Kinetic and Structural Effects of Mutations of the Catalytic Amino-Terminal Proline in 4-Oxalocrotonate Tautomerase†

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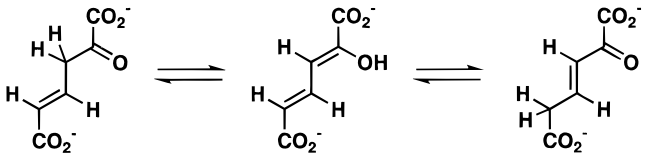
ABSTRACT: The catalytic general base, Pro-1, of the enzyme 4-oxalocrotonate tautomerase has been mutated to Gly, Ala, Val, and Leu, residues with aliphatic side chains. The Val mutant was partially (55%) processed by removal of the amino-terminal methionine to yield P1V/M1P2V, while the Leu mutant was not processed and completely retained methionine (M1P2L). The M1P2L mutant lost 2300-fold in k_{cat} with no change in K_m, and the residual activity of the unresolvable P1V/M1P2V mixture could be explained by the summation of two activities, one equal to that of M1P2L and the other equal to that of the P1G mutant. The P1G and P1A mutants showed 76- and 58-fold decreases in k_{cat} and much smaller decreases in K_m of 4- and 2.8-fold, respectively. The dissociation constant of the substrate analog *cis,cis*-muconate decreased 1.7-fold in the P1G mutant as determined by NMR titration. 2D $^1H-^{15}N$ HSQC spectra and 3D $^1H-^{15}N$ NOESY HSQC spectra of the ^{15}N-labeled P1G mutant showed no structural differences from the wild-type enzyme except for small changes in backbone ^{15}N and NH chemical shifts at the active site. Both the P1G and P1A mutants showed no change in overall conformation by circular dichroic spectroscopy. Both mutants and the wild-type enzyme generate the *S*-enantiomer of the product [5-^{2}H]-2-oxo-3-hexenedioate with comparable stereoselectivities indicating a largely intact active site. The P1G and P1A mutants showed 10- and 4-fold decreases, respectively, in catalysis of exchange of the C3 proton of the substrate 2-oxo-1,6-hexanedioate, consistent with the lower basicities of Gly-1 and Ala-1 compared to Pro-1. The pH dependences of k_{cat}/K_m for the P1G and P1A mutants revealed pK_a values of the general base of 5.3 and 5.9, respectively. NMR titration of the uniformly ^{15}N-labeled P1G mutant showed the pK_a of Gly-1 to be ≤5.6, in agreement with the kinetic data. As with the wild-type enzyme, the active site environments on the P1G and P1A mutants lower the pK_a of the general base by at least 2.5 units. It is concluded that the 2 order of magnitude decreases in k_{cat} in the P1G and P1A mutants result from both a decrease in basicity and an increase in flexibility of the general base. The greater $10^{3.4}$-fold decrease in k_{cat} found with the presence of an additional residue at the amino-terminus is ascribed to either the complete blockage or the drastically altered position of the general base.

4-Oxalocrotonate tautomerase (4-OT,[1] EC 5.3.2) catalyzes the isomerization of unconjugated α-keto acids such as 2-oxo-4-hexenedioate (**1**) to its conjugated isomer, 2-oxo-3-hexenedioate (**3**), through the dienol intermediate 2-hydroxy-2,4-hexadienedioate (**2**) (Scheme 1) (Whitman et al., 1991; Stivers et al., 1996a,b). The enzyme is elaborated by the soil bacterium *Pseudomonas putida mt-2* as part of a degradative pathway that converts aromatic hydrocarbons to intermediates in the Krebs cycle (Harayama et al., 1989). Stereochemical studies indicate that 4-OT catalyzes a suprafacial allylic rearrangement of **1** to **3** consistent with a one-base mechanism (Lian & Whitman, 1993). Affinity labeling (Stivers et al., 1996a), kinetic analysis (Stivers et al., 1996b), chemical synthesis (Fitzgerald et al., 1996), NMR (Stivers et al., 1996c,d), and crystallographic studies (Subramanya et al., 1996) identified the amino-terminal proline as the catalytic base in the 4-OT-catalyzed reaction with a pK_a value of ∼6.4.

Scheme 1



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[1] Abbreviations: 4-OT, 4-oxalocrotonate tautomerase; CCM, *cis,-cis*-muconate; ESI-MS, electrospray ionization mass spectrometry; HPLC, high-pressure liquid chromatography; HSQC, heteronuclear single quantum coherence; IPTG, isopropyl β-D-thiogalactoside; Kn, kanamycin; LB, Luria-Bertani medium; MALDI, matrix-assisted laser desorption-ionization; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TSP, sodium 3-(trimethylsilyl)-propionate-*2,2,3,3-d*$_4$.

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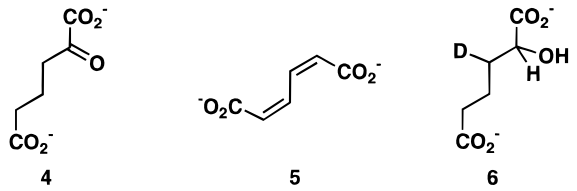
Chart 1



Proline is unique among the 20 common amino acids because it is a cyclic secondary amino acid. Its unusual role as a catalytic base raises questions about how its most notable features, the secondary amine and the rigidity of the ring system, contribute to the mechanism and the overall rate enhancement. Typically, questions about the role of a specific amino acid residue in a mechanism can be addressed by analyzing the mechanistic and structural consequences of site-directed mutations of such residues. This strategy is somewhat limited in the case of 4-OT because of the identity and position of the catalytic base. One obvious limitation is that alternative secondary amines cannot be introduced by mutagenesis. In addition, several common amino acids cannot be used in this position because those with nucleophilic groups on the side chain introduce a second potential general base which may obscure the role of the terminal amine and thereby complicate the analysis. Nonetheless, some issues such as the importance of the side chain rigidity can be examined using site-directed mutagenesis, and these results can guide the chemical synthesis of mutants using unnatural amino acids.

In this study we report the construction of four mutants of 4-OT in which the amino-terminal proline is replaced with a glycine, alanine, valine, and leucine. These alterations all result in an active enzyme, although significantly less active than the wild-type enzyme. Expression of the valine mutant results in the isolation of a mixture of enzymes with an unblocked and a methionine-blocked amino terminus (designated P1V and M1P2V, respectively),[2] while expression of the leucine mutant results only in the recovery of enzyme with a methionine-blocked amino-terminus (designated M1P2L). The small quantity of protein obtained for these mutants allowed only for a kinetic analysis. Expression of the P1G and P1A mutants, however, produced sufficiently large quantities of enzyme with an unblocked amino-terminus so that key kinetic and mechanistic properties could be studied in order to determine the cause for the decrease in activity. Accordingly, the kinetic parameters, the pH rate profiles, the stereochemical integrity utilizing **2** in D_2O, and the exchange rate of the C3 protons (for deuterons in D_2O) of an alternate substrate, 2-oxo-1,6-hexanedioate (**4**; Chart 1) were determined for these two mutants. In addition, the P1G variant was subjected to further scrutiny by NMR spectroscopy as the free enzyme and as a complex with *cis*,*cis*-muconate (**5**; Chart 1), a competitive inhibitor, in order to assess the effect of this mutation on the global structure and the active site. The results suggest that the rigidity of the pyrrolidine ring and the basicity of the secondary amine contribute to the efficient proton transfer between C3 and C5 of compounds **1** and **3**.

[2] For simplicity of the discussion, mutants with a blocked amino-terminus will be designated M1P2X while mutants with an unblocked amino-terminus will be designated P1X.

