Dehydroquinate Synthase from *Escherichia coli*: Purification, Cloning, and Construction of Overproducers of the Enzyme†

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ABSTRACT: Dehydroquinate synthase has been purified 9000-fold from *Escherichia coli* K-12 (strain MM294). The synthase is encoded by the *aroB* gene, which is carried by plasmid pLC29-47 from the Carbon–Clarke library. Construction of an appropriate host bearing pLC29-47 results in a strain that produces 20 times more enzyme than strain MM294. Subcloning of the *aroB* gene behind a *tac* promoter results in *E. coli* transformants that produce 1000 times more enzyme than MM294: the synthase constitutes 5% of the soluble protein of the cell. A laborious isolation from 50 g of wild-type *E. coli* cells yields 80 μg of impure enzyme, whereas 50 g of cells containing the subcloned gene yields 150 mg of homogeneous enzyme in a two-column purification. Dehydroquinate synthase is a monomeric protein of M_r 40 000–44 000. The chromosomal enzyme from *E. coli* K-12, the cloned enzyme encoded by the plasmid pLC29-47, and the subcloned inducible enzyme encoded by pJB14 all comigrate on polyacrylamide gel electrophoresis under denaturing conditions.

The biosynthesis of aromatic rings from carbohydrate precursors in plants and microorganisms involves a range of extraordinary chemical transformations that together constitute the shikimate pathway (Haslam, 1974). The second enzyme in this sequence, dehydroquinate synthase, catalyzes the cyclization of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP)[1] (**1**) to dehydroquinate (DHQ) (**2**). In the course of this conversion (Figure 1), the synthase apparently catalyzes an oxidation, a β-elimination, an intramolecular aldol condensation, and a reduction. DHQ synthase is thus an ideal candidate for investigation of multistep sequences mediated by single-enzyme systems, yet the unavailability of the protein in reasonable quantity has hindered mechanistic work on this enigmatic enzyme. Although isolation of the synthetase has been reported (Maitra & Sprinson, 1978), the enzyme occurs in nature at agonizingly low concentrations.

Subsequent to the initial report of its existence (Srinivasan et al., 1963), studies of DHQ synthase have centered on the stereochemistry of the cyclization reaction (Rotenberg & Sprinson, 1970), kinetic isotope effects (Rotenberg & Sprinson, 1978), steady-state quench experiments (Maitra & Sprinson, 1978), and substrate analogue studies (Le Marechal & Azerad, 1976a,b; Le Marechal et al., 1980). All but one of these studies were carried out with crude extracts or with partially purified enzyme. In our hands, the existing purification protocol (Maitra & Sprinson, 1978) did not yield the desired amounts of homogeneous protein. We report here the purification of dehydroquinate synthase from *Escherichia coli* K-12 and the construction of two plasmid-bearing strains that overproduce the enzyme by factors of 20 and 1000, respectively. The synthesis and the microbiological isolation of the enzyme substrate (3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate) are reported in the accompanying paper (Frost & Knowles, 1984).

Experimental Procedures

Assays. Assay solutions (2 mL) consisted of deionized, glass-distilled water containing 100 mM potassium 3-(*N*-morpholino)propanesulfonate buffer, pH 7.4, cobalt(II) chloride (1.2 mM), NAD^+ (0.12 mM), and DAHP (0.65 mM). After an incubation at 15 °C for 10 min, enzyme was added, and portions (0.2 mL) were withdrawn at intervals and quenched by the addition of 10% (w/v) trichloroacetic acid (0.1 mL).

Remaining substrate was determined by thiobarbituric acid (Sigma) visualization of the periodate cleavage products of DAHP (Gollub et al., 1971). Product inorganic phosphate was visualized by the method of Ames (1966). One unit of enzyme activity catalyzes the consumption of 1 μmol of DAHP or the liberation of 1 μmol of inorganic phosphate per min. Extracts of the *E. coli* K-12 strain MM294 and of strain JB12 (pLC29-47) required assay of 50–100 milliunits of enzyme at 1-min time points. Extracts of the subcloned transformant RB791(pJB14) were assayed with 15-s time points and 200–500 milliunits of enzyme activity. Protein concentrations were determined by the method of Lowry et al. (1951).

