Proc. Natl. Acad. Sci. USA
Vol. 75, No. 10, pp. 4810–4813, October 1978
Biochemistry

Iron, an essential element for biosynthesis of aromatic compounds

[3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (phospho-2-keto-3-deoxyheptonate aldolase)/phospho*enol*pyruvate/absorption spectrum/isoenzymes/*Escherichia coli*]

RUSSELL J. MCCANDLISS AND KLAUS M. HERRMANN*

Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907

Communicated by H. E. Umbarger, July 26, 1978

ABSTRACT **Homogeneous preparations of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase [7-phospho-2-keto-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate lyase (pyruvate phosphorylating), EC 4.1.2.15] isolated as the enzyme·phospho*enol*pyruvate complex from *Escherichia coli* are shown by atomic absorption analysis to contain approximately one mole of iron per mole of native enzyme. No cobalt was found, in contrast to suggestions of earlier workers. Pure enzyme preparations show a unique absorption maximum around 350 nm with an ϵ value of about 3500 M^{-1} cm^{-1}. The 350-nm band as well as the enzyme activity is lost when the enzyme is denatured with guanidine·hydrochloride, or when phospho*enol*pyruvate, the first substrate to bind to the enzyme, is totally removed from the enzyme by incubation with an excess of erythrose 4-phosphate, the second substrate to bind to the enzyme. The iron remains bound to the enzyme when phospho*enol*pyruvate is removed from the enzyme·phospho*enol*pyruvate complex.**

The first committed step in the biosynthesis of aromatic compounds in bacteria and plants is the condensation of *P-enol*pyruvate and erythrose-4-*P* to form 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) and inorganic phosphate (1). This reaction is catalyzed by DAHP synthase [7-phospho-2-keto-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate lyase (pyruvate phosphorylating), EC 4.1.2.15]. The enzyme has been purified to homogeneity from *Neurospora crassa* (2), *Bacillus subtilis* (3), *Escherichia coli* (4, 5), *Salmonella typhimurium* (6), and *Schizosaccharomyces pombe* (7). Partially purified preparations of this enzyme have been reported from several other sources.

Cells of wild type *E. coli* contain three DAHP synthase isoenzymes, the phenylalanine-sensitive DAHP synthase (Phe), the tyrosine-sensitive DAHP synthase (Tyr), and the tryptophan-sensitive DAHP synthase (Trp) (8, 9). DAHP synthase (Phe) and DAHP synthase (Tyr) are inhibited by chelating agents, and partially purified preparations of these enzymes are activated by Co^{2+} (10, 11). We have recently confirmed that partially purified preparations of the tyrosine-sensitive and the phenylalanine-sensitive isoenzymes are activated by Co^{2+}, and we have shown that this property is lost during the purification of both isoenzymes (4, 5). Pure enzyme preparations are unaffected by Co^{2+} below 4 mM and inhibited by higher Co^{2+} concentrations, as has been shown previously for the DAHP synthase (Tyr) from *S. typhimurium* (12).

It has been suggested that DAHP synthase is a metalloprotein containing cobalt (10). However, Simpson and Davidson reported that *E. coli* DAHP synthase (Phe) contains no cobalt (13). In this paper we show that DAHP synthase contains approximately one mole of iron per mole of enzyme, and that the native form of the enzyme has a unique absorption spectrum with a maximum at 350 nm.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Chemicals and Enzymes. *P-enol*pyruvate (14) and erythrose-4-*P* (15) were synthesized and assayed as described previously (4, 16); guanidine·hydrochloride, ultra pure, was from Schwarz/Mann; *m*-phenanthroline was from ICN-K + K Laboratories and was recrystallized twice from hot water. Bovine hemoglobin, twice crystallized, was from Miles Laboratories. All other chemicals were obtained commercially as the highest purity available and were used without further purification.

DAHP synthease (Phe) (5) and DAHP synthase (Tyr) (4) were the homogeneous preparations described previously; *P-enol*pyruvate carboxylase [orthophosphate:oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31] was about 30% pure and from Boehringer Mannheim.

Enzyme Assays and Protein Determinations. DAHP synthase was assayed by method A of Schoner and Herrmann (4) except that 1,3-propanediol was omitted from all buffers. The unit of enzyme activity is defined as the amount of enzyme catalyzing the appearance of 1.0 μmol of DAHP per min.

Protein was determined either by the method of Lowry *et al.* (17) with bovine serum albumin as a standard, or spectrophotometrically, using an $A^{1\%}_{1\,cm}$ at 280 nm of 10.87 (5).

