Expression, Purification, and Characterization of *Escherichia coli* Dihydrodipicolinate Reductase†

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ABSTRACT: Dihydrodipicolinate reductase catalyzes the NAD(P)H-dependent reduction of the carbon−carbon double bond of the α,β-unsaturated cyclic imine dihydrodipicolinate to form the cyclic imine tetrahydrodipicolinate. The enzyme is a component of the bacterial biosynthetic pathway forming L-lysine and diaminopimelate from L-aspartate. The gene encoding dihydrodipicolinate reductase, *dapB*, has been cloned and sequenced from *Escherichia coli* (Bouvier et al., 1984), and we have used this sequence information to generate an expression vector containing the *dapB* gene. Expression and purification of dihydrodipicolinate reductase to homogeneity have allowed us to characterize the kinetics, stereochemistry, and chemical mechanism of the enzymatic reaction. The kinetic mechanism is ordered, with reduced nucleotide binding preceding dihydrodipicolinate binding and presumably with tetrahydrodipicolinate dissociating prior to oxidized nucleotide release. The enzyme has a unique nucleotide specificity, with NADH being twice as effective as NADPH as a reductant. The enzyme catalyzes the stereospecific transfer of the 4*R* hydrogen atom of the reduced nucleotide, as a hydride ion, to the 4 position of dihydrodipicolinate. These results allow us to propose a chemical mechanism for the reaction catalyzed by dihydrodipicolinate reductase involving hydride transfer to the β carbon of the unsaturated imine. The resonance-stabilized C3 carbanion is protonated to generate the reduced product, tetrahydrodipicolinate.

In this paper, we have investigated the kinetic and chemical mechanism of dihydrodipicolinate reductase, the enzyme responsible for the biosynthesis of tetrahydrodipicolinate, a precursor for each of the three pathways of bacterial lysine biosynthesis. This enzyme was first purified and partially characterized from *Escherichia coli* (Tamir & Gilvarg, 1974). The genes encoding dihydrodipicolinate reductase, *dapB*, have been cloned and sequenced from *E. coli* (Bouvier et al., 1984), *Bacillus lactofermentum* (Pissabarro et al., 1993), and *Mycobacterium bovis* BCG (Cirillo et al., 1994). The predicted amino acid sequences are homologous over their entire length, although the *E. coli* enzyme contains a 22 amino acid insertion in the amino terminal portion. We report here the preparation of an expression plasmid for *E. coli* dihydrodipicolinate reductase and the purification of the enzyme to homogeneity. The kinetic and chemical mechanisms of the enzyme have been determined with this preparation. In an accompanying paper (Scapin et al., 1995), we report the determination of the three-dimensional structure of this enzyme at 2.2 Å resolution.

L-Lysine is an essential amino acid in mammals and must be obtained in the diet. In yeast and *Euglena*, L-lysine is synthesized from α-ketoglutarate via the α-aminoadipate pathway, while in bacteria, most algae, and higher plants, L-lysine is synthesized from L-aspartic acid via the diaminopimelate pathway (Gilvarg & Weinberger, 1970). Of the three stereoisomers of the symmetric diamino acid, diaminopimelate (DAP),[1] the *meso* compound is both the direct precursor to L-lysine and a component of the peptidoglycan structure in bacterial cell walls. The decarboxylation of diaminopimelate is catalyzed by a unique pyridoxal phosphate-dependent reaction occurring at the D stereocenter catalyzed by diaminopimelate decarboxylase with retention of configuration (Kelly & White, 1965). The cross-linking of adjacent peptide moieties of bacterial peptidoglycans similarly involves reactions at the D stereocenter, the amino group of *meso*-DAP displacing the covalently bound transpeptidase from the D-Ala−transpeptidase complex and forming the *meso*-DAP−alanine amide linkage between adjacent polysaccharide strands (Wietzerbin et al., 1974). Among bacteria, three pathways exist for the conversion of tetrahydrodipicolinate to *meso*-diaminopimelate. These include the succinylase and acetylase pathways (Gilvarg & Weinberger, 1970) as well as the less ubiquitous dehydrogenase pathway present in *Bacillus* species (Misona & Soda, 1980) and *Corynebacterium* (Schrumpf et al., 1991). Many bacteria contain enzymes for at least two of these pathways.

Due to these critical, and specific, functions in bacterial amino acid and cell wall biosynthesis, enzymes of the L-lysine biosynthetic pathway have been investigated as targets for bacterial inhibitor design. Thus, a tight-binding

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[1] Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; G6PDH, glucose-6-phosphate dehydrogenase; $NADP^+$, oxidized β-nicotinamide adenine dinucleotide phosphate; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; α-NADPH, reduced α-nicotinamide adenine dinucleotide phosphate; thio-NADPH, reduced β-thionicotinamide adenine dinucleotide phosphate; NHDPH, reduced β-nicotinamide hypoxanthine dinucleotide phosphate; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TEA, triethanolamine; TLCK, L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride; DHP, dihydrodipicolinate; THP, tetrahydrodipicolinate; DHPS, dihydrodipicolinate synthase; DHPR, dihydrodipicolinate reductase; Dapdh, diaminopimelate dehydrogenase; DAP, diaminopimelate.

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inhibitor (K_i = 58 nM) of tetrahydrodipicolinate *N*-succinyltransferase has been described (Berges et al., 1986), while mechanism-based approaches to inhibitor development have been attempted for diaminopimelate epimerase (Lam et al., 1988; Gelb et al., 1990). Because of its unique position in bacterial DAP/lysine biosynthesis, the results presented in this and the accompanying paper may be essential to the design and evaluation of dihydrodipicolinate reductase inhibitors as broad spectrum antibiotics.

MATERIALS AND METHODS

Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (type XXIV), hexokinase and glutathione reductase from yeast, NADH peroxidase from *Streptococcus faecalis*, D-[1-^{2}H]glucose (97 atom % ^{2}H), all oxidized pyridine nucleotides, 2,6-piperidinedicarboxylic acid, L-allylglycine, and all buffer components were purchased from Sigma. D-[1-^{3}H]Glucose and NaB^3H_4 were purchased from Amersham. [^{14}C]Pyruvate was from Dupont NEN research Products. D_2O (>99.8 atom % excess) was from Cambridge Isotopes Laboratory. 2,6-Pyridinedicarboxylic acid was from Aldrich. *E. coli* aspartate semialdehyde dehydrogenase was the generous gift of Dr. Ronald Viola (University of Akron, Akron, OH).

Preparation of 4S-Labeled Pyridine Nucleotides. The procedures described below were routinely used for the preparation of the reduced [(4*S*)-4-^{3}H]pyridine nucleotides. One-milliliter solutions of the oxidized pyridine nucleotides (5−25 mM) in 10 mM TEA·HCl, pH 7.8, were purified on a FPLC Mono Q anion-exchange column equilibrated in 10 mM TEA·HCl buffer, pH 7.8, and eluted isocratically with the same buffer as described previously (Orr & Blanchard, 1984). This prepurification was essential for obtaining pure reduced pyridine nucleotides. Reduced pyridine nucleotides were prepared and purified as described previously (Vanoni et al., 1990). The concentrations of the phosphorylated and nonphosphorylated nucleotides were determined by enzymatic end-point assays with bakers' yeast glutathione reductase or *S. faecalis* NADH peroxidase, respectively.

Preparation of 4R-Labeled NADPH. $NADP^+$ was reduced in a total volume of 5 mL in the presence of [2-^{2}H]- or [2-^{3}H]-D,L-malate (140−180% excess over pyridine nucleotide) and 2 mM Mg^{2+} by chicken liver malic enzyme. The reaction was monitored spectrophotometrically, and the pH was maintained at 7.8−8.0 by the addition of KOH. When the reaction was completed, the solutions were filtered through an Amicon YM 10 ultrafiltration membrane, and the reduced pyridine nucleotides were purified as described previously (Orr & Blanchard, 1984). Fractions that had $A_{260}/A_{340} \leq 2.3$ were pooled. The concentrations of these solutions were calibrated enzymatically using glutathione reductase, in the presence of excess glutathione, and the total change in absorbance was monitored at 340 nm. Solutions of reduced pyridine nucleotides were used within 24 h.

