Mechanistic Studies on *trans*-2,3-Dihydro-2,3-dihydroxybenzoate Dehydrogenase (Ent A) in the Biosynthesis of the Iron Chelator Enterobactin†

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ABSTRACT: The enzyme 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (2,3-diDHB dehydrogenase, hereafter Ent A), the product of the enterobactin biosynthetic gene *ent*A, catalyzes the NAD^+-dependent oxidation of the dihydroaromatic substrate 2,3-dihydro-2,3-dihydroxybenzoate (2,3-diDHB) to the aromatic catecholic product 2,3-dihydroxybenzoate (2,3-DHB). The catechol 2,3-DHB is one of the key siderophore units of enterobactin, a potent iron chelator secreted by *Escherichia coli*. To probe the reaction mechanism of this oxidation, a variety of 2,3-diDHB analogues were synthesized and tested as substrates. Specifically, we set out to elucidate both the regio- and stereospecificity of alcohol oxidation as well as the stereochemistry of NAD^+ reduction. Of those analogues tested, only those with a C3-hydroxyl group (but not a C2-hydroxyl group) were oxidized to the corresponding ketone products. Reversibility of the Ent A catalyzed reaction was demonstrated with the corresponding NADH-dependent reduction of 3-ketocyclohexane- and -cyclohexene-1-carboxylates but not the 2-keto compounds. These results establish that Ent A functions as an alcohol dehydrogenase to specifically oxidize the C3-hydroxyl group of 2,3-diDHB to produce the corresponding 2-hydroxy-3-oxo-4,6-cyclohexadiene-1-carboxylate (Scheme II) as a transient species that undergoes rapid aromatization to give 2,3-DHB. Stereospecificity of the C3 allylic alcohol group oxidation was confirmed to be 3*R* in a 1*R*,3*R* dihydro substrate, **3**, and hydride transfer occurs to the *si* face of enzyme-bound NAD^+.

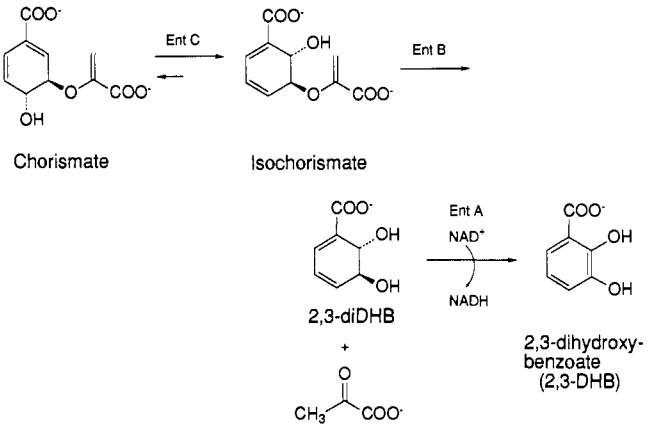
Organisms need iron for growth. When intracellular concentrations of iron fall, bacteria synthesize and secrete molecules termed siderophores, which strongly chelate ferric ions; the ferric chelate is then transported into the cell, where it releases iron for cellular metabolism. Enterobacteria such as *Escherichia coli* or *Salmonella* use two types of functional groups for Fe^{3+} chelation, hydroxamates and catechols, in such molecules as aerobactin and enterobactin, respectively (Neilands et al., 1981).

In the enterobactin pathway, the catechol functionality is presented by three 2,3-dihydroxybenzoate molecules in amide linkages to a serine trilactone scaffolding. The biosynthesis of the catecholic 2,3-dihydroxybenzoate occurs in three steps from the common aromatic pathway intermediate chorismate by consecutive action of isochorismate synthase (Ent C), isochorismatase (Ent B), and 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (Ent A) (Scheme I) (Young & Gibson, 1969; Young et al., 1971; Nahlik et al., 1987). The nucleotide sequence of the *ent*C, *ent*B, and *ent*A genes has been determined (Ozenberger et al., 1989; Elkins & Earhart, 1988; Liu et al., 1989; Nahlik et al., 1989). The three genes are clustered together in an iron-controlled regulon, and we have recently described their subcloning and the overproduction and purification to homogeneity of each of these three enzymes (Liu et al., 1989, 1990; Rusnak et al., 1990).

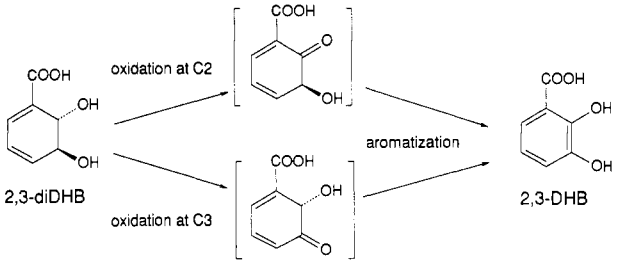
In the present study, we focus on the mechanism of Ent A, an NAD-linked dehydrogenase that catalyzes the aromatization of *trans*-2,3-dihydro-2,3-dihydroxybenzoate. Our expectation was that the enzyme functions as an alcohol dehydrogenase and proceeds by oxidation of either the C2- or C3-alcohol group of 2,3-diDHB to the corresponding ketone (Scheme II). The initial product tautomer, the 2- or the 3-dienone, presumably isomerizes very rapidly to the aromatic catechol and thereby renders cryptic the initial regiospecificity of enzyme oxidation.

To address such questions of regiospecificity and stereospecificity of this catechol-forming *trans*-dihydrodiol de-

Scheme I: Biosynthetic Pathway from Chorismic Acid to 2,3-DHB



Scheme II: Possible Mechanism for 2,3-DHB Formation from 2,3-diDHB Catalyzed by Ent A



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hydrogenase, we have prepared analogues of 2,3-dihydro-2,3-dihydroxybenzoate that contain a hydroxyl group at either C2 or C3 and used them to delineate several features of Ent A action.

Experimental Procedures

Materials

β-NAD (grade III), β-NADH (grade III, disodium salt), and *cis*-3,5-cyclohexadiene-1,2-diol (**8**) were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol-d_4 (99.9% atom % D), *tert*-butyldimethylsilyl chloride [*t*-Bu(Me)$_2$SiCl], (dimethylamino)pyridine (DMAP), pyridinium dichromate (PDC), tetrabutylammonium fluoride (*n*-Bu$_4$NF; 1 M solution in THF), 2-cyclohexen-1-ol (**10**), cyclohexanol (**11**), cyclohexanone (**25**), and 2-cyclohexen-1-one (**26**) were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). *m*-Hydroxybenzoic acid and 2,3-dihydroxybenzoic acid were purchased from Eastman Organic Chemicals (Rochester, NY). D_2O (99.9% atom % D) was purchased from Cambridge Isotope Laboratories (Woburn, MA). Rhodium(II) octanoate dimer [$Rh_2(Oct)_4$] was purchased from Strem Chemicals Inc. (Newburyport, MA). (*R*)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride [(+)-MTPA-Cl] was obtained as described (Dale et al., 1969). Silica gel 60 (70–230 mesh) for column chromatography, silica gel 60 thin-layer plates (for TLC; with F-254 fluorescent indicator and 0.25-mm layer thickness), and silica gel 60 preparative thin-layer plates (for PTLC; 0.50-mm layer thickness) were from Merck (Darmstadt, West Germany).

trans-2,3-Dihydro-2,3-dihydroxybenzoate dehydrogenase (Ent A) was isolated from a culture growth of *E. coli* JM105/pJLK-2 as described (Liu et al., 1989) and purified to a specific activity of 66 units/mg. Alanine racemase was isolated from a culture growth of *E. coli* W3110/pMDala-3 as described (Esaki et al., 1986) and purified to a specific activity of 255 units/mg. L-Alanine dehydrogenase (from *Bacillus subtilis*, 20 units/mg) and esterase (type I from porcine liver, 335 units/mg) were purchased from Sigma Chemical Co. (St. Louis, MO).

Synthetic Methodology

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on one of the following instruments: Varian XL-300, Varian Gemini 300, or Bruker WM-250. Chemical shifts are reported as δ values in ppm relative to tetramethylsilane (0.00 ppm). Unless otherwise indicated, ^{1}H NMR spectra were obtained in $CDCl_3$. Infrared (IR) spectra were recorded on a Perkin-Elmer 397 or Mattson FT infrared spectrometer. Mass spectral data were obtained on a Finnegan MAT8200 mass spectrometer by using electron-impact (EI) ionization at 70 eV.

