recombinant AHBA Synthase—Although the recombinant enzyme was obtained as a fusion protein and lacked the first six N-terminal amino acids, it showed very similar behavior to the enzyme isolated from A. mediterranei. The native molecular mass of the recombinant AHBA synthase was determined as 80 kDa and that of the denatured protein as 44 kDa by gel-filtration and SDS-PAGE. These values are somewhat low, compared with the molecular mass of 46,101 calculated from the DNA sequence. Nevertheless, the recombinant enzyme, as the native one, is most likely a dimer with subunits of identical molecular mass. The $K_m$ value of the recombinant enzyme for aminoDHS was 0.133 mM, compared with 0.164 mM for the native enzyme. The temperature and pH optima, 37 °C and 8.5, respectively, also differed only slightly or predictably from those of the native enzyme, 33 °C and 7.5.

The recombinant enzyme displayed an absorbance maximum at 418 nm, typical of a PLP-lysine Schiff’s base (53). Reduction with sodium borohydride abolished the enzymatic activity and led to a change in the absorbance maximum to 330 nm, characteristic of PLP derivatives. PLP forms an adduct with cysteine that absorbs at 330 nm, and this property was used to estimate the amount of PLP bound per mole of AHBA synthase holoenzyme (54). Solutions of AHBA synthase were mixed with 200 mM cysteine in 10 mM HCl, and the $A_{330}$ was measured. By comparison to standard solutions of PLP, the AHBA synthase was found to contain 0.6 $\pm$ 0.05 mol of PLP/mol of enzyme subunit. The protein concentration was measured by both the Bradford method and by UV, using the absorption coefficient calculated (55) from the deduced amino acid sequence of AHBA synthase. The low value compared with an expected one of 1 mol/mol of subunit left open the possibility that only one of the two subunits in the dimeric enzyme carries a PLP. This possibility was ruled out by ESI-MS analysis. ESI-MS was run on the NaBH$_4$-reduced and unreduced, then denatured protein and gave an average molecular mass of 46,075 $\pm$ 9 Da for the denatured apoenzyme and 46,297 $\pm$ 13 Da for the reduced, denatured holoenzyme. The difference, 222 Da, is close to the molecular mass of the phosphopyridoxyl moiety, 231 Da. Each sample gave only a single protein species, whereas in a spec-

![Fig. 4. Amino acid sequence alignment of AHBA synthase (AHBA) with nine putative PLP-dependent aminotransferase-like or pleiotropic regulatory proteins. The data were generated using the PILEUP program in the UWGCG package. Amino acids are presented in the one-letter code. Numbers to the left of the sequence show the position of amino acids. Identical residues in all proteins are shown in bold letters. The two conserved PLP-binding residues, an aspartate and a lysine, which presumably hydrogen-bond to N1 and form a Schiff’s base with the PLP cofactor, respectively, are indicated by vertical arrows. Aligned proteins are from A. mediterranei (AHBA) (the present work), B. griseus (StrC: a L-glutamine-dependent scyllo-inosose aminotransferase; 32.0% identity; 38.1% similarity) (30), B. pertussis (BplF: 31.8% identity; 42.5% similarity) (31), B. stearothermophilus (DegT: 30.4% identity; 40.9% similarity) (32), S. erythraea (EryC1: 36.3% identity; 44.6% similarity) (33), S. fradiae (TylB: 30.9% identity; 38.5% similarity) (34), S. griseus (StrS: a putative aminotransferase; 30.7% identity; 37.1% similarity) (30), S. peucetius (DnrJ: 30.4% identity; 41.0% similarity) (35), S. alboniger (Pur4: a putative aminotransferase; 28.7% identity; 35.8% similarity) (36), S. griseus (StsA: a putative L-alanine dependent N-amidino-3-keto-scyllo-inosamine aminotransferase; 31.7% identity; 39.8% similarity) (30). The values of identity and similarity refer to comparisons of the different proteins with AHBA.](image)
trum of the mixture the two species were clearly resolved, confirming that the holoenzyme must be a symmetrical dimer in which each subunit carries one molecule of PLP.