[2-^{2}H]- and [2-^{3}H]malates were prepared by the reduction of oxaloacetate with NaB^2H_4 and NaB^3H_4, respectively. [2-^{2}H]- and [2-^{3}H]malates were purified by adsorption to Dowex 1-X8 (formate form, 200−400 mesh) and eluted with a 0−4 N formic acid linear gradient. Fractions were assayed for malate content with malic enzyme, pooled, concentrated by rotary evaporation, and stored at −20 °C.

Coupling Enzymes. The *E. coli dapA* gene encoding dihydrodipicolinate synthase, cloned into the PTZ plasmid vector (Pharmacia) and present in the *E. coli* strain JM109, was obtained from Dr. Bill Karsten (North Texas State University). Cultures were grown on YT media and then transferred to M9 minimal media. Dihydrodipicolinate synthase was purified using anion-exchange and gel permeation chromatography. These steps yielded protein preparations which contained high levels of synthase activity with no contaminating reductase activity, under the experimental conditions. *Bacillus sphaericus* (ATCC 13805) was grown on YT media and harvested at an OD_{600} = 1.0. Diaminopimelate dehydrogenase (Dapdh) was purified as described above for the synthase. The active fractions were then applied to a 2′,5′-ADP column (Pharmacia). Elution with a nonlinear 0−1 M NaCl gradient in 20 mM TEA·HCl, pH 7.8, yielded homogeneous enzyme, as determined by SDS−PAGE with Coomassie blue staining.

Synthesis of Dihydrodipicolinate (DHP). L-Aspartic-β-semialdehyde (L-ASA) was prepared by the ozonolysis of L-allyl glycine according to the method of Black and Wright (1955) in 1 N HCl at 0 °C. L-ASA was purified by applying the solution to a 1 × 30 cm AG-X8 cation-exchange column (H^+ form, 200−400 mesh), washing with water, and eluting using a 0−1 M HCl gradient (Westerik & Wolfenden, 1974). Fractions were analyzed by thin-layer chromatography on silica gel, using butanol−acetic acid−water, 100:22:50, as the solvent and spraying with 0.3% ninhydrin. R_f values for aspartic acid, aspartic-β-semialdehyde, and allylglycine were determined to be 0.54, 0.65, and 0.80, respectively. Fractions exhibiting the appropriate R_f value were pooled and concentrated, and the concentration of ASA was calibrated enzymatically with *E. coli* aspartate semialdehyde dehydrogenase (Karsten & Viola, 1991). The ^{1}H and ^{13}C NMR spectra indicated that ASA exists predominantly as a hydrate (Wedler et al., 1984; Robins et al., 1993), as also observed previously for glutamate semialdehyde (Gough et al., 1989). Stock ASA solutions were stored in 4 N HCl at −20 °C and are stable for more than 1 year under these conditions. Before use, ASA solutions were titrated to pH 1−2 with sodium bicarbonate. Dihydrodipicolinate (DHP) is not stable and has never been isolated in a stable form (Gilvarg et al., 1965; Robins et al., 1993) and thus was prepared enzymatically *in situ*, by the condensation of ASA with sodium pyruvate using dihydrodipicolinate synthase.

PCR and Expression. The sequence of the *E. coli dapB* gene has been reported (Bouvier et al., 1984). Two oligonucleotides which were complementary to the amino-terminal coding and carboxy-terminal noncoding strands were synthesized and contained an *Nco*I and *Hin*dIII restriction sites, respectively, in noncomplementary overhangs. These were used to amplify the *E. coli dapB* gene from genomic *E. coli* K12 DNA using standard PCR conditions (Perkin-Elmer). The PCR product was purified by electrophoresis on low-melting agarose and ligated into a pET3d vector (Novagen), which had previously been treated with *Nco*I and *Hin*dIII. The plasmid was transformed first into *E. coli* strain JM105 and subsequently into BL21(DE3) (Novagen). Transformed cells were grown in 2 × LB media to an A_{600} = 1.0, induced with 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG), and incubated for an additional 5 h at 37 °C.

Purification of Dihydrodipicolinate Reductase. Six liters of 2 × LB media containing 0.05 mg/mL carbenecillin was inoculated with an overnight culture, grown to an OD_{600} of 1−1.5 and induced with 0.4 mM IPTG. After 5 h, approximately 20 g of cells was collected by centrifugation. Protease inhibitors were added directly to a 50% (w/v) suspension of cells (typically 2.3 mg/L leupeptin, 52 mg/L TLCK, 20 mg/L soybean trypsin inhibitor, 1.6 mg/L aprotinin, 1.1 mg/L pepstatin, and 36.2 mg/L PMSF). Cells were broken by two passages through a French press at 16 000 psi, and cell debris was removed by centrifugation for 45 min at 12 000 rpm. Nucleic acids were precipitated by the addition of streptomycin sulfate (1% w/v final) to the supernatant, and the cloudy solution was centrifuged for 45 min at 12 000 rpm to pellet the nucleic acids. The nucleic acid-free supernatant was dialyzed against 50 mM TEA·HCl, pH 7.8, for 4 h. During the dialysis, a precipitate forms which was removed by ultracentrifugation for 45 min. The clear supernatant was loaded onto a 400-mL fast-flow Q-Sepharose anion-exchange column (Pharmacia) which had been equilibrated in 25 mM TEA·HCl buffer, pH 7.8. The adsorbed protein was eluted at 2 mL/min with a 2-L linear 0−1 M NaCl gradient. The enzyme activity was eluted between 0.4 and 0.6 M NaCl. The active fractions were pooled, concentrated (PM 10, Amicon) to 10 mL, and applied to a 1.6 × 60 cm Superdex 200 (Pharmacia) gel filtration column equilibrated with 50 mM TEA·HCl, pH 7.8. The protein was eluted at 2 mL/min, and the active fractions were pooled, and applied to a 1 × 10 cm Mono Q (Pharmacia) high-performance anion-exchange column equilibrated with 20 mM TEA·HCl, pH 7.8. The enzyme was eluted between 0.4 and 0.5 M NaCl using a 130-mL nonlinear 0−1 M NaCl gradient. The active fractions, which exhibited a single band on SDS−PAGE with Coomassie blue staining, were pooled.

Analytical Methods. The subunit molecular weight was determined using SDS−PAGE to be approximately 28 000. The apparent native molecular weight of dihydrodipicolinate reductase was determined using a Superdex 200 gel filtration column (Pharmacia) calibrated using molecular weight standards (Bio-Rad). Automated amino-terminal sequencing were performed using an Applied Biosystems sequencer using standard Edman chemistry. The purified protein (100 pmol) was analyzed by electrospray ionization/mass spectrometry in an API III triple−quadrapole mass spectrometer (PE SCIEX). The data were deconvoluted by computer to determine the subunit molecular weight of the enzyme.

Enzymatic Assay Conditions. Determination of the initial rates of DHPR activity was based on the absorbance of NAD(P)H at 340 nm (ϵ_{340} = 6220 M^{-1} cm^{-1}). All spectrophotometric changes were monitored in 3-mL quartz cuvettes using a Gilford 260 spectrophotometer maintained at 25 °C with a circulating water bath and thermospacers. Absorbance changes were recorded on a 10-mV strip chart recorder with adjustable zero and multispeed drive. pH measurements were determined directly in the cuvette using a Radiometer PHM62 pH meter equipped with a combined microelectrode and standardized with calibrated buffers from Radiometer. Typical assays contained 100 mM Hepes, pH 7.8, 100 μM NADPH, 100 μM L-ASA, 1 mM sodium pyruvate, and 10 μg dihydrodipicolinate synthase (DHPS) in 50% (v/v) glycerol. Dihydrodipicolinate synthase activity was determined to be always in excess to DHPR activity. Reaction mixtures were incubated for 5 min, a period of time determined to be sufficient for the complete conversion of L-ASA to dihydrodipicolinate, and initiated by the addition of a small volume (5−10 μL) of DHPR to a temperature-equilibrated reaction mixture.