Atomic Absorption Spectrophotometry. Metals were determined with a Perkin–Elmer model 460 atomic absorption spectrophotometer equipped with a HGA-2200 graphite furnace. Duplicate 20-μl samples of bovine hemoglobin, $Fe(NO_3)_3$, $CoCl_2$, and $MnCl_2$ in glass-distilled water in the concentration range 0.25–2 μM were used to construct standard curves. Thus, the actual amounts of metals determined were on the order of 10^{-11} mol. Under these conditions buffers containing phosphate or sulfate were unsuitable because of background interference. Consequently, all samples were dialyzed before analysis against 0.01 M 1,3-bis[tris(hydroxymethyl)methylamino]propane/acetate, pH 7.0, containing 2 mM *P-enol*pyruvate.

Absorption Spectrophotometry. Spectra were recorded with a GCA/McPherson EU-700 series double-beam spectrophotometer.

RESULTS

Atomic Absorption Spectrophotometry of DAHP Synthase. Various amounts of *E. coli* DAHP synthase (Phe) in the concentration range 0.2–2.0 μM were analyzed for iron, cobalt, and manganese by atomic absorption spectrophotometry as described in *Materials and Methods*. The results are given in Table 1. The enzyme contains approximately one mole of iron per 140,000 daltons—i.e., one mole of iron per native tetramer. Cobalt and manganese were detected in insignificant amounts. During the purification of DAHP synthase (Phe) to apparent homogeneity, the iron-to-enzyme-activity ratio becomes con-

Abbreviation: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate.

* To whom reprint requests should be addressed.

Table 1. Metal content of DAHP synthase (Phe)

Element	mol/140,000 daltons
Fe	1.21
Co	<0.01
Mn	<0.01
Fe*	0.95

* Iron determination of enzyme preparations inactivated by incubation with excess erythrose-4-*P* and subsequently dialyzed against 0.01 M 1,3-bis[tris(hydroxymethyl)methylamino]propane/acetate, pH 7.0, containing 0.5 mM *P-enol*pyruvate.

stant after the phenyl-Sepharose step (5), while earlier fractions have higher iron-to-enzyme-activity ratios, presumably due to contamination by other iron-containing proteins.

Inhibition of DAHP Synthase (Phe) by Chelating Agents. When DAHP synthase (Phe) is incubated with 10 mM ethylenediaminetetraacetic acid at 0°, the enzyme activity is stable for extended periods of time. Incubation at 37° under otherwise identical conditions inactivates the enzyme. (Fig. 1). Similar results are obtained with *o*-phenanthroline, except that this iron chelator is a more potent inhibitor of the enzyme (Fig. 1). *m*-Phenanthroline has also been tested for its ability to inhibit the enzyme. No significant inhibition is found at concentrations at which *o*-phenanthroline gives 100% inhibition (Fig. 1). Attempts to reactivate the enzyme by removal of the chelating agent or addition of excess iron have so far been unsuccessful.

Absorption Spectrum of DAHP Synthase. The absorption spectrum of pure DAHP synthase (Phe) from *E. coli* (Fig. 2*A*) shows the usual protein absorption maximum around 279 nm and an additional absorption maximum around 350 nm. The two absorption bands have $A^{1\%}_{1\,cm}$ values of 10.87 and 1.01, respectively.

The absorption band centered at 350 nm disappears upon denaturation of the enzyme with 6 M guanidine·hydrochloride (Fig. 2*B*). This result makes it unlikely that the 350-nm absorption band is caused by the presence of a cofactor such as pyridoxal phosphate or NADH.

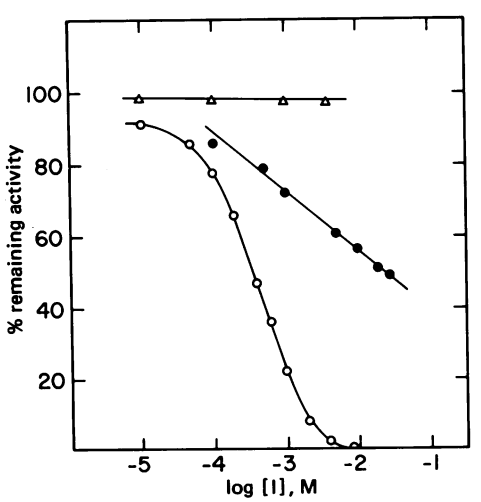

FIG. 1. Inhibition of DAHP synthase (Phe) by metal chelating agents. One unit of DAHP synthase (Phe) was incubated for 30 min at 37° in 0.05 M potassium phosphate, pH 6.5, containing 2 mM *P-enol*pyruvate, 0.5 mg of ovalbumin per ml, and the indicated amounts of chelator I. Enzyme activity was determined after the incubation. The percent of activity remaining, with enzyme treated as above but in the absence of chelator being 100%, is plotted versus the logarithm of chelator concentration. ○, *o*-Phenanthroline; ●, ethylenediaminetetraacetic acid; △, *m*-phenanthroline.

