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Analysis of the Metal Requirement of 3-Deoxy-D-*arabino*-heptulosonate-7-phosphate synthase from *Escherichia coli**

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The three isozymes of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase from *Escherichia coli* were overproduced, purified, and characterized with respect to their requirement for metal cofactor. The isolated isozymes contained 0.2–0.3 mol of iron/mol of enzyme monomer, variable amounts of zinc, and traces of copper. Enzymatic activity of the native enzymes was stimulated 3–4-fold by the addition of Fe^{2+} ions to the reaction mixture and was eliminated by treatment of the enzymes with EDTA. The chelated enzymes were reactivated by a variety of divalent metal ions, including Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}. The specific activities of the reactivated enzymes varied widely with the different metals as follows: $Mn^{2+} > Cd^{2+}$, $Fe^{2+} > Co^{2+} > Ni^{2+}$, Cu^{2+}, $Zn^{2+} \gg Ca^{2+}$. Steady state kinetic analysis of the Mn^{2+}, Fe^{2+}, Co^{2+}, and Zn^{2+} forms of the phenylalanine-sensitive isozyme (DAHPS(Phe)) revealed that metal variation significantly affected the apparent affinity for the substrate, erythrose 4-phosphate, but not for the second substrate, phosphoenolpyruvate, or for the feedback inhibitor, L-phenylalanine. The tetrameric DAHPS(Phe) exhibited positive homotropic cooperativity with respect to erythrose 4-phosphate, phosphoenolpyruvate, and phenylalanine in the presence of all metals tested.

The enzyme DAHP synthase (EC 4.1.2.15, phospho-2-dehydro-3-deoxyheptonate aldolase) catalyzes the condensation of phosphoenolpyruvate (PEP)[1] and erythrose 4-phosphate (E4P) to yield 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) and P_i (1). This reaction is the first committed step in the biosynthesis of chorismate in microorganisms and plants, from which tryptophan, tyrosine, phenylalanine, and aromatic cofactors such as folate and quinones are derived. DAHP synthase serves as the primary site for feedback regulation of carbon flow into chorismate synthesis. *Escherichia coli* contains three isoenzymic forms of DAHP synthase, each of which is subject to feedback regulation by one of the aromatic amino acids. The primary sequences of DAHP synthases from *E. coli* (2–4), *Salmonella typhimurium* (5), *Saccharomyces cerevisiae* (6), and *Solanum tuberosum* (potato) (7) have a high degree of similarity throughout the polypeptide, implying a common ancestry and conserved structural and functional properties.

It is now clear that DAHP synthases in general require a divalent metal cofactor for activity, although the role of the metal ion remains undefined. Though the DAHP synthase isozymes from *E. coli* have been studied most extensively, details of their metal requirement still remain confused due to an abundance of conflicting evidence. In earlier work, some enzyme preparations were found to be inhibited by chelating agents and reactivated by divalent metals (8–10), whereas others were unaffected by chelation or exogenous metals (12–14). More recent reports indicated that the three isozymes are iron-dependent (11, 15), although other evidence favored cobalt (9) or copper dependence (16). DAHP synthases from other bacteria (*i.e. S. typhimurium* DAHPS(Tyr) (17) and *Streptomyces aureofaciens* DAHPS(Trp) (18)) and from various eukaryotes (*i.e. S. cerevisiae* (6), *Neurospora crassa* (19), cauliflower (20), and *Vigna radiata* (21)) have generally been shown to be EDTA sensitive and stimulatable by various divalent cations, although the identity of the effective metal ions and the extent of stimulation varied.

We report here parallel studies of the metal requirement of the three *E. coli* DAHP synthases. Cloning of the genes encoding the enzymes into a plasmid vector designed for their overexpression facilitated rapid purification of the isozymes. Evidence is presented that the three isozymes have a common metal requirement that can be satisfied *in vitro* by a variety of divalent metal ions. It is also shown that the identity of the metal ion utilized significantly affects the kinetic properties of the enzyme. Possible roles for metals in DAHP synthase function are discussed.

MATERIALS AND METHODS[2]

RESULTS

Purification of E. coli DAHP Synthases—Plasmid vectors for overproduction of the three DAHP synthase isozymes were developed in order to facilitate rapid, high-yield enzyme production. The *aroG, aroF,* and *aroH* genes were inserted in separate constructions behind the *tac* promoter (*ptac*) of phagemid pttS7, a high copy number expression vector (Fig. 1). The *aroG* construction (pttG1) differed from the *aroH* and *aroF* plasmids in that it retained the native *aroG* promoter downstream of *ptac* (Fig. 1). Induction of DAHP synthase

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[1] The abbreviations used are: PEP, phosphoenolpyruvate; E4P, erythrose 4-phosphate; DAHP, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate; IPTG, isopropyl-β-D-thiogalactopyranoside; BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

[2] Portions of this paper, including "Materials and Methods", Figs. 1 and 2, and Table I, are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

gene expression with IPTG resulted in each of the enzymes becoming the most abundant polypeptide in cell extracts (Fig. 2). Expression of *aroG* was the most efficient due to the tandem promoter arrangement; a construction lacking the native *aroG* promoter yielded 40% less enzyme (not shown). It should be noted that the copy numbers of the expression vectors constructed here were significantly greater than those of the plasmids used recently for the production of DAHPS(Tyr) (17) and DAHPS(Trp) (11).

The three DAHP synthase isozymes were purified by similar two-step protocols (see "Materials and Methods"). Representative results are summarized in Table I and Fig. 2. The final preparation of DAHPS(Phe) (M_r 40,000) was estimated to be about 98% pure based on densitometric scanning of Coomassie-stained SDS-PAGE gels, with a single visible contaminant of M_r 26,000. The DAHPS(Trp) (M_r 42,000) and DAHPS(Tyr) (M_r 41,000) preparations were slightly less pure, each having several minor contaminating components. The three isozymes were stable for extended periods when stored on ice in BTP buffer containing 200 μM PEP.

Dependence of DAHP Synthase Activity on Divalent Metals—As was reported previously for DAHPS(Trp) (11), inclusion of Fe^{2+} ions in reaction mixtures stimulated the activity of the DAHP synthase isozymes in crude extract (Table I). Treatment with various chelating agents, including EDTA, 1,10-phenanthroline, 8-hydroxyquinoline, and 2,2′-dipyridyl, reduced activity by 60–90% (data not shown). The degree of metal activation of the isozymes increased during purification (Table I). Atomic absorption analysis of the purified isozymes found each to contain substoichiometric amounts of iron and zinc (*i.e.* less than 1 g-atom/enzyme monomer) and traces of copper, but no detectable cobalt or manganese (Table II). These results suggest that the metal sites of the enzymes were only partially saturated in crude extracts and that they lost metal during the process of purification.