Data Analysis. Reciprocal initial velocities were plotted against the reciprocal of the variable substrate concentration, and the data were fitted to the appropriate rate equations by the least-squares method, assuming equal variances of the velocity. The Fortran programs of Cleland (1979) were modified to run on a personal computer. Intersecting initial velocity patterns were fit to eq 1, while parallel initial velocity patterns were fit to eq 2. Competitive, uncompetitive, and noncompetitive inhibition was fit to eq 3−5, respectively.

$$v = VAB/(K_aB + K_bA + K_{ia}K_b + AB) \quad (1)$$

$$v = VAB/(K_aB + K_bA + AB) \quad (2)$$

$$v = VA/K(1 + I/K_{is}) + A \quad (3)$$

$$v = VA/K + A(1 + I/K_{ii}) \quad (4)$$

$$v = VA/(K(1 + I/K_{is}) + A(1 + I/K_{ii}) \quad (5)$$

Stereochemistry of Hydride Transfer. A 3.0-mL reaction mixture containing 100 μM [(4*S*)-4-^{3}H]NADPH (15 × 10^5 dpm), 1.0 mM ASA, 1.0 mM pyruvate, and [^{14}C]pyruvate (1 × 10^6 dpm), in 10 mM TEA·HCl, pH 7.8, and 20 μg of DHPS, was prepared in a cuvette. Before, and at fixed intervals after, the addition of 16 μg of DHPR, 100-μL aliquots were removed and immediately injected onto a DEAE-nucleogen HPLC column. The column was eluted with a nonlinear gradient using 10 mM sodium phosphate and 10 mM sodium phosphate containing 1 M NaCl, and the absorbance of the eluate was continuously monitored at 260 nm. [^{3}H]$NADP^+$ and [^{14}C]tetrahydrodipicolinate (THP) were located and quantitated by liquid scintillation counting using Hydroflour scintillation cocktail (National Diagnostics) in an LKB RackBeta liquid scintillation counter. In a second experiment [(4*R*)-4-^{3}H] NADPH was substituted for [(4*S*)-4-^{3}H]NADPH, and an experiment identical to that described above for the 4*S* isomer was performed.

Substrate Reduction. A 10-mL reaction mixture containing 20 mM [2-^{2}H]-D,L-malate, 5 mM ASA, 6 × 10^6 dpm of [^{14}C]pyruvate, 100 μM $NADP^+$, 2 mM Mg^{2+}, and 100 mM NH_4OAc was prepared in an Erlenmeyer flask. A total of 100 units of chicken liver malic enzyme was added to the reaction mixture to generate [(4*R*)-4-^{2}H]NADPH. Then 50 units of DHPS was added to generate DHP. Five minutes later, 50 units of DHPR was added to reduce DHP to THP. Ten minutes later, 50 units of DAPDH was added to convert THP to the stable *meso*-diaminopimelate. The reaction mixture was incubated at 37 °C overnight, ultrafiltered through an Amicon YM 10 membrane to remove enzymes, and concentrated by rotary evaporation to 3−4 mL. The reaction mixture was applied to 1 × 10 cm Mono Q anion-exchange column (Pharmacia) and eluted with a nonlinear gradient from 0.02 to 1 M NH_4OAc, pH 7.8. The radioactive fractions were counted by liquid scintillation, and 95% of the ^{14}C initially present as [^{14}C]pyruvate was recovered in fractions 7−12. These fractions were pooled, concentrated

Table 1: Purification of *E. coli* Dihydrodipicolinate Reductase

fraction[a]	total protein[b] (mg)	total units[c]	specific activity (units/mg)	% yield	x-fold purification
dialysate	12 970	592 000	45	100	1.0
Q-Sepharose	3 350	475 000	141	80.0	3.1
Superdex 200	1 270	447 000	352	76.0	7.8
Mono Q	1 100	438 000	398	74.0	8.8

[a] Pooled fractions after each step. [b] Determined using the Bradford dye-binding assay (Bio-Rad). [c] Units equal to 1 μmol min^{-1}. Assay conditions are as described in the text.

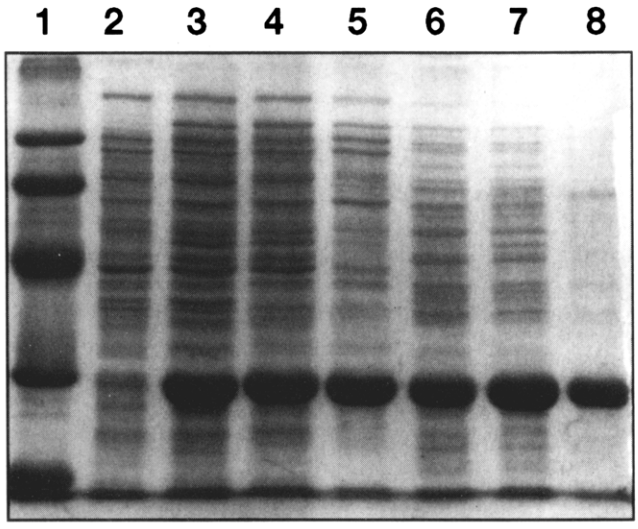


FIGURE 1: SDS−PAGE gel showing the purification of *E. coli* dihydrodipicolinate reductase at each step. Lanes: 1, low-range markers; 2, *E. coli* cell-free extract (before induction); 3, extract after induction with 0.4 mM IPTG for 5 h; 4, streptomycin sulfate supernatant; 5, supernatant after dialysis; 6, pooled fast-flow Q-Sepharose fractions; 7, pooled Superdex 200 fractions; 8, pooled Mono Q fractions.

by rotary evaporation, acidified to pH 2.0 with HCl, and applied to a 0.7 × 9.0 cm AG 1-X8 (200−400 mesh) cation-exchange column (H^+ form). The column was washed with 50 mL of water and then eluted with a linear 0−4 N HCl gradient. The radioactive fractions were counted, pooled, and concentrated to dryness by rotary evaporation. This procedure was repeated several times to remove HCl completely, and the residue was dissolved in 1.0 mL of 99.8% D_2O and taken to dryness. A portion of the residue was analyzed by FAB mass spectrometry, and the remaining residue was dissolved in 1.0 mL of 99.8% D_2O. This solution was placed into a 5-mm NMR tube and analyzed by ^1H NMR at 500 MHz on a Varian VXR-500 spectrometer.

RESULTS

Expression and Purification of Dihydrodipicolinate Reductase. Upon IPTG induction, BL21B cells containing the pET3d construct vector overproduced a soluble protein which had the same monomer molecular weight as the wild-type *E. coli* DHPR. Time−course studies revealed that a 5-h induction maximized enzyme expression in the cell-free extracts which exhibited substantially elevated dihydrodipicolinate reductase activity. Table 1 summarizes a typical purification of expressed dihydrodipicolinate reductase, and Figure 1 shows a SDS−PAGE gel of pooled fractions obtained after each step. Using this protocol, 1.1 g of DHPR has been purified 8.8-fold in 74% overall yield. The purified enzyme showed a single protein band of 28 kDa upon SDS−PAGE. The subunit molecular weight was determined to be 28 758 by electrospray mass spectrometry, a result consistent with the mass predicted from the previously reported amino acid sequence of dihydrodipicolinate reductase (Bouvier et al., 1984). Automated Edman amino-terminal sequencing confirmed that the first 18 residues of purified protein were identical to the derived amino acid sequence of *E. coli* DHPR. The apparent native molecular weight, determined by gel filtration, was approximately 120 000, suggesting that the native enzyme exists as a tetramer of identical 28 000 subunits. The purification could be completed in approximately 3 days.