All reactions were monitored by silica gel 60 (F-254) thin-layer chromatography with a UV lamp (254 nm), $KMnO_4$ solution (0.5 g dissolved in 100 mL of water) for olefins, PMA (5% in 95% ethanol) for alcohols, and/or 10% sulfuric acid solution. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials.

Preparation of 2,3-diDHB Analogues. The following compounds used in this study were synthesized according to the following procedures: (±)-*trans*-2,3-dihydro-2,3-dihydroxybenzoic acid (**1**) (DeMarinis et al., 1974; Quinn and Berchtold, manuscript in preparation); methyl (±)-2,3-dihydro-3-hydroxybenzoate (**2**) (McGowan & Berchtold, 1982; Kronis and Berchtold, unpublished results); (±)-*cis*-5-hydroxy-3-cyclohexene-1-carboxylic acid (**3**) (Kato et al., 1975); methyl (±)-3-hydroxy-1-cyclohexene-1-carboxylate (Gajewski et al., 1987); methyl (±)-3-hydroxy-1,4-cyclohexadiene-1-carboxylate (**5**) (Danishefsky et al., 1978; Pawlak & Berchtold, 1987); (±)-*cis*-3-hydroxycyclohexane-1-carboxylic aid (**6**) and (±)-*trans*-3-hydroxycyclohexane-1-carboxlic acid (**7**) (Noyce & Denney, 1952); (+)-*trans*-1,3-cyclohexadiene-5,6-diol (**9**) and (−)-*trans*-1,3-cyclohexadiene-5,6-diol (**21**) (Ganey et al., 1989); (±)-*trans*-3-cyclohexene-1,2-diol (**12**) (Ross et al., 1982); 2,4-cyclohexadien-1-ol (**13**) (Apparu & Barrelle, 1977); methyl (±)-2-acetoxy-3-hydroxy-6-cyclohexene-1-carboxylate (Quinn and Berchtold, manuscript in preparation); methyl (±)-(1α,5α,6α)-5-hydroxy-7-oxabicyclo[4.1.0]hept-3-ene-3-carboxylate (Hoare et al., 1983; Pawlak & Berchtold, 1987); (±)-*trans*-3,4-dihydro-3,4-dihydroxy-1-benzoic acid (**16**) (Ikota & Ganem, 1978; Padykula and Berchtold, unpublished results); methyl (±)-*cis*-2-hydroxy-3-cyclohexene-1-carboxylate (Ayral-Kaloustian et al., 1978); (±)-*cis*-2-hydroxycyclohexane-1-carboxylic acid (**18**) (Gabor et al., 1970); (±)-*trans*-2-hydroxycyclohexane-1-carboxylic acid (**19**) (Yang & Dinesho, 1988); 3-oxo-1-cyclohexene-1-carboxlic acid (**23**) (Bugg et al., 1988); methyl (±)-2-oxocyclohexane-1-carboxylate (Tsuda et al., 1986); methyl 2-oxo-3-cyclohexene-1-carboxylate (**28**) (Mander & Sethi, 1983); methyl 2-oxo-6-cyclohexene-1-carboxylate (**29**) (Liotta et al., 1981).

3-Oxocyclohexane-1-carboxylic acid (**22**) was prepared from **24** by hydrogenation over 5% palladium on carbon. ^{1}H NMR spectral data were consistent with those previously reported for this compound (Allan et al., 1981).

3-Oxo-4-cyclohexene-1-carboxylic acid (**24**) was prepared from **3** by Jones oxidation. ^{1}H NMR spectral data were consistent with those previously reported for this compound (Webster & Silverstein, 1988).

3-Hydroxy-1-cyclohexene-1-carboxylic Acid (**4**). To a solution of methyl 3-hydroxy-1-cyclohexene-1-carboxylate (2.0 g, 12.8 mmol) in THF/H_2O (2:1, 90 mL) at 0 °C was added dropwise NaOH (26.9 mL of a 1.0 N solution, 26.9 mmol, 2.1 equiv). The reaction mixture was stirred for 4 h at 0 °C and then concentrated to $^1/_3$ volume. Amberlite IR-120 (plus) acidic resin was added until pH 3 was reached. The resin was removed by suction filtration and rinsed with additional H_2O, and the filtrate was concentrated on a high-vacuum rotary evaporator to give 1.93 g (92%) of **4** as a waxy white solid: IR (KBr) ν_{max} 3394, 3130, 2986, 2538, 1698, 1402, 1262 cm^{-1}; ^{1}H NMR (300 MHz, CD_3OD) δ 6.83 (1 H, s), 4.27 (1 H, m), 2.18 (2 H, s), 1.78–1.93 (2 H, m), 1.48–1.61 (2 H, m); ^{13}C NMR (300 MHz, CD_3OD) δ 170.8, 144.1, 131.8, 70.8, 68.0, 24.6, 22.8; MS *m/z* (rel intensity) 142 (M^+, 14.4), 124 (27.9), 113 (6.5), 97 (100.0), 86 (16.6), 79 (25.3), 68 (28.1), 55 (20.7); HRMS calcd for $C_7H_{10}O_3$ (M^+) 142.0630, found 142.0630.

(±)-*trans-2,3-Dihydroxy-6-cyclohexene-1-carboxylic Acid* (**14**). To a solution of methyl 2-acetoxy-3-hydroxy-6-cyclohexene-1-carboxylate (0.521 g, 2.4 mmol) in THF/H_2O (2:1, 60 mL) at 0 °C was added dropwise NaOH (7.3 mL of a 1.0 N solution, 7.3 mmol, 3.0 equiv). The reaction mixture was stirred for 5 h at room temperature and then concentrated to $^1/_3$ volume. Amberlite IR-120 (plus) acidic resin was added until pH 3 was reached. The resin was removed by suction filtration and rinsed with additional H_2O, and the filtrate was concentrated on a high-vacuum rotary evaporator to give 0.338 g (89%) of **14** as an oily solid: IR (KBr) ν_{max} 3455, 3349, 3116, 2925, 1691, 1400, 1062 cm^{-1}; ^{1}H NMR (300 MHz, CD_3OD) δ 7.09 (1 H, t, J = 4 Hz), 4.31 (1 H, d, J = 4 Hz), 3.88 (1 H, quintet, J = 3 Hz), 2.40–2.15 (2 H, m), 1.92–1.82 (1 H, m), 1.66–1.75 (1 H, m); ^{13}C NMR (300 MHz, CD_3OD) δ 171.5, 141.8, 133.8, 66.7, 31.9, 25.2, 20.3; MS *m/z* (rel

intensity) 158 (M^+, 0.3), 140 (2.2), 114 (75.7), 96 (100.0), 77 (6.9), 68 (75.4); HRMS calcd for $C_7H_{10}O_4$ (M^+ 158.0579, found 158.0579.

(±)-(1α,5α,6α)-5-Hydroxy-7-oxabicyclo[4.1.0]hept-3-ene-3-carboxylic Acid (**15**). To a solution of methyl (±)-[1α,5α,6α]-5-hydroxy-7-oxabicyclo[4.1.0]hept-3-ene-3-carboxylate (0.486 g, 2.8 mmol) in THF/H_2O (2:1, 60 mL) at 0 °C was added dropwise NaOH (5.85 mL of a 1.0 N solution, 5.85 mmol, 2.05 equiv). The reaction mixture was stirred for 4 h at 0 °C and then concentrated to $^1/_3$ volume. Amberlite IR-120 (plus) acidic resin was added until pH 3 was reached. The resin was removed by suction filtration and rinsed with additional H_2O, and the filtrate was concentrated on a high-vacuum rotary evaporator to an oil. The oil was purified by flash chromatography (EtOAc with AcOH 1% increased to 5%) to give 0.35 g (81%) of **15** as an off-white solid: IR (KBr) ν_{max} 3320, 3140, 2950, 2582, 1691, 1400, 1273 cm^{-1}; ^1H NMR (300 MHz, CD_3OD) δ 6.64 (1 H, d, J = 2 Hz), 4.58 (1 H, t, J = 2 Hz), 3.46 (2 H, m), 2.9 (1 H, d, J = 20 Hz), 2.43 (1 H, dq, J = 20, 2 Hz); ^{13}C NMR (300 MHz, CD_3OD) δ 170.0, 138.3, 128.0, 66.5, 55.9, 52.3, 25.2; MS m/z (rel intensity) 156 (M^+, 1.1), 155 (2.3), 138 (11.0), 122 (83.6), 111 (36.7), 105 (100.0), 81 (24.7), 77 (81.1), 53 (44.5), 51 (45.7); HRMS calcd for $C_7H_8O_4$ (M^+) 156.0423, found 156.0422.

