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An Enzyme Aggregate Involved in the Biosynthesis of Aromatic Amino Acids in *Bacillus subtilis*. Its Possible Function in Feedback Regulation*

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ABSTRACT: Chromatographic and genetic evidence indicates that two enzymes of aromatic amino acid synthesis, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) synthetase and chorismate mutase, occur in a single aggregate in *Bacillus subtilis*. The possibility that a third enzyme of this pathway, shikimate kinase, is also a part of this aggregate was suggested by chromatographic data. All three enzyme activities are eluted in the same fractions following chromatography on DEAE-cellulose and filtration through Sephadex G-100. A single-step mutant was isolated which lacks both chorismate mutase and DAHP synthetase activities but does have shikimate kinase activity. Since DAHP synthetase and shikimate kinase are feedback inhibited by chorismate and prephenate, we suggest that this aggregate has a functional significance either in localization of inhibitors with control enzymes or in the utilization of a catalytic site for one enzyme as a regulatory site for another enzyme.

Another organism, *Staphylococcus epidermidis*, in which the DAHP synthetase is inhibited by prephenate and chorismate, may also have this enzyme and chorismate mutase in a complex.

Biochemical and genetic analyses of mutationally induced structural alterations of proteins have experimentally demonstrated the requirement for interaction between polypeptide chains for the expression of many enzyme activities (Reithel, 1963; Reed and Cox, 1966). These interactions may involve identical polypeptide chains, presumably coded by the same cistron. The enzyme alkaline phosphatase of *Escherichia coli* falls into this category (Schlesinger, 1964). In other cases, nonidentical subunits interact to form an enzymatically active protein. A well-studied example of this is the tryptophan synthetase of *E. coli* (Yanofsky, 1960). This enzyme consists of an A protein coded by one cistron and a B protein coded by an adjacent cistron. Neither protein by itself has any activity for the terminal physiological step of tryptophan synthesis, but the complex of two molecules of A protein to one molecule of B protein is enzymatically active (Wilson and Crawford, 1965). An enzyme complex or aggregate may possess more than one catalytic activity. Thus, Munkres (1965) has shown that the malate dehydrogenase and aspartate aminotransferase activities in *Neurospora crassa* are associated with the same protein. The subunit components of this complex are dictated by two unlinked genetic loci. Loper *et al.* (1964) have reported that two nonsequential enzymes of histidine biosynthesis are catalyzed by the same protein. In this system, however, only one genetic locus specifies the synthesis of the protein.

DeMoss and Wegman (1965) have shown that in *N. crassa* the activities of three enzymes of tryptophan biosynthesis are associated with a single enzyme aggregate, which thus far cannot be separated into smaller units which possess enzyme activity. Ito and Yanofsky (1966) and Bauerle and Margolin (1966) have shown interactions between enzymes of tryptophan biosynthesis in *E. coli* and *Salmonella*, respectively. Giles *et al.* (1965) have shown that five of the enzymes common to the synthesis of tryptophan, tyrosine, and phenylalanine are closely linked genetically in *Neurospora* and may occur in a single aggregate (N. H. Giles and C. W. H. Partridge, personal communication).

In the course of an investigation on the molecular

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TABLE I: Description of Organisms.

Organism	Genotype	Enzyme Defect	Source
B. subtilis			
WB 932	Aro^-	CM_3 chorismate mutase, DAHP synthetase	*N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine mutant of WB 746
WB 746	Prototroph		Spontaneous revertant of 168
Strain 168	Trp_2^-	Indoleglycerol phosphate synthetase	Dr. C. Yanofsky, Stanford University
SB 167	Aro^-	Dehydroquinate synthetase	
SB 163	Aro^-	DAHP synthetase	
WB 672	Prototroph		Spontaneous revertant of strain 23 (Thr^-)
WB 2100	$His_2^-CM_3^-$		
WB 698b	Prototroph excretes tyrosine		
S. epidermidis	Prototroph		University of Washington culture collection

forms of chorismate mutase in *Bacillus subtilis*, we observed that a single-step mutant which lacked chorismate mutase activity did not grow when supplied with the amino acid end products of this enzyme, tyrosine and phenylalanine, but did grow if tryptophan or shikimate were present. This report explores the reason for this anomalous nutritional requirement and presents evidence that chorismate mutase is complexed with another enzyme of aromatic amino acid synthesis, DAHP[1] synthetase, and possibly with a third enzyme involved in the same pathway, shikimate kinase.

Materials and Methods

Organisms. The relevant information on the organisms used in this investigation is given in Table I.

Growth of Cells and Preparation of Extracts. Cells were grown, harvested, and extracts were prepared according to the procedures described by Lorence and Nester (1967). In some cases, specifically noted, cells of *B. subtilis* were disintegrated by sonic disruption for three minutes at maximum power in an MSE ultrasonic disintegrator (Instrumentation Associates, Inc.). *Staphylococcus epidermidis* cells, grown in Spizizen's minimal medium plus 0.1% yeast extract, were treated for 10 min in the ultrasonic disintegrator at maximum power.

DNA Transformation Procedure. The techniques described by Nester *et al.* (1963) were followed.

Chemicals. The quality, source, and description of most chemicals used in this investigation are given in the accompanying paper (Lorence and Nester, 1967). TEAE-cellulose was obtained from Brown Co., Corvallis, Ore. Shikimic acid was obtained from Calbiochem, Los Angeles, Calif. [^{14}C]Shikimic acid (uniformly labeled) was obtained from New England Nuclear Corp., Boston, Mass.