Initial Velocity, Product, and Dead-End Inhibition Studies. Initial velocity studies, obtained by varying the concentration of L-DHP at several fixed levels of NADPH, exhibited an intersecting pattern, indicative of the sequential addition of these substrates. From these experiments, the steady-state K_m values of NADPH and L-DHP were determined to be 8 ± 2.5 and 50 ± 12 μM, respectively. $NADP^+$ was used as a product inhibitor and exhibited linear, competitive inhibition versus NADPH (K_{is} = 190 ± 35 μM) and linear, noncompetitive inhibition versus L-DHP (K_{is} = 0.71 ± 0.2 mM; K_{ii} = 24 ± 0.2 mM). 2,6-Pyridinedicarboxylic acid (2,6-PDC), an aromatic analogue of the substrate, was used as a dead-end inhibitor. 2,6-PDC was a linear, competitive inhibitor versus DHP, exhibiting a K_{is} of 26 ± 6 μM. 2,6-PDC exhibited linear, uncompetitive inhibition versus NADPH with a K_{ii} = 0.33 ± 0.05 mM at a fixed DHP concentration of 50 μM (Table 2).

Nucleotide Specificity and Binding. The kinetic parameters of eight reduced pyridine nucleotide substrates for dihydrodipicolinate reductase were determined at saturating DHP concentrations (Table 3). Relative maximum velocities were determined by comparing the maximal velocities of each substrate to that of NADPH at the same time under identical experimental conditions. The stoichiometry of nucleotide binding and dissociation constants for NADPH and NADH from DHPR were determined using titration calorimetry (Figure 2). Titration calorimetry was performed using an Omega differential titrating calorimeter (MicroCal Inc., Northampton, MA). Eighteen 2-μL aliquots of enzymatically calibrated NADH (panel A) and NADPH (panel B) (3.0 mM in 50 mM Hepes buffer, pH 7.5) were injected into the protein sample (2.0 mL of 20 μM DHPR in 50 mM Hepes buffer, pH 7.5), to a final NAD(P)H:protein mole ratio of 2.7. All titrations were carried out at 25 °C, with constant mixing at 350 rpm.

Nucleotide Stereochemistry. When [(4*S*)-4-^3H]NADPH (100 μM, 15 × 10^5 dpm) was incubated with 1.0 mM ASA, 1.0 mM pyruvate, and [^{14}C]pyruvate (1 × 10^6 dpm) in 10 mM TEA·HCl, pH 7.8, 20 μg of DHPS (excess over DHPR), and 15 μg of DHPR, and the products were separated on a DEAE-nucleogen column, 95% of the tritium originally present in [(4*S*)-4-^3H]NADPH was recovered as [4-^3H]-$NADP^+$. When an identical experiment was performed using [(4*R*)-4-^3H]NADPH, 94% of the tritium was recovered in the [^3H,^{14}C]THP. These results suggest that DHPR catalyzes the stereospecific transfer of the 4*R* hydrogen atom from NADPH to DHP to form THP.

Substrate Reduction. The mass spectrum of purified [^1H]-*meso*-DAP, synthesized enzymatically from ASA, pyruvate,

Table 2: Product and Dead-End Inhibition Studies

varied substrate	product inhibitor	dead-end inhibitor	K_{is} (μM)	K_{ii} (mM)	fixed substrate (μM)	inhibition[a]
NADPH	$NADP^+$		190 ± 35		L-DHP (100)	C
L-DHP[b]		2,6-PDC[c]	26 ± 6		NADPH (100)	C
L-DHP	$NADP^+$		700 ± 200	24 ± 0.2	NADPH (35)	NC
NADPH		2,6-PDC		0.33 ± 0.05	L-DHP (50)	UC

[a] C, competitive inhibition; NC, noncompetitive inhibition; UC, uncompetitive inhibition. [b] L-DHP, L-dihydrodipicolinate. [c] 2,6-PDC, 2,6-pyridinedicarboxylic acid.

Table 3: Kinetic Parameters of Nucleotide Substrates for *E. coli* Dihydrodipicolinate Reductase

nucleotide substrate	K_m (μM)	V_{rel}	V/K_{rel}
NADH	1.6 ± 0.5	62	217
β-NADPH	5.6 ± 0.9	100	100
α-NADPH	52 ± 13	110	12
NHDPH	9 ± 3	55	35
3-acetylpyridine	4 ± 2	45	66
3′-NADPH	80 ± 30	45	3
thio-NADPH	2.1 ± 1.7	32	85
2′,3′-cyclic NADPH	57 ± 17	24	13

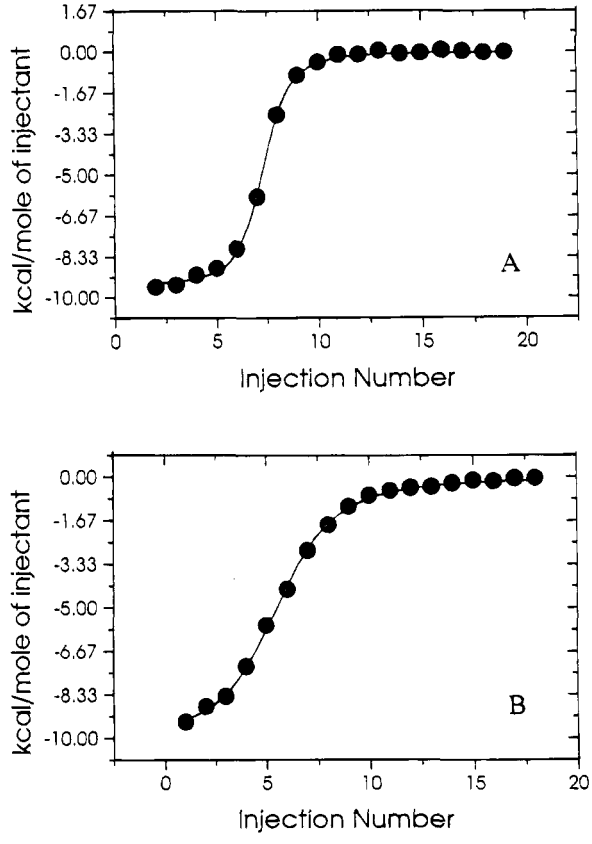


FIGURE 2: Direct binding studies by microcalorimetry. Titration calorimetry of NADH binding (panel A) and NADPH binding (panel B) to 20 μM *E. coli* dihydrodipicolinate reductase. NAD(P)H (3 μM) was added per each injection.

NH_4^+, and NADPH using DHPS, DHPR, and DAPDH, exhibited a *m/z* peak at 191.0. The ^1H NMR spectrum of this material is shown in Figure 3 (panel A). The α-amino C1 and C1′ protons resonate at 4.35 ppm and appear as a triplet due to their coupling to the C2, C2′ methylene protons. The C2 and C2′ protons appear as a complex multiplet centered at 2.3 ppm due to their coupling to both the C1 and C3 protons. The C3 methylene protons appear as two separated multiplets centered at 1.83 and 1.96 ppm. Integration yields the expected 2:4:1:1 signal ratio.

The mass spectrum of [2,4-^2H]-*meso*-DAP, synthesized as shown in Scheme 1, exhibited a *m/z* peak at 192.9, and the ^1H NMR spectrum of this material is shown in Figure 3 (panel B). DAP dehydrogenase has been previously shown to transfer the 4*S* hydrogen of NADPH to form the D-amino acid center of diaminopimelate (Misono & Soda, 1980). The L-α-amino C1 proton appears as a triplet at 4.35 ppm with half of the intensity observed for protio-DAP, due to deuteration at the D-α-amino position by the DAP dehydrogenase. The C2 and C2′ methylene protons appear as a multiplet at 2.3 ppm. A single C3 methylene proton appears at 1.96 ppm, while the other C3 methylene proton resonating at 1.83 ppm is lost due to deuterium transfer from [(4*R*)-4-^2H]NADPH to DHP catalyzed by DHPR (see Scheme 2). Integration of the C1, C2, and C3 peaks reveals a 1:4:1 ratio.

