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PURIFICATION AND METAL REQUIREMENTS OF 3-DEHYDROQUINATE SYNTHASE FROM *PHASEOLUS MUNGO* SEEDLINGS*

ETSUO YAMAMOTO†

Department of Biology, Tokyo Metropolitan University, Fukazawa, Setagaya-ku, Tokyo 158, Japan

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Key Word Index—*Phaseolus mungo*; Leguminosae; mung bean; dehydroquinate synthase.

Abstract—Dehydroquinate synthase of *Phaseolus mungo* seedlings was purified 4400-fold from the $(NH_4)_2SO_4$ fraction of a crude extract, the specific activity being 810 nkat per mg protein. When the purified enzyme was subjected to electrophoresis with or without sodium dodecyl sulfate, a single band was observed. The MW of the enzyme was estimated to be 67 000 by Sephadex G-100 gel chromatography and the minimum MW of the enzyme 43 000 by gel electrophoresis with sodium dodecyl sulfate. Atomic absorption analysis revealed that the purified enzyme contained small amounts of copper. Cobalt was not detected, although it has been implicated as a cofactor requirement.

INTRODUCTION

3-Dehydroquinate (DHQ) synthase catalyses the first cyclization step in the shikimate pathway for the biosynthesis of aromatic amino acids [1]. The enzyme was first found in extracts of *Escherichia coli* [2] and reported to require NAD and CO^{2+} as cofactors. The enzyme has since been found in various other microorganisms [3–5] and in higher plants [5–8]. Previously we reported that DHQ synthase from *Phaseolus mungo* required Co^{2+} and NAD but that some activity was observed even without these added cofactors as in the case of the *E. coli* enzyme [7]. However, cobalt has been thought to be inessential for the growth of higher plants [9]. It is interesting that the partially purified DHQ synthase from *P. mungo* requires Cu^{2+} in addition to Co^{2+} [8]. Some activity was observed in the partially purified enzyme without these metal ions but was lost on treatment with EDTA [8]. To determine whether activity without added Co^{2+} or Cu^{2+} is due to tightly bound metals or not, it is necessary to extensively purify the enzyme.

In the present work DHQ synthase from *P. mungo* has been highly purified by the use of phosphocellulose and Blue Sepharose, and analysed for its cobalt and copper contents.

RESULTS

Purification procedure

The DHQ synthase in the $(NH_4)_2SO_4$ fraction of etiolated *P. mungo* seedlings was purified *ca* 4400-fold by the procedure outlined in Table 1. Total activity of DHQ synthase was significantly increased with $(NH_4)_2SO_4$, presumably due to the removal of interfering substances [8]. The final colorless preparation retained some activity in the absence of exogenous cofactors, such as NAD and Co^{2+}. When the purified enzyme was subjected to electrophoresis on 7% gel, using a continuous buffering system of 5 mM Tris–glycine (pH 8.5), a single protein band, which coincided exactly with a single peak of the DHQ synthase activity on an identical gel obtained from the same run was observed: R_m value (mobility relative to the tracking dye) of this band was 0.46.

The apparent MW of native DHQ synthase, estimated by Sephadex G-100 chromatography, is 67 000. Using the SDS-gel electrophoresis system, the minimum MW of the enzyme was 43 000.

Metal requirement of DHQ synthase

Previous experiments with the partially purified enzyme indicated that the enzyme requires both Co^{2+} and Cu^{2+} as cofactors, although some activity was observed even in the absence of these metals [8]. After incubation of the highly purified enzyme with EDTA followed by dialysis against metal-free buffer, the activity was lost. On addition of 0.1 mM $CoCl_2$ or 10 μM $CuCl_2$, 35% of the enzyme activity was recovered, and activity was not increased by further preincubation of the enzyme with $CoCl_2$.

Metal content

The cobalt and copper contents of the DHQ synthase preparations at several steps in the purification process were measured using an atomic absorption spectrophotometer (Table 1). Prior to analysis, the

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† Present address: Botany Department, University of British Columbia, Vancouver, B.C., Canada V6T 1W5.