EXPERIMENTAL PROCEDURES

Materials. All reagents, buffers, and solvents were obtained from either Aldrich Chemical Co. or Sigma Chemical Co. unless noted otherwise. Tryptone and yeast extract were obtained from Difco (Detroit, MI). The YM-3 ultrafiltration membranes and Centricon (10 000 MW cutoff) centrifugal microconcentrators were obtained from Amicon. Isopropyl β-D-thiogalactoside (IPTG) and thin-walled PCR tubes were obtained from Ambion, Inc. (Austin, TX). The synthesis of 2-hydroxymuconate (**2**) is described elsewhere (Whitman et al., 1991). The construction of the plasmid pETOT which contains the gene for 4-OT under control of the T7 expression system (pET system; Novagen, Inc., Madison, WI) has been described (Stivers et al., 1996a). The expression vector pET24a was obtained from Novagen, Inc. Restriction enzymes, T4 DNA ligase, low-melting point agarose, the Magic PCR Preps DNA purification kit, and the Wizard Plus Minipreps DNA Purification System were obtained from Promega Corp. (Madison, WI). The Geneclean II kit was purchased from Bio 101, Inc. (La Jolla, CA). Reagents for the PCR were obtained from either GibcoBRL (Gaithersburg, MD) or Perkin Elmer (Norwalk, CT). Oligonucleotides for site-directed mutagenesis and DNA sequencing were synthesized by Oligos Etc. Inc. (Wilsonville, OR).

Strains. Escherichia coli strain JM109 was obtained from Promega Corp. and used for transformation of ligated plasmids. *E. coli* strain BL21(DE3)pLysS was obtained from Novagen and used for expression of the recombinant proteins. Cells for general cloning and expression were grown in LB media supplemented with kanamycin (50−100 μg/mL). The composition of LB medium is described in Sambrook et al. (1989).

General Methods. Techniques for restriction enzyme digestions, ligation, transformation, and other standard molecular biology manipulations were based on methods described elsewhere (Sambrook et al., 1989). DNA sequencing was done at The University of Texas (Austin) Sequencing Facility on a Perkin Elmer/ABI Prism 377 DNA sequencer according to the instructions provided with the ABI Prism Dye-Terminator kit. The base sequence is determined by analyzing fluorescent dye-labeled nucleotide fragments. Kinetic data were obtained on a Hewlett Packard 8452A diode array spectrophotometer. Enzyme activity was monitored by following the formation of **3** at 236 nm (Whitman et al., 1991). The cuvettes were mixed by a stir/add cuvette mixer. The kinetic data were fitted by nonlinear regression data analysis using the Grafit program (Erithacus Software Ltd., Staines, U.K.) obtained from Sigma Chemical Co. HPLC was performed on a Waters system using either a Bio-Gel Phenyl 5-PW hydrophobic column or a Pharmacia Superose 12 (HR 10/30) gel filtration column. Protein was analyzed by tricine sodium dodecyl sulfate−polyacrylamide gel electrophoresis under denaturing conditions on 12.5% gels on a vertical gel electrophoresis apparatus obtained from Gibco (Chen et al., 1992). Trichloroacetic acid was used instead of acetic acid in the staining and destaining solutions. Protein concentrations were determined using the method of Waddell (1956). Electrospray ionization mass spectra were acquired using a Sciex API-III quadrupole electrospray mass spectrometer. Matrix-assisted laser desorption-ionization (MALDI) mass spectrometry was performed on a

Massphoresis System from Ciphergen Biosystems (San Jose, CA). Circular dichroism spectra were recorded on either a Jasco J-600 or an AVIV 60DS spectropolarimeter as noted below.

Site-Directed Mutagenesis. The four mutants of 4-OT were prepared using the gene for 4-OT in the plasmid pETOT as the template (Stivers et al., 1996a). The gene is flanked by an *Nde*I restriction site and a *Sal*I restriction site. The mutations were made using the overlap extension polymerase chain reaction as described by Ho et al. (1989). The external PCR primers were oligonucleotides 5′-GATCTCGATCCCGCGAAATTAATACG-3′ (designated primer A) and 5′-CAGTGGTGGTGGTGGTGGTG-3′ (designated primer D). Primer A corresponds to the coding sequence of a region of the pET-24a(+) vector ~100 bp upstream from the *Nde*I restriction site, while primer D corresponds to the complementary sequence of the His·Tag region of the pET-24a(+) vector. For the P1G mutant, the internal PCR primers were oligonucleotides 5′-GGCAAT**GCC**CATATGTATATC-3′ (primer B) and 5′-GATATACATATG**GGC**ATTGCC-3′ (primer C). For the P1A mutant, the internal PCR primers were oligonucleotides 5′-AAT**GGC**CATATGTATATCTCC-3′ (primer B) and 5′-GGAGATATACATATG**GCC**ATT-3′ (primer C). For the M1P2V/P1V mutant, the internal PCR primers were oligonucleotides 5′-GGCAAT**AAC**CATATGTATATC-3′ (primer B) and 5′-GATATACATATG**GTT**ATTGCC-3′ (primer C). For the M1P2L mutant, the internal PCR primers were oligonucleotides 5′-GGCAAT**AAG**CATATGTATATC-3′ (primer B) and 5′-GATATACATATG**CTT**ATTGCC-3′ (primer C). In each set of primers, primer C contains an *Nde*I restriction site (underlined) followed by the codon for the desired mutation (boldface). The subsequent six bases correspond to the coding sequence of the gene for 4-OT. Primer B is the complementary primer.

PCR reactions were carried out in a Perkin Elmer DNA Thermocycler 480 using template DNA, synthetic primers, and the PCR reagents supplied in either the PCR reagent system or the Perkin Elmer Cetus GeneAMP kit. The template DNA was prepared using the Wizard Plus Minipreps DNA Purification System which provides the reagents for the extraction of DNA from bacterial cells by the alkaline lysis method described in Sambrook et al. (1989). Subsequently, the cleared bacterial lysate was passed through a column containing a proprietary resin suspended in 6 M guanidine hydrochloride. The bound DNA was eluted with sterile water. Each 100-μL reaction for the PCR contained 10× PCR buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3, 10 μL), dATP, dTTP, dGTP, and dCTP (2 μL each from 10 mM stock solutions), primers (5 μL each of 20 μM stock solutions), Taq DNA polymerase (1 μL of a 5 units/μL solution), $MgCl_2$ (6 μL of a 25 mM solution), and ~0.6 μg of template DNA. Reaction mixtures were carried out in sterile thin-walled PCR tubes overlaid with 3 drops of sterile silicone oil. The reaction protocol consisted of 25 cycles, a 5-min incubation period at 94 °C preceding the 25 cycles, and a 5-min incubation period at 72 °C following the 25 cycles. Each cycle consisted of three steps: denaturation at 94 °C for 1 min, annealing at 53 °C for 75 s, and elongation at 72 °C for 75 s.

In two separate PCRs, the AB and CD fragments were generated using the plasmid pETOT as template with primers A and B in one reaction and primers C and D in a second reaction. The PCR reaction mixtures were subjected to electrophoresis on a 1% agarose gel with TAE buffer (4.84 g of Tris base/L, 1.1 mL of glacial acetic acid/L, 2 mL of 0.5 M EDTA solution, pH 8.0/L), and the two fragments were extracted separately using the Magic PCR Preps DNA purification kit and dissolved in 50 μL of sterile water. Purification was achieved by the binding of DNA to a proprietary resin suspended in 6 M guanidine hydrochloride. The DNA was eluted using an 80% (v/v) 2-propanol solution. Subsequently, a second PCR was carried out on a mixture of the AB and CD fragments (5 μL each) using primers A and D. The PCR reaction mixture was purified as described above. The mutated DNA fragment and the pET24a(+) vector were digested with *Nde*I and *Sal*I restriction enzymes as described in Sambrook et al. (1989), purified on a 1% agarose gel, and extracted from the gel using the Geneclean II kit. The portion of the agarose gel containing the DNA fragment was solubilized into an aqueous mixture of a proprietary silica matrix and 4 M NaI. DNA binds to the matrix under these conditions and was eluted using sterile water. The linearized vector and mutant fragment were ligated using T4 DNA ligase (3 units) at 24 °C overnight as described in Sambrook et al. (1989). Aliquots of the resulting mixture were transformed into competent *E. coli* JM109 cells as described in Sambrook et al. (1989) and grown on LB/Kn (100 μg/mL) plates at 37 °C. Single colonies were chosen at random and grown in liquid LB/Kn media (50−100 μg/mL). The newly constructed plasmid was isolated and sequenced in order to verify the mutation. Subsequently, the mutated plasmid was transformed as described above into *E. coli* strain BL21(DE3)pLysS for protein expression.