DISCUSSION

L-lysine biosynthesis in *E. coli* occurs exclusively through the succinylase pathway (Gilvarg & Weinberger, 1970) although other bacteria use either the biochemically equivalent acetylase pathway or the dehydrogenase shunt. All of the enzymes in the succinylase pathway, Scheme 1, have been purified and partially characterized from *E. coli*, including the synthase (*dapA*; Shedlarski & Gilvarg, 1970), reductase (*dapB*; Tamir & Gilvarg, 1974), *N*-succinylase (*dapC*; Simms et al., 1984), transaminase (*dapD*; Peterkofsky & Gilvarg, 1961), desuccinylase (*dapE*; Lin *et al.*, 1988), epimerase (*dapF*; Wiseman & Nichols, 1984), and decarboxylase (*lysA*; Kelly & White, 1965). While some of the genes encoding these enzymes appear to be clustered in *Corynebacterium glutamicum* (Cremer et al., 1991) and *E. coli* (Richaud et al., 1986), others are scattered throughout the *E. coli* chromosome (Bachman, 1990). Both the ultimate product, L-lysine, and its biosynthetic precursor, *meso*-diaminopimelate, are required for protein synthesis and cell wall biosynthesis, respectively, suggesting that inhibitors of the biosynthetic pathway would be potent antibacterial agents. We have thus felt that a complete mechanistic and structural understanding of the enzymes of this pathway would be enlightening and report here, and in the accompanying paper, our results on dihydrodipicolinate reductase.

Enzyme Expression and Purification. The reported gene sequence of *E. coli* dihydrodipicoline reductase, encoded by *dapB* (Bouvier et al., 1984) was used to design oligonucleotide primers that were used to PCR the complete gene from genomic *E. coli* DNA. The primers were designed with noncomplementary overhangs which contained appropriate restriction sites for the in-frame insertion of the gene into a pET3d vector. This afforded a plasmid which, when transformed into an appropriate host strain, resulted in a high level of expression of the soluble reductase when induced

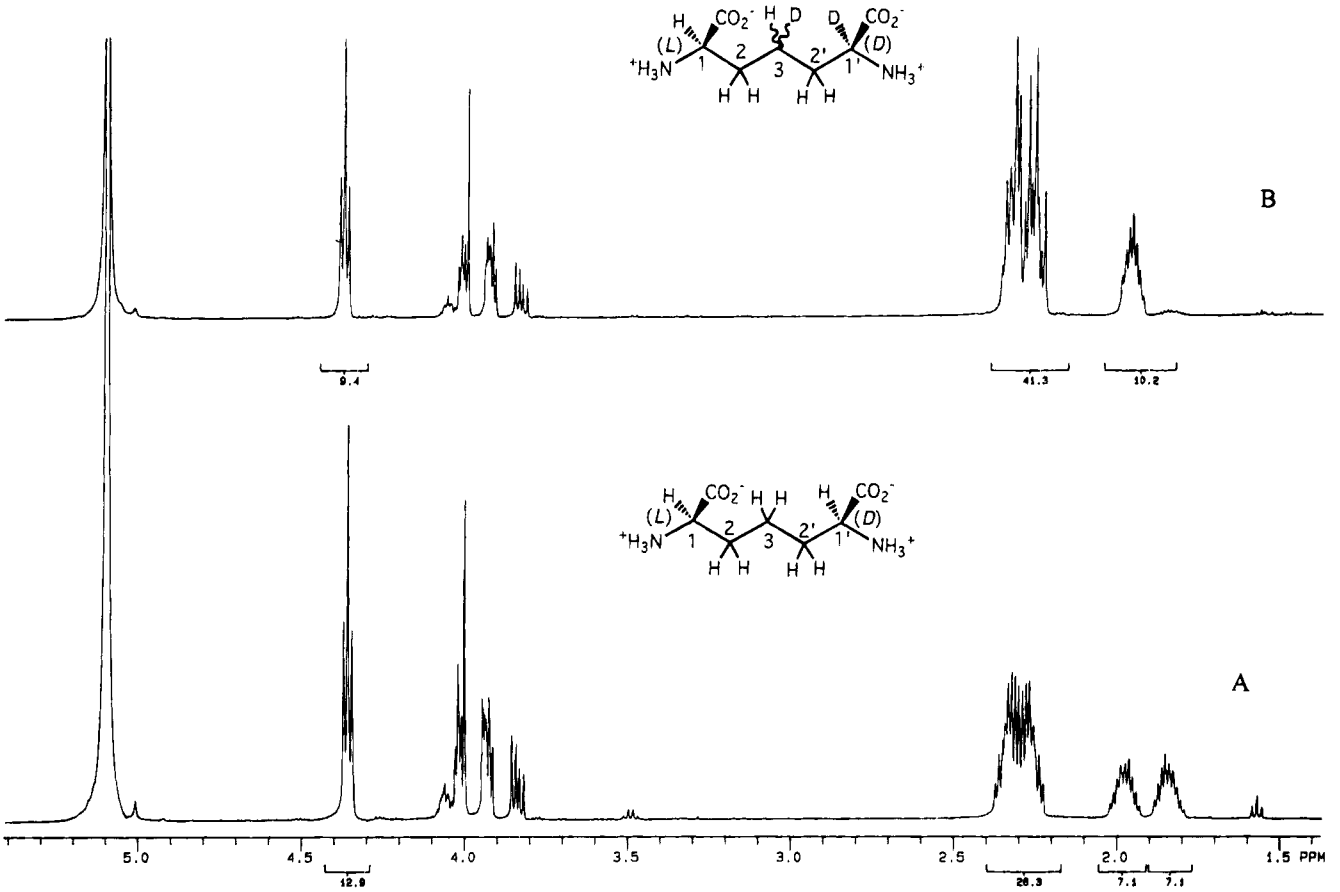


FIGURE 3: 500 MHz ^{1}H NMR spectrum of diaminopimelate synthesized as in Scheme 2 using L-malate (panel A) or [2-^{2}H]-D,L-malate (panel B). The samples were prepared in D_2O, pH = 2.5, and chemical shifts are measured relative to the external DDS.

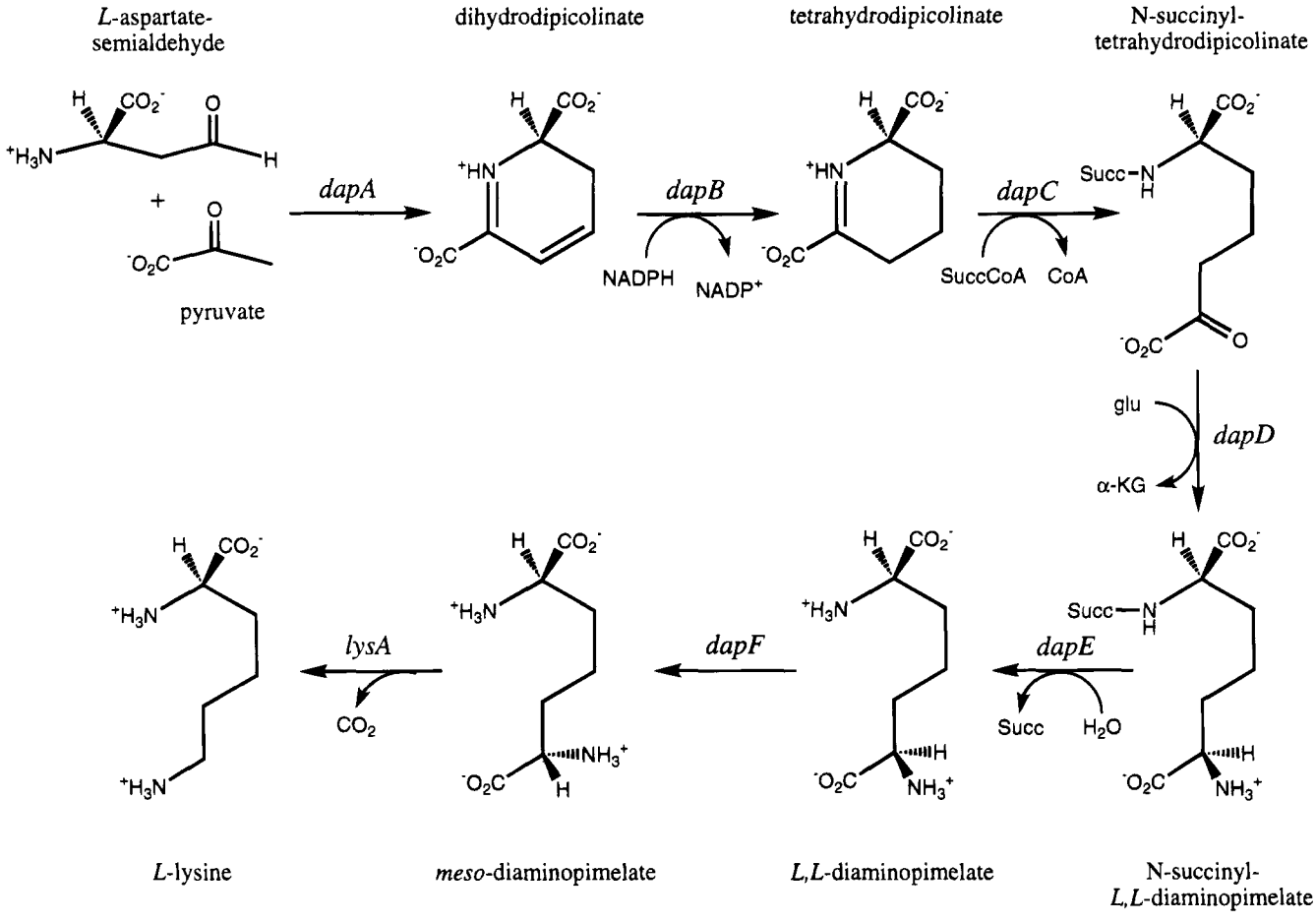
with IPTG. This strain was used for the subsequent purification of the enzyme.