Enzyme Assays. Chorismate mutase was assayed as described by Lorence and Nester (1967); DAHP synthetase by the procedure of Srinivasan and Sprinson (1959) as modified by Jensen and Nester (1966a). Shikimate kinase was determined using ^{14}C-labeled shikimic acid as substrate, a method suggested by Dr. M. Green of the University of California at San Diego. The reaction mixture consisted of (final concentration in micromoles per milliliter) Tris-HCl (pH 8.3), 50; ATP, 1; $MgCl_2$, 5; KF, 3; shikimic acid, 1; and [^{14}C]shikimic acid, 0.02 μc. The mixture was incubated for varying lengths of time at 37°. The protein was removed by centrifugation at room temperature after precipitation with 0.1 ml of 50% TCA. Measured amounts of the supernatant (usually 0.8 ml) were pipetted into 1 ml of saturated $Ba(OH)_2$ and a drop of a saturated solution of $(NH_4)_2SO_4$ was added as a coprecipitant. The precipitate was collected on a membrane filter, dried, suspended in 10 ml of Liquifluor (Nuclear-Chicago, Des Plaines, Illinois), and counted in a Packard Tri-Carb scintillation counter. Under the conditions used, the assay was linear with respect to time and protein concentration.

Chromatography on DEAE-cellulose and Sephadex G-100 was performed by the procedures described by Lorence and Nester (1967). Hydroxylapatite was prepared according to the procedure of Siegelman *et al.* (1965). A thick slurry, in 0.03 M potassium phosphate buffer (pH 6.8), was poured into a 2-cm column

[1] Abbreviations used: DAHP, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate; CM, chorismate mutase; TSY, trypticase soy broth (BBL) supplemented with dipotassium phosphate (2.5 g/l.) and yeast extract (10 g/l.); TCA, trichloroacetic acid; Shk, shikimate; Sk, shikimate kinase.

and allowed to pack without pressure until a bed height of 20 cm was achieved. The column was washed with approximately ten volumes of this same buffer at 4°. When the bed had reached incipient dryness, a 2-ml sample of extract, approximately 40 mg of protein, prepared by sonication in 0.03 M potassium phosphate buffer (pH 6.8), was applied. After the sample had entered the resin completely, 6 ml of 0.03 M potassium phosphate buffer (pH 6.8) was placed on top of the bed, and the column was connected to a mixing device described by Hegenauer *et al.* (1965) containing 125 ml of 0.03 M potassium phosphate buffer (pH 6.8) in the mixing chamber and 125 ml of 0.4 M potassium phosphate buffer (pH 6.8) in the reservoir. A linear gradient was established and 2.3 ml/tube was collected.

Sucrose density gradients were made using a linear gradient of sucrose from 10 to 30%. The internal standard employed was human hemoglobin. Fractions of 0.1 ml were collected in approximately 50 tubes. Other conditions of the gradient technique were the same as described by Lorence and Nester (1967).

Protein Determinations. Protein was determined in the eluents from the various columns by reading absorbancy at 280 mμ. When more accurate determinations were required, the Lowry *et al.* (1951) method was used with bovine serum albumin as the standard protein.

Experimental Results

Growth Requirements of a Chorismate Mutase Negative Mutant. The data in the accompanying paper (Lorence and Nester, 1967) indicate that there are three chromatographically distinct molecular species of chorismate mutase designated CM_1, CM_2, and CM_3. A mutant, WB 932, derived from a strain having only the CM_3 molecular species had no chorismate mutase activity. Contrary to our expectation, this mutant did not grow if the minimal medium was supplemented with tyrosine and phenylalanine. The results of Figure 1 show that this strain requires either tryptophan or shikimate in addition to tyrosine and phenylalanine. This observation suggested that, in addition to the deficiency in chorismate mutase, this strain lacks an enzyme activity prior to the synthesis of shikimate. The results given in Table II show that WB 932 has almost no DAHP synthetase activity; at most a few per cent of the fully repressed activity of the strain 168. We next explored the most obvious possibilities which would account for this low activity of the first enzyme in aromatic amino acid synthesis.

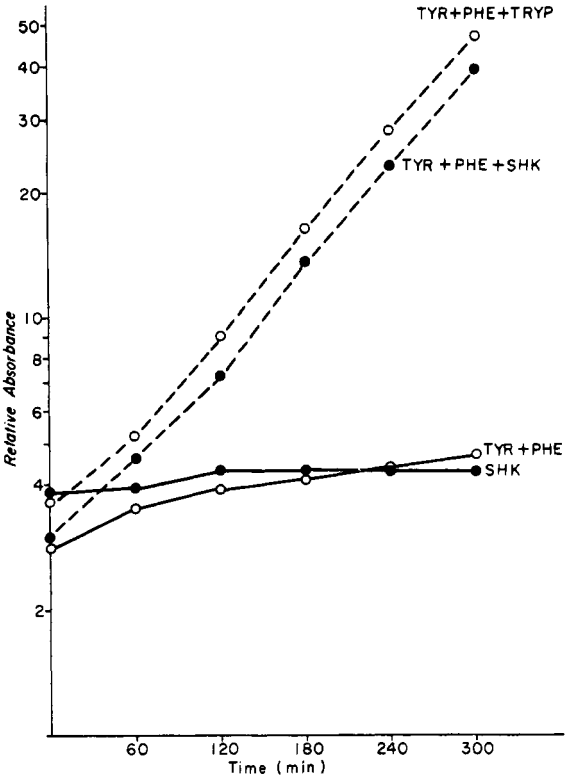

FIGURE 1: Strain WB 932 was grown for 18 hr in minimal medium supplemented with tyrosine, phenylalanine, and tryptophan (20 μg/ml each) and shikimic acid (50 μg/ml). The culture was centrifuged, and the cells were washed two times in minimal medium and resuspended at an appropriate dilution in 10 ml of minimal medium with the noted supplements in a 125-ml erlenmeyer flask fitted with a side arm for determining the optical density of the cultures. The Klett readings were taken using a 660-mμ filter and plotted as relative absorbance.

TABLE II: Specific Activity of DAHP Synthetase in Strains WB 932 and 168.[a]

Strain	Sp Act. of DAHP Synthetase
WB 932	<0.5
168 (derepressed)	106.0
168 (repressed)	35.8

[a] Strain WB 932 was grown in minimal medium containing tyrosine, phenylalanine, and tryptophan (20 μg/ml). Strain 168 was grown in minimal medium containing tryptophan (20 μg/ml) or tyrosine, phenylalanine, and tryptophan (50 μg/ml each) in the case of the repressed culture. Extracts were prepared by lysozyme treatment, processed, and assayed as described. In all cases, specific activity is defined as millimicromoles of product formed per minute per milligram of protein.