Overexpression and Purification of Recombinant Enzymes. A single colony of the expression strain containing the desired plasmid was used to inoculate 25 mL of LB/Kn medium (50−100 μg/mL). After overnight growth at 37 °C, 3 mL of the culture was used to inoculate 500 mL of LB/Kn medium (50−100 μg/mL) in a 2-L Erlenmeyer flask. Cultures were grown to an OD_{600} of ~0.6 at 37 °C with vigorous shaking and then induced with IPTG (1 mM final concentration). Incubation was continued for 4 h at 37 °C. Cells were harvested by centrifugation (7000*g*, 12 min) and stored at −80 °C. Typically, 4 L of culture grown under these conditions yields 15−20 g of cells.

4-Oxalocrotonate tautomerase and the mutant enzymes were purified to homogeneity using a modification of a published procedure (Chen et al., 1992). In a typical procedure, the cells (~8 g) were thawed and suspended in 20 mM NaH_2PO_4 buffer (16 mL, pH 7.3). The cells were disrupted at 4 °C by sonication with one pulse (1 min) from a Heat Systems W-385 sonicator equipped with a 0.5-in. tapped horn delivering approximately 330 W/pulse. After the solution was made 2 mM in phenylmethanesulfonyl fluoride, 1 mM in 6 aminocaproate, 50 μM in leupeptin, and 0.067 μM in aprotonin, sonication was continued for 10 min using 5-s pulses spaced at 5-s intervals. After centrifugation (17000*g*, 30 min), the supernatant was made 2 M in ammonium sulfate and stirred for 2 h at 4 °C. Subsequently, the solution was centrifuged (17000*g*, 30 min), and the supernatant was injected in two portions (~6−8 mL each) into a Phenyl 5-PW column (150 × 21.5 cm) attached to a Waters HPLC system. The column had previously been equilibrated with 10 mM ethylenediamine buffer, pH 7.3,

containing 2 M ammonium sulfate at a flow rate of 5 mL/min. The protein was eluted using the gradient conditions described previously (Chen et al., 1992). Fractions containing 4-OT activity were pooled, concentrated by ultrafiltration, exchanged into 50 mM NaH_2PO_4 buffer, pH 7.3, and chromatographed on a Superose 12 gel filtration column equilibrated with 50 mM NaH_2PO_4 buffer, pH 7.3. At a flow rate of 0.4 mL/min, the recombinant proteins eluted at ~32−38 min. Typically, the yield of purified enzyme (>95% as assessed by SDS−PAGE) per liter is ~80 mg of 4-OT, ~10 mg of P1G, ~16 mg of P1A, ~0.3 mg of M1P2V and P1V, and 1 mg of M1P2L.

Mass Spectrometry. The masses of purified wild-type 4-OT and the purified mutant proteins were determined by either electrospray ionization mass spectrometry (ESI-MS) using a Sciex API-III quadrupole electrospray mass spectrometer or MALDI mass spectrometry using a Ciphergen Biosystems Massphoresis System time-of-flight mass spectrometer. Samples for ESI-MS were analyzed in a solution of 30% (v/v) acetonitrile in water, 0.1% TFA at concentrations of approximately 5 μM. For MALDI mass spectrometry, the matrix consisted of saturated α-cyano-4-hydroxycinnamic acid in 30% (v/v) acetonitrile in water with 0.1% TFA. The mutant (1 μL of a 1 mg/mL solution in 20 mM NaH_2PO_4 buffer, pH 7.4) was mixed with 20 μL of the matrix solution. A portion (1 μL) was applied to the stainless steel target, air-dried, and introduced into the spectrometer.

Circular Dichroism Spectroscopy. Circular dichroism spectra of wild type, P1G, and P1A were measured in 50 mM sodium borate buffer (pH 7.0) at a concentration of approximately 10 μM using a 1.0-mm optical path length. CD spectra were recorded on the AVIV 60DS spectropolarimeter.

*4-OT-, P1G-, and P1A-Catalyzed Exchange of the Proton at C3 of 2-Oxo-1,6-hexanedioate (**4**) with D_2O.* Reactions (0.6 mL) measuring the rate of exchange of the proton at C3 of 2-oxo-1,6-hexanedioate (**4**) with D_2O catalyzed by 4-OT, P1G, and P1A were performed at 23 °C in 100 mM Na_2DPO_4 buffer, pD ~ 9.7. Addition of **4** as the free acid dissolved in (dimethyl sulfoxide)-d_6 (30 μL) lowered the pD to 6.3 (no enzyme), 6.8 (4-OT), 6.8 (P1G), and 6.9 (P1A). The final concentration of **4** in each reaction was 40 mM (no enzyme, 4-OT, P1A) and 43 mM (P1G). The individual reactions were initiated by the addition of sufficient enzyme (4-OT, 0.4 mg; P1G, 2.1 mg; P1A, 1.8 mg) to complete the exchange of approximately one proton at C3 in about 120 min. Enzyme solutions had previously been exchanged by repeated dilution and concentration in 20 mM NaD_2PO_4 (pD ~ 6.8) buffer in a Centricon-10 microconcentrator and stored overnight. The progress of each reaction was monitored by recording successive ^{1}H NMR spectra on a Varian Unity INOVA-500 spectrometer at 23 °C. The intensity of the resonance observed at C3 (δ = 2.66 ppm) was measured at timed intervals every 2 min (4-OT, P1A), 3 min (P1G), and 4 min (no enzyme). The initial intensity of the resonance which was observed at 3 min (no enzyme), 2 min (4-OT), 3 min (P1G), and 2 min (P1A) after the addition of enzyme was set equal to one proton. Subsequent measurements were divided by this initial intensity to give the fraction of the proton remaining. First-order rate constants were obtained from these data by measuring the observed $t_{1/2}$ values as the fraction of protons decreased to 0.5, 0.25, and 0.125 (Moore & Pearson, 1981). In order to compare the observed rate constants, they were normalized to the amount of enzyme used in the wild-type experiment.

pH Rate Profiles of P1G and P1A. The dependences of the rate of ketonization of **2** to **3** by the P1G and P1A mutants were determined in 50 mM sodium phosphate buffer (pH 4.8−11) at 23 °C as described previously (Stivers et al., 1996b) using the following modifications. To facilitate the measurement of the reduced rates between the pH values 4.8 and 6, the enzyme concentration was increased to 1.5 μM. The final concentration of enzyme in the pH range 6−10.5 was 0.4 μM. The reactions were initiated by the addition of a quantity of **2** (final concentration 0.025−0.75 mM) from a stock solution made up in ethanol. The pH dependences of the kinetic parameters were fitted and analyzed as described previously (Stivers et al., 1996b).

*Enzymatic Ketonization of **2** to [5-^{2}H]-**3** in D_2O and Conversion to [2-^{2}H]Glutarate*. The stereochemical analysis of [5-^{2}H]-**3** generated by the enzymatic ketonization of **2** in D_2O catalyzed in individual reactions by either 4-OT, P1G, or P1A was based on the chemical conversion of [5-^{2}H]-**3** to a stereoselectively monodeuteriated glutarate according to the procedure of Whitman et al. (1992) with the following modifications. The ketonization of **2** to **3** requires a sufficiently large amount of enzyme in order to minimize the facile nonenzymatic ketonization of either **2** to **3** or **2** to **1** and the resulting stereorandom incorporation of a deuterium at either C3 or C5. Hence, the amount of enzyme was determined for 4-OT and for each P1 mutant by preliminary monitoring of a small-scale reaction by ^{1}H NMR spectroscopy on a Bruker AM-250 spectrometer as previously described (Whitman et al., 1992). Subsequently, several identical reactions (~30) were processed using the appropriate amount of enzyme. An individual reaction mixture consisted of **2** (4-OT, 4.6 mg; P1G, 4.0 mg; P1A, 3.0 mg) dissolved in (dimethyl sulfoxide)-d_6 (10 μL) and enzyme (4-OT, 0.1 mg; P1G, 0.5 mg; P1A, 0.35 mg). All reactions were carried out in 100 mM Na_2DPO_4 (0.6 mL, pD 9.4). The addition of **2** lowered the pD to ~6.8 (4-OT), 7.0 (P1G), and 7.2 (P1A). The individual reaction mixtures were allowed to run for 2.5 min (4-OT), 6 min (P1G), or 5 min (P1A), quenched with $NaBH_4$, pooled, and subjected to anion-exchange chromatography on a Dowex-1 (formate) column (25 × 2.0 cm). The product was eluted using a linear gradient of aqueous formic acid (0−2 M, 600 mL total) followed by a wash of 2 M formic acid (100 mL). The product eluted at about 2 M formic acid. In this manner, 33 mg (4-OT), 39 mg (P1G), or 28 mg (P1A) of [5-^{2}H]-**3** was recovered as the major product.