Dihydrodipicolinate reductase has been previously purified from wild-type *E. coli,* requiring a 2000-fold purification to obtain small amounts of enzyme (Tamir & Gilvarg, 1974). In the present study, we have been able to obtain large amounts of enzyme by the overexpression of the enzyme and the sequential use of anion-exchange, gel filtration, and high-resolution anion-exchange chromatography. The enzyme appears homogeneous on SDS−PAGE, exhibiting a subunit molecular weight of approximately 28 000. This molecular weight has been confirmed by electrospray mass spectrometry, which yields a subunit molecular weight of 28 758 ± 8, in close agreement with the mass of 28 757 predicted from the amino acid sequence of the enzyme derived from the gene sequence (Bouvier et al., 1984). The native enzyme elutes from gel filtration columns at the position expected for a 120 kDa protein, suggesting that it exists as a homotetramer in solution. Automated amino-terminal sequencing of the first 18 residues confirms the identity of the expressed enzyme as *E. coli* dihydrodipicolinate reductase.

Kinetic Mechanism. The chemical synthesis of dihydrodipicolinate, from aspartate semialdehyde and oxaloacetate, has been reported (Gilvarg & Farkas, 1965). We found that the yields of dihydrodipicolinate obtained in this way were extremely variable and elected to generate the reducible substrate *in situ* by the addition of dihydrodipicolinate synthase to assay mixtures containing variable amounts of aspartate semialdehyde and excess pyruvate. Under our conditions, the reaction proceeds to completion in several minutes, and the addition of NADPH and dihydrodipicolinate reductase results in the stoichiometric reduction of dihydrodipicolinate to tetrahydrodipicolinate. We have thus used this *in situ* generation system in all subsequent kinetic studies.

In initial velocity experiments where the concentration of dihydrodipicolinate was varied at several fixed concentrations of NADPH, an intersecting pattern was observed, suggesting the sequential addition of the two substrates. Product inhibition by $NADP^+$ was linearly competitive versus NADPH and linearly noncompetitive versus dihydrodipicolinate. The aromatic analog of dihydrodipicolinate, 2,6-pyridinedicarboxylate, had previously been shown to be an inhibitor of dihydrodipicolinate reductase. 2,6-Pyridinedicarboxylate exhibits linear, competitive inhibition versus dihydrodipicolinate (K_{is} = 26 ± 6 μM) and linear, uncompetitive inhibition versus NADPH. These initial velocity, product, and dead-end inhibition studies are compatible with an ordered, sequential mechanism in which the binding of NADPH is followed by dihydrodipicolinate binding and with tetrahydrodipicolinate release preceding $NADP^+$ release (Figure 4). Both ordered and random sequential kinetic mechanisms have been reported for pyridine nucleotide-dependent oxidoreductases. Examples of enzymes catalyzing the chemically similar reduction of α,β-unsaturated keto compounds by ordered sequential kinetic mechanisms include the steroid 5α-reductases (Liang et al., 1983; Houston et al., 1987) and the fatty acyl enoyl-CoA reductases (Simon & Buhler, 1982). Our inability to demonstrate the enzyme-catalyzed $NAD(P)^+$-dependent oxidation of tetrahydrodipicolinate has precluded a more detailed kinetic mechanism determination.

Scheme 1: Lysine Biosynthetic Pathway in *E. coli*



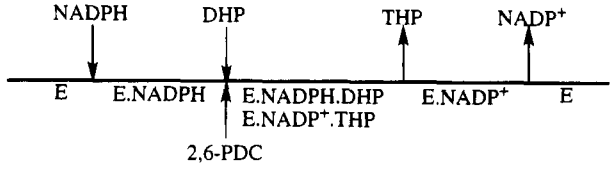


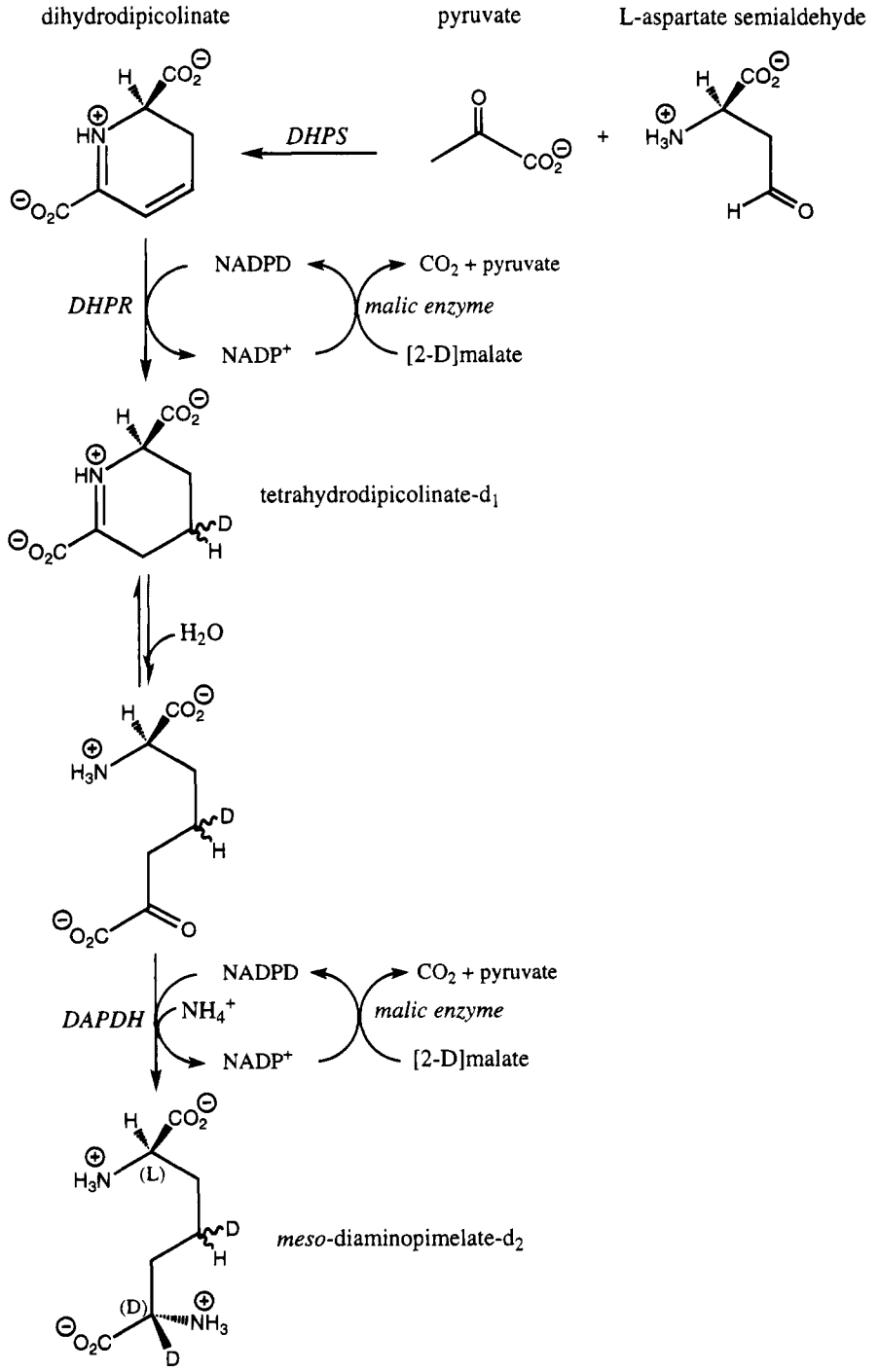
FIGURE 4: Schematic representation of the kinetic mechanism of *E. coli* dihydrodipicolinate reductase.