Inhibition by Chorismate. A mutant lacking chorismate mutase activity would be expected to accumulate chorismate. Since this compound is known to be an effective feedback inhibitor of DAHP synthetase, its accumulation would decrease the activity of this enzyme. However, this explanation is not tenable since various treatments designed to remove chorismate from the

extract, such as dialysis through a cellophane bag or gel filtration through a Sephadex G-25 column, did not restore enzyme activity. Further, the addition of a crude extract of WB 932 to an extract containing DAHP synthetase did not decrease this enzyme activity. Thus, the absence of this enzymatic activity cannot be accounted for by the presence of an inhibitor.

If chorismate were the corepressor of DAHP synthetase, then its accumulation would be expected to result in a lowered DAHP synthetase specific activity. This explanation is unlikely on several grounds. First, the activity of DAHP synthetase in WB 932 is 20-fold lower than the lowest activity we have ever observed in any strain under conditions of maximal repression (Table II). The amounts of the aromatic amino acids added to the growth medium for 168 (repressed) ensures that an excess of these compounds exists at any time in the growth cycle of the organism. In addition, the cells were harvested during the logarithmic growth phase in order that conditions of maximal repression were met. Second, if chorismate did accumulate in a concentration sufficient to result in repression, it should be converted readily to anthranilate since anthranilate synthetase is functional in WB 932 and there would be no requirement for tryptophan. Consequently, the most tenable explanation is that WB 932 has a defective DAHP synthetase.

Since this mutant was made by treatment of the parent strain with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, a potent mutagen, we considered the possibility that WB 932 carried two independent mutations, one in the gene concerned with chorismate mutase and the other in the gene of DAHP synthetase. If this were the case, then the two genetic lesions should be separable by genetic recombination. To test this hypothesis, WB 932 was transformed with DNA extracted from its parent, the prototroph WB 746. Transformants were plated on minimal medium supplemented with shikimic acid, thereby selecting for cells with a functional chorismate mutase but not necessarily an operative DAHP synthetase. The colonies were then scored for a functional DAHP synthetase by replica plating to minimal medium and determining their growth response.

When 200 transformants were so analyzed, all transformants selected on shikimate were able to grow on minimal medium. The mutant locus responsible for the loss of DAHP synthetase activity cannot be separated from the site concerned with the loss of chorismate mutase activity. Therefore, we tentatively conclude that a single mutant site is involved.

Reversion Studies. If a single mutation has resulted in the loss of both enzyme activities, both enzyme activities should be restored by a single-step reversion. This can be readily accomplished. When WB 932 is plated on minimal medium, approximately one colony arises for every 10^7 cells plated. Table III indicates that these revertants have regained activity for both enzymes. In all 15 revertants analyzed, the restored chorismate mutase activity has a specific activity characteristic of CM_3 rather than $CM_1 + CM_2$. In some instances a strain has regained activity approximately equal to that of the parent. Most revertants, however, have regained only partial activity for each enzyme. Nevertheless, these partial activities are enough to allow a rate of growth equal to that of the wild-type organism.

TABLE III: Chorismate Mutase and DAHP Synthetase Activity of Prototrophic Revertants of WB 932.[b]

Mutant Strain	Specific Activity	
	Chorismate Mutase	DAHP Synthetase
1	1.1	9.5
2	0.8	10.6
3	1.7	*a*
7	1.0	*a*
8	1.5	17.1
9	1.4	11.0
10	1.2	15.9
11	2.0	9.3
12	1.4	9.9
13	2.2	16.7
14	1.6	16.9
15	1.4	13.1
16	2.4	28.7
17	2.2	11.8
18	2.6	22.5
168	2.8	35.8

[a] Strains not tested. [b] WB 932 was grown in TSY broth, the cells were resuspended in minimal medium to the original volume and 0.1 ml was spread on an agar plate containing glucose salts. After incubation for 48 hr at 45° the colonies which appeared were purified, cultures were grown on unsupplemented minimal medium, and extracts were prepared and assayed for DAHP synthetase and chorismate mutase as described in Methods.

Partial Revertants. In addition to revertants which have regained both enzyme activities, it is theoretically possible to select revertants which must have regained either one or the other activity. Thus, by plating WB 932 on minimal medium supplemented with tyrosine plus phenylalanine, revertants which have regained some DAHP synthetase activity but not necessarily chorismate mutase activity, would be selected. When 10^7 cells of WB 932 were plated on such a medium, approximately ten colonies (one of which was a prototroph) arose. The majority of the revertants would only grow if the two aromatic supplements were supplied. The results in Table IV for six such revertants show that they have regained approximately 5% of the level of wild-type DAHP synthetase

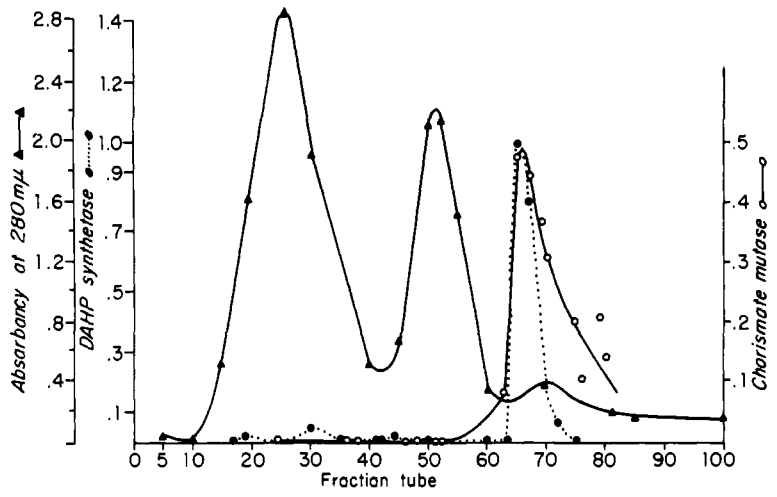

FIGURE 2: DEAE-cellulose chromatography of chorismate mutase and DAHP synthetase activity in an extract of SB 167. An extract of cells of SB 167 grown on shikimic acid (100 μg/ml) was prepared by lysozyme treatment and processed by the procedure described in Methods. The column was 2 × 20 cm and after application of approximately 40 mg of the extract protein a linear gradient of 0.05 M NaCl–0.5 M NaCl in 0.05 M potassium phosphate buffer (pH 6.8) was established. Volumes of 125 ml were used for each buffer. The flow rate of the column was approximately 25 ml/hr and 2-ml samples were collected. The eluents were assayed for DAHP synthetase and chorismate mutase activities by the described procedures.