Metal-free preparations of the three purified isozymes were prepared by dialysis against buffer containing 1 mM EDTA, followed by dialysis against metal-free buffer to remove the EDTA. Atomic absorption analysis verified the absence of iron, zinc, copper, cobalt, and manganese in the EDTA-treated enzymes. The chelated enzymes were essentially devoid of catalytic activity but were readily reactivated by the inclusion of Fe^{2+} as well as numerous other divalent metal ions in the reaction mixture, including Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}. The range of activities restored by the different metals varied more than 10-fold (Table III). Mn^{2+} gave the highest activity with all three isozymes and Ca^{2+} the lowest. With few exceptions, the hierarchy of activities supported by the different metals was the same with the three isozymes. It was also noted that the specific activity of DAHPS(Phe) with each metal was consistently 2–3-fold greater than that of DAHPS(Trp) or DAHPS(Tyr). Other cations tested, including Mg^{2+}, Na^+, and K^+, were ineffective

TABLE II

Metal content of purified DAHP synthases

Atomic absorption analysis of samples of purified isozymes was carried out in triplicate as described under "Materials and Methods." Concentrations of the enzyme preparations analyzed were as follows: DAHPS(Phe), 38.4 μN; DAHPS(Trp), 9.2 μN; DAHPS(Tyr), 5.4 μN. The detection limits for the metals were as follows: iron, 48 nM; zinc, 1.1 μM; copper, 8 nM; manganese, 18 nM; cobalt, 3 nM.

Isozyme	Iron	Zinc	Copper	Manganese	Cobalt
	g-atoms/mol enzyme monomer				
DAHPS(Phe)	0.23	0.03	0.01	<0.005	<0.005
DAPHS(Trp)	0.26	0.28	0.04	<0.005	<0.005
DAPHS(Tyr)	0.19	0.29	0.02	<0.005	<0.005

TABLE III

Activities of metal-free DAHP synthases reactivated by different metals

Activities were determined at least in triplicate with enzymes prepared and assayed as described under "Materials and Methods." Standard deviations are indicated. All metals were at 50 μM, except Ca^{2+} which was at 1 mM and, in the case of DAHPS(Tyr), Zn^{2+} which was at 5 μM (see text). Activities with Ca^{2+}, Cu^{2+}, and Ni^{2+} were determined by preincubation of the enzyme with metal, PEP, and buffer for 10 min at 23 °C and initiation of the reaction by the addition of E4P. Activities with the other metals were determined by initiating the reaction with enzyme.

Metal	DAHPS(Phe)	DAHPS(Trp)	DAHPS(Tyr)
	units/mg	*units/mg*	*units/mg*
None	0.73 ± 0.3	0.23 ± 0.2	0.12 ± 0.1
Mn^{2+}	93.3 ± 5.0	40.9 ± 2.8	33.3 ± 1.8
Cd^{2+}	66.5 ± 1.7	26.6 ± 0.2	24.1 ± 2.3
Fe^{2+}	61.6 ± 4.3	27.7 ± 1.9	22.1 ± 0.5
Co^{2+}	39.8 ± 3.2	15.4 ± 0.3	15.5 ± 0.9
Ni^{2+}	23.8 ± 1.7	9.3 ± 0.9	8.0 ± 1.1
Cu^{2+}	22.7 ± 1.3	16.9 ± 0.8	6.3 ± 0.2
Zn^{2+}	17.8 ± 0.6	7.8 ± 0.2	8.0 ± 0.2
Ca^{2+}	6.6 ± 2.2	1.6 ± 0.2	1.1 ± 0.2

in restoring activity at concentrations up to 5 mM. Although the assessment of activation by Cr^{3+} and Fe^{3+} was complicated by interference of these ions with the spectrophotometric assay, Cr^{3+} at concentrations up to 500 μM appeared to be completely ineffective, while Fe^{3+} at concentrations up to 100 μM was weakly active (≤20% of the activity obtained with Fe^{2+}). The level of Fe^{2+} in the Fe^{3+} preparation used was not assessed.

Some metals (Cu^{2+}, Ni^{2+}, and Ca^{2+}) were slow in apoenzyme activation. When apoenzymes were added to reaction mixtures containing PEP, E4P, and Cu^{2+} (50 μM), Ni^{2+} (50 μM), or Ca^{2+} (1 mM), the reaction rate was initially very low but increased steadily for 3–5 min before becoming constant (not shown). However, if the enzymes were preincubated for several minutes at 23 °C with buffer, PEP, and one of these metals before initiating the reaction with E4P, maximum velocity was attained much more rapidly. The length of the initial lag period was inversely related to metal concentration and preincubation time, and under optimal conditions was usually no more than 30 s.

DAHPS(Tyr) had a unique response to Zn^{2+} activation. Concentrations of Zn^{2+} greater than 5 μM caused a progressive decline in the reaction rate during the first minute of the reaction; the rate of this decline increased with increasing Zn^{2+} concentration. This was not due to substrate exhaustion or product accumulation but resulted directly from interaction of the enzyme with Zn^{2+}, as preincubation of enzyme with 50 μM Zn^{2+} (5 min at 23 °C) reduced activity to about 10% of that attained with 5 μM Zn^{2+}. No such decline in activity was observed with DAHPS(Phe) or DAHPS(Trp) in the presence of Zn^{2+} at concentrations as high as 60 μM, and preincubation of these two isozymes with 500 μM Zn^{2+} caused no more than a 25% reduction in activity relative to that obtained with 5 μM Zn^{2+}.

To examine the stoichiometry of metal binding in activated enzyme, a 10-fold molar excess of Mn^{2+} or Zn^{2+} was added to metal-free DAHPS(Phe), after which unbound metal ions were removed by gel filtration chromatography (see "Materials and Methods"). The activity of the Mn^{2+}-treated preparation was stimulated an additional 25% by the addition of 50 μM Mn^{2+} to the assay mixture, indicating only 80% saturation of metal-binding sites. In contrast, the Zn^{2+}-treated preparation was not activated further by exogenous Zn^{2+}. Atomic absorption analysis revealed 0.8 g-atoms of man-

ganese and 1.5 g-atoms of zinc bound/mole enzyme monomer in the respective preparations, suggesting a functional stoichiometry of one metal ion/enzyme monomer in the fully activated enzymes.

It is clear from these results that the three *E. coli* DAHP synthases have a common requirement for a metal cofactor which can be similarly satisfied by a range of divalent metal ions. In view of this, subsequent experiments to define further the role of the metal ion in enzyme function were carried out with only one of the isozymes, DAHPS(Phe).