Culture Media. All media were prepared in distilled, deionized water. Medium Z is minimal A (Miller, 1972) and contains (in 1 L) K_2HPO_4 (10.5 g), KH_2PO_4 (4.59), $(NH_4)_2SO_4$ (1 g), and sodium citrate dihydrate (0.5 g). Medium Y was identical with medium Z, except that it additionally contained L-tyrosine (8 mg), L-phenylalanine (8 mg), L-tryptophan (4 mg), *p*-aminobenzoic acid (4 μg), *p*-hydroxybenzoic acid (4 μg), L-histidine (40 mg), L-isoleucine (40 mg), L-valine (40 mg), and kanamycin sulfate (75 mg). Medium X was identical with medium Y, except that less $MgSO_4 \cdot 7H_2O$ (0.12 g) was used and tetracycline (25 mg) was substituted for the kanamycin. Medium W contained, in 1 L, Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NH_4Cl (1 g), NaCl (0.5 g), glucose (0.5% w/v), $MgSO_4$ (0.12 g), thiamin (5 mg), L-histidine (8 mg), L-isoleucine (8 mg), L-valine (8 mg), L-tryptophan (0.5 mg), and kanamycin (75 mg). Medium V is LB (Miller, 1972) and contains Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g) in 1 L. Medium U was identical with medium V except that it additionally contained ampicillin (100 μg/mL). Medium T is YT (Miller, 1972) and

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[1] Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate; DHQ, dehydroquinate; NAD^+, oxidized nicotinamide adenine dinucleotide; IPTG, isopropyl 1-thio-β-D-galactopyranoside; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Ap^R, resistance to ampicillin; Kan^R, resistance to kanamycin; Tet^R, resistance to tetracycline; Tris, tris(hydroxymethyl)aminomethane.

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Table I: Bacterial Strains

strain	genotype	reference/source
MM294	F⁻ *endA1 hsdR17* (r_{K^-}, m_{K^+}) *supE44 thi-1*	Meselson & Yuan, 1968
JP2312	F⁻ *tyrR trpS* His⁻,Ile⁻,Val⁻	J. F. Morrison
NK6626	F⁻ *aroB351 tsx-354 mal-354*	N. Kleckner
NK5304	Hfr P045 *srl-300*::Tn10 *recA56 ilv-318 thr-300 thi-1 rel-1 spc-300*	N. Kleckner
NK7293	Δ(*lac-pro*)XIII *str mal recA56* λ^R(thi-1) F′ *lacL8I^{q}Z*::Tn10 (*tps*⁻) *proAB*	N. Kleckner
JA200(pLC29-47)	Δ*trpE5 recA1 thr leuB6 lacY*/F⁺/pLC29-47	B. Bachmann
JB5	JP2312 F⁻ *aroB351 malT*::Tn5	this study
JB11	JB5 F⁻ *srl-300*::Tn10 (*tps*⁻) *recA56*	this study
JB12(pLC29-47)	JB11/F⁺/pLC29-47	this study
JB14	NK6626/F′ *lacL8I^{q}Z*::Tn10(*tps*⁻)*proAB*	this study
RB791	W3110 *lacL8I^q*	R. Brent

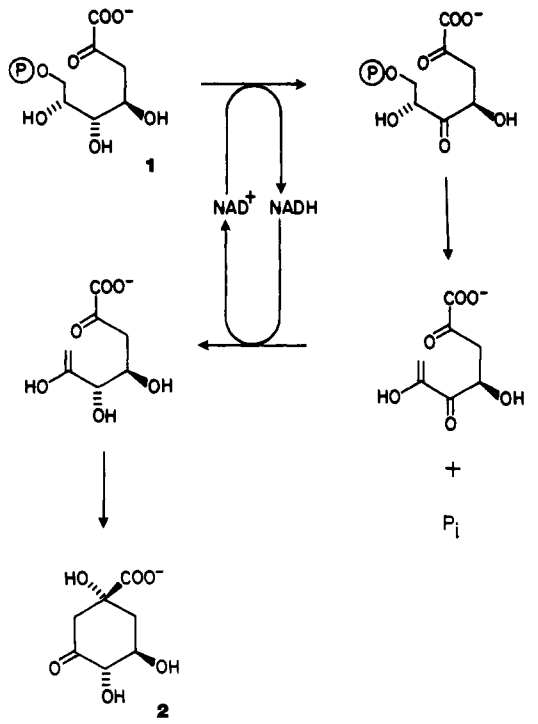

FIGURE 1: Possible mechanistic pathway for DHQ synthase (following Sprinson).

contains Bacto tryptone (8 g), Bacto yeast extract (5 g), and NaCl (5 g) in 1 L. Medium S was identical with medium T except that the concentration of all solutes was doubled.