activity. There may be a proportionate regain of chorismate mutase activity, although at these low enzyme levels there is considerable inaccuracy in this assay. It is not surprising that these strains grow quite well on tyrosine plus phenylalanine when only 5% of their DAHP synthetase activity has been restored, since the only aromatic amino acid which must be synthesized under these conditions is tryptophan, quantitatively the least significant of the aromatic amino acids. The other products of this pathway, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, as well as an elusive sixth factor (Davis, 1952), are required in only vitamin amounts.

TABLE IV: Chorismate Mutase and DAHP Synthetase Activity of Partial Revertants of WB 932.[a]

Strain	Specific Activity	
	Chorismate Mutase	DAHP Synthetase
D1	0.1	1.3
D2	0.1	1.6
D3	0.2	2.0
D4	0.1	1.4
D5	0.5	1.2
D6	0.5	1.8
168	2.8	35.8

[a] WB 932 was grown in TSY broth, the culture was resuspended in minimal medium to the original volume, and 0.1 ml was spread on an agar plate containing glucose salts supplemented with tyrosine and phenylalanine (20 μg/ml). After incubation for 48 hr at 45°, the colonies which appeared were purified, cultures was grown on minimal medium containing tyrosine and phenylalanine (20 μg/ml), extracts were prepared and processed, and enzyme activity was determined as described in Methods.

When spontaneous revertants were selected for chorismate mutase activity by plating on shikimate, all of the colonies which arose were prototrophs. No colonies were isolated which would grow only in the presence of shikimate. These reversion studies indicate that the regain of either enzyme activity generally results in a proportional regain of the other. More extensive attempts are being made to obtain revertants preferential for chorismate mutase activity.

Linkage of CM_3 and DAHP Synthetase. Since WB 932 had lost both DAHP synthetase as well as chorismate mutase activity, we considered the possibility that the mutation in this strain was in the gene specifying DAHP synthetase, and that secondarily the chorismate mutase activity was affected. That this is not the case rests mainly on the fact that the mutation in WB 932 can be mapped in relation to a known mutation in the locus concerned with DAHP synthetase. Strain SB 163 is deficient in DAHP synthetase activity but still possesses a normal level of CM_3 activity. Apparently this mutation affects the catalytic site of DAHP synthetase, but does not alter its complexing with CM_3. Strain WB 932 recipient cells were transformed with DNA isolated from strain 163 (Table V).

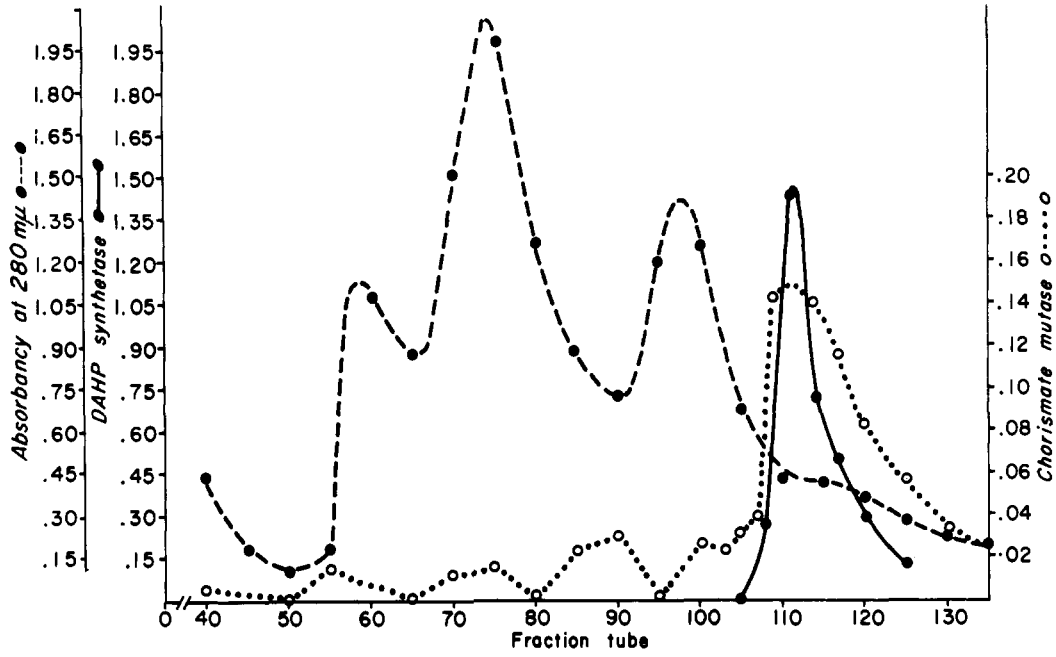

FIGURE 3: TEAE-cellulose chromatography of chorismate mutase and DAHP synthetase. An extract of cells of SB 167 grown on shikimic acid (100 μg/ml) was prepared by lysozyme treatment and processed by the procedure described in Methods. The column was 2 × 20 cm and a linear gradient of 0.05 M NaCl–0.5 M NaCl in 0.05 M potassium phosphate buffer (pH 6.8) was established. Volumes of 125 ml were used for each buffer. The flow rate of the column was approximately 25 ml/hr and 2-ml samples were collected. The eluents were assayed for DAHP synthetase and chorismate mutase activities by the described procedures.