Metal Competition Experiments—The relative affinity of DAHPS(Phe) for various metals was assessed by examining the ability of metals to compete with one another for activation of the metal-free apoenzyme. First, initial enzymatic activities were measured after the enzyme was exposed simultaneously in assay mixtures to all possible pairs of six different activating metals (namely, Mn^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, and Ca^{2+}). In all cases the activities observed with the metal pairs were never significantly higher or lower than the activity in the presence of either of the metals alone (Table IV), indicating that they competed for binding to a single functional site. By comparing the activity of the enzyme in the presence of each metal pair with that of the individual metals, it was found that Zn^{2+} was favored over all other metals, followed in turn by Mn^{2+}, Fe^{2+}, Co^{2+}, Ca^{2+}, and Cu^{2+}. The low initial activities in the presence of Ca^{2+} and Cu^{2+} and the weak competition of Ca^{2+} and Cu^{2+} with other metals are consistent with the slow activation of DAHP synthase activity noted above for these two ions.

In a second set of experiments the ability of one metal to displace another already bound to the enzyme was tested. DAHPS(Phe) apoenzyme was preincubated with PEP and one metal for 5 min at 23 °C, after which the metal competitor was added, followed immediately by E4P to start the reaction. The competition patterns obtained were somewhat different from those of the first experiment (Table IV). Enzyme first exposed to Fe^{2+}, Co^{2+}, or Zn^{2+} exhibited activities characteristic of these metals no matter which competitor was added, indicating that stable metal-enzyme complexes had formed during the preincubation. In contrast, when the Mn^{2+}, Cu^{2+}, and Ca^{2+} enzymes were challenged with other metals, observed activities were in general very similar to those obtained by simultaneous exposure of the enzymes to the respective metal pairs. This suggests that these latter three metal-enzyme complexes were unstable and that the bound metals were readily displaced by other metals. It should be pointed out that the intermediate activity of the Mn^{2+}-enzyme complex challenged with Zn^{2+} was an artifact of the assay procedure, rather than an exception. It was found in this case that enzymatic activity decreased continuously over the course of the assay and approached that characteristic of the Zn^{2+} enzyme, revealing a slow displacement of Mn^{2+} by Zn^{2+}.

Kinetic Analysis of Metal Utilization—Apparent K_m values for Mn^{2+}, Fe^{2+}, Co^{2+}, and Zn^{2+} were determined with DAHPS(Phe) in steady state kinetic experiments in the presence of saturating concentrations of PEP and E4P (Table V). Zn^{2+} had the highest apparent affinity, which was more than 10 times that of Co^{2+}, Fe^{2+}, and Mn^{2+}. In fact, the K_m for Zn^{2+} was too low to be measured under the conditions employed, as the lowest feasible concentration of DAHPS(Phe) (30 nN) achieved 90% of V_{max} at equimolar Zn^{2+}. Thus, it appears that one Zn^{2+} atom/enzyme monomer is sufficient for full activity. The V_{max} values of the enzyme with the various metals, presented in Table V as k_{cat} constants, were essentially iden-

TABLE IV

Competition between metals for activation of DAHPS(Phe)

	Mn^{2+} [a]	Fe^{2+}	Co^{2+}	Zn^{2+}	Cu^{2+}	Ca^{2+}
			units/mg			
	94.4 ± 1.4	56.8 ± 1.6	38.5 ± 1.2	17.5 ± 1.8	5.0 ± 3.3	3.3 ± 1.3
Mn^{2+}		80.9 ± 2.8 (36%)	87.6 ± 1.2 (12%)	22.8 ± 0.8 (93%)	96.0 ± 2.3 (0%)	93.4 ± 1.2 (1%)
Fe^{2+}	80.9 ± 2.8 (64%)		49.2 ± 2.2 (41%)	18.1 ± 0.7 (98%)	57.8 ± 0.8 (4%)	54.6 ± 0.8 (4%)
Co^{2+}	87.6 ± 1.2 (88%)	49.2 ± 2.2 (59%)		18.3 ± 0.8 (96%)	38.3 ± 1.3 (1%)	36.5 ± 1.2 (6%)
Zn^{2+}	22.8 ± 0.8 (7%)	18.1 ± 0.7 (2%)	18.3 ± 0.8 (4%)		17.9 ± 1.7 (0%)	16.1 ± 1.3 (13%)
Cu^{2+}	96.0 ± 2.3 (100%)	57.8 ± 0.8 (96%)	38.3 ± 1.3 (99%)	17.9 ± 1.7 (100%)		2.8 ± 2.2 (100%)
Ca^{2+}	93.4 ± 1.2 (99%)	54.6 ± 0.8 (96%)	36.5 ± 1.2 (94%)	16.1 ± 1.3 (87%)	2.8 ± 2.2 (0%)	
Competing metal	Preincubation metal[b]					
	Mn^{2+}	Fe^{2+}	Co^{2+}	Zn^{2+}	Cu^{2+}	Ca^{2+}
			units/mg			
None	92.1 ± 1.2	58.0 ± 2.7	37.6 ± 1.2	18.3 ± 1.5	12.2 ± 2.0	4.4 ± 2.4
Mn^{2+}		55.2 ± 1.7	36.7 ± 1.0	16.7 ± 1.4	84.9 ± 2.1	82.5 ± 3.1
Fe^{2+}	78.5 ± 1.4		35.9 ± 2.7	14.9 ± 1.7	45.2 ± 6.8	48.3 ± 0.6
Co^{2+}	84.5 ± 1.7	55.2 ± 1.4		17.6 ± 1.7	31.5 ± 2.2	30.5 ± 3.0
Zn^{2+}	58.5 ± 6.7	53.7 ± 2.2	36.6 ± 1.5		18.2 ± 1.8	15.4 ± 1.0
Cu^{2+}	89.1 ± 2.5	53.3 ± 3.2	36.9 ± 1.3	16.4 ± 1.1		6.4 ± 1.8
Ca^{2+}	88.9 ± 2.2	54.6 ± 1.6	36.5 ± 2.2	17.9 ± 2.0	14.3 ± 3.1	

[a] Activity of DAHPS(Phe) apoenzyme exposed simultaneously to two metal ions. All reactions were initiated by the addition of enzyme. Values represent DAHP synthase activity over the first minute of the reaction and are the mean of at least three independent determinations. Percentages in parentheses indicate calculated saturation of enzyme sites with the metal ion listed at the head of each column (see "Materials and Methods"). Metal ions were at 50 μM except Ca^{2+} which was at 1 mM.

[b] Initial activity of DAHPS(Phe) apoenzyme preincubated with one metal, PEP, and buffer for 5 min at 23 °C, then exposed to the competing metal. The reaction was initiated immediately after addition of the competing metal by the addition of E4P. Metal ion concentrations were as in top of table.

TABLE V

Kinetic properties of DAHPS(Phe) with different metals

Methods for determination of kinetic parameters are described under "Materials and Methods." The standard error of all values was ≤±15% except for $S_{0.5}^{PEP}$, which was ≤±40%.