Nucleotide Specificity and Stereochemistry. Most pyridine nucleotide-dependent oxidoreductases exhibit a strong preference for either 2′-phosphorylated (NADPH) or nonphosphorylated (NADH) nucleotide substrates (Dixon & Webb, 1979). Previous studies of the *E. coli* dihydrodipicolinate reductase (Tamir & Gilvarg, 1974) demonstrated that both NADPH and NADH were substrates, although no detailed kinetic analysis was reported. As seen in Table 3, NADH is a better substrate than NADPH on the basis of its approximately 2-fold higher relative *V/K* value. While exhibiting a maximum velocity 40% lower than NADPH, NADH exhibits a steady-state K_m value approximately 4 times lower. This is an unusual result, since the 2-phosphate of NADPH generally contributes substantially to the binding interaction between enzymes and their nucleotide substrate. Thus, in most enzymes which do exhibit dual nucleotide specificity, the steady-state K_m values of NADPH are significantly lower than the equivalent values for NAD(H) (e.g., *L. mesenteroides* glucose-6-phosphate dehydrogenase; Olive & Levy, 1967). In order to confirm that the differences in the steady-state K_m values of NADH and NADPH were truly reflective of the stronger binding of NADH, we performed titration calorimetry studies. As seen in Figure 2, both NADPH and NADH bind stoichiometrically, and noncooperatively, to dihydrodipicolinate reductase. Fits of the data yielded K_d values of 1.8 ± 0.09 and 0.26 ± 0.02 μM for NADPH and NADH, respectively. The ratio of these K_d values demonstrates that the difference in the apparent steady-state K_m values of the two nucleotides is due to *bona fide* tighter binding of NADH. The ability to use either reduced nucleotide, with essentially equal efficiency, may indicate the critical biological function of dihydrodipicolinate reductase.

The kinetic parameters of eight phosphorylated nucleotide analogs were determined to assess their interactions with the reductase. The α anomer of NADPH was shown to be a competent substrate, exhibiting a higher maximum velocity than the natural substrate, β-NADPH, but also a 10-fold higher K_m value. This is also an unusual, but precedented, observation. Thus α-NADPH exhibits a higher maximum velocity than β-NADPH with Old Yellow enzyme (Massey & Schopfer, 1986), presumably because of its 20 mV lower redox potential (−340 mV; Oppenheimer, 1982). All other phosphorylated nucleotide analogs exhibit lower maximum velocities than β-NADPH. Nucleotide substrates which contain a 3′-phosphate monoester or 2′-cyclic phosphodiester function exhibit significantly higher steady-state K_m values than their 2′-phosphate monoester analogs.

The stereochemistry of reduced pyridine nucleotide oxidation by dihydrodipicolinate reductase was investigated by preparing [(4*S*)-4-^{3}H]- and [(4*R*)-4-^{3}H]NADPH and chromatographically determining the radioactivity of the sepa-

Scheme 2: Enzymatic Synthesis of Diaminopimelate from ASA and Pyruvate[a]



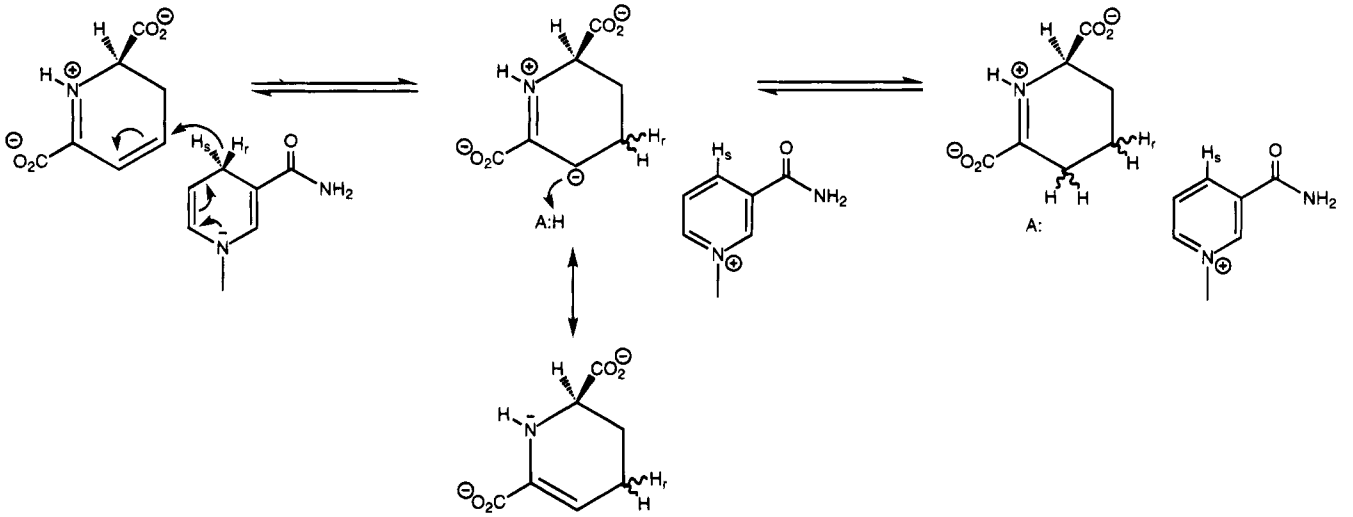
[a] Use of malic enzyme and [2-^{2}H]-D,L-malate to recycle the nucleotide allowed the determination of the position of hydride transfer to DHP by dihydrodipicolinate reductase to be determined.

rated products, $NADP^+$ and tetrahydrodipicolinate. Previous studies using [(4*S*)-4-^{3}H]NADPH suggested that dihydrodipicolinate reductase catalyzed the transfer of the *pro-R* hydrogen to substrate (Tamir & Gilvarg, 1974). Our studies confirm this result and have demonstrated the nearly complete (95%)[2] retention of tritium in $NADP^+$, when [(4*S*)-4-^{3}H]NADPH is used as substrate, and the nearly complete (94%) transfer of tritium to tetrahydrodipicolinate when [(4*R*)-4-^{3}H]NADPH is used as substrate. These studies have allowed us to analyze the position of hydride transfer to dihydrodipicolinate using [(4*R*)-4-^{2}H]NADPH.

Chemical Mechanism. The mechanism of the dihydropicolinate reductase catalyzed reduction of the C−C double bond of dihydrodipicolinate, in particular the position of hydride transfer from NADPH, was investigated by taking advantage of the demonstrated transfer of the 4*R* hydrogen of NADPH to the product, tetrahydrodipicolinate. Tetrahydrodipicolinate, while more stable than the substrate, is capable of existing in both cyclic and open-chain forms (Robins et al., 1992) and was considered to be unsuitable

[2] [^{3}H]NADPH is unstable and is nonenzymatically broken down into numerous products during the incubation. This accounts for our inability to demonstrate 100% retention or transfer of ^{3}H into the product.