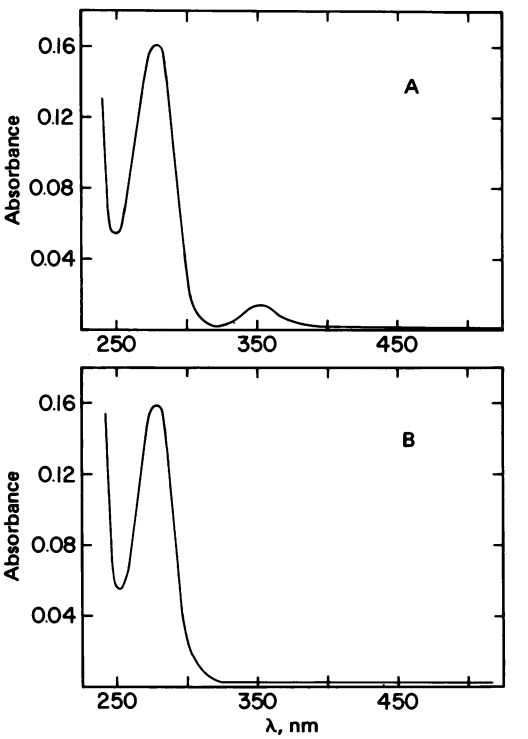

FIG. 2. Absorption spectrum of DAHP synthase (Phe). Enzyme solutions were 1.4 μM in 0.05 M potassium phosphate, pH 6.5, containing 2 mM *P-enol*pyruvate. (*A*) Native enzyme; (*B*) enzyme after denaturation with 6 M guanidine·hydrochloride, or after incubation with 4 mM erythrose-4-*P* at 37° for 30 min.

The absorption band centered at 350 nm also disappears when the enzyme is incubated with an excess of erythrose-4-*P* (Fig. 3). This disappearance of the 350-nm absorption band presumably results from a conformational change in the enzyme structure that follows removal of *P-enol*pyruvate by conversion to DAHP. The disappearance of the 350-nm band should not be taken as a measure of either erythrose-4-*P* binding or DAHP formation. The half-time of over 1 min (Fig. 3) is representative not of a catalytically meaningful reaction, but rather more likely of the conformational change that occurs in the absence of *P-enol*pyruvate. The 350-nm absorption band cannot be restored simply by addition of more *P-enol*pyruvate as shown in Fig. 3. The enzyme from which all *P-enol*pyruvate has been removed in this way is inactive and cannot be reactivated by addition of *P-enol*pyruvate or by dialysis against *P-enol*pyruvate-containing buffers. Elimination of the 350-nm

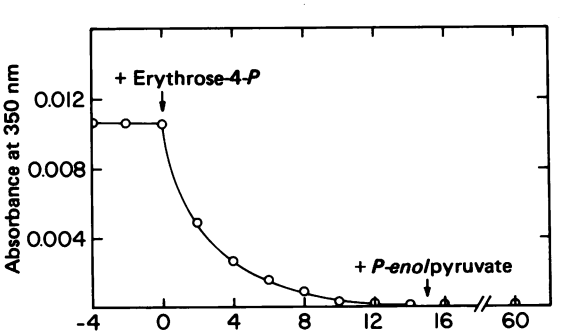

FIG. 3. Effect of erythrose-4-*P* on the 350-nm absorbance band of DAHP synthase (Phe). DAHP synthase (Phe) (0.4 nmol) in 0.5 ml of 0.05 M potassium phosphate, pH 6.5, containing 0.4 mM *P-enol*pyruvate was incubated at 37°. At 0 time, erythrose-4-*P* was added to give a final concentration of 0.8 mM. At 15 min, *P-enol*pyruvate was added to give a final concentration of 1 mM.

absorption band by removal of *P-enol*pyruvate, however, does not result in the loss of iron. Atomic absorption analysis (Table 1) showed that the iron content of the enzyme was virtually unchanged when *P-enol*pyruvate had been removed from the enzyme·*P-enol*pyruvate complex by incubation with an excess of erythrose-4-*P*.