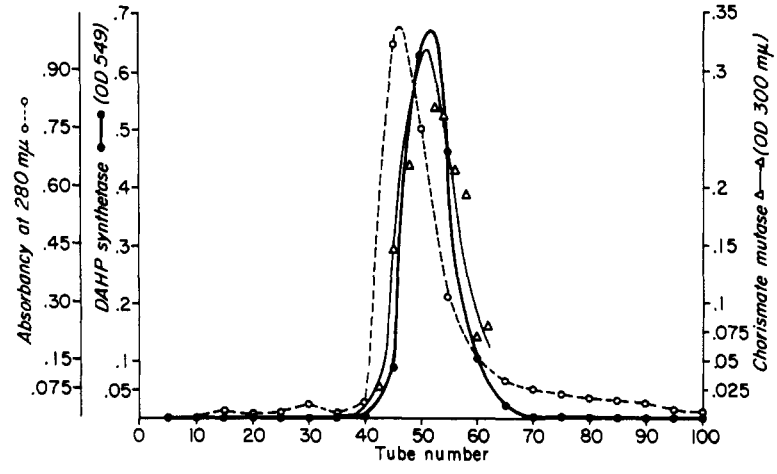

FIGURE 4: Chromatography of an extract of SB 167 on hydroxylapatite. SB 167 was grown on shikimic acid (100 μg/ml) and an extract was prepared by lysozyme treatment. The extract was processed as described in Methods. Approximately 40 mg of extract protein was added to the 1 × 20 cm column and allowed to enter the bed. Potassium phosphate (6 ml of 0.03 M at pH 6.8) was placed on top of the bed and the column was connected to a mixing device containing 125 ml each of 0.03 M potassium phosphate (pH 6.8) and 0.4 M potassium phosphate (pH 6.8) in the two chambers. The flow rate of the column was approximately 25 ml/hr and 2.3 ml/tube was collected. DAHP synthetase and chorismate mutase activities were assayed by the described procedures.

Transformants were selected on shikimic acid supplemented medium and replica plated to unsupplemented minimal medium. About 25% of the transformants was capable of growth on minimal medium. Since these recombinants must have resulted from a crossover event between the two mutant sites, the mutation in WB 932 and the gene for DAHP synthetase are closely linked. These two markers are transferred

TABLE V: Linkage of CM_3 to DAHP Synthetase.[a]

Medium Supplementation for Primary Selection	Transformant Classes (per 100 colonies)		Cotransfer Index
	CM_3^+-DAHP$^-$	CM_3^+-DAHP$^+$	
Shikimic acid	73	27	0.58

[a] DNA was prepared from SB 163 and added to the recipient cells, WB 932. The recipient culture was plated on minimal medium supplemented with shikimic acid (50 μg/ml). Transformants (100) were picked to nutrient agar and replica plated to minimal medium to determine the remainder of their genotype. DNA concentration = 0.005 μg/ml of recipient culture.

The cotransfer index =

$$\frac{(CM_3^+DAHP^-)}{2(CM_3^+DAHP^+) + (CM_3^+DAHP^-)}$$

SB 163 --x SB 932

Cross:

$CM_1^-CM_3^+DAHP^-$ --x $CM_1^-CM_3^-DAHP^-$

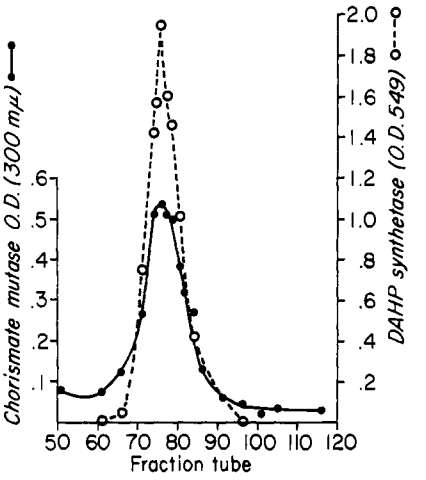

FIGURE 5: Sephadex G-100 chromatography of an extract of SB 167. The extract was prepared from cells grown on shikimic acid (100 μg/ml) and processed as described in Methods. The column was 2.5 × 100 cm and approximately 100 mg of protein was applied to the bed. The protein was eluted with 0.05 M potassium phosphate (pH 7.5) with a flow rate of approximately 1 drop every 15–20 sec. Samples of 2 ml were collected. The column was calibrated with cytochrome C, human hemoglobin, bovine γ-globulin, and lyophilized catalase. The peak tube fractions for the standards were catalase and γ-globulin, 70; hemoglobin, 112; and cytochrome C, 162.

simultaneously at about the same frequency as the Trp_2 and His_2 markers. Therefore, although they are linked, we infer that at least one locus separates the genes of DAHP synthetase and chorismate mutase.

Relationship between the Enzymes of DAHP Synthetase and Chorismate Mutase. An explanation consistent with all of the above facts is that both enzyme activities are catalyzed by a single molecular species. If this is true, then both enzyme activities should travel together when chromatographed if the subunits are in a stable aggregate. Extracts from SB 167 ($CM_1^-CM_2^-CM_3^+$) were chromatographed on DEAE-cellulose (Figure 2), TEAE-cellulose (Figure 3), and hydroxylapatite (Figure 4). In each, the peak of enzyme activity of the chorismate mutase CM_3 molecular species coincided exactly with that of DAHP synthetase activity. In some chromatographic procedures, there are apparent small peaks of enzyme activity (Figure 3). We have not been able to see a reproducible pattern with these and their significance is questionable. In all three procedures, the bulk of the extract protein traveled quite differently from these activities, suggesting that this coincidence is not a result of a nonspecific aggregation of extract protein.

An extract of SB 167 was also chromatographed on Sephadex G-100 which had been calibrated for molecular weight determinations according to the procedures given by Andrews (1964) (Figure 5). Again the peak (CM_3) of chorismate mutase activity coincides with the peak of DAHP synthetase activity. The molecular weight of the complex was estimated to be approximately 138,000. Centrifugation of crude extracts of SB 167 in a sucrose gradient also revealed an exact coincidence of the chorismate mutase and DAHP synthetase activity.