Solutions of inorganic salts, of magnesium salt, and of glucose were autoclaved separately and then mixed. Antibiotics, thiamin, amino acids, and aromatic compounds were introduced by syringe, through sterilizing Millex-GS 0.22-μm (Millipore) membranes. Agar plates were made from Difco agar (1.5% w/v).

Host Manipulations. The genotypes of the *E. coli* strains used in this study are listed in Table I. The techniques of P1 transduction, Hfr × F⁻ mating, and bacterial episome transfer were performed according to Miller (1972).

E. coli strain JB12(pLC29-47) was constructed as follows. First, a *tyrR* mutant, JP2312, was made AroB⁻ by P1 transduction from NK6626 (*aroB malT*::Tn5) in which the *aroB* mutation is tightly linked to Tn5 (which contains a kanamycin-resistance gene) inserted in the *malT* locus. Selection of transductants for Kan^R and screening for AroB⁻ yielded strain JB5 (*tyrR aroB malT*::Tn5). Mating of NK5304 (Hfr *recA srl*::Tn10), in which the *recA* mutation is tightly linked to Tn10 (which contains a tetracycline-resistance gene) inserted into the *srl* locus, with JB5 then gave strain JB11 (*tyrR aroB malT*::Tn5 *recA srl*::Tn10) by selection for Kan^R and Tet^R and screening for RecA⁻ by nitrofurantoin sensitivity (McEntee, 1977). Finally, transfer of the F episome and pLC29-47 [pLC29-47 carries the *aroB* gene (Takeda et al., 1981)] from JA200(pLC29-47) into JB11 yielded JB12-(pLC29-47) by selection for Kan^R and AroB⁺.

E. coli strain JB14 (*aroB malT*::Tn5/F′*lacI^{q}Z*::Tn10 *tps*⁻) was constructed by episome transfer from NK7293 (F′*lacI^{q}Z*::Tn10 *tps*⁻) into NK6626 (*aroB malT*::Tn5), selecting for Kan^R (from Tn5) and Tet^R (from Tn10).

Construction of pJB14. All restriction enzymes, T4 DNA ligase, *E. coli* DNA polymerase I (large fragment), and *Cla*I linker oligonucleotide (5′-CATCGATG) were obtained from New England Biolabs. T4 polynucleotide kinase was from New England Nuclear. The *tac* promoter vector pKK223-3 was the generous gift of Dr. Jürgen Brosius. All recombinant DNA techniques were performed essentially as described by Maniatis et al. (1982).

The plasmid pJB14 was constructed as follows. First, a derivative of pKK223-3, pJB1, was made by insertion of *Cla*I linkers into the unique *Eco*RI site downstream from the *tac* promoter. Plasmid pKK223-3 was linearized with *Eco*RI, the 5′-protruding ends were filled in with DNA polymerase, and 5′-phosphorylated *Cla*I linkers were ligated to the resulting blunt ends. After digestion with *Cla*I, the plasmid was separated from small fragments by gel filtration over Sepharose CL-4B (Sigma) and circularized. The new plasmid, pJB1, which confers ampicillin resistance (Ap^R) and contains the strongly inducible *tac* promoter, was purified from JB14 cells and tested for a unique *Cla*I site before serving as the cloning vector in subsequent experiments.

The *aroB* gene of pLC29-47 was cloned into the *Cla*I site of pJB1 as follows. The *Cla*I cohesive ends are complementary to those generated by two four-base recognizing enzymes, *Taq*I and *Msp*I, and partial digests of purified pLC29-47 (10 μg) were made with each of these two enzymes. Restriction fragments of 2–4 kilobases were extracted from a gel of low-melting agarose (Sigma). These fragments were ligated into pJB1 at the *Cla*I site, and the resulting DNA was used to transform competent JB14 cells. Nineteen transformants having Ap^R AroB⁺ were selected from the *Msp*I-derived recombinants. No colonies with the Ap^R AroB⁺ phenotype were obtained from insertion of the *Taq*I fragments. Assays of French-press lysates of the 19 Ap^R AroB⁺ transformants revealed that production of DHQ synthase was strongly induced by IPTG in three of the subclones. One candidate [JB14-(pJB14)] had approximately 1000 times more DHQ synthetase activity when fully induced, compared with the *E. coli* K-12 strain. Finally, the plasmid pJB14 was purified from this candidate and used to transform RB791 cells, to avoid any problems with host genotype stability arising from transposition of Tn5 in JB14.