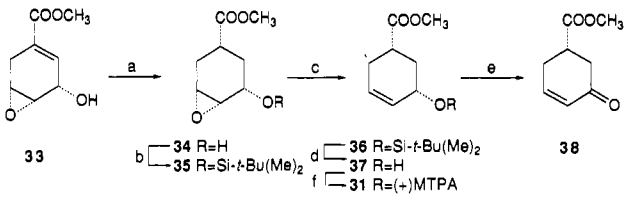
Methyl 3-Deuterio-3-hydroxy-1-cyclohexene-1-carboxylate (**39**). To a solution of methyl 3-oxo-1-cyclohexene-1-carboxylate (2.25 g, 14.6 mmol) in dry MeOH (30 mL) at 0 °C under N_2 was added sodium borodeuteride (98%, 0.642 g, 15.3 mmol, 1.05 equiv). The reaction mixture was stirred for 30 min at 0 °C, diluted with ethyl acetate (60 mL), and poured into saturated NH_4Cl (30 mL). The layers were separated, and the aqueous phase was extracted with ethyl acetate (3 × 50 mL). The organic layers were combined, dried ($MgSO_4$), and filtered. The filtrate was concentrated in vacuo and purified by flash chromatography (elution with EtOAc/petroleum ether 1:4 to 1:1) to give **39** (1.76 g, 77%) as a pale yellow oil contaminated with 4% nondeuterated compound: ^1H NMR (300 MHz) δ 6.90 (1 H, s), 3.77 (3 H, s), 2.49–2.60 (1 H, br s), 2.28 (2 H, m), 1.79–2.00 (2 H, m), 1.54–1.71 (2 H, m).

3-Deuterio-3-hydroxy-1-cyclohexene-1-carboxylic Acid (**40**). To a solution of methyl 3-deuterio-3-hydroxy-1-cyclohexene-1-carboxylate (**39**) (1.76 g, 11.2 mmol) in THF/H_2O (2:1, 80 mL) at 0 °C was added dropwise NaOH (23.5 mL of a 1.0 N solution, 23.5 mmol, 2.1 equiv). The reaction mixture was stirred for 3 h at 0 °C and then concentrated to $^1/_3$ volume. Amberlite IR-120 (plus) acidic resin was added until pH 3 was reached. The resin was removed by suction filtration and rinsed with additional H_2O, and the filtrate was concentrated on a high-vacuum rotary evaporator to give 1.44 g (90%) of **40** as a waxy white solid: IR (KBr) ν_{max} 3386, 3122, 2952, 2649, 1697, 1399, 1267 cm^{-1}; ^1H NMR (300 MHz, CD_3OD) δ 6.83 (1 H, s), 2.20 (2 H, s), 1.78–2.20 (2 H, m), 1.46–1.61 (2 H, m); ^{13}C NMR (300 MHz, CD_3OD) δ 171.2, 141.9, 133.7, 66.2 (t, J = 22 Hz), 31.7, 25.1, 20.2; MS m/z (rel intensity) 143 (M^+, 16.2), 125 (29.6), 98 (100.0), 87 (16.2), 80 (19.0), 69 (37.2); HRMS calcd for $C_7D_1H_9O_3$ (M^+) 143.0692, found 143.0700.

Preparation of Optically Active Compounds **37** *and* **38**. The synthetic outlines are shown in Scheme III.

Methyl (1R,3R,5S,6S)-5-Hydroxy-7-oxabicyclo[4.1.0]-heptane-3-carboxylate (**34**). To a solution of α,β-unsaturated ester **33** [$[\alpha]^{25}_D$ +50.5° (c 1.0, $CHCl_3$), 98% ee; 500 mg, 2.9 mmol] (Pawlak & Berchtold, 1987) in MeOH (30 mL) was

Scheme III: Preparation of Optically Active Compounds



[a] H_2 (50 psi), Pd–C, MeOH. [b] t-Bu(Me)$_2$SiCl, DMAP, Et_3N, CH_2Cl_2. [c] N_2=C(COOCH$_3$)$_2$ $Rh_2(OAc)_4$, C_6H_6. [d] n-Bu_4NF, THF. [e] PDC, CH_2Cl_2. [f] (+)-MTPA-Cl, Et_3N, DMAP.

added 10% Pd–C (50 mg). The reaction mixture was shaken at room temperature under H_2 (50 psi) for 60 min. The catalyst was removed by filtration through a Celite pad, and the filtrate was concentrated in vacuo to give an oily residue, which was purified by column chromatography on silica gel (elution with EtOAc/hexane 1:1 to 2:1) to give **34** (200 mg, 40%) as a colorless oil: $[\alpha]^{25}_D$ −8.2° (c 1.0, $CHCl_3$); IR (NaCl film) ν_{max} 3420, 3010, 2960, 1738, 1441 cm^{-1}; ^1H NMR (300 MHz) δ 4.08 (1 H, m), 3.70 (3 H, s), 3.34 (2 H, m), 2.58 (1 H, br d, J = 7 Hz), 2.42 (1 H, m), 2.12 (2 H, m), 1.97 (1 H, ddd, J = 13, 7, 4 Hz), 1.66 (1 H, ddd, J = 13, 11, 9 Hz); ^{13}C NMR (300 MHz, $CDCl_3$) δ 174.6, 67.4, 55.3, 53.8, 52.0, 38.0, 30.1, 25.4; MS m/z (rel intensity) 173 ([M + H]$^+$, 1.3), 155 (1.4), 142 (10.0), 128 (18.0), 116 (40.3); HRMS calcd for $C_8H_{12}O_4$ ([M − OH]$^+$) 155.0708, found 155.0709.

Methyl (1R,3S,5S,6S)-5-[(tert-Butyldimethylsilyl)oxy]-7-oxabicyclo[4.1.0]heptane-3-carboxylate (**35**). To a solution of alcohol **34** (166 mg, 0.97 mmol) in dry CH_2Cl_2 (6 mL) was added Et_3N (270 μL, 1.93 mmol), DMAP (54 mg, 0.44 mmol), and *tert*-butyldimethylsilyl chloride (291 mg, 1.93 mmol). The mixture was stirred at room temperature for 15 h and extracted several times with ether. The combined organic phase was washed with brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give an oily residue, which was purified by column chromatography on silica gel (elution with EtOAc/hexane 15:1 to 10:1) to give **35** (270 mg, 98%) as a colorless oil: $[\alpha]^{25}_D$ +8.8° (c 1.0, $CHCl_3$); IR (NaCl film) ν_{max} 2970, 2870, 1740, 1465, 1445 cm^{-1}; ^1H NMR (300 MHz) δ 4.10 (1 H, ddd, J = 11, 5, 2 Hz, 3.68 (3 H, s), 3.26 (1 H, dd, J = 5, 4 Hz), 3.16 (1 H, br d, J = 4 Hz), 2.35 (1 H, m), 2.10 (1 H, m), 2.03 (1 H, m), 1.83 (1 H, ddd, J = 12, 4, 2 Hz), 1.64 (1 H, ddd, J = 12, 12, 11 Hz), 0.91 (9 H, S), 0.13 (3 H, s), 0.11 (3 H, s); ^{13}C NMR (300 MHz) δ 174.5, 69.4, 55.7, 52.7, 51.8, 38.6, 30.0, 25.6, 24.9, 17.9, −4.8, −4.9; MS m/z (rel intensity) 255 ([M − OCH_3]$^+$, 7.1), 229 (53.4), 211 (8.7), 197 (17.5); HRMS calcd for $C_{14}H_{26}O_4Si$ ([M − CH_3]$^+$) 271.1366, found 271.1367.