Activating metal	k_{cat}	K_m^M	k_{cat}/K_m^M	$S_{0.5}^{E4P}$	$S_{0.5}^{PEP}$
	s^{-1}	μM	$s^{-1}\ M^{-1}$	μM	μM
Mn^{2+}	61	0.56	1.1×10^8	170	2.0
Fe^{2+}	40	0.19	2.1×10^8	67	1.8
Co^{2+}	26	0.14	1.9×10^8	36	1.1
Zn^{2+}	12	<0.03	$>4 \times 10^8$	16	2.1

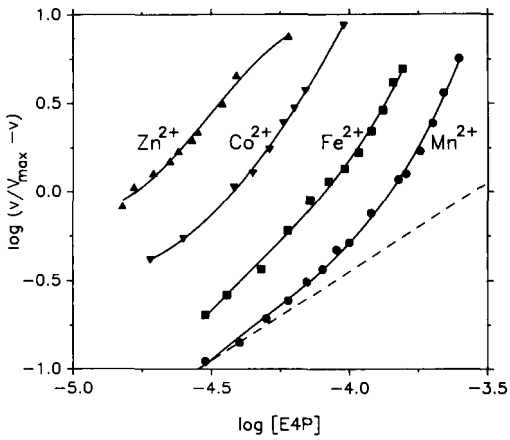

FIG. 3. **Hill plot of the kinetics of erythrose 4-phosphate utilization by DAHPS(Phe) in the presence of various metals.** Initial PEP concentration was 100 μM. Metal ions were at 50 μM. Slope of *dashed* reference line is 1.0. ●, Mn^{2+}; ■, Fe^{2+}; ▼, Co^{2+}; ▲, Zn^{2+}.

tical to the specific activities determined under standard assay conditions (Table III). The catalytic efficiency of the enzyme with respect to each metal (k_{cat}/K_m^M) was very high, in each case exceeding $10^8\ s^{-1}\ M^{-1}$.

There was no indication in the above results of cooperative interaction between metal-binding sites in the enzyme, a possibility considering its tetrameric structure (22).[3] However, cooperative kinetics were observed in experiments in which the concentration of E4P was varied at subsaturating levels in the presence of saturating concentrations of PEP and metal (Mn^{2+}, Fe^{2+}, Co^{2+}, or Zn^{2+}). Hill plots of the data are presented in Fig. 3. Strong positive cooperativity for E4P was evident with all four metals. For example, with Mn^{2+}, the slope of the curve (n_H^{app}) approached 1.0 at low concentrations of E4P, was about 2.1 at E4P concentrations yielding 50% of V_{max}, and increased further to about 3.8 at concentrations yielding 80–95% of V_{max}. It was also found that $S_{0.5}^{E4P}$ of the enzyme varied 10-fold depending on the identity of the metal (Fig. 3 and Table V).

Apparent positive cooperativity was also noted for PEP utilization by the enzyme in the presence of the same four metals, as Hill plots of the kinetic data indicated $n_H^{app} \approx 1.4$ between 50–90% of V_{max} (not shown). $S_{0.5}^{PEP}$ values approximated from the Hill plots varied little with the different metals, indicating that the apparent affinity of the enzyme for PEP was not significantly affected by the nature of the activating metal.

Feedback inhibition of DAHPS(Phe) activity by phenylalanine was analyzed in the presence of the four metals. At fixed PEP, E4P, and metal concentrations, sigmoidal inhibition kinetics were observed. Hill plots of these data produced symmetrical curves with a slope of −1.9 to −2.0 at the midpoint (Fig. 4). The phenylalanine concentration for half-maximal inhibition ($[I]_{0.5}$) was 10–12 μM with Fe^{2+}, Co^{2+}, and Mn^{2+} as the activating metal and 28 μM with Zn^{2+}. Covariation of phenylalanine and E4P concentrations showed that phenylalanine significantly increased $S_{0.5}^{E4P}$ but had little effect on V_{max} (Fig. 5). Furthermore, phenylalanine reduced the cooperativity of E4P utilization, with n_H^{app} for E4P approaching 1.0 at the highest concentrations of the inhibitor (Hill plot not shown).

Absorbance Properties of DAHPS(Phe)—Concentrated solutions of native DAHPS(Phe) had a pinkish coloration prior to metal removal, as was previously noted with DAHPS(Trp) (11). The absorbance properties of DAHPS(Phe) were examined to determine whether metals might serve as chromophores (Fig. 6). The absorption spectrum of the metal-free apoenzyme in the 300–600-nm range was undistinctive. Ad-

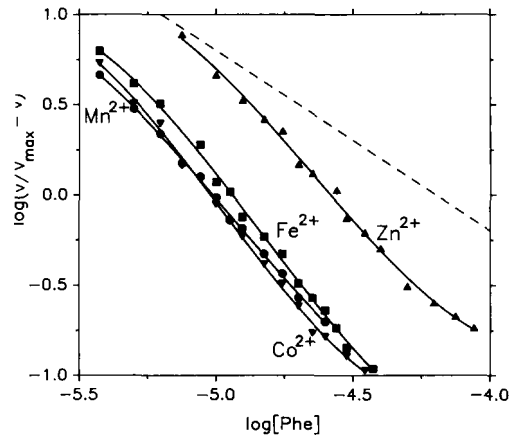

FIG. 4. **Hill plot of the kinetics of phenylalanine inhibition of DAHPS(Phe) in the presence of different metals.** Initial substrate concentrations were: PEP, 100 μM; E4P, 480 μM. Metal ions were at 50 μM. Slope of *dashed* reference line is −1.0. ●, Mn^{2+}; ■, Fe^{2+}; ▼, Co^{2+}, ▲, Zn^{2+}.

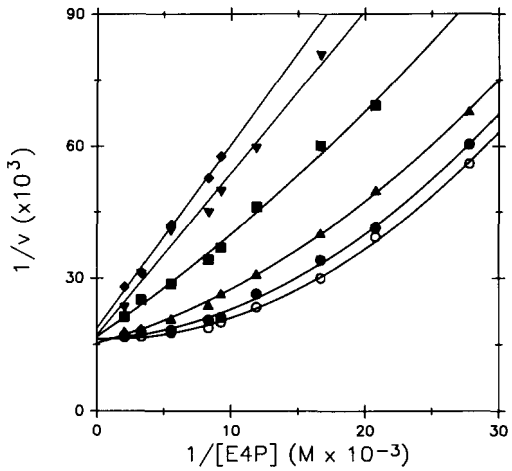

FIG. 5. **Lineweaver-Burk plot of phenylalanine inhibition of DAHPS(Phe) with E4P as the varied substrate.** Initial concentration of PEP was 100 μM. Concentration of Fe^{2+} was 50 μM. Phenylalanine concentrations: ○, none; ●, 2 μM; ▲, 4 μM; ■, 6 μM; ▼, 8 μM; and ◆, 10 μM. Each data point represents the average of at least two determinations. The curves shown are regression lines (second order for 0, 2, 4, and 6 μM phenylalanine; first order for 8 and 10 μM phenylalanine) generated by the Sigmaplot software (Jandel Scientific).

[3] We have confirmed the tetrameric state of DAHPS(Phe) in our preparations by size exclusion chromatography (data not shown).