Cell Growth. All cells were harvested by centrifugation at 8000*g* for 10 min. *E. coli* K-12 strain MM294 was grown up

from a single colony in medium Z (200 mL). This was used to inoculate medium S (6 L) in a rotary fementor. Gentle oxygen aeration and rotation at 200 rpm for 8 h afforded 70 g of wet cell paste.

E. coli JB12(pLC29-47) was grown up from a single colony in medium W (600 mL). This was used to inoculate six 4-L shaker flasks, each containing medium V (1 L). Growth for 11 h yielded 19 g of wet cell paste.

E. coli RB791(pJB14) was grown up from a single colony in medium U (180 mL). This was used to inoculate six 4-L shaker flasks, each containing medium U (1 L). After growth for 1 h, IPTG (0.238 g) was added to each flask, and growth was continued for 8 h. The yield of wet cell paste was 39 g.

Enzyme Isolation. (*A*) *Buffers.* All buffers were made up in distilled deionized water. Buffer components were of the highest grade commercially available. NAD^+ (grade AA1) was from Sigma. The buffers used were as follows: (A) 10 mM β-glycerophosphate, pH 6.6, containing $CoCl_2$ (0.25 mM) and NAD^+ (0.5 mM); (B) 75 mM potassium phosphate, pH 6.6, containing β-glycerophosphate (10 mM), $CoCl_2$ (0.25 mM), and NAD^+ (0.5 mM); (C) 10 mM potassium phosphate, pH 6.6, containing $CoCl_2$ (0.25 mM); (D) 25 mM potassium phosphate, pH 6.6, containing $CoCl_2$ (0.25 mM) and NAD^+ (0.5 mM); (E) 150 mM potassium phosphate, pH 6.6, containing $CoCl_2$ (0.25 mM) and NAD^+ (0.5 mM); (F) same as buffer D but at pH 6.1; (G) same as buffer D but at pH 7.1; (H) same as buffer D but at pH 7.6; (J) 10 mM β-glycerophosphate, pH 6.6.

(*B*) *Chromatography Columns.* Dyematrex Red A (Amicon)– and Reactive Blue 2 (Sigma)–agarose gels were each eluted with 10 column volumes of 8 M urea at room temperature and then left overnight. Dyematrex Red A was then eluted with 10 column volumes of buffer C. The gel was removed from the column, cooled to 4 °C, and briefly degassed under high vacuum. The material was then repacked in a column. Elution with an additional 10 column volumes of buffer C at 4 °C preceded protein application. After the urea treatment, columns of Reactive Blue 2–agarose were eluted with 10 column volumes of buffer F, cooled to 4 °C, and eluted with an additional 10 column volumes of buffer F at 4 °C.

Hydroxylapatite (Bio-Rad) was slurried in degassed buffer J before column packing. After elution with 10 column volumes of degassed buffer J, the column was equilibrated with 2 column volumes of degassed buffer A.

(*C*) *General.* Crude cell lysates were prepared by passing cell suspensions through a French press at 13 000 psi. Cell debris was then removed by centrifugation at 27000*g* for 30 min. Concentration of all protein solutions utilized PM-10 Diaflo ultrafiltration membranes (Amicon). Dialysis tubing (12 000–14 000 M_r cutoff from Fisher) was soaked in buffer C for at least 24 h and thoroughly washed before use. All manipulations were done at 4 °C.

(*D*) *Isolation of DHQ Synthase from E. coli K-12 Strain MM294.* Cells (140 g) were suspended in buffer A (100 mL), and a crude lysate was prepared. After dialysis overnight against multiple changes of buffer A, the extract was loaded on to a column (800 mL) of hydroxylapatite. The column was washed with buffer A (800 mL), followed by elution with a linear gradient of buffer A (4 L) plus buffer B (4 L). The fractions containing DHQ synthase were concentrated and then dialyzed overnight against multiple changes of buffer A. This material was applied to a column (120 mL) of hydroxylapatite, which was then washed with buffer A (100 mL). Enzyme was eluted with a linear gradient of buffer A (500 mL) plus buffer B (500 mL). The fractions containing synthase were concentrated and then dialyzed overnight against multiple changes of buffer D. This material was loaded on a column (120 mL) of Dyematrex Red A, and the column was washed with buffer D (100 mL). Enzyme was eluted with a linear gradient of buffer D (500 mL) plus buffer E (500 mL). The active fractions were concentrated and then dialyzed against buffer F overnight. The solution was finally loaded on a column (3 mL) of Reactive Blue 2–agarose, and the column was eluted successively with buffer F (9 mL), buffer D (9 mL), buffer G (9 mL), and buffer I (9 mL). The active fractions were pooled and concentrated.