The saturated compound, 2-hydroxy[5-^{2}H]hexanedioate (**6**, Chart 1), was obtained by hydrogenation of [5-^{2}H]-**3** in the presence of Wilkinson's catalyst (Whitman et al., 1992). In a typical procedure, $(Ph_3P)_3RhCl$ (16 mg, 0.02 mmol) was added to a hydrogenation reaction containing a solution of [5-^{2}H]-**3** (33 mg, 0.2 mmol), isolated from the 4-OT reaction mixture, in 1:1 ethanol/benzene (14 mL). The mixture was shaken vigorously under H_2 (40 psi) in a Parr apparatus at 23 °C for 4 days and worked up as previously described (Whitman et al., 1992).

Oxidative decarboxylation of **6** by lead tetraacetate (in acetic acid) generated glutaric semialdehyde, which was then oxidized to glutaric acid by potassium permanganate (Whitman et al., 1992). Subsequently, the reaction mixture was extracted with ethyl acetate; the ethyl acetate extracts were

combined, dried over Na_2SO_4, and evaporated to dryness. The crude glutaric acid was purified by chromatography on a Dowex-1 (formate) column and eluted with a formic acid gradient (0−4 M formic acid). Glutaric acid eluted at 1.4−2.0 M formic acid. Appropriate fractions were pooled and evaporated to dryness. The residue was subjected to further purification by HPLC using a semipreparative reverse-phase column (Alltech Econosil C18 column, 10 μm, 2.2 × 25 cm) with a mobile phase of 89% water/10% methanol/1% trifluoroacetic acid at a flow rate of 5 mL/min. The effluent was monitored at 210 nm. Glutarate eluted at 18.3 min. A ^{1}H NMR spectrum taken at 250 MHz on a Bruker AM 250 spectrometer corresponded to the previously reported spectrum (Whitman et al., 1992). Circular dichroism spectra were recorded on a Jasco J-600 spectropolarimeter.

Structural NMR Methods. Unless otherwise stated, solution conditions for structural studies of the P1G mutant enzyme by NMR were uniformly ^{15}N-labeled enzyme, 3.6 mM in subunits, 8 mM sodium phosphate buffer, pH 6.50, in 0.6 mL of H_2O/D_2O (90:10) at 42 °C. The NMR data were collected on a Varian Unity Plus 600 NMR spectrometer equipped with *z* gradient capabilities, using a Varian 5-mm triple-resonance probe. Data were processed on a Silicon Graphics Personal IRIS 4D/35 workstation using the FELIX software package (Biosym Technologies, Inc.). Multidimensional data sets were collected using the States-TPPI method (Marion et al., 1989) in all of the indirect dimensions, with relaxation delays of 0.9 s. These acquired domain data points were extended by one-third of the original size by the forward linear prediction routine in FELIX. Shifted (65−90°) sine bell filters were used in the first and subsequent dimensions, respectively, prior to zero-filling and Fourier transformation. The observed ^{1}H chemical shifts were measured with respect to the H_2O signal, which was 4.61 ppm downfield from external TSP at 42 °C, and are reported with respect to TSP. The ^{15}N chemical shifts were measured with respect to external $^{15}NH_4Cl$ (2.9 mM in 1 M HCl) at 20 °C, which was 24.93 ppm downfield from liquid ammonia (Levy & Lichter, 1979), and are reported with respect to liquid ammonia.

3D ^{1}H−^{15}N NOESY HSQC of the P1G Mutant. A 3D ^{1}H−^{15}N NOESY HSQC experiment was recorded on a 3.6 mM solution of ^{15}N-labeled P1G mutant, 3.6 mM in subunits, containing the other components as described above, with a 50-ms mixing time, using the pulse sequence described by Kay et al. (1992). The data were obtained at 600 MHz with spectral widths of 6400, 1800, and 8000 Hz in f_1 (^{1}H), f_2 (^{15}N), and f_3 (^{1}HN), respectively, and with 128, 48, and 1024 complex points, respectively, in the t_1, t_2, and t_3 dimensions. A total of four transients were acquired for each hypercomplex t_1,t_2 pair. The final data matrix was 512 × 128 × 512 real points for the f_1 (^{1}H), f_2 (^{15}N), and f_3 (^{1}HN) dimensions, respectively.

^{1}H−^{15}N HSQC Spectra of P1G Mutant. ^{1}H−^{15}N HSQC spectra were recorded on the same sample using a pulse sequence in which the HSQC detection scheme was optimized to avoid water saturation (Mori et al., 1995). The data were obtained at 600 MHz with spectral widths of 1600 and 8000 Hz in f_1 (^{15}N) and f_2 (^{1}H), respectively, and with 192 and 1024 complex points, respectively, in the t_1 and t_2 dimensions. A total of four transients were acquired for each hypercomplex t_1 point with ^{1}H and ^{15}N carriers positioned at 4.61 and 119 ppm, respectively. The final data matrix was 1024 × 1024 real points for the f_1 (^{15}N) and f_2 (^{1}H) dimensions, respectively.

^{1}H−^{15}N HSQC Titration of P1G Mutant with cis,cis-Muconate. Since the complex of the substrate analog, *cis,cis*-muconate (**5**), with the P1G mutant is in fast exchange on the chemical shift time scale (see Results), the dissociation constant (K_D) and the amide ^{15}N and ^{1}H assignments for the complex were determined by titration of the enzyme with **5** and following the changes in the ^{1}H and ^{15}N chemical shifts in a series of ^{1}H−^{15}N HSQC spectra obtained as described above. The absolute values of the chemical shift changes ($\Delta\delta_{obs} = |\delta - \delta_0|$) for three well-resolved resonances with significant $\Delta\delta_{max}$ values (R11, V40, and G51) were plotted against the total concentration of **5** (L_{tot}). Such plots were used to determine K_D according to eq 1:

$$\Delta\delta_{obs} = \Delta\delta_{max}((K_d + E_{tot} + L_{tot}) - ((K_D + E_{tot} + L_{tot})^2 - (4E_{tot}L_{tot}))^{1/2}) \quad (1)$$

taking into account the small changes in total enzyme concentration, E_{tot}, due to dilution of the NMR sample upon addition of ligand, as described (Stivers et al., 1996d).

1D ^{15}N NMR Spectroscopy. 1D ^{15}N NMR was used to determine an upper limit to the pK_a of the amino group of glycine-1 in the P1G mutant by monitoring the pH dependence of the ^{15}N chemical shift of the amino nitrogen resonance. The ^{15}N chemical shifts were referenced to external liquid ammonia as described (Weber et al., 1993). A Varian Unity-Plus 600 NMR spectrometer operating at 50.659 MHz for ^{15}N was used. Spectra were acquired without proton decoupling using a 5-mm broad-band detection probe. The titrations were performed using samples which were 3.6 mM in subunits of the uniformly ^{15}N-labeled P1G mutant enzyme at 30 °C by adding small amounts of 1 M HCl or NaOH to the sample. The acquisition parameters for the enzyme titration were as follows: spectral width, 12 001.2 Hz; acquisition time, 0.683 s; relaxation delay, 0.1 s; total number of transients, 10 000−100 000. The titration was found to be reversible over the pH range 5.7−7.2, and the enzyme retained ≥85% of its initial activity at the conclusion of the experiment. The ^{15}N chemical shift of the amino nitrogen resonance was not detectable at pH values below 5.7 preventing the determination of δ_1, the limiting chemical shift at low pH. A limiting pK_a value was therefore determined from a nonlinear least-squares fit of the data to eq 2:

$$\delta\ (\text{ppm})^{app} = (\delta_1 + \delta_2(10^{pH-pK_a})^n)/((10^{pH-pK_a})^n + 1) \quad (2)$$

using set values of $n = 1$ for the Hill coefficient, $\delta_2 = 18$ ppm for the limiting chemical shift at high pH, and differing trial values of $\delta_1 = 20-25$ ppm for the limiting chemical shift at low pH.