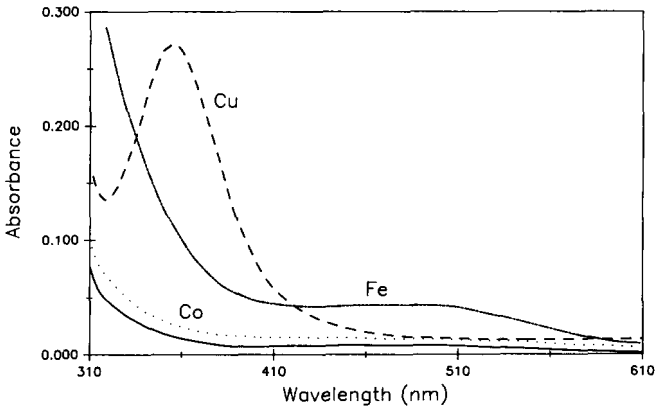

FIG. 6. **Absorption spectra of different metal forms of DAHPS(Phe).** DAHPS(Phe) concentration was 100 μN. Metal ions were added to 100 μM and the enzyme was incubated at 25 °C for 2 h, then stored at 4 °C for 14 h. The spectra shown were obtained at the end of these incubations. The *solid* curve is that of the metal-free apoenzyme.

dition of Cu^{2+} resulted in the slow appearance of a well-defined absorbance peak at 350 nm ($\epsilon^{N}_{350\,nm}$ = 2800). The $t_{1/2}$ for generation of the band was approximately 20 min at 25 °C when 100 μM $CuSO_4$ was added to 100 μN enzyme. Addition of Fe^{2+} to the enzyme resulted in an increase in absorbance at 310–410 nm (adding a shoulder to the 280-nm aromatic peak), and in a broad band centered at 485 nm. Addition of Co^{2+} caused marginal absorbance increases in the visible region, while Mn^{2+} and Zn^{2+} had no demonstrable effect (not shown).

DISCUSSION

Metal Requirement of DAHP Synthases—It has been demonstrated that the three *E. coli* DAHP synthase isozymes have essentially identical requirements for metal cofactor. Purified preparations of each isozyme contained the same metals (Table II), were inactivated upon removal of endogenous metal by treatment with EDTA, and were reactivated to similar extents by the same group of divalent metal ions (Table III). Although the specific activity of reactivated DAHPS(Phe) was about two times that of the DAHPS(Trp) and DAHPS(Tyr) isozymes with any given metal, the hierarchy of activities supported by the various metals was essentially the same with the three enzymes. Thus, it can be concluded that the catalytic activity of DAHP synthases is determined both by the specific properties of the activating metal ion and by the intrinsic properties of each isozyme.

The metal-enzyme stoichiometries of reactivated DAHPS(Phe) apoenzyme indicate that a single metal ion is associated with each enzyme monomer, as was previously concluded from the results of similar analyses of DAHPS(Trp) (11) and DAHPS(Tyr) (16). Furthermore, competition experiments involving sequential addition of metals demonstrated that Zn^{2+}, Fe^{2+}, and Co^{2+} are not readily displaced by other metals when bound to the enzyme. These results demonstrate that a functional metal-enzyme complex can be maintained for multiple catalytic cycles and argues against the possibility that a free metal-substrate complex is utilized by DAHP synthase. The observed patterns of competition between different metals for occupancy of the apoenzyme (Table IV) may primarily reflect differences in the k_{off} value of each metal, as the magnitude of the apparent second order rate constants for enzyme:metal association (*i.e.* k_{cat}/K^{M}_{m}) (Table V) indicates that the enzyme:metal interaction (k_{on}) is diffusion-controlled (23).

It appears likely that, of the various metals found to activate the *E. coli* DAHP synthases *in vitro*, Fe^{2+} and perhaps Zn^{2+} are preferred metal cofactors *in vivo*. This conclusion is based on the presence of these two metals in the purified isozymes, on the high apparent affinity of the DAHPS(Phe) isozyme for these ions *in vitro*, and on their relatively high bioavailability. Indeed, it is somewhat paradoxical that the isolated enzymes did not contain predominantly zinc, considering the preference shown by DAHPS(Phe) for Zn^{2+} *in vitro*. In apparent disagreement with the results presented here, Baasov and Knowles (16) recently described a purified preparation of *E. coli* DAHPS(Tyr) that contained primarily copper, was inactivated by cyanide (an effective copper ligand), and upon chelation was reactivated by Cu^{2+}, Cd^{2+}, and Zn^{2+}, but *not* by Fe^{2+}. The conditions for enzyme production in this case were significantly different and presumably less efficient than those used here. The *aroF* gene was carried on a plasmid of lower copy number and was expressed under the sole control of its native promoter. Also, the producing strain was grown in a complex medium (Luria-Bertani broth), rather than in supplemented minimal salts, and cells were harvested in the mid-logarithmic stage of growth. Thus, it may be that significant differences in the metal content of native DAHP synthase preparations can arise by variations in metal availability during cellular growth.[4] However, it remains unclear why the DAHPS(Tyr) preparation of Baasov and Knowles was not reactivatable by Fe^{2+} after chelation by EDTA. We consistently observed rapid and efficient reactivation of all three *E. coli* apoenzymes by Fe^{2+}; similar Fe^{2+} reactivation has also been demonstrated with EDTA-treated DAHPS(Phe) from *S. cerevisiae* (6).

Spectroscopic Properties of DAHPS(Phe)—The spectroscopic properties of the DAHPS(Phe) isozyme (Fig. 3) indicate that metals serve as chromophores in DAHP synthases. The pinkish coloration of solutions of the purified enzyme is probably due to its iron content, as this property was regenerated only when iron was added back to the apoenzyme. However, it is likely that the spectral features of the iron form of the enzyme result from oxidation of bound Fe^{2+} ions to Fe^{3+}, as absorption increased slowly and continuously under the aerobic conditions employed, whereas Fe^{2+} was found to bind and activate DAHPS(Phe) essentially instantaneously. Similar conclusions were drawn from spectroscopic studies of the DAHPS(Trp) isozyme (11).

Several inferences concerning the nature of the metal-binding site can be derived from the DAHPS(Phe) absorption spectra. The lack of strong absorbance features between 500–700 nm in the Co^{2+} enzyme, the range characteristic of d-d electronic transitions, suggests octahedral coordination of the metal ion (24). Furthermore, the absorption peak at 350 nm displayed by the Cu^{2+}-containing enzyme is suggestive of a ligand-to-metal charge transfer originating from thiolate and/or imidazolate ion(s) of the enzyme (25). In this regard, we have recently shown by directed mutagenesis of DAHPS(Phe)[5] that conservative changes in Cys-61, 1 of the 2 invariant cysteine residues in the DAHP synthases of known sequence (4, 6), destroy enzymatic activity, eliminate metal binding and prevent the appearance of the 350-nm absorbance band in purified metal-free enzyme upon addition of Cu^{2+}.[6]

[4] However, atomic absorption analysis of Luria-Bertani broth prepared in this laboratory did not reveal an abundance of copper. The metal content was as follows: iron, 12.7 μM; zinc, 12.7 μM; cobalt, 0.4 μM; copper, 0.3 μM; and manganese, 0.2 μM.