Methyl (1S,5S)-5-[(tert-Butyldimethylsilyl)oxy]-3-cyclohexene-1-carboxylate (**36**). To a solution of silyl ether **35** (240 mg, 0.84 mmol) in dry benzene (10 mL) was added rhodium(II) octanoate dimer (20 mg, 0.04 mmol). To this light green solution was added dimethyl diazomalonate (525 mg, 2.8 mmol) in benzene (10 mL) via cannula, and the mixture was stirred at room temperature under N_2. Vigorous evolution of N_2 was evident, and the solution turned brown. After 60 min the entire mixture was subjected to column chromatography on silica gel (elution with EtOAc/hexane 1:10) to give **36** (169 mg, 75%) as a colorless oil: $[\alpha]^{25}_D$ −6.2° (c 1.0, $CHCl_3$); IR (NaCl film) ν_{max} 2970, 2945, 2860, 1745, 1465, 1440, 1395 cm^{-1}; ^1H NMR (300 MHz) δ 5.73 (1 H, br d, J = 10 Hz), 5.62 (1 H, br d, J = 10 Hz), 4.38 (1 H, m), 3.71 (3 H, s), 2.66 (1 H, m), 2.25 (3 H, m), 1.67 (1 H, ddd, J = 12, 12, 11 Hz), 0.92 (9 H, s), 0.12 (3 H, s), 0.11 (3 H, s); ^{13}C NMR (300 MHz) δ 175.9, 132.6, 126.8, 68.1, 52.0, 38.9, 35.1,

27.6, 26.0, 18.3, −4.5, −4.6; MS *m/z* (rel intensity) 255 ([M − CH_3]$^+$, 1.5), 213 (38.6), 181 (59.1); HRMS calcd for $C_{14}H_{26}O_3Si$ ([M − CH_3]$^+$) 255.14165, found 255.1422.

Methyl (1S,5S)-5-Hydroxy-3-cyclohexene-1-carboxylate (**37**). To a solution of silyl ether **36** (139 mg, 0.51 mmol) in dry THF (5 mL) was added tetrabutylammonium fluoride (1.0 mL, 1.0 M solution in THF, 1.0 mmol). The mixture was stirred at room temperature for 30 min and extracted several times with EtOAc. The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give an oily residue, which was purified by column chromatography on silica gel (elution with EtOAc/hexane 1:2 to 1:1) to give **37** (65.0 mg, 81%) as a colorless oil: $[\alpha]^{25}_D$ −0.1° (*c* 1.0, $CHCl_3$); IR (NaCl film) ν_{max} 3400, 3040, 2965, 1735, 1455, 1440 cm^{-1}; ^{1}H NMR (300 MHz) δ 5.78 (2 H, br s), 4.30 (1 H, br s), 3.71 (3 H, s), 2.73 (1 H, dddd, *J* = 14, 10, 7, 3 Hz), 2.30 (3 H, m), 2.23 (1 H, br s), 1.75 (1 H, ddd, *J* = 12, 10, 8 Hz); ^{13}C NMR (300 MHz) δ 176.0, 131.0, 127.0, 65.9, 51.8, 37.6, 34.0, 27.2; MS *m/z* (rel intensity) 138 ([M − H_2O]$^+$, 6.3), 125 (4.6), 123 (2.4), 107 (6.1), 97 (95.4); HRMS calcd for $C_8H_{12}O_3$ ([M − H_2O]$^+$) 138.0681, found 138.0681.

Methyl (1S)-3-Oxo-4-cyclohexene-1-carboxylate (**38**). To a solution of alcohol **37** (35 mg, 0.24 mmol) in dry CH_2Cl_2 (5 mL) was added PDC (56 mg, 0.15 mmol). The mixture was stirred at room temperature for 2 h. To the mixture were added powdered anhydrous $MgSO_4$ and ether. After being stirred for 10 min, the reaction mixture was filtered. The filtrate was concentrated in vacuo to give an oily residue, which was purified by column chromatography on silica gel (elution with EtOAc/hexane 1:1 to 3:1) to give **38** (26.4 mg, 76%) as a colorless oil: $[\alpha]^{25}_D$ +82.3° (*c* 1.0, $CHCl_3$); IR (NaCl film) ν_{max} 3500, 2960, 1740, 1685, 1440, 1395 cm^{-1}; ^{1}H NMR (300 MHz) δ 6.98 (1 H, ddd, *J* = 10, 4, 4 Hz), 6.08 (1 H, ddd, *J* = 10, 2, 2 Hz), 3.73 (3 H, s), 3.11 (1 H, dddd, *J* = 14, 10, 7, 3 Hz), 2.7 (4 H, m); ^{13}C NMR (300 MHz) δ 197.4, 173.8, 148.0, 130.0, 76.6, 52.1, 39.5, 27.7; MS *m/z* (rel intensity) 154 (M$^+$, 11.0), 123 (13.0), 95 (93.2); HRMS calcd for $C_8H_{10}O_3$ (M$^+$) 154.0630, found 154.0630.

Preparation of the Mosher Ester **31**. *Methyl (1S,5S)-5-[(R)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetyl]-3-cyclohexene-1-carboxylate* (**31**). To a solution of alcohol **37** (10 mg, 0.06 mmol) in dry CH_2Cl_2 (2 mL) were added Et_3N (10 μL, 0.08 mmol), DMAP (5 mg, 0.04 mmol), and (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (20 mg, 0.08 mmol). The mixture was stirred at room temperature for 1 h and extracted several times with $CHCl_3$. The combined organic phase was washed with brine, dried ($MgSO_4$), and filtered. The filtrate was concentrated in vacuo to give an oily residue, which was subjected to PTLC (EtOAc/hexane 1:3) to give **31** (21.4 mg, 90%) as a colorless oil: IR (NaCl film) ν_{max} 2960, 1745, 1455, 1445 cm^{-1}; ^{1}H NMR (300 MHz) δ 7.52 (2 H, m), 7.40 (3 H, m), 5.89 (1 H, m), 5.66 (1 H, m), 5.60 (1 H, br d, *J* = 9 Hz), 3.69 (3 H, s), 3.54 (3 H, s), 2.76 (1 H, dddd, *J* = 14, 9, 7, 3 Hz) 2.50 (1 H, ddd, *J* = 12, 7, 4 Hz), 2.32 (2 H, m), 1.85 (1 H, ddd, *J* = 12, 12, 9 Hz); ^{13}C NMR (300 MHz) δ 174.9, 166.9, 132.8, 130.7, 130.2, 129.0, 127.9, 126.0, 72.2, 55.7, 52.2, 38.0, 30.4, 27.3; MS *m/z* (rel intensity) 372 (M$^+$, 0.02), 341 (0.02), 313 (0.1), 287 (0.3), 262 (0.5), 232 (0.6); HRMS calcd for $C_{18}H_{19}O_5F_3$ (M$^+$) 372.1185, found 372.1184.

Ent A Activity Measurement. All kinetic analyses were performed with a Perkin-Elmer Lambda 5 UV/vis spectrophotometer linked to a constant-temperature water bath at 37 °C in 1.0-mL total volume, 1.0-cm path length cuvettes. Ent A activity was monitored by continuous detection of the production of NADH from NAD$^+$ at 340 nm (ϵ_{340} = 6.22 mM^{-1} cm^{-1}) as described (Liu et al., 1989). Assay buffer in method A contained 50 mM potassium phosphate (KP_i, pH 7.4), 10 mM NAD$^+$, and Ent A (0.8–0.008 unit). Assay buffer in method B contained 50 mM KP_i (pH 7.4), 10 mM NAD$^+$, 64 units of esterase, and Ent A (0.8–0.008 unit). Assay buffer in the reverse reaction contained 50 mM KP_i (pH 7.4), 0.2 mM NADH, and Ent A (7.9–0.08 unit). These reaction mixtures were preincubated at 37 °C for 10, 20, and 10 min, respectively, before reaction was initiated with the addition of Ent A. Initial velocities were estimated by regression during the linear phase of the reaction. Standard errors ranged from 10 to 20%. The Michaelis constants (K_m) and turnover numbers (k_{cat}) were calculated with the nonlinear least-squares fitting routine HYPER (Cleland, 1979). Deuterium isotope effects on initial velocity were obtained by using method A buffer.

Inhibition Kinetics Determination. Inhibition constants were measured for certain inhibitors by determining K_m and V_{max} for 2,3-diDHB at various substrate and inhibitor concentrations (Segel, 1975). Assays were run according to the procedure given for Ent A activity measurement (vide supra). Single-point K_i values were determined by measuring Ent A activity with 2,3-diDHB (610 μM; $2K_m$) in the presence of 10 mM of each inhibitor. Approximate K_i values were calculated from $V_I = V_{max}[S]/\{K_m(1 + [I]/K_i) + [S]\}$.