RESULTS

Production, Expression, and Characterization of the Mutants. Four mutants were constructed by overlap extension PCR, expressed in *E. coli* strain BL21(DE3)pLysS, and purified to homogeneity (as judged by SDS−PAGE) using a modification of the previously described procedure (Chen et al., 1992). The sequence of each mutant was confirmed by DNA sequencing. The range of overproduction (per liter

Table 1: Kinetic Parameters for 4-OT and P1 Mutants[a]

enzyme	K_m, μM	k_{cat}, s^{-1}	k_{cat}/K_m, M^{-1} s^{-1}	relative k_{cat}	relative k_{cat}/K_m
wild type	280 ± 60	3500 ± 500	1.3×10^7	1.00	1.00
P1G	70 ± 14	46 ± 3	6.6×10^5	0.013	0.051
P1A	100 ± 12	60 ± 3	6.0×10^5	0.017	0.046
M1P2L	250 ± 40	1.5 ± 0.1	6.0×10^3	0.00043	0.00046
P1V/M1P2V	250 ± 35	24 ± 2	9.6×10^4	0.0069	0.0074

[a] The steady-state kinetic parameters were determined at 23 °C. Errors are standard deviations.

of culture) varied from 0.3 mg (M1P2V/P1V) to 80 mg (4-OT). The yields of protein in the M1P2V/P1V mixture and M1P2L (1 mg) mutant were poor, while the yields of the P1G (10 mg) and P1A (16 mg) mutants were moderate in comparison to wild type. SDS−PAGE of roughly equal quantities of crude lysate (data not shown) revealed that the intensity of the bands increased in the following order: P1G (least intense), P1A, M1P2V/P1V, and wild type (most intense).

The purified recombinant proteins were also subjected to either electrospray ionization mass spectral analysis or matrix-assisted laser desorption-ionization mass spectral analysis in order to determine whether the initiating methionine had been cleaved. Typically, the P1G and P1A mutants generate one major peak which corresponds to an expected molecular mass of a 62-amino acid species beginning with either glycine or alanine indicating that the N-terminal methionine is post-translationally cleaved in *E. coli*. The same observation is made for the wild-type enzyme (Chen et al., 1992). Samples of M1P2V/P1V typically generate two major peaks which correspond to the expected molecular masses of a 62-amino acid species beginning with valine (∼55%) and a 63-amino acid species beginning with methionine (∼45%). Attempts to separate these two species in solution have thus far not been successful. Samples of the M1P2L mutant typically generate one major peak which is consistent with an expected molecular mass of a 63-amino acid species beginning with methionine. In both the M1P2V/P1V and M1P2L mutants, there is no mass spectral evidence for the presence of a formyl group, indicating that the methionine was post-translationally deformylated.

Kinetic Properties of the P1 Mutants. The steady-state kinetic parameters for 4-OT and the P1 mutants were determined using **2** as substrate. The observed values for K_m, k_{cat}, and k_{cat}/K_m at 23 °C are given in Table 1. Two trends emerge from these data. First, the value of K_m is not greatly affected by these mutations. A small R group at the α-carbon (Gly or Ala) results in a 2.8−4.0-fold decrease in K_m. The M1P2L mutant, in which two structural changes have been made, and the P1V/M1P2V mixture show no significant changes in K_m from the wild-type enzyme. Second, these mutations have substantial effects on the values of k_{cat}. Significant decreases in k_{cat} are observed for the P1G mutant (77-fold) and the P1A mutant (58-fold). The largest decrease in k_{cat} (2300-fold) is observed for the M1P2L mutant which has an additional amino acid in the sequence and has altered Pro to Leu. The k_{cat} value of the unresolvable mixture P1V/M1P2V may be analyzed by assuming equal activities of the M1P2V component with that of M1P2L. Assuming this to be the case and making use of the composition of the mixture determined by mass spectrometry as 45% methionine-blocked yield a k_{cat} value for P1V of 42 s^{-1} comparable to those of P1G and P1A. Since k_{cat} is dominated by the chemical step (**2** → **3**) (Stivers et al., 1996b), these decreases in k_{cat} reflect the introduction of kinetic barriers to this step by the mutations. The magnitude of these barriers is 2.6 kcal/mol for the P1G mutant, 2.4 kcal/mol for the P1A mutant, and 4.6 kcal/mol for the M1P2L mutant in which two structural changes exist. The values of k_{cat}/K_m, which are dominated by the effects on k_{cat}, decrease ∼20-fold for the P1G and P1A mutants and ∼2200-fold for the M1P2L mutant.

4-OT-, P1G-, and P1A-Catalyzed Exchange of the Proton at C3 of 2-Oxo-1,6-hexanedioate (4) with D_2O. The steady-state kinetic constants listed in Table 1 were obtained by measuring the formation of product (**3**) from **2** (increase in concentration of **3** at 236 nm) which reflects the rate of ketonization of the intermediate dienol. In order to obtain insight into the effect of these mutations on the formation of the intermediate dienol (**1** → **2**), the slow exchange of the protons of an alternate substrate, 2-oxo-1,6-hexanedioate (**4**), was examined with the wild-type enzyme, the P1G mutant, and the P1A mutant since these mutants had only a single structural change. The data show that the exchange rate for the P1G and P1A mutants are respectively 10- and 4.5-fold less than the rate observed for the wild-type enzyme.

pH Dependence of the Kinetic Parameters for the P1G and P1A Mutants. The values of k_{cat} and k_{cat}/K_m for the P1G- and P1A-catalyzed ketonization of **2** suggest that the enzyme−substrate complex is in rapid equilibrium with the free enzyme and substrate. Hence, the pH dependence of the steady-state kinetic parameters for **2** for both mutants can be analyzed using rapid equilibrium assumptions as previously described (Stivers et al., 1996b). For both the P1G and P1A mutants, a plot of $\log(k_{cat}/K_m)$ for **2** (Figure 1) shows a bell-shaped dependence on pH with limiting slopes of 2 and 1 on the ascending and descending limbs, respectively. The slope of 2 on the ascending limb indicates that two ionizable groups in the free enzyme or substrate are important for activity below pH values <6 and one ionizable group is important for activity at pH values >9. If it is assumed that the dianionic form of **2** binds to the P1G and P1A mutants, an assumption made for wild-type enzyme (Stivers et al., 1996b), then one pK_a on the ascending limb most likely corresponds to the ionization of the 6-carboxylic acid group (pK_a = 5.4). A nonlinear least-squares fit of the pH dependence of k_{cat}/K_M to the logarithmic form of eq 3:

$$k_{cat}/K_m = (k_{cat}/K_m)^{max}/(1 + [H^+]/K_a^S)(1 + [H^+]/K_{H_2E} + K_{HE}/[H^+]) \quad (3)$$

which includes a constant for the ionization of **2**, gave pK_a values for the free enzyme of 5.3 ± 0.1 and 9.5 ± 0.1 for the P1G mutant and 5.9 ± 0.3 and 9.7 ± 0.2 for the P1A mutant. For both mutants, the pK_a value for the basic residue involved in catalysis is below that observed for the wild-type enzyme (6.2) while the pK_a value for the acidic residue involved in catalysis is above that observed for the wild-type enzyme (9.0) (Stivers et al., 1996b).