CM_1 and DAHP Synthetase Activity. Since the data suggest that CM_3 and DAHP synthetase form a complex, we were interested in determining whether the CM_1 or CM_2 chorismate mutase species would also complex with the DAHP synthetase polypeptide and yield an enzymatically functional DAHP synthetase. Therefore, we constructed a strain which had the CM_1 and CM_2 molecular species, but lacked CM_3. This was done by the insertion of 672 DNA ($CM_1^+CM_2^+CM_3^+$-His_2^+) into WB 2100 ($CM_1^-CM_2^-CM_3^-His_2^-$) recipient cells.

Since the CM_1 and CM_2 loci are closely linked to His_2 (Lorence and Nester, 1967) a high proportion of the His_2^+ transformants will also acquire the CM_1 and CM_2 locus. If either of these loci were able to substitute for the CM_3 locus, then the majority of His_2^+ transformants would produce a functional DAHP synthetase and be able to grow on minimal medium. If, however, the CM_1 locus cannot substitute for the CM_3 locus, then the strain would require shikimate for growth. When transformants from this cross selected on shikimate were tested for their ability to grow on minimal medium, 98 out of 100 did not grow. When extracts were prepared from two of the trans-

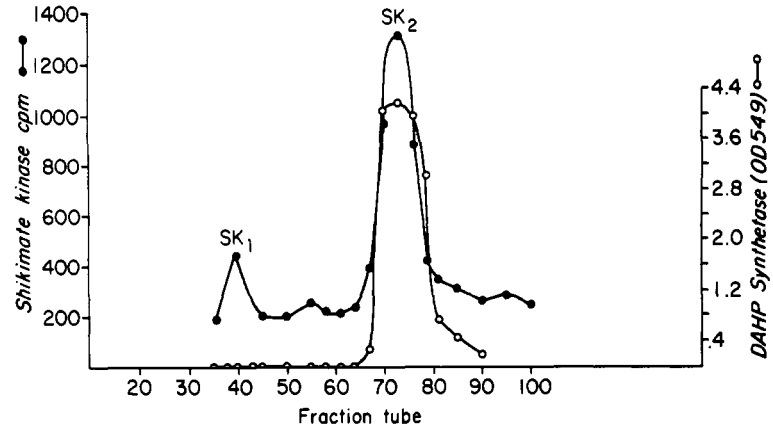

FIGURE 6: DEAE-cellulose chromatography of DAHP synthetase and shikimate kinase activity in an extract of strain WB 698b. The cells were grown in minimal medium, harvested, washed once, and resuspended in 0.1 M Tris-HCl (pH 8.3) buffer containing 1×10^{-4} M EDTA, 1×10^{-2} M $MgCl_2$, and 6×10^{-3} M mercaptoethanol. The cells were broken by sonication for 4 min with a Branson sonicator turned to maximum power at a setting of 5, and the extract was centrifuged at 25,000 *g* for 30 min. The extract was treated with protamine sulfate prior to addition to a 20 × 2 cm DEAE-cellulose column previously equilibrated with the same buffer. A linear gradient was established between 0 and 0.5 M NaCl in 0.1 M Tris-HCl buffer (pH 8.3) plus the additions in the same concentrations as listed above and the column eluted at 4°. Samples of 2 ml were collected.

formants which were not capable of growth on minimal medium, both CM_1 and CM_2 were readily demonstrable. This strongly suggests that neither CM_1 nor CM_2 can complex with the polypeptide product of the DAHP synthetase locus to yield a functional DAHP synthetase. These data provide strong evidence for the hypothesis that the polypeptide specified by CM_1 is structurally different from the polypeptide of CM_3.

Relationship of Shikimate Kinase to the Complex

Although DAHP synthetase and chorismate mutase are not sequential enzymes of aromatic amino acid synthesis, nevertheless, they do bind the same low molecular weight metabolites, chorismate and prephenate, since chorismate and prephenate feedback inhibit DAHP synthetase (Jensen and Nester, 1965). D. S. Nasser and E. W. Nester (manuscript in preparation) observed that shikimate kinase is also feedback inhibited by these two metabolites. In order to determine whether this enzyme might also be an integral part of this aggregate, extracts were chromatographed on DEAE-cellulose and Sephadex G-100, and the eluents were assayed for DAHP synthetase and shikimate kinase activity. The two enzymes elute in the same fractions from DEAE-cellulose (Figure 6) and Sephadex G-100 (Figure 7). These and similar chromatograms also demonstrate the presence of two molecular forms of shikimate kinase. These results suggest that one of the molecular forms of shikimate kinase forms a part of the complex with DAHP synthetase and chorismate mutase. More extensive experiments to definitely establish this point are now being carried out in our laboratory. WB 932 does have shikimate kinase activity but whether this activity is a result of the presumed aggregated species or the unaggregated species of the enzyme is not yet known. The detailed analysis of the regulatory properties of shikimate kinase in this mutant, as well as in the wild-type strain, will be presented in a subsequent paper.

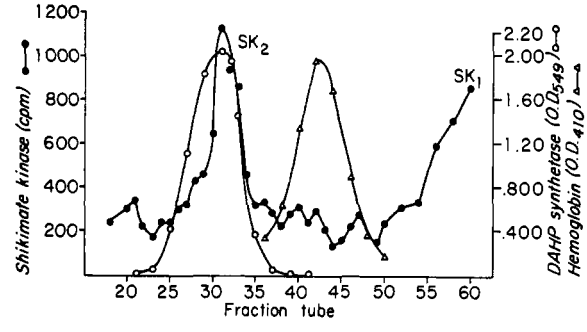

FIGURE 7: Sephadex G-100 chromatography of an extract of 698b for shikimate kinase and DAHP synthetase activity. The extract was prepared as described for Figure 6. The extract was added to a 60 × 2.5 cm Sephadex G-100 column. Samples of 2 ml were collected.