(*E*) *Isolation of Cloned DHQ Synthase from E. coli JB12(pLC29-47).* Cells (19 g) were suspended in buffer A (25 mL), and a crude lysate was prepared. After dialysis against buffer A, the material was applied to a column (400 mL) of hydroxylapatite. The column was washed with buffer A (400 mL), and the protein was eluted with a linear gradient of buffer A (2 L) plus buffer B (2 L). The active fractions were concentrated and then dialyzed overnight against multiple changes of buffer D. The solution was next loaded onto a column (100 mL) of Dyematrex Red A. The column was washed with buffer D (100 mL), and the protein was eluted with a linear gradient of buffer D (500 mL) plus buffer E (500 mL). The active fractions were concentrated and then dialyzed overnight against multiple changes of buffer F. This material was loaded on a column (20 mL) of Reactive Blue 2 and eluted successively with buffer F (60 mL), buffer D (60 mL), buffer G (60 mL), and buffer H (60 mL). The active fractions were pooled and concentrated.

(*F*) *Isolation of Subcloned DHQ Synthase from E. coli RB791(pJB14).* Cells (39 g) were suspended in buffer A (50 mL), and the crude lysate was prepared. After dialysis against multiple changes of buffer A, the solution was loaded on to a column (400 mL) of hydroxylapatite. The column was washed with buffer A (400 mL), and the protein was eluted with a linear gradient of buffer A (2 L) plus buffer B (2 L). The active fractions were pooled and concentrated, and the solution was then dialyzed overnight against buffer D. This material was applied to a column (500 mL) of Dyematrex Red A, and the column was washed with buffer D (500 mL). The protein was then eluted with a linear gradient of buffer D (2 L) plus buffer E (2 L). The active fractions were then pooled and concentrated. Concentrated solutions of purified DHQ synthase were quick-frozen in liquid nitrogen and stored at −70 °C. When required, this material was thawed at 15 °C with gentle agitation.

(*G*) *Molecular Weight Determination.* Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Laemmli (1970). The stacking gel was buffered with Tris-HCl at pH 6.8, and the separating gel (12.5% acrylamide) was buffered with Tris-HCl at pH 8.8. Typically, 20–50 μg of protein was loaded per channel. Gels were fixed with trichloroacetic acid solution and visualized with Coomassie Brilliant Blue. The standards used (from Sigma) were α-lactalbumin (14 200), trypsin inhibitor (20 100), trypsinogen (24 000), carbonic anhydrase (29 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), egg albumin (45 000), and bovine serum albumin (66 000). Log M_r was plotted against R_f, and gave an M_r for DHQ synthase of 40 000.

Molecular weight determination under nondenaturing conditions was carried out by high-pressure gel permeation chromatography with a Protein Pak 300 column (Waters). A high ionic strength eluant was used [Na_2SO_4 (0.1 M), NaH_2PO_4 (0.02 M), pH 6.8]. The column was calibrated with

Table II: Purification of DHQ Synthase from Wild-Type *E. coli* K-12 Strain MM294

step	total units	sp act. (units/mg)	x-fold purification	yield (%)
crude lysate (from 70 g of cells)	16	0.0018	1	(100)
hydroxylapatite 1	16	0.30	170	100
hydroxylapatite 2	13	0.35	190	81
Dyematrex Red A	4	17	9400	25
Reactive Blue 2	2	16	8900	13

standards (from Bio-Rad) including bovine thyroglobulin (670 000), bovine γ-globulin (158 000), chicken ovalbumin (44 000), horse myoglobin (17 000), and vitamin B-12 (1350). DHQ synthase coeluted with chicken ovalbumin.

Results and Discussion

Enzyme Assay. The most important prerequisite of any protein purification is a kinetic assay suitable for both crude cell extracts and purified enzyme. The literature assay method for crude DHQ synthase involves measurement of the disappearance of DAHP after incubation with enzyme for 30 min, using the color produced by the reaction of thiobarbiturate with periodate-oxidized DAHP (Gollub et al., 1971). When the time course of the enzymic reaction was followed more carefully, we observed substantial curvature in the rate of loss of substrate DAHP. As the enzyme purification progressed, this curvature in the assay became even more pronounced, and it was found that at 37 °C the homogeneous enzyme lost all activity rather rapidly. All assays were therefore performed at 15 °C, at which temperature the rate assays are linear. The enzyme may also be assayed (after some purification) by monitoring P_i release as a function of time. When this assay was used, the normal assay buffer (which contains P_i and β-glycerophosphate to stabilize the enzyme) was replaced by MOPS. When used with partially purified enzyme (free from nonspecific phosphatases), the two assays (thiobarbiturate to follow the loss of substrate and P_i release to follow appearance of product) agreed to within 10%. The thiobarbiturate assay for substrate loss was normally used during the course of the enzyme purification. The stability of DHQ synthase is markedly improved by the presence of cobalt(II) chloride and of NAD^+, as earlier found by Maitra & Sprinson (1978).