Mechanistic Role of Pyridoxal Phosphate—To demonstrate a role of PLP in the catalytic mechanism of AHBA synthase, we examined the effect of an inhibitor. Gabaculine (5-amino-1,3-cyclohexadien-1-carboxylic acid), a naturally occurring amino acid isolated from \textit{Streptomyces toyocaenis} (56), is an irreversible inhibitor of many PLP-requiring aminotransferases (57). Rinehart \textit{et al}. (58) and Ganem (59) had, in fact, suggested that this compound might be an intermediate on the biosynthetic pathway to mC7N units. In view of the structural similarity of gabaculine and aminoDHS, we checked the effect of gabaculine on AHBA synthase. Incubation of the recombinant enzyme with gabaculine at various concentrations led to irreversible inactivation of the enzyme. The inactivated enzyme did not recover activity upon dialysis against buffer, buffer containing PLP, or buffer containing substrate. The irreversible inhibition of AHBA synthase by gabaculine was time-dependent and showed a biphasic pattern in which the initial, more rapid phase was followed by a second phase of slower decline in activity (data not shown). At an inhibitor concentration of 15 mM, the $t_{1/2}$ (the preincubation time required for loss of 50% of enzyme activity) was about 20 s at 33 °C. The gabaculine-inactivated enzyme showed a UV absorption maximum at 330 nm. These data clearly implicate the enzyme-bound PLP in the catalytic mechanism of the enzyme (Scheme II).

The involvement of a PLP Schiff’s base in the catalytic process was further demonstrated by reduction of the enzyme-Gabaculine complex using DTT. The data were generated using the PILEUP program in the UWGCG package. Amino acids are presented in the one-letter code. Numbers to the left of the sequence show the position of amino acids. Identical residues in all proteins are shown in bold letters. The AHBA lysine residue (Lys-188), which might form a Schiff base with PLP, is indicated by a vertical arrow. The cysteine residues that are thought to represent an iron-sulfur binding motif are outlined. Representative PMP-dependent AscC-like proteins are from \textit{Vibrio cholerae} (Orf43x9: 25.6% identity; 34.9% similarity) (37), \textit{E. coli} (WbdK: 21.7% identity; 28.3% similarity) (38), \textit{Y. pseudotuberculosis} (AscC: a CDP-4-keto-6-deoxy-D-glucose-3-dehydrase; 23.2% identity; 28.2% similarity) (39, 40), \textit{Salmonella typhimurium} (RfbH: 26.8% identity; 34.4% similarity) (41), \textit{Streptomyces violaceoruber} (GraG: 27.8% identity; 33.9% similarity) (D. Tornus, K. Ichinose, D. J. Bedford, D. A. Hopwood, and H. G. Floss, and H. G. Floss, unpublished work), \textit{A. mediterranei} (Rif Orf6: 23.6% identity; 30.7% similarity) (P. R. August, T.-W. Yu, R. Müller, L. Heide, and H. G. Floss, unpublished work). The values of identity and similarity refer to comparisons of the different proteins with AHBA synthase (the present work).

| TABLE III | Purification of recombinant AHBA synthase |
|------------|----------------------------------------|
| **Step**   | **Volume** | **Activity** | **Recovery** |
|            | **Total units*** | **Units/mg of protein** | **Purification** |
| Crude extract | 42 | 59,854 | 5.2 |
| 35-55% (NH$_4$)$_2$SO$_4$ | 8 | 4,047 | 71 | 68 |
| DE 52 | 18 | 2,556 | 21.3 |
| His resin | 18 | 2,730 | 195 |

* Nanomoles of AHBA produced per minute.
3-Amino-5-hydroxybenzoic Acid Synthase

Following the establishment of a possible pathway for the formation of AHBA, the precursor of the mC₇N starter unit of ansamycin antibiotics, by cell-free experiments (9), we decided to focus on AHBA synthase as the first enzyme of this pathway to investigate in detail. Two considerations prompted this choice. 1) AHBA synthase was the only enzyme that seemed unique to this pathway; unlike the other presumed enzymes it has no equivalent in the normal shikimate pathway. Hence, it was expected that the gene encoding it would represent the most unique probe to isolate ansamycin and mitomycin biosynthetic gene clusters. 2) The chemical aromatization of aminoDHS by acid, base, or buffer treatment proceeds in the same way as that of DHS (61, 62), i.e. it produces exclusively protocatechuic acid (63). Since AHBA synthase generates quantitatively AHBA from the same substrate, the enzyme must completely redirect the aromatization chemistry of aminoDHS; the mechanism by which it achieves this was not obvious.