For the P1G mutant, a plot of log k_{cat} vs pH for **2** shows a bell-shaped dependence on pH with limiting slopes of 1 on the ascending and descending limbs (Figure 1A). A fit of the pH dependence of k_{cat} to the logarithmic form of eq 4:

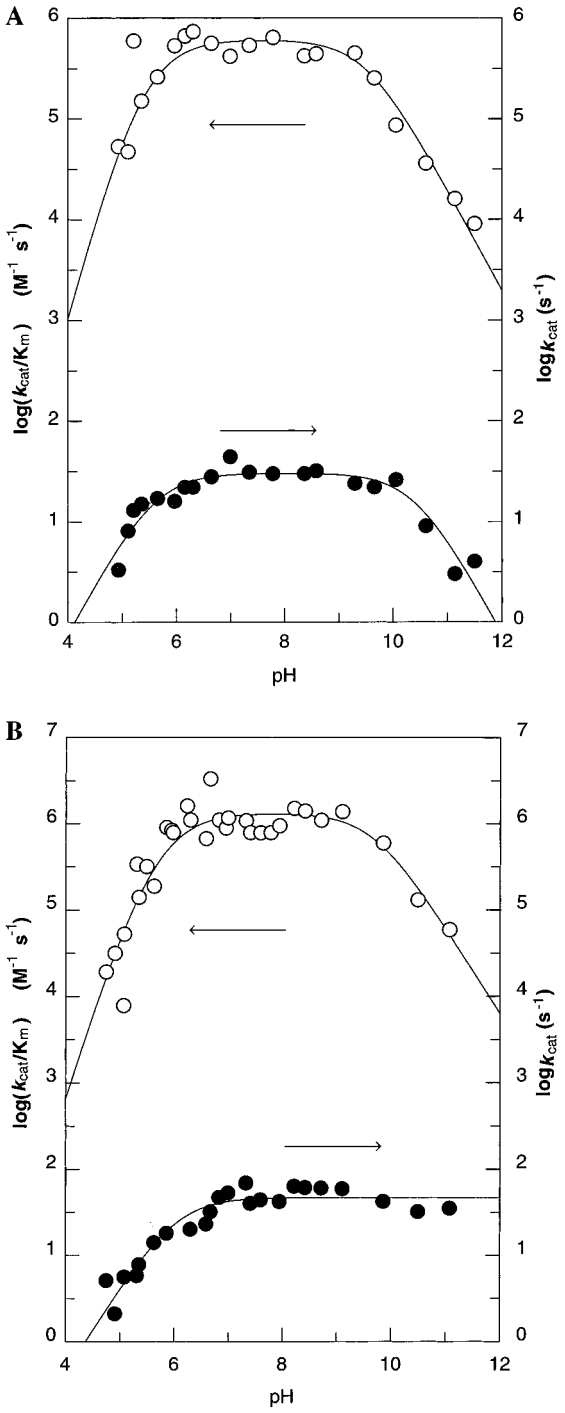


FIGURE 1: pH dependence of the kinetic parameters for the P1G and P1A mutant enzymes. The pH dependences of log(k_{cat}/K_m) (○) and log k_{cat} (●) are shown for the (A) P1G mutant enzyme and (B) P1A mutant enzyme. The curves were computed from a nonlinear least-squares fit of the data to eqs 3−5 as described in the text. The pK_a values are reported in the text.

$$k_{cat} = (k_{cat})^{max}/1 + [H^+]/K_{H_2ES} + K_{HES}/[H^+] \quad (4)$$

gave pK_a values for the enzyme−substrate complex of 5.6 ± 0.1 and 10.4 ± 0.1. For the P1A mutant, a plot of log k_{cat} vs pH for **2** shows a single ascending limb with a slope of 1 (Figure 1B). A fit of the pH dependence of k_{cat} to the logarithmic form of eq 5:

$$k_{cat} = (k_{cat})^{max}/1 + [H^+]/K_{H_2ES} \quad (5)$$

gave a pK_a value for the enzyme−substrate complex of 6.0 ± 0.1. If there is a pK_a for an acidic group, it must be greater than 11. The k_{cat} value of the wild-type enzyme showed pK_a values of 6.5 ± 0.2 and 9.6 ± 0.3. Thus, in the enzyme−substrate complex, the mutations at Pro-1 decreased the acidic pK_a and increased the basic pK_a.

Table 2: Molar Ellipticity of 2-Deuterioglutaric Acids Derived from Enzymatic Reactions

source of (*S*)-[2-^{2}H]glutaric acid	$[\theta]_{211}$[a]
synthetic sample[b]	108
wild type	183
P1G	216
P1A	195

[a] Circular dichroic spectra were recorded in 95% ethanol on a Jasco J-600 automatic recording spectropolarimeter at 25 °C. The units of $[\theta]$ are in deg M^{-1} cm^{-1}, and the errors are ±10 deg M^{-1} cm^{-1}. [b] Whitman et al. (1992).

Stereochemical Analysis of the P1G- and P1A-Catalyzed Reactions. The stereoselectivity of the P1G- and P1A-catalyzed ketonization of **2** to [5-^{2}H]-**3** in D_2O was examined in separate reactions. The stereoselectivity of the 4-OT-catalyzed reaction was reinvestigated as a control. The stereochemical analysis of [5-^{2}H]-**3** was based on its chemical conversion to a stereoselectively monodeuteriated glutarate according to the procedure of Whitman et al. (1992) with the modifications noted in Experimental Procedures. The three samples of purified monodeuteriated glutarate from these reactions were analyzed by circular dichroism (Table 2). It has been previously established that glutaric acid isolated from the same sequence of reactions using 4-OT from *P. putida* exhibits a positive Cotton effect in its circular dichroism spectrum, with a molar ellipticity $[\theta]_{211} = +183°$ at 25 °C. It was concluded from this result that the *S*-enantiomer of [5-^{2}H]-**3** is generated. The monodeuteriated glutarates derived from the 4-OT-, P1G-, and P1A-catalyzed reactions also exhibit positive Cotton effects with comparable values. Hence, it can be concluded that the *S*-enantiomer of [5-^{2}H]-**3** is generated with comparable stereoselectivity in these reactions, i.e., changing the general base from the rigid Pro-1 to the more flexible Gly-1 or Ala-1 did not alter the stereoselectivity of substrate protonation.

Structural Properties of the P1G and P1A Mutant Enzymes. It is important to determine whether the loss of catalytic activity in a mutant enzyme is due to the specific alteration of the catalytic residue or to the loss of native-like protein structure. CD spectra of the P1G and P1A mutants were indistinguishable from that of the wild-type enzyme, indicating that no gross conformational changes had occurred (data not shown). ^{1}H−^{15}N HSQC spectra of the P1G mutant enzyme indicate that 93% of the backbone ^{15}N resonances are unshifted (i.e., are within the error limits of 0.5 ppm of those of the wild-type enzyme) and 73% of the backbone NH resonances are unshifted (i.e., are within the error limit of 0.05 ppm of those of the wild-type enzyme) (Figure 2). The backbone amide ^{15}N and ^{1}H assignments for the P1G mutant were confirmed by comparison of NOE cross-peaks to the backbone NH resonances, obtained by 3D ^{1}H−^{15}N NOESY HSQC, to those of wild-type 4-OT in which complete ^{1}H, ^{15}N, and ^{13}C chemical shift assignments have been made (Stivers et al., 1996c). ^{1}H−^{15}N NOESY HSQC spectra showed little change in NOE intensities for *all* residues indicating very similar conformations for wild-type and P1G mutant enzymes (data not shown). While the

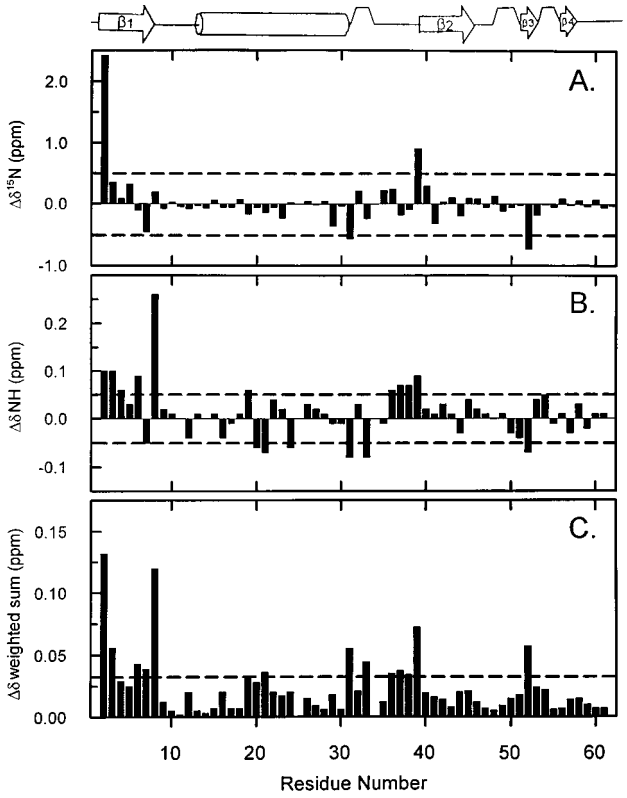


FIGURE 2: Backbone ^{15}N and NH chemical shift differences between the P1G mutant and wild-type 4-OT enzymes. Chemical shift differences were calculated on the basis of the chemical shift values obtained from ^{1}H−^{15}N HSQC spectra and are plotted versus the residue number and secondary structure (Stivers et al., 1996c): (A) ^{15}N chemical shift differences, (B) NH chemical shift differences, (C) sum of the absolute magnitudes of the ^{15}N and NH chemical shift changes which were weighted according to the backbone amide chemical shift dispersion in the ^{15}N and ^{1}H dimensions (27.36 and 2.29 ppm, respectively). The dashed lines indicate the error limits.

overall 3D fold is preserved in the P1G mutant, selective changes in ^{15}N or NH chemical shifts in the ^{1}H−^{15}N HSQC spectrum were observed for Ile-2, Leu-8, Leu-31, Arg-39, and Ile-52 (Figure 2). X-ray (Subramanya et al., 1996) and NMR (Stivers et al., 1996c) studies show that these residues are located near the active site and are in close proximity to the site of mutation.