[5] C. Stephens and R. Bauerle, submitted for publication.

[6] Interestingly, the thiol group of Cys-61 appears not to be readily susceptible to oxidation in the DAHPS isozymes, since the addition of reducing agents such as dithiothreitol or mercaptoethanol does not enhance enzymatic activity and is not required for stabilization of the enzymes during preparation and storage.

It appears that the copper-dependent absorbance band at 350 nm is identical to that previously described for the DAHPS(Tyr) apoenzyme in the presence of Cu^{2+} (16) and for native preparations of DAHPS(Phe) and DAHPS(Tyr) (15). In the latter cases, metal analysis indicated the presence of iron in both enzymes at a stoichiometry of 0.25–0.5 g-atom/enzyme monomer; in retrospect it seems likely that the enzymes also contained copper, as in our experience this feature is unique to copper-containing DAHP synthase isozymes.

Possible Roles for Metal Ions—The effects of metal identity on k_{cat} and $S_{0.5}^{E4P}$ of DAHPS(Phe) suggest an active site role for the metal ion. By analogy to other PEP-utilizing enzymes (26), one possibility is involvement in substrate binding by coordination of the phosphate or carboxylate oxygens of PEP or the phosphate of E4P. Active roles in catalysis are also conceivable, although uncertainty as to the mechanistic details of the condensation event (27) renders these highly speculative. In one proposed mechanism (28), nucleophilic attack by an enzyme component at C-2 of PEP generates a tetrahedral intermediate at this position, enabling C-3 to undertake nucleophilic attack of the aldehyde carbon (C-1) of E4P. A metal ion could facilitate such an attack by coordinating the E4P carbonyl oxygen to further polarize the C = O bond, increasing the electrophilicity of C-1. Our observation that metal ions affect $S_{0.5}^{E4P}$ but not $S_{0.5}^{PEP}$ is consistent with this model. Bond polarization to facilitate attack of a carbonyl group is a role ascribed to metal ions in a number of enzymes, including aldolase (29), carboxypeptidase A (30), and phospholipase A_2 (31).

It is also possible that the metal serves a structural role in DAHP synthase, as many proteins require bound metal to attain an active conformation (32, 33). Of course, an active site location and a structural role for metals in DAHP synthase are not necessarily exclusive. The results of chemical modification experiments with DAHPS(Phe) in the presence and absence of metal[5] are consistent with an active site location for the metal ion, but also suggest that the metal has significant effects on enzyme conformation. Clearly, more extensive examination of the *E. coli* DAHP synthase isozymes by a range of kinetic and biophysical approaches is necessary to provide further insight into the positioning and function of the metal in this enzyme.

Kinetic Properties of DAHPS(Phe)—Previous steady state kinetic studies of DAHPS(Phe) have resulted in values for K_m^{E4P} ranging from 0.55 to 1 mM and for K_m^{PEP} varying from 0.08 mM to 1 mM (8, 12, 34), although in one case it was concluded that K_m^{PEP} was below 10 μM (22). It is possible that the higher substrate affinities and cooperativity found here were the result of improvements in the methods of purification and assay. The rapidity of the purification regimen might facilitate maintenance of the native DAHPS(Phe) structure. In contrast to some earlier work, phosphate buffers were avoided, as P_i inhibits DAHP synthase; also the continuous assay used allows more rapid initial velocity measurements than the alternative discontinuous method (38). Perhaps most importantly, the identity of the metal cofactor of DAHP synthase was carefully controlled by using metal-free reagents and by extracting endogenous metals from the purified enzyme, permitting the reconstitution of enzyme with homogeneous metal content. It is noteworthy that the DAHPS(Trp) isozyme, expressed and purified using nearly identical methods and assayed in the presence of Fe^{2+}, has been found to have similarly low Michaelis constants (K_m^{PEP} of 10 μM and K_m^{E4P} of 50 μM).[7]

Although DAHPS(Phe) has previously been described as displaying typical Michaelis-Menten kinetics (8, 12), experiments performed here examining the effects of metal identity on the kinetic properties of the enzyme revealed significant positive homotropic cooperativity for both E4P and PEP (Fig. 3 and text). On the other hand, the cooperativity of phenylalanine inhibition of DAHPS(Phe) activity (Figs. 4 and 5) has been noted by others in the past (34, 35). The heterotropic effect of phenylalanine (*i.e.* reduction of E4P cooperativity) is phenomenologically similar to the elimination of cooperativity in fructose 6-phosphate binding in the *E. coli* phosphofructokinase by its feedback inhibitor, PEP (36). Kinetic and binding studies of phosphofructokinase showed that the behavior of the enzyme was consistent with the concerted transition model of allosterism (37), with the stipulation that the inhibitor-bound T state of the enzyme retains a low but nevertheless significant affinity for substrate. Similar binding studies with DAHPS(Phe) are necessary in order to fit the kinetic behavior of this enzyme to an allosteric model.

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[7] J. Ray and R. Bauerle, unpublished results.

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SUPPLEMENTARY MATERIAL TO:

ANALYSIS OF THE METAL REQUIREMENT OF 3-DEOXY-D-*ARABINO*-HEPTULOSONATE-7-PHOSPHATE SYNTHASE FROM *ESCHERICHIA COLI*.

Craig Stephens and Ronald Bauerle

MATERIALS AND METHODS

Chemicals and enzymes. 1,3-bis[tris(hydroxymethyl)-methylamino]propane (BTP), PEP (cyclohexylammonium salt), and E4P (sodium salt) were from Sigma. L-phenylalanine was from Calbiochem. Analytical grade $FeSO_4$, $CdSO_4$, $CoCl_2$, $CuSO_4$, $CaCl_2$, and $MgCl_2$ were from Aldrich, and $MnCl_2$, $Zn(C_2H_3O_2)_2$, $CrCl_3$, $NiCl_2$, and EDTA were from Mallinckrodt. Trace metals were removed from water, buffers and substrate solutions with Chelex 100 chelating resin (Bio-Rad Laboratories) as directed by the manufacturer. Acid titration of BTP showed only a slight loss of buffering capacity after Chelex treatment. Solutions of metal salts were prepared in chelexed water immediately before use. Enzymes utilized in DNA cloning manipulations were obtained from Bethesda Research Laboratories and were used as recommended by the manufacturer.