The studies reported here have revealed this mechanism (Scheme IV) by showing that the enzyme employs PLP catalysis. The genetic evidence that the enzyme is a PMP or, more likely, PLP enzyme was corroborated by analysis of the recombinant protein which showed the presence of a PLP-Schiff's base. NaBH₄ reduction experiments confirmed the involvement of a Schiff's base between the cofactor and the amino group of the substrate and ruled out a possible alternative mechanism proceeding through a Schiff's base between a PMP form of the enzyme and the C-3 carbonyl group of the substrate. The reaction mechanism of this enzyme thus can be suggested to resemble those of PLP enzymes catalyzing α,β-elimination reactions in amino acid metabolism, such as serine dehydratase or tryptophanase, i.e. the initial enzyme-substrate Schiff's base first undergoes deprotonation at the α-carbon to give a quinoid intermediate, which then ejects the electronegative β-substituent, possibly after protonation to make it a better leaving group (64). The other required part of the reaction, abstraction of a proton from C-6 in a vinylogous enolization, is also catalyzed by the enzyme, as evidenced by the fact that this deprotonation is stereospecific. However, it is not evident from the data whether this step occurs before or after the 4,5-dehydration of the substrate.

Interestingly, the enzyme fits the general paradigm of PLP enzymes that, with few exceptions, all the group (proton) transfer reactions take place on only one face of the enzyme-substrate complex (65, 66). The proton at C-5, the OH-group at C-4, and the pro-S proton abstracted from C-6 are all located on the same face of the substrate molecule. Although this may well be fortuitous, it is tempting to suggest that it could reflect the operation of a proton recycling mechanism in which the same enzyme base situated, as in other PLP enzymes, on the Si face of the planar PLP-substrate complex successively mediates the deprotonation at C-5, the protonation of the leaving group 4-OH and the deprotonation at C-6 (65, 66). On the other hand, the enzyme may well only catalyze the deprotonations at C-5 and C-6; if the vinylogous enolization precedes abstraction of the C-5 proton, then the resonance energy gained by aromatization should provide enough driving force for the spontaneous ejection of the 4-OH, i.e. the PLP catalysis may be limited to the generation of the resonance-stabilized carbanion at C-5.

In this context, it is interesting to note that the sequence homology between AHBA synthase and the PLP enzymes catalyzing α,β-elimination and β-replacement reactions is not particularly strong. Rather, AHBA synthase is most closely related to the deduced amino acid sequences of the products of a family of genes (they have been called the secondary metabolic amino-transferase or “SMAT” genes; Ref. 44), many of which are found

**DISCUSSION**

substrate complex with tritiated sodium borohydride followed by denaturation of the enzyme, acidic hydrolysis of the products and treatment with alkaline phosphate. The major tritiated product from the reaction of AHBA synthase preincubated with aminoDHS was identified by comparison with an independently synthesized unlabeled sample as 3-(N-pyridoxyl-amino)-5-hydroxybenzoic acid (N-pyridoxyl-AHBA) (Scheme III). A second, unidentified product was noted; this was not identical with an authentic reference sample of 5-deoxy-5-(N-pyridoxylamino)shikimic acid. The isolation of tritiated N-pyridoxyl-AHBA provides strong support for the intermediacy of this substrate complex with tritiated sodium borohydride followed by derivatization and analysis of the recombinant protein which showed the presence of a PLP-Schiff's base. NaBH₄ reduction experiments confirmed the involvement of a Schiff's base between the cofactor and the amino group of the substrate and ruled out a possible alternative mechanism proceeding through a Schiff's base between a PMP form of the enzyme and the C-3 carbonyl group of the substrate. The reaction mechanism of this enzyme thus can be suggested to resemble those of PLP enzymes catalyzing α,β-elimination reactions in amino acid metabolism, such as serine dehydratase or tryptophanase, i.e. the initial enzyme-substrate Schiff's base first undergoes deprotonation at the α-carbon to give a quinoid intermediate, which then ejects the electronegative β-substituent, possibly after protonation to make it a better leaving group (64). The other required part of the reaction, abstraction of a proton from C-6 in a vinylogous enolization, is also catalyzed by the enzyme, as evidenced by the fact that this deprotonation is stereospecific. However, it is not evident from the data whether this step occurs before or after the 4,5-dehydration of the substrate.