Confirmation of Absolute Stereochemistry of **30** *and* **31** *during Ent A Reaction.* A mixture containing **3** (13.0 mg, 0.09 mmol), NAD$^+$ (130 mg, 0.20 mmol), and 100 units of Ent A in 10 mL of 50 mM KP_i buffer (pH 7.4) was incubated at 37 °C. When no further change in A_{340} was observed, the enzyme was removed by ultrafiltration with an Amicon PM10 membrane filter. The filtrate was adjusted to pH 3.0 with 1 N HCl and extracted with EtOAc several times. The combined organic phase was dried ($MgSO_4$) and concentrated in vacuo. To a ethereal suspension of the residue at 0 °C was added diazomethane in ether. The mixture was stirred at 0 °C for 10 min and concentrated in vacuo to give an oily residue, which was applied to PTLC (EtOAc/hexane 3:1) to give 5 mg of the corresponding ketone **30** (38%) and 8 mg of starting materials. All spectroscopic data of the ketone, thus obtained, were identical with those of the synthetic authentic compound except for optical rotation {$[\alpha]^{25}_D$ −83.5° (*c* 0.5, $CHCl_3$); authentic sample $[\alpha]^{25}_D$ +82.3° (*c* 1.0, $CHCl_3$)}.

The recovered starting material (8.0 mg, 0.05 mmol) was dissolved in dry CH_2Cl_2 (2 mL). To this solution were added Et_3N (10 mL, 0.08 mmol), DMAP (5 mg, 0.04 mmol), and (*R*)-(+)-α-methoxy-α(trifluoromethyl)phenylacetyl chloride [(+)-MTPA-Cl; 20 mg, 0.08 mmol]. The reaction mixture was stirred at room temperature for 15 h and extracted several times with $CHCl_3$. The combined organic phase was washed with brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give an oily residue, which was applied to PTLC (EtOAc/hexane 1:2) to give the (+)-MTPA ester (17.6 mg, 90%) as a mixture of diastereomers.

Determination of Stereospecificity of Hydride Transfer to NADH. The stereochemistry of the hydride transfer to NAD$^+$ with Ent A was determined by direct comparison with an authentic [4*R*-^{2}H]NADH prepared by the modification method as described by Esaki (1989).

Authentic [4*R*-^{2}H]NADH was prepared in the following manner. A mixture containing D-alanine (8.7 mg, 1 mmol), NAD$^+$ (14.6 mg, 0.22 mmol), 250 units of alanine racemase (140 μL; enzymes were dialyzed two times by rapid Centricon

filtration against ^2H_2O adjusted to p^{2}H 10), and 2.3 units of L-alanine dehydrogenase (100 μL) in 3 mL of ^2H_2O (adjusted to p^{2}H 10 with NaO^{2}H) was incubated at 37 °C. When no further change in A_{340} was observed, the enzyme was removed by ultrafiltration with an Amicon PM10 membrane filter, and [4*R*-^{2}H]NADH was isolated with a Pharmacia LKB Biotechnology Inc. fast protein liquid chromatography system equipped with a Mono Q HR 5/5 anion-exchange column. The flow rate was 0.5 mL/min. The chromatography was programmed in the following sequence: washing with water (1–7 min), linear gradient from 0 to 0.14 M NH_4HCO_3 (7–19 min), linear gradient from 0.14 to 0.25 M NH_4HCO_3 (19–23 min), linear gradient from 0.25 to 0.50 M NH_4HCO_3 (23–24 min), and washing with 0.5 M NH_4HCO_3 (24–30 min).

Isolation and Characterization of NAD^{2}H Prepared by Ent A. A reaction mixture containing [3-^{2}H]-3-hydroxy-1-cyclohexene-1-carboxylate (**40**) (10 mg, 0.7 mmol; 96% isotope purity), NAD^+ (13.3 mg, 0.22 mmol), and 2.5 units of Ent A (30 μL) in 3 mL of 50 mM potassium phosphate buffer (pH 7.4) was incubated at 37 °C. When no further change in A_{340} was observed, the enzyme was removed by ultrafiltration with an Amicon PM10 membrane filter and the NAD^{2}H was isolated as described above.

Results

The enzyme 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase has only recently been purified to homogeneity subsequent to the cloning, sequencing, and expression of the encoding *ent*A gene of *E. coli* (Nahlik et al., 1989; Liu et al., 1989). No mechanistic studies have previously been reported. The most probable chemical mechanism for conversion of the dihydrodiol to the catechol product is oxidation of either the C2- or the C3-alcohol moiety to the corresponding cyclohexadienone regioisomer by hydride transfer to NAD^+, shown in Scheme II. Either of these would then be expected to undergo facile aromatization to the catecholic 2,3-dihydroxybenzoate.

Regiochemistry of Ent A Oxidation. To probe the reaction pathway, a variety of 2,3-diDHB analogues have been synthesized and examined for enzyme activity. The data of Table I summarize the steady-state parameters for a number of these compounds. Compounds **1–7** are 3-hydroxy-1-carboxylate analogues that function as substrates. By contrast, the 2-hydroxycyclohexane- and -cyclohexenecarboxylates **17–19** are inhibitors but not substrates. Thus it is clear that Ent A carries out regiospecific oxidation at the C3-alcohol position. In addition to monitoring substrate-dependent NADH formation, we confirmed the identities of the enzymic oxidation products from compounds **2–5** as 3-hydroxybenzoate (from compounds **2** and **5**), 5-oxo-3-cyclohexenecarboxylic acid (from **3**), and 3-oxo-1-cyclohexenecarboxylic acid (from **4**) by thin-layer chromatography, ^{1}H NMR, and/or mass spectrometry as compared with authentic synthetic compounds. The conversion of the esters **2** and **5** to 3-hydroxybenzoate by Ent A requires prior treatment with pig liver esterase to yield the 3-hydroxycyclohexadienecarboxylic acids. (Chemical efforts to deblock the ester were not successful due to aromatization.)

Compounds **2–7** show decreasing catalytic efficiency (k_{cat}/K_m) compared to that of the natural substrate **1**. For example, the absence of the C2-alcohol has a 5-fold effect, expressed mostly in k_{cat}, whereas removal of either one of the olefinic links, as in the isomeric allylic alcohols **3** and **4**, had substantial effects on K_m as well as k_{cat}. The shape of **3** and **4** vs that of **1** and **2** will clearly differ. On the other hand, the ketone products from **3** and **4**, in contrast to those from **1**, **2**, and **5**, are stable and isolable and suggest that oxidation is separable from the epiphenomenon of aromatization. Furthermore, the oxidation of the saturated 3-hydroxycyclohexane-1-carboxylates **6** and **7** indicates that an allylic alcohol is not required, although both **6** and **7** are not fully oxidized to the corresponding 3-ketone products under the assay condition described (see Experimental Procedures). In addition, 1,3-cis-substituted substrate **6** gives about a 40-fold higher k_{cat}/K_m value than the trans substrate **7**.

To further probe the minimal alcohol structure for catalytic processing by Ent A, the decarboxy analogues **8–13** were tested and shown to be only poor substrates. Thus, while the C1-carboxyl group and allylic olefin are not strictly required, as exemplified by cyclohexanol (**11**), it is a barely detectable substrate. A direct comparison of **10** with **3** and **9** with **1** shows that the absence of the carboxylate moiety provides a 30–50-fold elevation of K_m and a 20–90-fold decrease in k_{cat}. Lastly, the enzyme only poorly tolerates substituents at the 4 and 5 positions, suggesting interference with side chains in the active site (compounds **15**, **16**, and **20**).

Reversibility of Ent A Oxidations. During the biosynthesis of enterobactin in times of iron limitation, the Ent A reaction is irreversible physiologically. In vitro with 2,3-dihydro-2,3-dihydroxybenzoate, it is also irreversible due to the aromatization of the nascent cyclohexadien-3-one product. To assess the reversibility with ketocyclohexane and ketocyclohexene substrates, compounds **22–29** were prepared and analyzed for enzyme-dependent NADH oxidation (Table II). The regiospecificity of the enzyme is corroborated in the back direction since none of the 2-ketocyclohexane- or -cyclohexenecarboxylates **27–29** were reduced while the 3-keto analogues **22–24** were processed to the corresponding alcohols. The best substrate was 3-oxocyclohexanecarboxylate (**22**), with 8–40-fold better catalytic efficiency for reduction than the enones **23** and **24**. As in the alcohol oxidation direction, ketone reduction could be detected for cyclohexanone itself and for 2-cyclohexenone, but again the absence of the carboxylate group lowered k_{cat}/K_m by 10^4–10^5-fold (compounds **25** and **26**).