If the 350-nm band indicates *P-enol*pyruvate binding to the active site of the enzyme, one could imagine this absorption to be a more common feature of enzymes with *P-enol*pyruvate as a substrate. *P-enol*pyruvate carboxylase catalyzes the formation of a single carbon–carbon bond between C3 of *P-enol*pyruvate and CO_2, which contains a highly oxidized carbon, with *P-enol*pyruvate phosphorolysis as the driving force for the reaction. These features are also found in the reaction catalyzed by DAHP synthase. Yet the spectrum of the native *P-enol*pyruvate carboxylase does not show a particular absorption band around 350 nm (results not shown), indicating that the 350-nm band is not characteristic for all enzymes that have *P-enol*pyruvate as a substrate.

DISCUSSION

E. coli DAHP synthase (Phe) purified to electrophoretic homogeneity contains approximately one mole of iron per mole of active enzyme. The tyrosine-sensitive isoenzyme from *E. coli* purified to apparent homogeneity as described previously (4) also contains iron (A. DeLucia and K. M. Herrmann, unpublished data). Therefore, one might expect that all DAHP synthases contain iron and not, as has been suggested by several earlier workers, cobalt. DAHP synthase (Phe) is strongly inhibited by *o*-phenanthroline. This inhibition appears to be due to the chelating ability rather than the aromatic or basic structure of *o*-phenanthroline, because *m*-phenanthroline, an analog that lacks the ability to chelate metal ions (18), is not an inhibitor of DAHP synthase (Phe).

The iron content of DAHP synthase deserves attention in one other context. DAHP synthase is the first enzyme in the common aromatic pathway that leads not only to the synthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan but also to substances necessary for iron transport—e.g., in *E. coli* the dihydroxybenzoylserine trimer enterochelin (19). We have shown that the biosynthesis of at least one DAHP synthase isoenzyme in *E. coli* is dependent upon the iron content of the growth medium, with the mechanism of the iron-mediated derepression still speculative (20). Results presented in this paper indicate that the biosynthesis of all aromatic compounds is directly dependent upon iron by virtue of the iron content of the first enzyme in the pathway. It is tempting to speculate that plants regulate their aromatic biosynthesis through the mineral content of the available nutrients, because all efforts to demonstrate feedback inhibition of plant DAHP synthases by aromatic amino acids have failed thus far (21).

The absorption spectrum of DAHP synthase (Phe) shows a maximum around 350 nm with an ϵ of about 3500 M^{-1} cm^{-1}. No other absorption bands with ϵ values exceeding 100 are detectable towards the red end of the spectrum. Thus, heme iron is excluded. Iron associated with acid-labile sulfur is also very unlikely, because all so-called non-heme-iron proteins contain more than one mole of iron per polypeptide chain. The iron environment within DAHP synthase (Phe) might be similar to hemerythrin, which also shows an absorption band around 350 nm (22). Hemerythrin contains two iron atoms per polypeptide chain, the ϵ value for the 350 nm absorption band is about 7000 M^{-1} cm^{-1}, and the 350 nm band does not appear in deoxyhemerythrin or iron-free hemerythrin (22).

For DAHP synthase, maintenance of the 350-nm absorption band and of enzyme activity requires the native conformation of the enzyme and the presence of *P-enol*pyruvate. That the native conformation of the enzyme is required is demonstrated by the loss of the 350-nm band after denaturation of the enzyme with guanidine·hydrochloride. The presence of small molecules such as NADH or pyridoxal phosphate, which themselves have absorption maxima around 350 nm, is ruled out because these molecules should exhibit the absorption even in 6 M guanidine·hydrochloride. The requirement of *P-enol*pyruvate is demonstrated by the fact that, upon removal of *P-enol*pyruvate from the enzyme preparation by incubation with an excess of erythrose-4-*P*, the absorbance at 350 nm disappears and enzyme activity is lost. It has been shown for both DAHP synthase (Phe) (5) and DAHP synthase (Tyr) (4) that *P-enol*pyruvate is required to maintain an active enzyme preparation. In addition, extensive kinetic analysis of DAHP synthase (Tyr) (4, 12) has shown that *P-enol*pyruvate is the first substrate to bind to the enzyme. For DAHP synthase (Phe) and DAHP synthase (Tyr) the Michaelis constants for *P-enol*pyruvate are about 5 μM. Because the intracellular concentration of *P-enol*pyruvate, which varies according to growth conditions (23), is never lower than 88 μM, or about 15 times K_m, it is likely that DAHP synthase is never in an environment containing subsaturating amounts of *P-enol*pyruvate.

As shown by the presence of iron in inactivated enzyme, iron alone in the enzyme does not give rise to the absorption band at 350 nm. Therefore, we conclude that the 350-nm band is indicative of a complex formed by *P-enol*pyruvate with the iron-containing polypeptide chain. Which amino acid residues contribute to the complex formation can only be guessed at this stage. Cysteine residues might be good candidates, because earlier workers showed that partially purified enzyme preparations were inhibited by *p*-hydroxymercuribenzoate (1) and 5,5′-dithio-bis(nitrobenzoic acid), with *P-enol*pyruvate protecting against inhibition by the latter (10).