Bacterial plasmids and strains. The plasmid expression vector pttS7 (2.8 kb) (Fig. 1) was constructed from plasmid ptacterm, a high copy number vector containing the *tac* promoter (*ptac*), the *rpoC* terminator, and the ampicillin resistance gene (*amp*) (39). The *Eco*RI and *Hin*dIII sites of ptacterm were destroyed in successive manipulations by digestion with the respective enzymes, elimination of overhanging ends with S1 nuclease, and religation. The *Bam*HI/*Sal*I fragment between *ptac* and the terminator was replaced with a multiple cloning site containing *Bam*HI, *Sma*I, *Pst*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Cla*I, and *Sal*I restriction sites. The M13 origin of replication was then inserted on a 0.5 kb segment located between *ptac* and *amp*. Expression vectors for the *aroG*, *aroF*, and *aroH* genes were constructed from pttS7. Restriction maps and nucleotide sequences of these genes have been reported (2,3,4). The *aroG* gene with its native promoter and terminator was inserted on a 1.5 kb *Bgl*II/*Xmn*I fragment into the *Eco*RV site of pttS7, creating pttG1 (4.3 kb) (Fig. 1). The *aroF* gene on a 1.4 kb *Hpa*II/*Bgl*II fragment was inserted without its promoter into the *Eco*RV site of pttS7, creating pCFG4 (4.2 kb). The *aroH* gene on a 1.3 kb *Dde*I fragment was inserted without its promoter into the *Eco*RV site of pttS7, creating pCHA1 (4.1 kb). These plasmids were maintained in *E. coli* strain CB198 (W3110 *tna trpR lacI*^{q}), routinely grown in L broth containing 50 mg/l ampicillin.

DAHP synthase purification. The three DAHPS isozymes were purified by slight modifications of a method developed earlier for DAHPS(Trp) (11). Cultures of the plasmid-bearing strains were grown in minimal salts medium (4) supplemented with 5 mg/l glucose, 1 mg/l casein acid hydrolysate (United States Biochemical), and 25 mg/l ampicillin, with vigorous shaking in baffled flasks at 37°C. IPTG was added at 1 mM to mid-logarithmic phase cultures (A_{550nm} = 0.5) to induce transcription from *ptac*. Cells were harvested by centrifugation 3 h after IPTG induction ($A_{550nm} \approx 1.2$). Subsequent manipulations were carried out at 0-4°C. The cell pellet was resuspended in BPP buffer (10 mM BTP, pH 6.8, 200 μM PEP). Cells were ruptured by sonification at 70 W for 45 s with a Branson sonifier (Branson Sonic Power Co.), and cell debris was removed by centrifugation at 50000 x g for 30 min. Streptomycin sulfate (20% solution) was added to the extract to a final concentration of 1%. After stirring for 10 min, precipitated material was removed by centrifugation at 12000 x g for 10 min.

For the purification of the DAHPS(Phe) isozyme, cold acetone was added to the supernatant to a final concentration of 30%. After 15 min, the precipitate was collected by centrifugation at 12000 x g for 20 min. The pellet was drained, dissolved in 6 ml BPP, and clarified by passage through a 0.22 μm pore cellulose acetate centrifuge filtration unit. The filtrate was fractionated by anion exchange chromatography over a Mono Q HR 5/5 FPLC column (Pharmacia LKB) using a Shimadzu HPLC system. The column was equilibrated with 10 mM BTP, pH 6.8, and 1 ml of the enzyme preparation (5-10 mg total protein) was loaded onto the column in each run. The column was developed with a gradient of 0 to 500 mM KCl in 10 mM BTP, pH 6.8 over 25 min at a flow rate of 1 ml/min. DAHPS(Phe) was eluted at approximately 275 mM KCl. PEP was immediately added to enzyme-containing fractions to a final concentration of 200 μM. Fractions from successive runs were pooled.

For the purification of the DAHPS(Trp) and DAHPS(Tyr) isozymes the same protocol was followed except that DAHPS(Trp) was precipitated with 25% acetone and DAHPS(Tyr) was recovered as a 20-35% acetone precipitate. DAHPS(Trp) was eluted from the Mono Q column at approximately 175 mM KCl and DAHPS(Tyr) at 250 mM KCl.

Enzyme assays. DAHP synthase activity was measured by the continuous spectrophotometric method (13). Overproduction of DAHP synthases minimized the problem of background activity from other PEP-consuming enzymes, as E4P-independent consumption of PEP was ≤ 5% of the total activity in crude extracts and was undetectable in purified preparations. DAHP synthase activities reported were corrected for any E4P-independent activity present. Reaction mixtures contained 10 mM BTP (pH 6.8), 150 μM PEP, 600 μM E4P, and unless indicated otherwise, 50 μM metal in a total volume of 1.0 ml. Disappearance of PEP was monitored at 232 nm in 1 cm path-length quartz cuvettes in a Varian DMS200 double-beam spectrophotometer equipped with a thermostatted cuvette holder held at 23°C. Rates were measured over successive 6 s intervals and were averaged over the linear portion of the reaction; results from multiple assays were averaged. One activity unit is defined as the disappearance of 1 μmole of PEP per minute at 23°C. Reactions were generally initiated by addition of 1-5 μl enzyme solution (10-40 pmoles) to the reaction mixture. In assays using $CuSO_4$, $NiCl_2$, or $CaCl_2$, maximal activity was achieved by preincubating enzyme with PEP and these salts for 10 min at 23°C, and then initiating the reaction with E4P. All pipetting was done with metal-free pipet tips (BioRad Laboratories), and cuvettes were cleaned with metal-free water between uses.

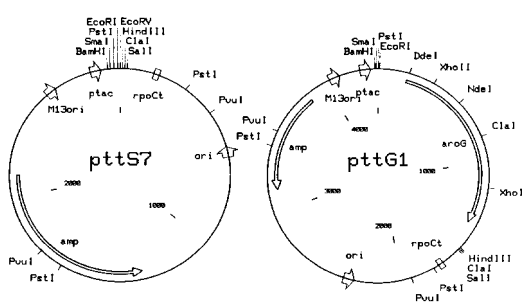

Fig. 1. Physical maps of plasmids pttS7 and pttG1. *ptac* indicates the *tac* promoter, *rpoCt* the *rpoC* transcriptional terminator, *ori* the plasmid origin of replication, *M13ori* the phage origin, and *amp* the β-lactamase gene.

Saturating concentrations of the unvaried components in steady-state kinetic experiments were 100 μM PEP, 50 μM metal salt, and 600 μM E4P (with Mn^{2+}), or 480 μM E4P (with Fe^{2+}, Co^{2+}, or Zn^{2+}). In experiments using subsaturating concentrations of PEP, a significant portion (10% to 40%) of the PEP was consumed during the minimum assayable interval (6 s). Velocities were thus plotted as functions of the calculated mean concentration of PEP present during the interval (40), rather than the initial concentration. The small amount of PEP carried over with the enzyme was taken into account in calculation of the initial concentration of PEP. Apparent K_m values for metal ions were calculated by the Enzfitter non-linear regression data analysis software (Elsevier). For velocity plots showing evidence of significant cooperative effects, V_{max} was determined graphically from double-reciprocal plots, and $S_{0.5}$ and n_H[40] values were determined from Hill plots, with the aid of SigmaPlot and SigmaScan software (Jandel Scientific).