Complexes in Other Organisms. In a comparative study of the regulation of DAHP synthetase in a wide variety of microorganisms, R. A. Jensen and D. S. Nasser (manuscript in preparation) observed that *S. epidermidis* was also feedback inhibited by chorismate and prephenate. We were interested in determining whether this organism might also have DAHP synthetase and chorismate mutase in a complex. Figure 8 indicates that the two enzymes do chromatograph

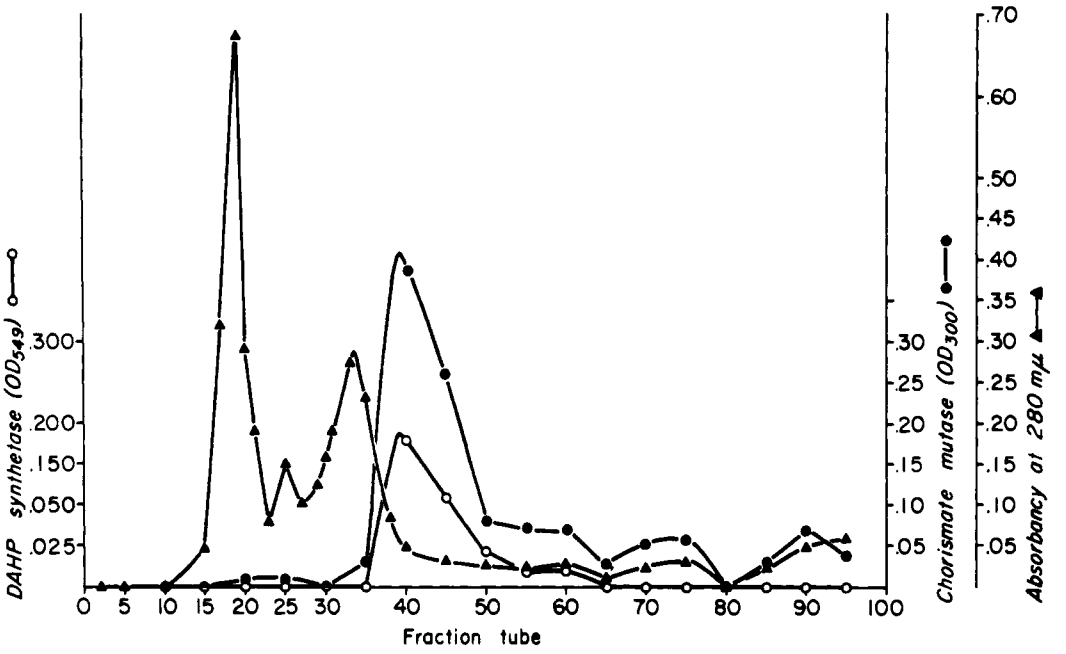

FIGURE 8: DEAE-cellulose chromatography of an extract of *S. epidermidis* for chorismate mutase and DAHP synthetase activity. *S. epidermidis* was grown in Spizizen's (1958) minimal medium supplemented with 0.5% glucose and 0.1% yeast extract. The organism was grown at 37° on a rotary shaker. After 18-hr incubation, the culture was centrifuged, washed one time in minimal medium, resuspended in 4 ml of 0.05 M Tris-HCl buffer (pH 8.1), and the cells were disrupted by sonic vibration in an MSE ultrasonic disintegrater at maximum power for 8 min. The suspension was centrifuged at 25,000 *g* for 30 min and the supernatant was applied to the top of a 2 × 20 cm DEAE-cellulose column previously equilibrated with 0.05 M Tris-HCl (pH 8.1). A linear gradient was established between 0.05 and 0.5 M NaCl in 0.05 M Tris-HCl (pH 8.1) with 125 ml of each buffer. Samples of 2 ml were collected.

together on DEAE-cellulose and Figure 9 shows the coincidence of the enzyme activities in the fractions collected from a sucrose gradient. These results indicate that in *S. epidermidis* as in *B. subtilis* the two enzymes form a stable complex.

Discussion

A survey of the recent literature makes it abundantly clear that enzyme aggregation is of widespread occurrence (Reed and Cox, 1966). In the majority of cases thus far studied, the enzymes which are found in an aggregate do operate sequentially on a given substrate. Current thinking on the physiological significance of enzyme complexes emphasizes the fact that the assembly of a series of enzymes may increase the efficiency of the over-all process. The encounter of an intermediate produced by one component with the next catalytic site in the sequence should be more probable in an organized complex than in a mixed solution of separate enzymes. In some instances, apparently enzymes must be organized into a catalytically active unit before they can carry out an entire reaction sequence (Wagner *et al.*, 1964).

In the present study, the enzymes which have been shown to occur in a multienzyme complex are nonsequential and represent only a few of the enzymes concerned with aromatic amino acid biosynthesis. Accordingly, it seems unlikely that the physiological significance of this complex, if our observations have significance *in vivo*, lies in an increased efficiency of the over-all process of aromatic amino acid biosynthesis because of the close proximity of substrate, product, and succeeding enzymes. The one feature which these three enzymes do have in common is a binding site for chorismate and prephenate. Jensen and Nester (1966b) characterized DAHP synthetase as an allosteric protein of the V type (Changeux, 1964) in which both prephenate and chorismate served as feedback effectors. D. S. Nasser and E. W. Nester (manuscript in preparation) have shown that shikimate kinase is also feedback inhibited by these same metabolites. Thus, we feel that this enzyme complex is designed for the efficient feedback control of DAHP synthetase and possibly shikimate kinase. One possibility for the functioning of the complex involves the likelihood that the prephenate produced by the catalytic activity of chorismate mutase would be available, perhaps without diffusion, to the allosteric sites of the other enzyme(s) in the complex. Presumably, the excessive production of prephenate would result in its binding to DAHP synthetase and shikimate kinase, thereby decreasing the flow of low molecular weight metabolites into the pathway.