Scheme 3: Proposed Chemical Mechanism of *E. coli* Dihydrodipicolinate Reductase



for ^{1}H NMR analysis. We thus elected to convert the product of the reductase reaction, tetrahydrodipicolinate, to *meso*-diaminopimelate by the action of purified *B. sphaericus* *meso*-diaminopimelate dehydrogenase. This enzyme converts tetrahydrodipicolinate, ammonia, and NADPH to *meso*-diaminopimelate and has been demonstrated to transfer the *pro-S* hydrogen to the substrate to form the D stereocenter (Misono & Soda, 1980). As seen in Scheme 2, this sequence of coupled enzymatic reactions generates the dideuterated *meso*-diaminopimelate product. Mass spectrometric analysis of the product confirmed that the isolated *meso*-diaminopimelate contained two deuterium atoms. The 500 MHz ^{1}H NMR spectrum of all-protio *meso*-diaminopimelate, prepared identically to dideuterated *meso*-diaminopimelate, exhibited peaks centered at 4.35, 2.30, 1.96, and 1.83 ppm, which were assigned to the C1 and C1′ (2H's), C2 (4H's), C3 (1H), and C3′(1H) protons, respectively. A cluster of peaks between 3.8 and 4.1 ppm were not observed in commercial samples of diaminopimelate and appear to be impurities present in, or generated during, the enzymatic synthetic reactions. The 500 MHz ^{1}H NMR spectrum of dideuterated *meso*-diaminopimelate, synthesized as shown in Scheme 2, exhibited peaks centered at 4.35 (1H), 2.30 (4H's), and 1.96 (1H) ppm. The reduction in intensity of the 4.35 ppm peak from 2 protons to 1, due to deuterium transfer from NADPD to the imine of DAP, catalyzed by diaminopimelate dehydrogenase, and the loss of the 1.83 ppm peak, due to the transfer of deuterium from NADPD to the β position of the α,β-unsaturated imine by dihydrodipicolinate reductase, allow us to write a chemical mechanism for dihydrodipicolinate reductase.

The chemical mechanism for *E. coli* dihydrodipicolinate reductase involves hydride ion transfer from the C_{4R} position of NAD(P)H to the C4 position of dihydrodipicolinate (Scheme 3). The resulting carbanion is resonance stabilized by the adjacent imine and presumably can form the more stable enamine intermediate pictured in Scheme 3. Tautomerization and enzymatic-assisted protonation at C3 yields the product, tetrahydrodipicolinate. While we currently favor this stepwise mechanism, it is possible that the carbon–carbon double bond is reduced in a concerted fashion. In the formally analogous reaction catalyzed by steroid 5α-reductase, the potential existence of an enolate intermediate, analogous to the enamine intermediate pictured in Scheme 3, has been used to design stable steroidal acrylates, which are effective inhibitors of the enzyme (Levy et al., 1990). Future studies will focus on the details of this chemical mechanism, including the identity of groups responsible for acid/base assistance, and the determination of rate-limiting steps in the chemical mechanism. These studies will be aided by the detailed three-dimensional structure of this enzyme reported in the following paper.

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REFERENCES

Bachmann, B. J. (1990) *Microbiol. Rev. 54*, 130–197.

Berges, D. A., Dewolf, W. E., Dunn, G. L., Grappel, S. F., Newmann, S. J., Taggart, J. J., & Gilvarg, C. (1986) *J. Med. Chem. 29*, 89–95.

Black & Wright (1955) *J. Biol. Chem. 213*, 39–50.

Bouvier, J. C., Richaud, F., Richaud, J. C. P., & Stragier (1984) *J. Biol. Chem. 259*, 14829–14834.

Cirrilo, J. D., Weisbrod, T. R., Banerjee, A., Bloom., B. R., & Jacobs, W. R., Jr. (1994) *J. Bacteriol. 176*, 4424–4429.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.

Couper, L., Robins, D. J., & Chrystal, E. J. T. (1992) *Tetrahedron Lett. 33*, 2717.

Cremer, J., Eggeling, L., & Sahm, H. (1991) *Mol. Gen. Genet. 220*, 478–480.

Dixon, M., & Webb, E. C. (1979) *Enzymes*, 3rd ed., pp 684 and 761, Longman Group, London.

Gelb, M. H., Lin, Y., Pickard, M. A., Song, Y., & Vederas, J. C. (1990) *J. Am. Chem. Soc. 112*, 4932–4942.

Gilvarg, C., & Farkas, W. (1965) *J. Biol. Chem. 240*, 4717–4722.

Gilvarg, C., & Weinberger, S. (1970) *J. Bacteriol.* 101, 323–324.

Gough, S. P., Kannangara, C. G., & Bock, K. (1989) *Carlberg Res. Commun. 54*, 99–108.

Houston, B. B., Chisholm, G. D., & Habib, F. H. (1987) *Steroids 49*, 355–369.

Karsten, W. E., & Viola, R. E. (1991) *Biochim. Biophys. Acta 1077*, 209–219.

Kelly, B., & White, P. J. (1965) *Biochem. J. 96*, 75−84.
Lam, L. K. P., Arnold, L. D., Kalantar, T. H., Kelland, J. G., Lane-Bell, P. M., Palcic, M. M., Pickard, M. A., & Vederas, J. C. (1988) *J. Biol. Chem. 263*, 11814−11819.
Levy, M. A., Brandt, M., & Greway, A. T. (1990) *Biochemistry 29*, 2808−2815.
Liang, T., Heiss, E., Ostrove, S., Rasumusson, G. H., & Cheung, A. H. (1983) *Endocrinology 112*, 1460−1468.
Lin, Y., Myhrman, R., Schrag, M. L., & Gelb, M. H. (1988) *J. Biol. Chem. 263*, 1622−1627.
Massey, V., & Schopfer, L. M. (1986) *J. Biol. Chem. 261*, 1215−1222.
Misono, H., & Soda, K. (1980) *J. Biol. Chem. 255*, 10599−10605.
Olive, C., & Levy, R. C. (1967) *Biochemistry 6*, 730−736.
Oppenheimer, N. J. (1982) in *The Pyridine Nucleotide Coenzymes* (Everse, J., Anderson, B., & You, K., Eds.) pp 51−81, Academic Press, New York.
Orr, G. A., & Blanchard, J. S. (1984) *Anal. Biochem. 142*, 232−234.
Peterkofsky, B., & Gilvarg, C. (1961) *J. Biol. Chem. 236*, 1432−1438.
Pissabarro, A., Malumbres, M., Mateos, L. M., Oguiza, J. A., & Martin, J. F. (1993) *J. Bacteriol. 175*, 2743−2749.
Richaud, F., Richaud, C., Ratet, P., & Patte, J. C. (1986) *J. Bacteriol. 166*, 297−300.
Robins, D. J., Couper, L., & Chrystal, E. J. T. (1992) *Tetrahedron Lett. 33*, 2717−2720.
Robins, D. J., Tudor, D. W., & Lewis, T. (1993) *Synthesis*, 1061.
Scapin, G., Blanchard, J. S., & Sacchettini, J. C. (1995) *Biochemistry 34*, 3502−3512.
Schrumpf, B., Schwarzer, Kalinowski, J., Puhler, A., Eggrling, L., & Sahm, H. (1991) *J. Bacteriol. 173*, 4510−4516.
Shedlarski, J. G., & Gilvarg, C. (1970) *J. Biol. Chem. 245*, 1362−1373.
Simms, S. A., Voige, W. H., & Gilvarg, C. (1984) *J. Biol. Chem. 259*, 2734−2741.
Simon, H., & Buhler, M. (1982) *Hoppe-Seyler's Z. Physiol. Chem. 363*, 609−625.
Tamir, H., & Gilvarg, C. (1974) *J. Biol. Chem. 249*, 3034−3040.
Vanoni, M. A., Wong, K. K., Ballou, D. P., & Blanchard, J. S. (1990) *Biochemistry 29*, 5790−5796.
Wedler, F. C., & Shames, S. L. (1984) *Arch. Biochem. Biophys. 235*, 359−370.
Westerik, J. O. C., & Wolfenden, R. (1974) *J. Biol. Chem. 249*, 6351−6353.
Wietzerbin, J., Das, B. C., Petit, J. F., Lederer, E., Leyh-Bouille, M., & Ghuysen, J. M. (1974) *Biochemistry 13*, 3471−3476.
Wiseman, J. S., & Nicholos, J. S. (1984) *J. Biol. Chem. 259*, 8907−8914.

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