Stereospecific Oxidation of Allylic Alcohol **3**. When 2,3-dihydro-2,3-dihydroxybenzoate was isolated as a natural product by Young et al. (1969), it was assigned the 2*S*,3*S* configuration. We noted in Table I that allylic alcohols **3** and **4**, in racemic form, were reasonable substrates and assumed that a single isomer would be accepted. In compounds **1**, **2**, and **4**, the 1,6 double bond imposes a pseudoequatorial orientation on the carboxylate, whereas in **3**, with the allylic double bond in the 4,5 locus, the C1 center can be *R* or *S*. To test that the enzyme was processing (±)-**3** with the anticipated 1*R*,3*R* stereospecificity, we first determined that the racemic 1*R*,3*R*/1*S*,3*S* mixture proceeded to less than 50% consumption, consistent with single diastereomer utilization. Racemic **3** was then treated on a large scale (13 mg) with Ent A and NAD^+ until no further change in absorbance at 340 nm occurred (Scheme IV). The product was then extracted, esterified, and separated from starting material to give **30** as product (38%) and recovered starting material. The optical rotation of **30**, $[\alpha]^{25}_D = -83.5°$ (*c* 0.5, $CHCl_3$), was equal but opposite in sign to that of the 1*S* isomer **38**, $[\alpha]^{25}_D = +82.3°$, synthesized in good yield as summarized in Scheme IV from the known chiral compound **33** (98% ee). Since the optical rotation of the methyl ester alcohol **37** was too small to use to determine the absolute stereochemistry of nonutilized starting material, the Mosher esters of both the standard and the enzymic samples were also synthesized and examined by NMR. As shown in Figure 1, ^{1}H NMR of the diastereomeric

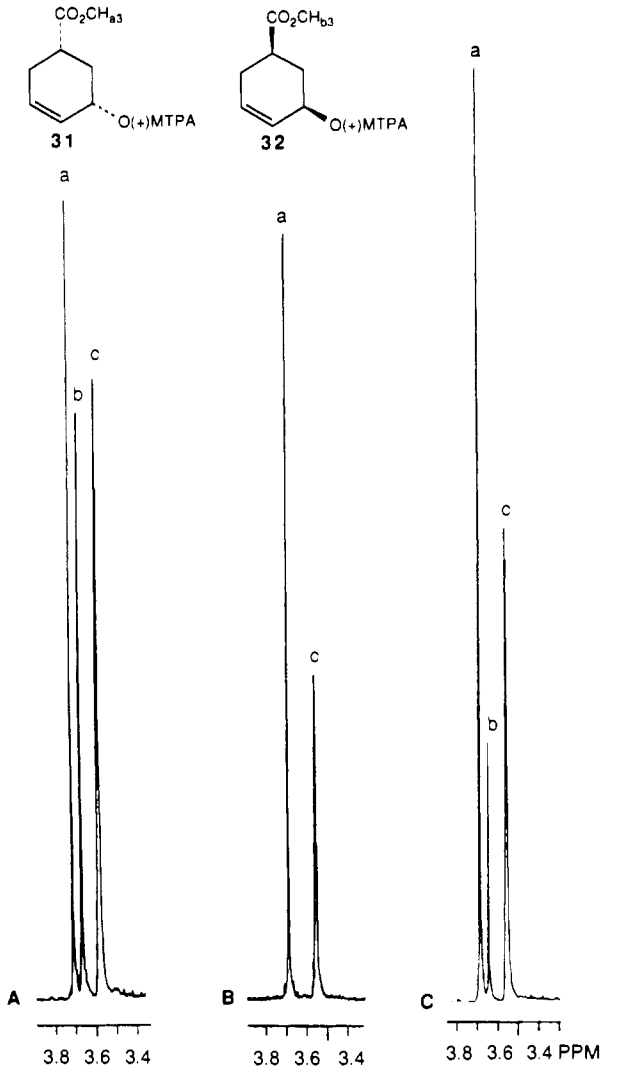
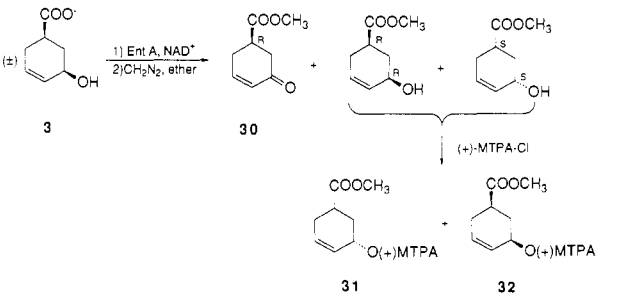


FIGURE 1: Methoxy group region of the ^1H NMR spectra of (+) Mosher esters. (Only the 3.3–3.9 ppm region is illustrated.) (A) Spectrum of racemic compound [a, methoxy group of methyl ester of 1*S*,3*S* compound; b, methoxy group of methyl ester of 1*R*,3*R* compound; c, methoxy group of (+) Mosher ester]. (B) Spectrum of 1*S*,3*S* authentic compound. (C) Spectrum of the recovered starting materials after Ent A reaction.

Scheme IV: Confirmation of Absolute Stereochemistry during Ent A Reaction



methoxy group of the methyl ester of (1*S*,3*S*)-**31** yields a peak at 3.70 ppm, whereas the Mosher ester of the 1*R*,3*R* isomer gives a peak at 3.65 ppm. Figure 1C shows that, in the enzymic oxidation of racemic **3**, there is indeed selective consumption of the 1*R*,3*R* isomer.

Stereospecificity of NAD⁺ Reduction. Having confirmed the stereospecific 3*R* oxidation of substrate **3**, we next turned our attention to the determination of hydride transfer stereochemistry by analysis of whether NAD^+ was reduced from the *re* or *si* face. This could be addressed by using a 3-deuterio

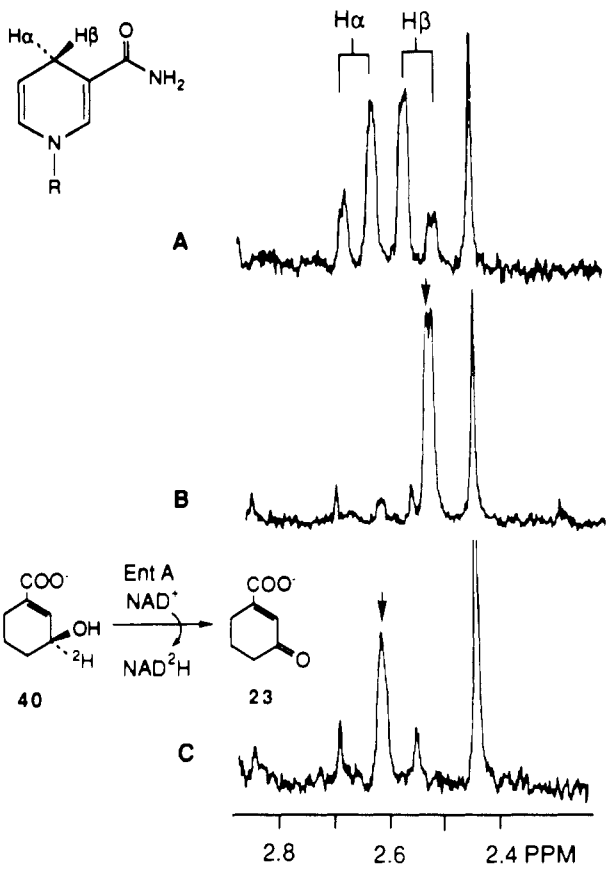


FIGURE 2: Determination of stereospecificity of hydride transfer to NAD^+. (Only the 2.2–2.9 ppm region is illustrated.) (A) ^1H NMR spectrum of NADH. (B) ^1H NMR spectrum of [4*R*-^2H]NADH prepared with alanine racemase and L-alanine dehydrogenase. (C) ^1H NMR spectrum of [4-^2H]NADH prepared by Ent A action shown on left side. All samples (2–5 mg) were lyophilized twice from 99% ^2H_2O and then dissolved in 0.7 mL of 50 mM KP_i (p^2H 8.5) buffer with 1 mM EDTA.