Enzyme Purification. As a first column in all the purifications, commercial hydroxylapatite was extremely useful. A substantial purification with high unit yield is achieved. The presence of stabilizing cobalt(II) in all buffers presents a complication, since hydroxylapatite removes most of the cobalt from the eluting buffers. The purification factor can drop off dramatically as the leading portion of the column becomes increasingly loaded with cobalt(II). The hydroxylapatite column should therefore be equilibrated with the prescribed buffer volumes, and the packing material discarded after use. Subsequent to the hydroxylapatite column, Procion Red HE-3B (Dyematrex Red A) and Cibacron Blue F3GA (Reactive Blue 2) immobilized on cross-linked agarose provided purification sufficient to obtain near or complete protein homogeneity.

The *E. coli* K-12 strain used for the purification of the chromosomal enzyme was selected for its good growth characteristics. Despite the 9000-fold purification obtained from the cell lysate (Table II), it was clear from both the specific catalytic activity and analysis by polyacrylamide gel electrophoresis that the enzyme was still not homogeneous (Figure 2). The small amounts of DHQ synthase in wild-type *E. coli* K-12 cells and the low yield of the purification procedure

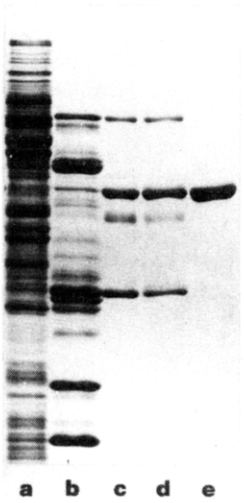

FIGURE 2: Isolation of DHQ synthase from wild-type *E. coli* K-12 strain MM294: (lane a) crude extract; (lane b) after hydroxylapatite; (lane c) after red dye; (lane d) after blue dye; (lane e) subcloned homogeneous enzyme.

Table III: Purification of DHQ Synthase from the Clone *E. coli* JB12(pLC29-47)

step	total units	sp act. (units/mg)	x-fold purification	yield (%)
crude lysate (from 19 g of cells)	62	0.040	1	100
hydroxylapatite	98	2.0	49	158
Dyematrex Red A	59	16	390	95
Reactive Blue 2	39	22	550	64

prompted us to explore ways of increasing the amount of DHQ synthetase in cultured cells.

We expected that a bacterial strain with multiple copies of a plasmid carrying the *aroB* (dehydroquinate synthase) gene would overproduce dehydroquinate synthase. Fortunately, it was known (Takeda et al., 1981) which plasmid from the Carbon-Clarke genomic library (Clarke & Carbon, 1976) carries the *aroB* gene, and an appropriate host (JB12) was constructed for this plasmid (pLC29-47). This host strain has two important phenotypes: $AroB^-$ (to allow maintenance of the *aroB* gene when the cell is grown on minimal medium) and $RecA^-$ (to prevent reversion of the *aroB* allele to wild type by a recombination event). P1 transduction of *aroB* (which is tightly linked to a Tn5 kanamycin-resistance insert) from NK6626 into JP2312 gave a host that was then made $RecA^-$ by mating with a donor in which *recA* is tightly linked to a Tn10 tetracycline-resistance insert. This host was then mated with the appropriate donor from the Carbon-Clarke bank [JA200(pLC29-47)] to give *E. coli* JB12(pLC-47).

E. coli JB12(pLC29-47) afforded a 20-fold increase in the activity of DHQ synthase in the crude lysate, compared with wild-type cells. Isolation of the enzyme (Table III) was achieved with a 550-fold purification and yielded nearly 2 mg of almost homogeneous enzyme (Figure 3). The increased levels of enzyme greatly reduced the amount of cells required for purification and allowed a 5-fold improvement in the overall unit yield compared to wild-type *E. coli*. However, strain JB12(pLC29-47) grows poorly on minimal medium (which is required to maintain the plasmid), and when grown on rich medium on the large scale, the amount of DHQ synthase is only 10 times higher than in wild-type cells. So although some improvement in DHQ synthase yield could be realized by using JB12(pLC29-47), we decided to subclone the *aroB* gene behind a strong promoter in a more readily maintained vector carrying