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in antibiotic biosynthesis gene clusters, which contain a typical PLP or PMP binding motif and seem to be involved primarily in the formation of antibiotic deoxysugar moieties, but some possibly also in regulatory functions (Refs. 29 and 42; cf. Refs. 43–45). As the alignments in Figs. 4 and 5 show, these genes neatly fall into two subgroups. One carries a modified PLP binding motif with two adjacent histidines in place of the conserved active site lysine, and most of them also encode a series of cysteines which represent an iron-sulfur cluster motif (Fig. 5). This group mostly seems to be involved in 3-deoxygenation reactions of deoxysugars; it includes \( \text{ascC} \), the gene encoding the well characterized (46–48) enzyme E1 from \( \text{Y. pseudotuberculosis} \), which catalyzes the 3-deoxygenation reaction in the biosynthesis of ascarylose. Another member of this subgroup, \( \text{graG} \), has recently been shown by inactivation experiments to be involved in the biosynthesis of the 2,3,6-trideoxysugar, \( \alpha \)-rhodinose.\(^2\) The second subgroup (Fig. 4) encodes a typical PLP binding site and lacks the iron-sulfur cluster motif. Its members have been suggested to be involved in transamination reactions leading to amino deoxysugars and aminocyclitols. Only recently has transaminase activity of one gene product from this family been demonstrated. The protein expressed from \( \text{stsC} \) from \( \text{Streptomyces griseus} \) was shown to catalyze the

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\(^2\) D. Tornus and H. G. Floss, unpublished results.
transamination between glutamic and scyllo-inosose to give scyllo-inosamine, the precursor of the aminocyclothyiol moiety of streptomyacin (30). As the phylogenetic tree in Fig. 7 illustrates, these two subgroups clearly represent two separate branches of this superfamily of genes which diverged some time ago. AHBA synthase not only shares the PLP, as opposed to the PMP binding motif with the proposed transaminases, but is in general much more closely related to the PLP than to the PMP subgroup, although the dehydration chemistry it catalyzes formally resembles part of the chemistry of E1 more than the chemistry of transamination. The membership of an enzyme like AHBA synthase in this subgroup of PLP genes/enzymes suggests that some other members of this subgroup may also encode enzymes that catalyze reactions other than transaminations.

The cloning of the AHBA synthase gene and the demonstration that this gene is essential for rifamycin biosynthesis provides the means for the isolation of gene clusters encoding the biosynthesis of rifamycin as well as other antibiotics biosynthesized from AHBA. Sequence analysis of cosmid clones of A. mediterranei DNA isolated with the AHBA synthase gene as a probe and extended by chromosome walking has revealed numerous other genes involved in rifamycin biosynthesis. Their sequence and functional analysis will be reported in forthcoming publications.3 The AHBA synthase gene from A. mediterranei has also been used, in a collaboration with the group of E. Leistner, Bonn, to clone homologous genes from Streptomyces collinus Tu 1892 presumably involved in the biosynthesis of ansatrienin (mycotrienin) and naphthomycin, and from Actinosynnema pretiosum, presumably involved in ansamitocin biosynthesis. Similarly, D. Sherman and co-workers have used the AHBA synthase gene from A. mediterranei to clone the mitomycin biosynthetic gene cluster from S. lavendulae. The AHBA synthase gene thus represents another useful addition to the growing inventory of strategic gene probes, which can serve to identify biosynthetic pathways for key natural product classes and to isolate the gene clusters encoding them.

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