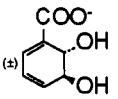
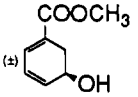
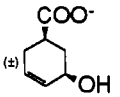
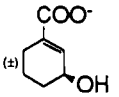
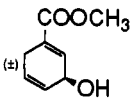
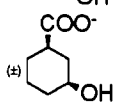
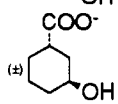
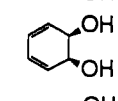
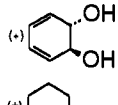
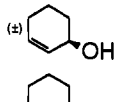
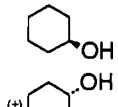
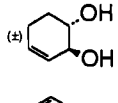
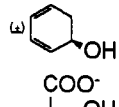
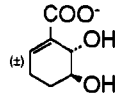
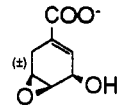
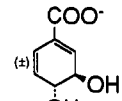
alcohol substrate to produce the [4-^2H]NADH product, the chirality of which could be determined by using ^1H NMR. It was found that 3-oxo-1-cyclohexene-1-carboxylate (**23**) could be reduced to racemic [3-^2H]-**40**, an isotopic variant of allylic alcohol **4**. The (±)-[3-^2H] alcohol **40** was used to reduce NAD^+ via Ent A in 21% yield after purification (see Experimental Procedures). The [4-^2H]NADH sample was lyophilized twice from 99% ^2H_2O and dissolved in 0.7 mL of 50 mM KPi (p^2H 8.5) buffer with 1 mM EDTA to suppress any line broadening from paramagnetic metals. An authentic sample of a chiral [4-^2H]NADH, specifically [4*R*-^2H]NADH, was prepared as an NMR standard by the recently described method of Esaki et al. (1989), using alanine racemase and L-alanine dehydrogenase in ^2H_2O. The data of Figure 2 clearly demonstrate that the NAD^2H produced by Ent A action, with a resonance due to H4 at 2.62 ppm, has the 4*S* configuration.

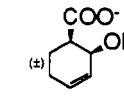
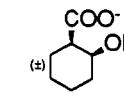
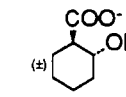
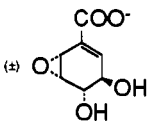
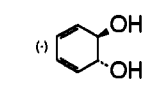
Kinetic Deuterium Isotope Effect. Given the availability of [3-^2H]-**40**, kinetic isotope effects on k_{cat} and k_{cat}/K_m were determined for its oxidation compared to that of [3-^1H]-**4**. There was no effect on K_m, but a deuterium isotope effect of 2.9 on k_{cat} (and therefore k_{cat}/K_m) was observed, indicating a significantly rate-limiting hydride transfer transition state with this substrate.

Discussion

In times of iron limitation, *E. coli* coordinately turn on an interdigitated bidirectional four-regulon cluster comprised of the *ent*, *fep*, and *fes* genes for the synthesis, export, and uptake of ferric enterobactin and its hydrolytic cleavage to release

Table I: Steady-State Parameters K_m and k_{cat} for Oxidation of 2,3-diDHB Analogues by Ent A

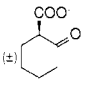
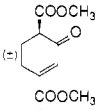
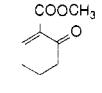
Substrate	Numbering	K_m (μM)	Turnover k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M^{-1} min^{-1}$)	k_{cat}/K_m (% of 2,3-diDHB)
	1[a]	300	5550	18.5	100
	2[b]	260	1050	4.0	22
	3[a]	2800	1380	0.49	2.6
	4[a]	1900	300	0.16	0.86
	5[b]	1700	180	0.11	0.59
	6[a]	4100	300	0.07	0.38
	7[a]	25200	44	1.7×10^{-3}	0.009
	8[a]	168000	1000	6.0×10^{-3}	0.03
	9[a]	16500	60	3.6×10^{-3}	0.02
	10[a]	83300	60	7.2×10^{-4}	3.9×10^{-3}
	11[a]	positive[c]			
	12[a]	positive[c]			
	13[a]	positive[c]			
	14[a]	positive[c]			
	15[a]	positive[c]			
	16[a]	positive[c]			

Nonsubstrate	Numbering	K_i (μM), Type
	17[d]	1400, Non
	18[e]	3300
	19[e]	2300
	20[e]	21000
	21[e]	negative

[a] Method A: 10 mM NAD^+ and Ent A in 50 mM KP_i (pH 7.4). [b] Method B: 10 mM NAD^+, esterase, and Ent A in 50 mM KP_i (pH 7.4). [The utility of utilizing pig liver esterase to provide for the carboxylate substrates in measurements to determine the steady-state kinetic parameters was demonstrated with methyl 2,3-diDHB (methyl ester of **1**). The kinetic parameters obtained in this fashion were identical within experimental error with those reported in Table I for the free carboxylate **1** (data not shown).] [c] These compounds produce an enzyme-mediated reduction of NAD^+ but require ca. 100–1000-fold higher levels of enzyme than the preceding compds. and thus it is not possible to measure K_m and k_{cat}. [d] Inhibition constant was measured by determining K_m and V_{max} for 2,3-diDHB at various substrate and inhibitor concentrations in the presence of 10 mM NAD^+ and Ent A. [e] Inhibition constant was determined by measuring a single-point Ent A activity in the presence of 10 mM NAD^+, 0.61 mM 2,3-diDHB, Ent A, and 10 mM inhibitor.

Fe^{3+} within the cell (Elkins & Earhart, 1988; Pettis et al., 1988; Jajal & Van der Helm, 1989; Pettis & McIntosh, 1987; Ozenberger et al., 1987). Of the six *ent* biosynthetic genes, the first three, *ent*C, *ent*B, and *ent*A, act sequentially to divert chorismate molecules to produce 2,3-dihydroxybenzoate (Scheme I). While isochorismate synthase (Ent C) and iso-

Table II: Steady-State Parameters K_m and k_{cat} for Reduction of 3-Keto Analogues by Ent A

Substrate	Numbering	K_m (μM)	Turnover k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	k_{cat}/K_m (% of **22**)	Nonsubstrate	Numbering
	**22**[a]	200	260	1.30	100		**27**[a]
	**23**[a]	200	33	0.17	13		**28**[b]
	**24**[a]	700	23	0.033	2.5		**29**[b]
	**25**[a]	45000	14	3.1×10^{-4}	0.024		
	**26**[a]	30500	0.4	1.3×10^{-5}	0.001		

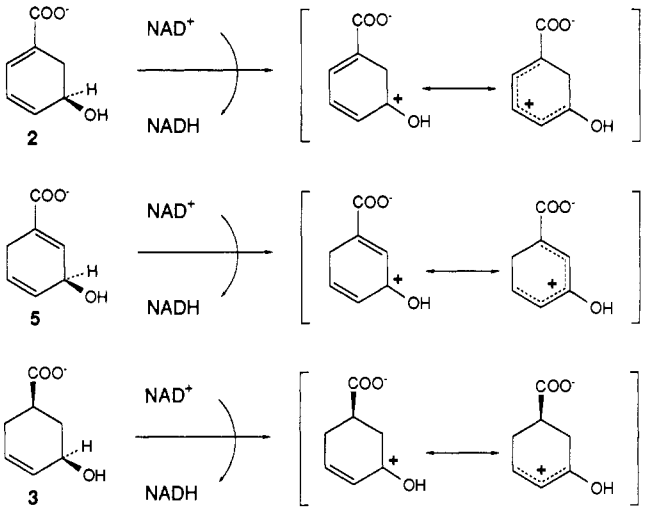
[a] Reactions were carried out by using 0.2 mM NADH and Ent A in 50 mM KP_i (pH 7.4). [b] Reactions were carried out by using 0.2 mM NADH, esterase, and Ent A in 50 mM KP_i (pH 7.4).

chorismatase (Ent B) carry out unusual transformations on the dihydroaromatic skeleton, it is the third enzyme, Ent A, that creates the iron-chelating catechol moiety that is the business end of enterobactin by oxidation of the dihydrodiol 2,3-dihydro-2,3-dihydroxybenzoate to 2,3-dihydroxybenzoate (Young et al., 1971; Nahlik et al., 1987). Until recently none of these three enzymes had been available for study, but we have now reported the subcloning of these genes and the overproduction and purification of all three enzymes to homogeneity in quantity for mechanistic study (Liu et al., 1989, 1990; Rusnak et al., 1990).