Determination of protein concentration. Protein concentrations were determined using the Bio-Rad Laboratories protein assay reagent with bovine serum albumin as the standard. Quantitative amino acid analysis of acid hydrolysed enzyme samples indicated that this method overestimates the concentration of the purified *E. coli* DAHP synthase isozymes. Correction factors of 0.65 for DAHPS(Phe), 0.64 for DAHPS(Trp), and 0.86 for DAHPS(Tyr) were derived and used to convert results from Bio-Rad assays on purified isozymes into actual concentrations. Enzyme concentrations are presented as normal (N) values, indicating *monomer* concentrations.

Metal analysis. Enzyme and buffer samples were analyzed with a Perkin-Elmer model 5100 atomic absorption spectrometer using a graphite furnace (iron, cobalt, and manganese determinations) or a flame analyzer (zinc). Values listed are the average of triplicate determinations. The limits of detection were as follows: manganese, 18 nM; iron, 48 nM; cobalt, 3 nM; copper, 8 nM; zinc, 1.1 μM. Enzyme concentrations in the samples were 10-70 μN. For determination of metal:enzyme stoichiometries in fully activated DAHPS(Phe), a 10-fold molar excess of $MnCl_2$ or $ZnCl_2$ was added to 1 ml of 10-20 μM metal-free enzyme. After approximately 30 min, unbound metal was separated from the enzyme by gel filtration over a 20 ml Sephadex G-50 column which had been washed with 1 mM EDTA, followed by metal-free BPP buffer. The desalted enzyme was then analyzed by atomic absorption spectrometry as described above.

Removal of metals from DAHP synthases. Metals were removed from the purified enzymes by dialysis of 1 ml samples (2-5 mg) against 400 ml BPP containing 1 mM EDTA. Dialysis tubing (Spectra/Por 2, molecular weight cutoff of 14000) was prepared by boiling in 1 mM EDTA followed by rinsing in metal-free water. For DAHPS(Phe), dialysis was carried out for 2 h at 23°C or 8-12 h at 0°C. (Inactivation by chelation was found to be more rapid at higher temperatures.) DAHPS(Trp) and DAHPS(Tyr) were unstable at 23°C and thus were dialyzed against BPP/EDTA for 8-12 h at 0°C. Samples were then further dialyzed against two changes of metal-free BPP (400 ml), and transferred to sterile polypropylene test tubes.

For enzyme samples to be analyzed by UV/visible absorption spectroscopy, metals were removed by incubation of the enzyme solutions for 8-12 h at 0°C with one-tenth volume of neutralized Chelex resin, followed by dialysis of the sample (including resin) against metal-free BPP. This treatment was slightly less effective than EDTA, but avoided potential spectral artifacts due to residual metal:EDTA complexes.

Metal competition experiments. In one set of competition experiments, DAHPS(Phe) apoenzyme was added to standard assay mixtures containing two different metal salts, each at 50 μM except in the case of $CaCl_2$, which was at 1 mM. Activity over the first 1 min interval was averaged for three independent assays. In a second set of experiments, DAHPS(Phe) apoenzyme was added to a standard assay mixture lacking only E4P and containing a single metal (concentrations same as above) and then incubated for 5 min at 23°C. The competitor metal was added, the cuvette mixed, and E4P added immediately to start the reaction. Activities were then recorded and averaged as above.

The apparent saturation of enzyme binding sites with each metal in both experiments was calculated by the following equation: % *Metal A* bound = 100 $[1-(a - x)/(a - b)]$; where a = activity with *Metal A* alone, b = activity in the presence of *Metal B* (competitor) alone, and x = activity in the presence of both metals. As shown in Table V, x was occasionally not within the limits defined by a and b but in no case was the deviation from the limit value greater than the standard error of the a (or b) and x values.

SDS-PAGE. Denaturing polyacrylamide gel electrophoresis was done by the method of Laemmli (41). Denatured samples (1-5 μg protein) were loaded on 10% polyacrylamide gels and electrophoresed at 25 milliamps until the bromphenol blue dye had run off the gel. Proteins were visualized by staining with Coomassie Brilliant blue, followed by destaining in 20% methanol/7% acetic acid.

TABLE I

PURIFICATION OF DAHP SYNTHASE ISOZYMES

DAHPS(Phe) was purified as described under "*Materials and Methods*" from a 500 ml culture, whereas DAHPS(Trp) and DAHPS(Tyr) were purified from 200 ml cultures. Enzymes were assayed without added metal and in the presence of 50 μM $FeSO_4$.

DAHPS(Phe)

Preparation	Activity (*u/mg*)		Purification (*fold*)	Protein (*mg*)
	No metal	$+Fe^{2+}$		
Crude extract	8.7	19.3	1.0	45.3
Streptomycin supernatant	5.5	20.2	1.0	42.3
Acetone precipitate	8.0	22.7	1.2	27.5
Mono Q peak	9.5	36.2	1.9	10.2

DAHPS(Trp)

Preparation	Activity (*u/mg*)		Purification (*fold*)	Protein (*mg*)
	No metal	$+Fe^{2+}$		
Crude extract	1.5	1.9	1.0	58.0
Streptomycin supernatant	1.2	2.2	1.2	47.8
Acetone precipitate	2.8	3.9	2.1	14.5
Mono Q peak	4.6	13.2	7.1	2.0

DAHPS(Tyr)

Preparation	Activity (*u/mg*)		Purification (*fold*)	Protein (*mg*)
	No metal	$+Fe^{2+}$		
Crude extract	1.5	4.8	1.0	37.5
Streptomycin supernatant	1.3	6.8	1.4	28.1
Acetone precipitate	5.0	18.8	3.9	10.2
Mono Q peak	5.8	24.6	5.1	3.4

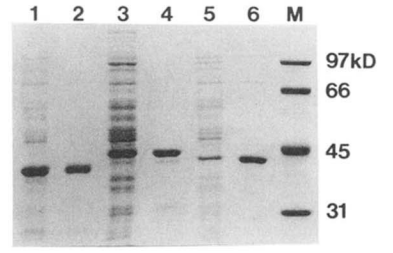

Figure 2. Purification of DAHP synthase isozymes. Contents of the lanes are as follows: (1) DAHPS(Phe) in crude extract, (2) purified DAHPS(Phe), (3) DAHPS(Trp) in crude extract, (4) purified DAHPS(Trp), (5) DAHPS(Tyr) in crude extract, (6) purified DAHPS(Tyr), (M) molecular weight standards. 2.5 to 5 μg total protein was loaded in lanes containing crude extracts; approximately 1 μg protein was loaded in each lane with purified isozymes.