Table 1. Purification of DHQ synthase from *Phaseolus mungo* seedlings

Step	Total protein (mg)	Total activity (μ kat)	Specific activity (nkat/mg protein)	Cu* content (ng/mg protein)	Yield (%)
Crude extract	122 000	3.75	0.0307	—	100
Ammonium sulfate	28 900	5.32	0.184	62*	142
DE-23	2920	3.31	1.13	63	88
Sephadex G-100	772	1.96	2.54	42*	52
P-11	9.10	0.742	76.5	316	20
Sephacryl S-200	3.59	0.676	188	381	18
Blue Sepharose	0.448	0.364	813	310	10

*No cobalt was detected in these fractions.

enzyme preparations were subjected to exhaustive dialysis against 5 mM Hepes–KOH, pH 7.4. Cobalt was not detected in the enzyme from $(NH_4)_2SO_4$ or Sephadex, but the copper content of the enzyme preparations increased to *ca* 0.31 μg/mg protein in the final preparation.

DISCUSSION

Highly purified DHQ synthase from *P. mungo* seedlings had a maximum specific activity of 810 nkat per mg protein, much greater than that of other preparations reported so far [6, 8, 10, 11]. Electrophoresis of the purified enzyme on polyacrylamide gel showed a single peak of DHQ synthase activity, although the final preparation retained the activity without added cofactors. This result showed that enzyme activity without metal is not derived from the presence of any isomers of the enzyme. The results from SDS-gel electrophoresis of the purified enzyme also suggest that the enzyme preparation is homogenous or nearly homogenous.

Cobalt is not considered to be necessary for the growth of higher plants [9] and there are few reports on the participation of Co^{2+} in the biochemical reactions of higher plants. As far as I can ascertain, aminoacylase (EC 3.5.1.14) is the only plant enzyme which is exclusively activated by Co^{2+} [12]. In earlier experiments crude preparations of DHQ synthase from *P. mungo*, Co^{2+} was found to activate the enzyme similarly to the bacterial enzyme. This finding was confirmed in the present experiments using purified enzyme. In this connection it is of interest that Cu^{2+}, at the very limited concentration ranges, was equivalent to Co^{2+} as a cofactor of DHQ synthase from *P. mungo* and that the enzyme retains *ca* 30% of the activity without the addition of these metal ions [8]. These facts suggest that a bound metal is present in DHQ synthase since the activity without added Co^{2+} is completely inhibited by EDTA [8]. This assumption is further supported by the results from EDTA treatment of the enzyme, followed by exhaustive dialysis against metal-free buffer. Atomic absorption analysis clearly showed the absence of any significant quantity of the cobalt and the presence of a small, but significant, amount of copper (Table 1). Calculation based on the gel electrophoresis data indicated that the copper content of the final preparation is 0.20 atom per mol of enzyme. Since this value is unreasonable stoichiometrically, the value obtained may be attributable to nonspecific binding of copper to the enzyme. Alternatively, the figure may represent copper bound to the active site of the enzyme throughout the purification process.

EXPERIMENTAL

Materials. Ca^{2+} salts of DAHP and DAHP-[1-^{14}C] were prepared by the method of ref. [13], and converted to the K^+ salts before use. Uniselex-30 resin was a kind gift from Unitika Co. (Tokyo, Japan).