In one sense, as an NAD-linked alcohol dehydrogenase, one anticipates Ent A to be a somewhat prosaic redox catalyst, but it deals with a relatively unstable substrate and presumably yields an even more labile initial product, a cyclohexadienone tautomer of the catechol. Other dihydrodiol dehydrogenases converting *cis*-dihydrodiols to catecholic products have been described, albeit in bacterial oxidative transformations. Benzene *cis*-dihydrodiol dehydrogenase (Axcell et al., 1973) and the toluene *cis*-dihydrodiol dehydrogenase (Rogers et al., 1971) have been purified and certified to yield the catechol products. The benzene *cis*-dihydrodiol dehydrogenase gene sequence has been determined, but on comparison with the *ent*A gene sequence only the NAD^+ binding motif signature regions display significant homology. Furthermore, there is very little sequence homology between *ent*A and the *E. coli* shikimate dehydrogenase gene, *aro*E (Anton & Coggins, 1988), an enzyme earlier in the chorismate pathway that catalyzes an oxidation with similar stereo- and regiochemical requirements to that catalyzed by Ent A. We note that Ent A, unlike the other two diol dehydrogenases, uses a *trans*-diol substrate. Until now no mechanistic studies on any of these dihydrodiol dehydrogenases have been reported.

Perhaps the major initial mechanistic questions are the regiochemistry of dehydrogenation and whether dehydrogenation is indeed uncoupled from aromatization. The tautomeric lability of the anticipated 2- or 3-keto group in the cyclohexadienediol framework (Scheme II) renders the initial regiochemistry cryptic. We have resorted to deoxy substrates to establish regiochemistry and have thereby conclusively shown regiospecificity for oxidation at C3. With regard to catalysis, the presence of an allylic system lowers the activation energy of the transition state since the developing carbonium ion character may be delocalized as an allyl cation. For the physiological substrate, if the cyclohexadiene ring is essentially

Scheme V



planar in the transition state, the dienyl cation will be even more stabilized (Scheme V), and this may be a crucial factor in the catalytic efficiency of Ent A. On the other hand, the diene system in **5**, after treatment with esterase to yield the carboxylate form of substrate, can given an alternate dienyl cationic transition state (Scheme V). This is a poorer substrate than **2** by some 40-fold by k_{cat}/K_m catalytic efficiency assessment. Even the simple allylic alcohol cyclohexen-3-ol is a substrate at a k_{cat} of 1 s^{-1}, but now without the carboxylate group it has a ca. 300-fold deficit in relative binding ability.

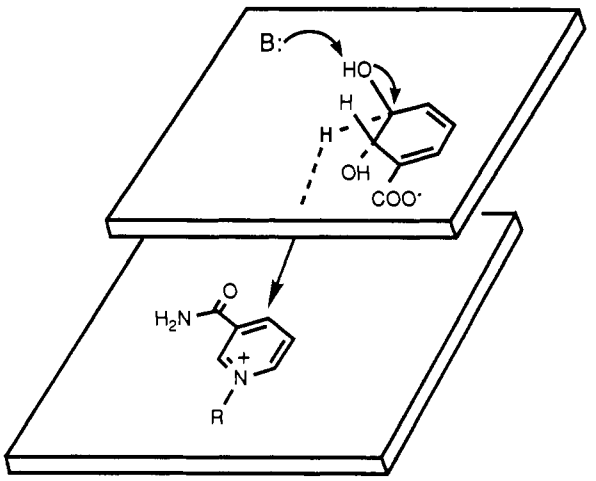
While one cannot study the back-region with the natural product 2,3-dihydroxybenozate (no reaction detectable) since the 3-oxocyclohexadienecarboxylate does not exist in appreciable concentrations, we were able to demonstrate the exclusive regiospecific reduction of 3-ketocyclohexene- and -cyclohexanecarboxylates. Now the 3-ketocyclohexenes show 8–10-fold lower k_{cat} values than the 3-ketocyclohexane system, consistent with a higher barrier for reduction and disruption of the conjugated system.

While we have not yet prepared [3-^{2}H]-2,3-dihydro-2,3-dihydroxybenzoate to evaluate to what extent the chemical step is in fact rate-limiting in catalysis, we have used [3-^{2}H]-**4** to show that for this substrate a 2.9-fold k_H/k_D confirms that hydride transfer is a kinetically significant transition state.

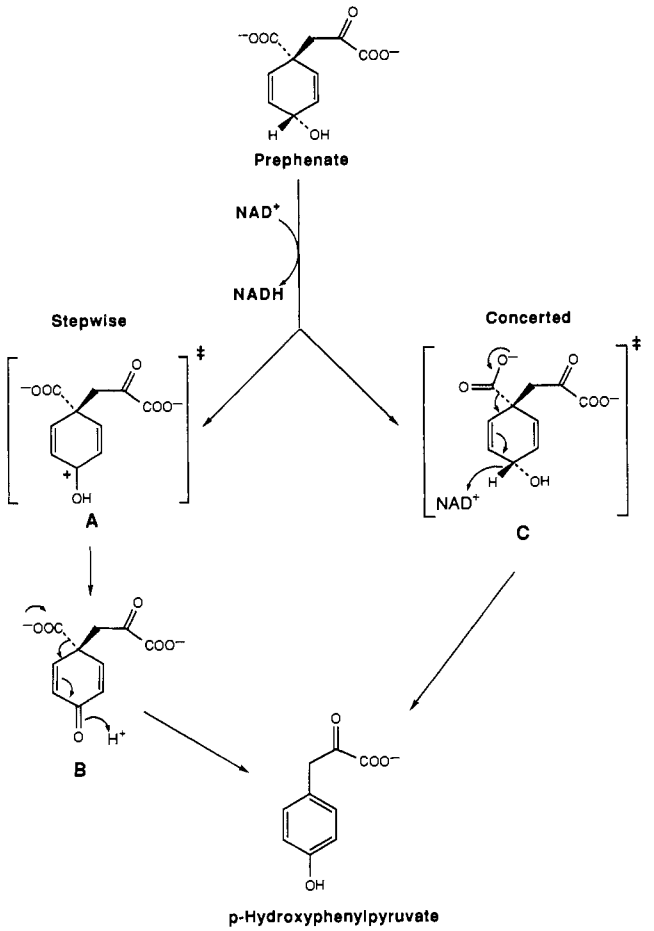
With the regiospecificity of Ent A action established, we turned to a study of the stereochemical outcome of the dehydrogenation sequence. Although natural 2,3-dihydro-2,3-dihydroxybenzoate was reported to have a 2*S*,3*S* configuration, we wanted to prove that Ent A was specific in oxidation of the allylic 3-alcohol substrates since it was possible to deuterate one of these, **4**, conveniently in racemic form. It was demonstrated that the enzyme utilizes only the 1*R*,3*R* diastereomer of **3**, consistent with the 3*S* stereochemistry (due to priority changes in the numbering of **1** vs **4**) of the physiological substrate and strongly suggesting the 3*R* isomer of synthetic [3-^{2}H]-**4** is also recognized. With NMR analysis to establish that the migrating deuteride produces [4*S*-^{2}H]NADH, one can write, by extention to **3**, the relative orientation for the physiologial substrate alcohol and NAD^+ partners in the Ent A active site as shown in Scheme VI.

As a net aromatizing dehydrogenase via oxidation of a dihydroaromatic dienol substrate, Ent A bears some formal analogy to another aromatizing dehydrogenase earlier in the chorismate pathway, namely, prephenate dehydrogenase, which processes prephenate to *p*-hydroxyphenyl pyruvate. One can write the reaction as indicated with a cyclohexadienyl

Scheme VI



Scheme VII



cationic transition state (A) in Scheme VII in analogy to that in Scheme V for the Ent A reaction. The product of the reaction would ensue from decarboxylation, and that could occur subsequent to formation of the transition state through the dienone intermediate B, or it could occur in a single transition state concerted with hydride loss (Scheme VIIC). The use of dihydro analogues of prephenate allowed isolation of the enone product without any decarboxylation, consistent with the stepwise mechanism, but double isotope effect studies on prephenate itself led Cleland and colleagues to favor the concerted transition state C with the physiological substrate (Hermes et al., 1984), indicating a switch in mechanism from concerted to stepwise for the dihydro analogue when the aromatizing driving force was not present. These precedents raise the issue of whether in 2,3-dihydro-2,3-dihydroxybenzoate itself the Ent A oxidation/aromatization may be concerted, but in this instance there is no comparable concerted mechanism one could write that inveles oxidation of the C3 alcoholic group and aromatization to catechol. A concerted mechanism for Ent A would have to involve C2H loss as a proton and ejection of C3 as a hydride to form the 2,3 olefinic link and the aromatic system without discrete formation of a C3 ketone group. To test this proposal, a double isotope effect study with dihydro-DHB deuterated separately (at C2 or C3) and together may be in order. In the absence of such an observation, the current results strongly support straightforward regio- and stereospecific dehydrogenation of the *trans*-dihydrodiol system at C3 with aromatization either following with enzyme assistance or occurring as an epiphenomenon away from the active site.

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