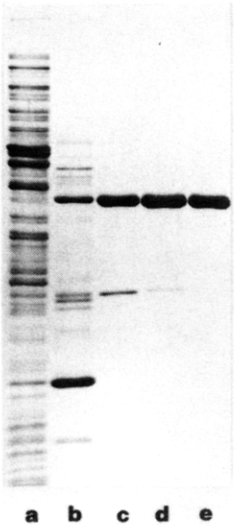

FIGURE 3: Purification of DHQ synthase from the clone *E. coli* JB12(pLC29-47): (lane a) crude extract; (lane b) after hydroxylapatite; (lane c) after red dye; (lane d) after blue dye; (lane e) subcloned homogeneous enzyme.

Table IV: Purification of DHQ Synthase from the Subclone *E. coli* RB791(pJB14)

step	total units	sp act. (units/mg)	*x*-fold purification	yield (%)
crude lysate (from 39 g of cells)	6400	1.6	1	100
hydroxylapatite	8300	29	18	129
Dyematrex Red A	5500	44	27	86

a drug-resistance marker and to use a healthier and less engineered host for this plasmid.

The *aroB* gene from pLC29-47 was therefore inserted behind a *tac* promoter, in a plasmid (pKK223-3) carrying the β-lactamase (ampicillin-resistance) gene. [The *tac* promoter is a hybrid of the −35 base-pair region of the *trp* promoter and the −10 base-pair operator region of the *lacUV5* promoter. It is therefore under the same control as *lac*.] The subclone (pJB14) was found by selection for ampicillin resistance and for IPTG-inducible expression of DHQ synthase. The host, *E. coli* strain RB791, carries the *lacI*^{q} mutation that overproduces the *lac* repressor so that the genes downstream from the *tac* operator are not fully expressed until induction by the lactose analogue IPTG. In this way, healthy growth of the transformant RB791(pJB14) could be realized, and induction by IPTG in mid-log phase resulted in the synthesis of large amounts of DHQ synthase (Figure 4). The IPTG-induced cells produced nearly 1000 times as much DHQ synthase activity as the wild-type *E. coli* strain MM294, and the enzyme constituted ~5% of the water-soluble protein (Figure 4). Purification of DHQ synthase from RB791(pJB14) required only two chromatographic steps (Table IV) to yield homogeneous enzyme (Figure 4).

In summary, cells carrying pJB14 in which the *aroB* gene is transcribed from the *tac* promoter have three advantages over cells carrying pLC29-47: (i) pJB14 is maintained by ampicillin resistance rather than by a nutritional requirement that adversely affects cell growth; (ii) the *aroB* gene has been excised from some 7 kilobases of superfluous DNA in pLC29-47; (iii) the powerful *tac* promoter allows a massive increase in synthase gene transcription, controlled by the exogenous inducer IPTG.

It is evident from Table II and Figure 2 that a purification of around 20 000-fold would be necessary to obtain homogeneous DHQ synthase from wild-type *E. coli*. The production of amounts of the enzyme for mechanistic study needing other than catalytic quantities would therefore be a heroic exercise. The successive cloning and subcloning of the DHQ synthetase gene reported here [see also Duncan & Coggins (1984)] remove these difficulties and allow the isolation of more than 100 mg of homogeneous enzyme from 50 g of *E. coli* cells (Table IV). The homogeneous enzymes isolated from the cloned gene [in JB12(pLC29-47)] and the subcloned gene [in RB791(pJB14)] are identical (see Figures 3 and 4), though the isolated enzyme is smaller (40 000–44 000 daltons, as distinct from 57 000 daltons) and appears to show a greater thermal instability and a considerably higher specific catalytic activity [44 units/mg, as distinct from 3.6 units/mg (adjusted for differences in assay temperature)] than the values cited earlier (Maitra & Sprinson, 1978). While the lower specific activity may arise from activity losses during the necessarily lengthier purification procedures of the earlier work, the apparently smaller size of our cloned and subcloned enzyme raised the concern that the cloned genes had been clipped during the generation of the Carbon-Clarke plasmid pLC29-47 or during our subsequent manipulations of it. To check this point, we have isolated DHQ synthase derived from the *wild-type* gene in *E. coli* K-12 and find that the major band in this preparation (Figure 2) runs precisely with the enzyme isolated from our subcloned gene on polyacrylamide gel electrophoresis under denaturing conditions (Figures 2–4). Furthermore, the enzymes from wild-type cells and from the clone and subclone have identical chromatographic characteristics on all the columns used. It appears, therefore, that our overproducing strain is indeed synthesizing a polypeptide identical with the wild-type gene product.