The 350-nm absorption may be a unique feature resulting from *P-enol*pyruvate bound to the active site of DAHP synthase. It might be reasonable, therefore, to expect such an absorption band for other DAHP synthases. The homogeneous preparation of DAHP synthase (Tyr) from *E. coli* (4) purified from a different strain of this organism by a different procedure shows an absorption spectrum very similar to the one of DAHP synthase (Phe) shown in Fig. 2*A* (A. DeLucia and K. M. Herrmann, unpublished data). Again, upon removal of *P-enol*pyruvate by incubation with an excess of the second substrate, the 350-nm absorption band disappears. Virtually identical results are obtained with a highly purified, yet not homogeneous, preparation of DAHP synthase (Trp) from *E. coli* (data not shown). Thus, all three isoenzymes show a 350-nm absorption band.

We thank Dr. W. R. Featherston, Department of Animal Sciences, Purdue University, and Bill Harris and Toby Rodney, Eli Lilly & Co., Indianapolis, for the use of atomic absorption spectrophotometry equipment, and M. D. Poling and J. A. Suzich, for technical assistance. These studies were supported by U.S. Public Health Service Training Grant GM-1195 to R.J.M. and by Grant GM-17678 from the National Institute of General Medical Sciences to K.M.H. This is paper No. 7067 of the Purdue University Agricultural Experiment Station.

1. Srinivasan, P. R. & Sprinson, D. B. (1959) *J. Biol. Chem.* **234**, 716–722.
2. Hoffmann, P. J., Doy, C. H. & Catcheside, D. E. A. (1972) *Biochim. Biophys. Acta* **268**, 550–561.
3. Huang, L., Nakatsukasa, W. M. & Nester, E. (1974) *J. Biol. Chem.* **249**, 4467–4472.
4. Schoner, R. & Herrmann, K. M. (1976) *J. Biol. Chem.* **251**, 5440–5447.
5. McCandliss, R. J., Poling, M. D. & Herrmann, K. M. (1978) *J. Biol. Chem.* **253**, 4259–4265.

6. Hu, C.-Y. & Sprinson, D. B. (1977) *J. Bacteriol.* **129,** 177–183.
7. Bracher, M. & Schweingruber, M. E. (1977) *Biochim. Biophys. Acta* **485,** 446–451.
8. Doy, C. H. & Brown, K. D. (1965) *Biochim. Biophys. Acta* **104,** 377–389.
9. Pittard, J. & Gibson, F. (1970) in *Current Topics in Cellular Regulation*, eds. Horecker, B. L. & Stadtman, E. R. (Academic, New York), Vol. 2, pp. 29–63.
10. Staub, M. & Denes, G. (1969) *Biochim. Biophys. Acta* **178,** 599–608.
11. Dusha, I. & Denes, G. (1976) *Biochim. Biophys. Acta* **438,** 563–573.
12. DeLeo, A. B., Dayan, J. & Sprinson, D. B. (1973) *J. Biol. Chem.* **248,** 2344–2353.
13. Simpson, R. J. & Davidson, B. E. (1976) *Eur. J. Biochem.* **70,** 493–500.
14. Clark, V. M. & Kirby, A. J. (1963) *Biochim. Biophys. Acta* **78,** 732.
15. Sieben, A. S., Perlin, A. S. & Simpson, F. J. (1966) *Can. J. Biochem.* **44,** 663–669.
16. Herrmann, K. M. & Poling, M. D. (1975) *J. Biol. Chem.* **250,** 6817–6821.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
18. Slater, J. P., Mildvan, A. S. & Loeb, L. A. (1971) *Biochem. Biophys. Res. Commun.* **44,** 37–43.
19. O'Brien, I. G., Cox, G. B. & Gibson, F. (1970) *Biochim. Biophys. Acta* **215,** 393–402.
20. McCray, J. W., Jr. & Herrmann, K. M. (1976) *J. Bacteriol.* **125,** 608–615.
21. Huisman, O. C. & Kosuge, T. (1974) *J. Biol. Chem.* **249,** 6842–6848.
22. Klotz, I. M., Klippenstein, G. L. & Hendrickson, W. A. (1976) *Science* **192,** 335–344.
23. Lowry, O. H., Carter, J., Ward, J. B. & Glaser, L. (1971) *J. Biol. Chem.* **246,** 6511–6521.