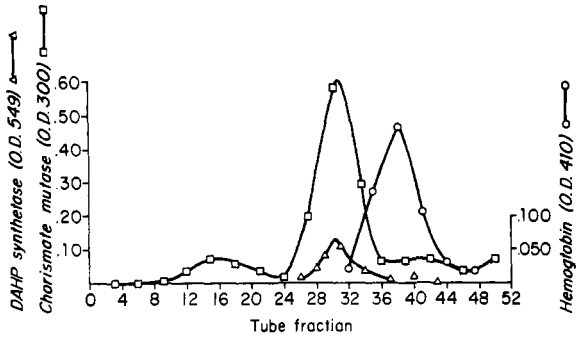

FIGURE 9: Sucrose gradient profile of an extract of *S. epidermidis* for chorismate mutase and DAHP synthetase activities. The extract was prepared as described for Figure 8. The extract (0.3 ml) was layered on top of a 10–30% sucrose gradient and the tubes were centrifuged at 39,000*g* for 16 hr at 4°. Fractions of 8 drops were collected and the activities were assayed as described in Methods. Human hemoglobin was used as an internal standard.

Another reasonable, but yet experimentally unassessed hypothesis concerning the functioning of this complex as a unit of feedback control can be entertained. It is possible that the catalytic site on chorismate mutase binding chorismate and prephenate is the allosteric site for control of the other enzyme(s) in the aggregate. This model is, in principle, analogous to the experimentally demonstrated situation in the aspartate–transcarbamylase system (Gerhart and Pardee, 1963; Gerhart and Schachman, 1965). In their system, there are two distinct and separable polypeptide chains, a catalytic subunit and a regulatory subunit. In our system, the regulatory subunit has a catalytic function—the conversion of chorismate to prephenate. The binding of the chorismate or prephenate at the catalytic site of chorismate mutase may change the conformation of the entire complex, thereby affecting the molecular activity of the other components of the complex. Our data have no bearing as to whether this model could be best fit to the induced-fit hypothesis of Koshland (1958, 1963), Atkinson (1966), or the model proposed by Monod *et al.* (1965). If our model has any merit, we should theoretically, be able to demonstrate that the removal of the chorismate mutase from the complex desensitizes DAHP synthetase and shikimate kinase to their allosteric effectors. Attempts to obtain mutants with altered feedback properties are now in progress.

The observations made on *S. epidermidis* suggest that the complex of DAHP synthetase and chorismate mutase may have a regulatory significance in other genetically unrelated organisms which have the same feedback control of DAHP synthetase. Additonal studies on other genera should clarify whether we can correlate the occurrence and composition of enzyme aggregates from the pattern of control of DAHP synthetase and shikimate kinase.

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Crystal Spectra of a Heme and Some Heme–Protein Complexes*

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ABSTRACT: The crystal spectra of mesoporphyrin IX iron(III) methoxide, ten myoglobin derivatives, and cytochrome *c* peroxidase have been obtained. The comparison between these spectra and the corresponding solution spectra show that in all cases there is a reduction in intensity of absorption bands in the solid state but that in most cases the energy of the spectrum is virtually unchanged. Myoglobin fluoride and cytochrome *c* peroxidase are exceptions. It is proposed that the crystallization of a heme protein always causes a shift in the equilibrium position, high-spin form $\rightleftharpoons$ low-spin form, toward low-spin forms but that changes in conformation can also be involved. The use of polarized single-crystal spectra has permitted an assignment of all the absorption bands in the spectra. The transitions are all polarized in the heme plane, showing them to have the same character as the $\pi-\pi^*$ transitions.

The crystal spectra of both small molecules and proteins are of interest in two very different fields of study. First an unpolarized crystal spectrum can be compared directly with a solution spectrum. Any changes which are then observed can be interpreted in terms of conformation changes, restrictions of vibrations, or cooperative effects. Details of one such study on a small model compound, $PtCl_4^{2-}$, are described by Day *et al.* (1965). The application of this comparison to protein molecules is particularly desirable for at present it is assumed that X-ray crystal studies in the solid state are directly relevant to solution studies despite the fact that this has not been put to careful experimental test. In this paper we shall examine the crystal spectra of several myoglobin derivatives of known crystal structure in an effort to establish whether or not distinct differences between solid and solution phases need to be postulated. We have also examined the spectra of cytochrome *c* peroxidase in both phases although the crystal structure of this protein is not known. In both proteins the established sensitivity of the spectra of high-spin iron(III) porphyrin compounds to changes in the environment of the iron atom make the systems ideal for our studies. Furthermore, it is already known that there are differences in reactivity between solid and solution phases of myoglobin compounds (Chance *et al.*, 1966).

The second field of interest concerns the theory of the absorption spectra of metal porphyrins. Although the *visible* spectra of porphyrins have been extensively studied and subjected to theoretical treatment (Braterman *et al.*, 1964; Day *et al.*, 1964; Gouterman, 1961; Weiss *et al.*, 1965), the near-infrared region has been neglected. The origin of the weak band observed at about 10,000 cm^{-1} in high-spin iron(III) porphyrins has not been conclusively established, and $\pi-\pi^*$ triplets have not been observed in absorption. Polarized single-crystal spectra are of particular value in making such assignments as we shall see below.

Materials

The mesoporphyrin IX iron(III) methoxide (dimethyl ester) was kindly supplied by Dr. W. S. Caughey. Myoglobin crystals were a gift from Dr. C. Nobbs. The myoglobin derivatives were obtained by bathing crystals of the acid metmyoglobin in solutions of different simple salts. The concentrations of the solutions were chosen so as to give at least 99.0% formation of the required derivative as estimated from solution equilibrium data. The treatment of the crystals lasted at least 24 hr and in the case of the thiocyanate it was shown that bathing for longer periods did not alter the crystal spectrum. Each compound was measured at least twice. The cytochrome *c* peroxidase crystals were a gift from Professor T. Yonetani.

The single-crystal polarized spectra were obtained using an apparatus which is essentially a microspectrophotometer employing a reflection microscope and a polarizer. With it crystals between 0.2 and 1.0 mm in length can be studied. The instrument will be described in detail later.

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