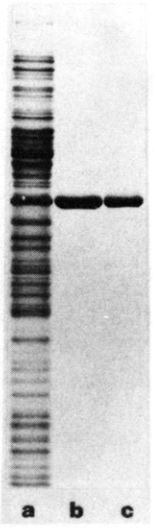

FIGURE 4: Purification of DHQ synthase from the subclone *E. coli* RB791(pJB14): (lane a) crude extract; (lane b) after hydroxylapatite; (lane c) after red dye.

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Registry No. Dehydroquinate synthase, 37211-77-1.

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Interaction of Purified Nicotinamidenucleotide Transhydrogenase with Dicyclohexylcarbodiimide†

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ABSTRACT: The inhibition of the energy-linked nicotinamidenucleotide transhydrogenase (TH; EC 1.6.1.1) by dicyclohexylcarbodiimide (DCCD) has been further studied because of its important mechanistic implications. We had shown earlier that TH bound to submitochondrial particles from bovine heart is inhibited by DCCD and that NAD(H) protects the enzyme against this inhibition [Phelps, D. C., & Hatefi, Y. (1981) *J. Biol. Chem. 256*, 8217–8221]. By contrast, Pennington and Fisher [Pennington, R. M., & Fisher, R. R. (1981) *J. Biol. Chem. 256*, 8963–8969] working with purified TH concluded that NAD(H) does not protect against DCCD inhibition and that DCCD inhibition involves the TH proton channel rather than the nucleotide-binding active site. The present study shows that NAD(H) as well as AMP and ADP, which are known to bind to the NAD(H) binding site from competitive inhibition studies, protect the purified TH against inhibition by DCCD, whereas 2′-AMP and 3′-AMP, which bind to the NADP(H) site on TH, do not protect. In addition, it is shown that whereas the unmodified TH binds to NAD–agarose such that it is elutable by buffer containing NADH, the DCCD-modified enzyme does not bind to NAD–agarose. These results suggest strongly that DCCD binds at or near the NAD(H) binding site on TH. Another less likely possibility is that NAD(H) and DCCD bind to separate sites, but their bindings are mutually exclusive. With the use of [^{14}C]DCCD, it has been shown that 100% activity inhibition corresponds to 0.5 mol of DCCD binding per mol of TH ($M_r \sim 11 \times 10^4$). Both the inhibition and DCCD binding are pseudo first order with respect to the time of TH exposure to DCCD and follow a parallel course when binding is plotted on the basis of saturation at 0.5 mol of DCCD/mol of TH. Since the purified TH and the membrane-bound TH appear to be dimeric, these results suggest a case of half-of-the-sites reactivity in which only the active protomer is capable of rapid DCCD binding. This conclusion agrees with the possibility of DCCD and NAD(H) binding at the same site. Thus, the protomer that cannot bind DCCD might be inactive because it cannot bind NAD(H) either.

Mitochondrial nicotinamidenucleotide transhydrogenase (TH; EC 1.6.1.1)[1] is a membrane-bound enzyme, which catalyzes the transfer of a hydride ion between NAD and NADP. This reaction is coupled to transmembrane proton translocation as shown in eq 1 where H^+_c and H^+_m denote

$$NADH + NADP + nH^+_c \rightleftharpoons NAD + NADPH + nH^+_m \quad (1)$$

protons on the cytosolic and matrix sides of the inner membrane. The number of protons translocated (n) per mole of hydride ion transferred has been estimated to be close to unity (Earle & Fisher, 1980). The mitochondrial transhydrogenase (TH) has been isolated and highly purified (Hojeberg & Rydström, 1977; Wu et al., 1982). The enzyme has a molecular weight of (11–12) × 10^4 and is considered to occur in the membranes as a dimer (Anderson & Fisher, 1981; Wu & Fisher, 1983).

Previous studies have shown that TH is inhibited by DCCD (Phelps & Hatefi, 1981; Pennington & Fisher, 1981), a property that is shared by all proton-linked ATP synthases (Senior & Wise, 1983), ubiquinol-cytochrome c reductases

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[1] Abbreviations: AcPyAD, 3-acetylpyridine adenine dinucleotide; DCCD, *N,N′*-dicyclohexylcarbodiimide; TH, nicotinamidenucleotide transhydrogenase; SMP, submitochondrial particles; ATPase, adenosinetriphosphatase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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