*^{1}H−^{15}N HSQC Titration of P1G Mutant 4-OT with cis,-cis-Muconate (**5**).* The binding of the competitive inhibitor **5** to the P1G mutant was monitored by ^{1}H−^{15}N HSQC titrations. The final ^{15}N and NH $\Delta\delta$ values ($\delta_{complex} - \delta_{free}$) at saturating **5** are plotted according to residue number in Figure 3A−C. In this titration, tracing the changes in chemical shifts of the amide resonances permitted the assignment of the ^{15}N−H amide cross-peaks of all 60 of the non-proline residues of the P1G mutant in the complex. Using eq 1 and the $\Delta\delta$(NH) values for Arg-11, Val-40, and Gly-51, a $K_D = 0.35 \pm 0.07$ mM for the binding of **5** to the P1G mutant was determined (Figure 3D). This value decreased 1.7-fold from the K_D value of 0.59 ± 0.14 mM found for wild-type 4-OT (Stivers et al., 1996d).

pH Titration of Amino Proton of Gly-1 in P1G Mutant 4-OT. The kinetically determined pK_a value for k_{cat}/K_m (5.3 ± 0.1) of the P1G mutant (Figure 1A) was confirmed by direct pH titration of the uniformly ^{15}N-labeled enzyme using ^{15}N NMR (Figure 4). The ^{15}N chemical shift of the amino nitrogen of Gly-1 (18−20 ppm) over the range pH 5.7−7.2 is upfield and well-resolved from all other ^{15}N resonances of the enzyme (>35 ppm). The limiting ^{15}N chemical shift of Gly-1 at high pH (δ_2) was 18 ppm. The ^{15}N resonance of Gly-1 was not detectable due to broadening at pH values below 5.7, precluding a determination of δ_1, the limiting chemical shift at low pH. Therefore the titration data were fitted to eq 2 to obtain the pK_a using trial values of δ_1 ranging from 20 to 25 ppm and fixed values for the Hill coefficient ($n = 1$) and limiting chemical shift at high pH ($\delta_2 = 18$ ppm). Figure 4 shows that the best fit to the data yielded values of $\delta_1 \geq 22$ ppm and a limiting p$K_a \leq 5.6$. In contrast, the pK_a of the ammonium group of glycinamide is 8.1. Thus, the enzymatic environment of the P1G mutant lowers the pK_a of the ammonium group of Gly-1 by ≥2.5 units in comparison with its model compound, glycinamide. Similarly, the pK_a of the ammonium group of Pro-1 in wild-type 4-OT is lowered by 3.0 units in comparison with its model compound, prolinamide (Stivers et al., 1996b).

DISCUSSION

The initial strategy for exploring the role of proline in the 4-OT-catalyzed reaction was to examine the properties of four mutants (P1G, P1A, P1V, and P1L) with aliphatic side chains so that only the primary α-amino group could act as the catalytic base. The valine and leucine mutants were isolated with a methionine-blocked amino-terminus but without a formyl group, while the glycine and alanine mutants were isolated with unblocked amino-termini. These observations are consistent with two previous reports concerning the amino-terminal processing. First, the formyl group and the methionine residue of the initiating N-formylmethionine are removed sequentially in a two-step process. The deformylation reaction is much faster so that it may be more tightly associated with the translation process than the removal of the methionine (Pine, 1969; Hirel et al., 1989). Second, in *E. coli*, there is a clear correlation between removal of the methionine by the methionyl aminopeptidase and the presence of glycine, alanine, or proline in the penultimate position. If valine occupies the penultimate position, the likelihood of methionine removal is less. If leucine (or isoleucine) is in this position, methionine excision is very low (Hirel et al., 1989; Schrock & Lloyd, 1993) and was undetectable by mass spectroscopy in our preparations. These observations also have implications for the amino acids that can occupy the amino-terminal position. If the amino-terminus of an enzyme has evolved to play a critical role in the mechanism, then post-translational cleavage of the initiating methionine is essential and the specificity of the methionyl aminopeptidase may dictate which amino acids can occupy this position (Hirel et al., 1989).

An examination of the kinetic properties of the P1G and P1A mutants reveals that the major effects of these mutations are on the values of both k_{cat} and k_{cat}/K_m. Thus, the replacement of the rigid secondary amine Pro-1 with the flexible primary amine Gly-1 or Ala-1 introduces kinetic barriers to k_{cat} of 2.4−2.6 kcal/mol, to k_{cat}/K_m of 1.8−1.9 kcal/mol, and to the slow exchange of protons from the alternative substrate **4** of 0.9−1.4 kcal/mol. The effect on both k_{cat} and k_{cat}/K_m indicates that the mutations have affected the reaction chemistry, the release of product, or both the reaction chemistry and product release. Preliminary experiments[3] on the P1G and P1A mutants show a significant solvent isotope effect over the pH-independent range on both