Enzyme purification. All operations described were carried out at 2–5°. The initial $(NH_4)_2SO_4$ fraction obtained from 10 kg of frozen 2-day-old *P. mungo* seedlings [8], was resuspended in a small vol. of 10 mM K-Pi buffer, pH 7.4, containing 10 mM 2-mercaptoethanol and dialysed against the same soln. The dialysed soln (60 g protein) was applied to a Whatman DE-23 column (4.7×49 cm) equilibrated with 10 mM K-Pi buffer, pH 7.4. After washing the column with 2.55 l. of 50 mM K-Pi buffer, pH 7.4, the enzyme was eluted with a linear gradient of 50–300 mM K-Pi buffer, pH 7.4 (4.4 l.). Active fractions (*ca* 1 l.) were combined and the enzyme was recovered by precipitation with $(NH_4)_2SO_4$ at 70% satn. The ppt. (*ca* 3 g protein) was dissolved in 12 ml 50 mM K-Pi buffer, pH 7.4, and applied to a Sephadex G-100 column (5×100 cm) equilibrated with the same buffer. The enzyme was eluted with the same buffer at a flow rate of 200 ml/hr. The most active fraction was pooled (175 ml) and protein was precipitated with 70% $(NH_4)_2SO_4$. The ppt. (*ca* 0.77 g) was dialysed against 10 mM K-Pi buffer, pH 6.2, and then applied to a Whatman P-11 column (2.5×45 cm) equilibrated with the same buffer. After washing the column with 550 ml of the above buffer, the DHQ synthase was eluted by linear gradient of 0–0.5 M KCl (1.1 l.) at a flow rate of *ca* 50 ml/hr.

The active fractions (*ca* 170 ml) were immediately concentrated to *ca* 20 ml by ultrafiltration with a Diafilter G-10 membrane (Bioengineering Co., Japan), because the enzyme is very unstable at low protein concn. After further concn to *ca* 1.1 ml in a collodion bag *in vacuo*, the enzyme soln was dialysed against 10 mM K-Pi buffer, pH 7.4. The supernatant after centrifugation (18 000 g for 30 min) was applied to a Sephacryl S-200 column (1.25×41 cm) equilibrated with 50 mM K-Pi buffer, pH 7.4. Elution was accomplished with the same buffer at a flow rate of *ca* 6.5 ml/hr. The combined active fractions were concd to 10% and dialysed against 10 mM K-Pi buffer, pH 7.4. The dialysed soln (*ca* 3 mg protein) was applied to Blue Sepharose CL-6B column (1.9×16 cm) equilibrated with 10 mM K-Pi buffer. After standing for 30 min, the column was washed with 25 ml of the same buffer. DHQ synthase was eluted by a linear gradient of 10–300 mM K-Pi buffer, pH 7.4 (50 ml) at a flow rate of 10 ml/hr. The combined active fractions were immediately concd to *ca* 0.2 ml, and dialysed against 5 mM K-Pi buffer, pH 7.4. The enzyme soln was centrifuged and the resulting supernatant was stored at −20°. The conc Blue Sepharose-purified enzyme was fairly stable at −20° for 1 month, but lost activity after repeated freezing and thawing.

Enzyme assay. The activity of DHQ synthase was determined by measuring the disappearance of DAHP according to ref. [2]. The incubation mixture contained (in μmol) K-Pi buffer, pH 7.4, 10; NAD, 0.002; $CoCl_2$, 0.02; DAHP, 0.06; and 1.6 mU of the enzyme soln in a total vol. of 0.2 ml. The mixture was preincubated at 30° for 5 min and the reaction initiated by adding DAHP. After 20 min it was stopped by adding 0.1 ml of 10% TCA.

Removal of activity by EDTA. Native enzyme was treated with EDTA in a similar way to the prepn of apo-enzyme from aldolase [14]. Enzyme preparation (6 mg) after Sephadex treatment, was dissolved in 0.1 M EDTA, pH 7.4 and incubated at 25° for 1 hr. The enzyme was exhaustively dialysed against metal-free (Uniselex UR-30 resin) 50 mM Hepes buffer, pH 7.7.

Analytical. Disc polyacrylamide gel electrophoresis with or without SDS was carried out as described earlier [15, 16]. Enzyme activity was detected by cutting developed gels into 1mm sections and using the assay mixture described above. Estimation of MW was performed by gel chromatography on Sephadex G-100 superfine [17]. Protein was estimated according to ref. [18].

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