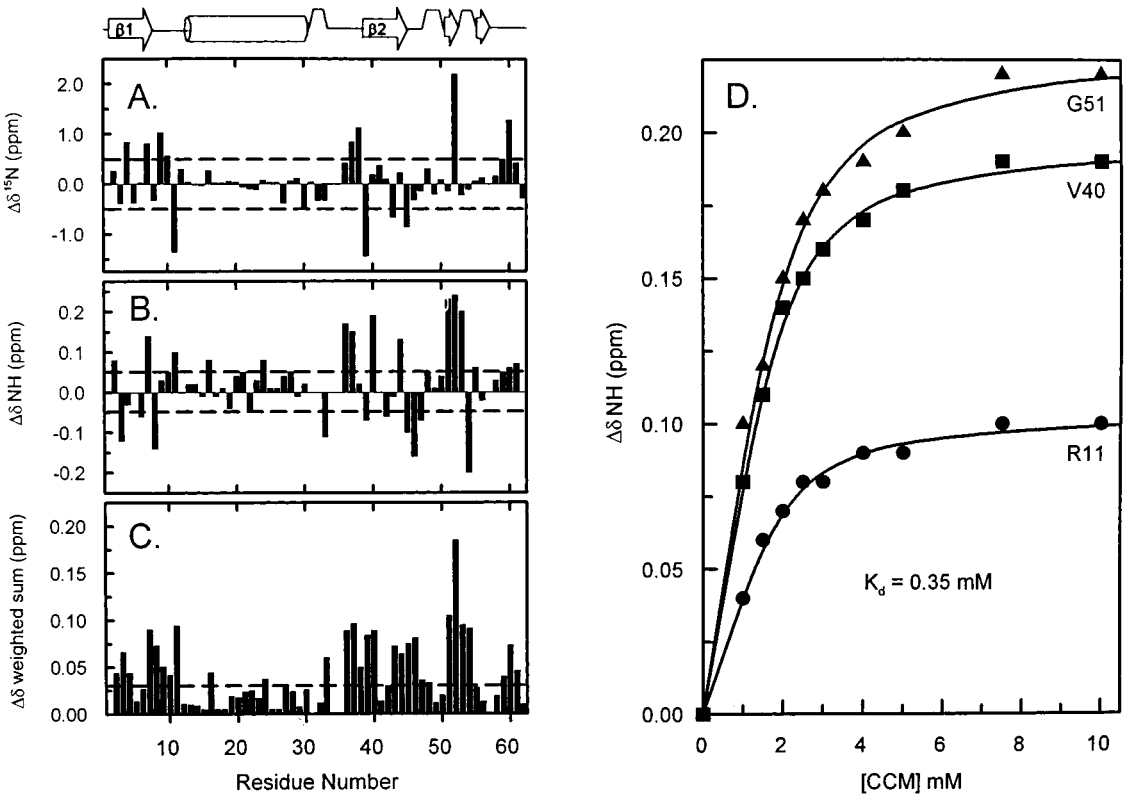


FIGURE 3: Changes in the backbone amide chemical shifts of the P1G mutant upon binding of *cis,cis*-muconate (**5**) and determination of the dissociation constant for the complex. Chemical shift changes ($\Delta\delta = \delta_{complex} - \delta_{free\ enzyme}$) were calculated on the basis of the chemical shift values obtained from $^1H-^{15}N$ HSQC spectra and plotted versus the residue number and solution secondary structure of wild-type 4-OT (Stivers et al., 1996c): (A) ^{15}N chemical shift differences; (B) NH chemical shift differences; (C) sum of the absolute magnitudes of the ^{15}N and NH chemical shift changes which were weighted according to the backbone amide chemical shift dispersion in the ^{15}N and ^1H dimensions (26.93 and 2.91 ppm, respectively), where dashed lines indicate the error limit; (D) determination of the dissociation constant for the complex. The amide proton chemical shift changes of Arg-11 (●), Val-40 (■), and Gly-51 (▲) were followed in $^1H-^{15}N$ HSQC spectra as the enzyme was titrated with **5**. The curves were generated by eq 1 using dissociation constants $K_D = 0.34 \pm 0.06$ mM (●), $K_D = 0.32 \pm 0.03$ mM (■), and $K_D = 0.40 \pm 0.07$ mM (▲), which yield a mean value of $K_D = 0.35 \pm 0.07$ mM for the binding of **5** to the P1G mutant enzyme.

mutant enzyme-catalyzed reactions suggesting that the effect is on the reaction chemistry, i.e., the rate of delivery of a proton to C5 of **3**.

The results of 3D $^1H-^{15}N$ NOESY HSQC and $^1H-^{15}N$ HSQC experiments on the P1G mutant indicate that the overall structure of this mutant is very similar to that of the wild-type enzyme. While some changes in backbone ^{15}N and NH chemical shifts occurred at the active site of the P1G mutant, the structural alterations at the active site are very small as indicated by four observations. First, a small, 4-fold decrease in K_m of the substrate **2** was found (Table 1). Second, a very small, 1.7-fold decrease in K_D of the substrate analog **5** was measured (Figure 3). Third, a comparable decrease in the pK_a of the catalytic base compared to model compounds was found (Figures 1A and 4) for both Gly-1 in the mutant and Pro-1 in the wild-type enzyme (Stivers et al., 1996b). Fourth, the stereochemistry of proton delivery to **3** is unaltered from that found with the wild-type enzyme. Hence, the 76-fold decrease in k_{cat} found in the P1G mutant (Table 1) is probably a direct effect of the substitution of glycine for proline as the general base. Similarly, the P1A mutant showed a small decrease in K_m but a 58-fold decrease in k_{cat} (Table 1), likely due to a direct effect of the change in the catalytic base.

Lower basicities of the primary amines in the P1G and P1A mutants, as compared to the secondary amine of Pro-1, are indicated in the pH rate profiles (Figure 1) by the lower pK_a values of the general base by 0.9 unit for Gly-1 and 0.3 unit for Ala-1.[4] With a β-value of unity, these decreases in basicity would predict 7.9- and 2.0-fold decreases in catalysis by the P1G and P1A mutants, respectively, which are comparable with the 10.3- and 4.5-fold decreases observed in the rates of solvent deuterium exchange catalyzed by these mutants into the partial substrate **4**. The order of magnitude greater effects of these mutations on k_{cat} of 76- and 58-fold may reflect, in addition to the decreases in basicity, the greater flexibility of the primary amines Gly-1 and Ala-1 compared to the secondary amine Pro-1. The flexibility could result in less than optimal positioning of the catalytic base. Such poor positioning of the catalytic base may be responsible for the 2300-fold decrease in k_{cat} observed in the M1P2L mutant with methionine as an additional residue at the amino-terminus, which significantly alters its location. Alternatively, the profound decrease in k_{cat} with the M1P2L

[3] R. M. Czerwinski and C. P. Whitman, unpublished observations, 1997.

[4] The decrease in the pK_a for the Gly-1 mutant compared to the Pro-1 enzyme agrees, within error, with the difference in the pK_a value between the amino acids themselves which is 0.8 unit. The decrease in the pK_a for the Ala-1 mutant is not as great as that predicted by the difference in the pK_a value between alanine and proline which is 0.7 unit (Dawson et al., 1986).

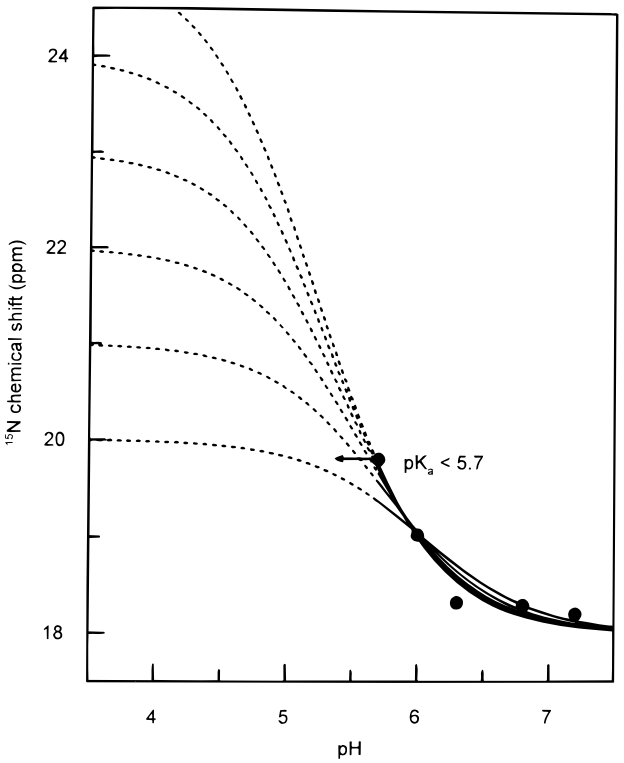


FIGURE 4: Determination of an upper limit pK_a for the amino group of glycine-1 in the P1G mutant by ^{15}N NMR spectroscopy (T = 30 °C). The lines are described by eq 2 using a Hill coefficient of $n = 1$, a limiting chemical shift at high pH of $\delta_2 = 18$ ppm, and pK_a = 6.04, 5.74, 5.55, 5.42, 5.31, and 5.23 for limiting chemical shifts at low pH of $\delta_1 = 20$, 21, 22, 23, 24, and 25 ppm, respectively. Best fits are obtained with $\delta_1 \geq 22$ ppm and p$K_a \leq$ 5.55.

mutant may reflect the complete loss of general base catalysis.

CONCLUSIONS

Proline-1, the general base in 4-OT, was mutated to nonpolar aliphatic residues, and several kinetic and structural parameters were measured and compared to those of the wild-type enzyme. Major effects were found only on k_{cat} which was decreased $10^{1.9}$-fold in the P1G mutant and $10^{1.8}$-fold in the P1A mutant. The P1G mutant showed small structural changes at the active site and no structural changes elsewhere in the molecule as determined by backbone ^{15}N and NH chemical shifts, nuclear Overhauser effects, and CD spectroscopy. An order of magnitude decrease in k_{cat} may result from the lower basicities of the primary amine Gly-1 or Ala-1 compared to the secondary amine Pro-1, as reflected in the lower pK_a's of these residues detected in pH rate profiles and also by direct titration in the case of the P1G mutant. The additional order of magnitude decrease in k_{cat} may result from the greater flexibility of the catalytic base in the mutants. Methionine as an additional residue at the amino-terminus profoundly decreases k_{cat} by a factor of $10^{3.4}$ due either to the drastically altered location of the catalytic base or to the complete blockage of general base catalysis.

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