Catalytic Role of the Amino-Terminal Proline in 4-Oxalocrotonate Tautomerase: Affinity Labeling and Heteronuclear NMR Studies†

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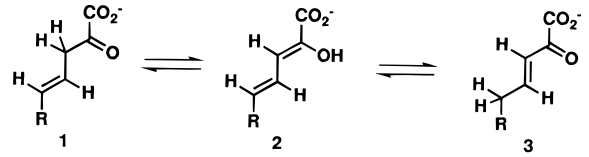
ABSTRACT: 4-Oxalocrotonate tautomerase (EC 5.3.2-; 4-OT), a hexamer consisting of 62 residues per subunit, catalyzes the isomerization of unsaturated α-keto acids, converting unconjugated ketones to the conjugated isomers via a dienolic intermediate. The recently solved crystal structure of an isozyme of 4-OT suggests that the amino-terminal proline is the catalytic base [Subramanya, H. S., Roper, D. I., Dauter, Z., Dodson, E. J., Davies, G. J., Wilson, K. S., & Wigley, D. B. (1996) *Biochemistry 35*, 792−802]. In support of this proposed role, we have found that the active-site-directed irreversible inhibitor 3-bromopyruvate (3-BP) blocks the amino terminus of 4-OT to Edman degradation and results in the disappearance of the ^{15}N resonance of Pro-1 (δ = 49.2 ppm at pH 6.40 and 42 °C) in the ^{15}N NMR spectrum of uniformly ^{15}N-labeled 4-OT. Furthermore, covalent bonding between a ^{15}N resonance of 4-OT and the methylene carbon of the reduced, 3-^{13}C-labeled lactyl adduct derived from [3-^{13}C]-bromopyruvate was then directly demonstrated using two heteronuclear NMR methods, an ^{1}H−^{13}C HSQC experiment and a novel inverse correlation experiment which we call H(C)N. The chemical shift of the modified ^{15}N resonance (δ = 86.5 ppm) is consistent with that of an alkylated and cationic, amino-terminal proline. Affinity labeling with 2-^{14}C-labeled bromopyruvate indicates that the ultimate stoichiometry of modification is 1 equiv of 3-BP per 4-OT monomer. However, an analysis of the residual enzyme activity after differing extents of fractional modification with 3-BP indicates that modification of three active sites per hexamer abolishes essentially all activity of the hexamer. Thus, 4-OT exhibits half-of-the-sites stoichiometry with 3-BP. Finally, the pH dependence of k_{inact}/K_I for affinity labeling by 3-BP yields a pK_a value of 6.7 ± 0.3, in reasonable agreement with the pK_a values found for k_{cat}/K_M for the non-sticky substrate 2-hydroxy-2,4-pentadienoate and by direct NMR titration of Pro-1 [Stivers, J. T., Abeygunawardana, C., Mildvan, A. S., Hajipour, G., & Whitman, C. P. (1996) *Biochemistry 35*, 814−823]. These results strongly implicate the amino-terminal proline as the general-base catalyst on 4-OT.

4-Oxalocrotonate tautomerase[1] (EC 5.3.2-; 4-OT) from *Pseudomonas putida mt*-2 catalyzes the near diffusion-controlled isomerization of unconjugated α-keto acids, such as **1a** to the conjugated isomer **3a** via a dienolic intermediate **2a** (Scheme 1), accelerating the rate of this reaction by a factor of ∼10^8 (Whitman *et al.*, 1991). The enzyme is a hexamer (Roper *et al.*, 1994) with six identical subunits, each consisting of only 62 amino acid residues. Stereochemical studies indicate that this enzyme-catalyzed allylic rearrangement is a suprafacial process consistent with a single base mechanism (Lian & Whitman, 1993). As with other non-metal-requiring keto−enol tautomerases, such as ketosteroid isomerase (Kuliopulos *et al.*, 1989, 1990) and triose phos-

Scheme 1



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[1] Abbreviations: 4-OT, 4-oxalocrotonate tautomerase; **1a**, 2-oxo-4-hexenedioate; **2a**, 2-hydroxy-2,4-hexadienedioate; **3a**, 2-oxo-3-hexenedioate; **2b**, 2-hydroxy-2,4-pentadienoate; 3-BP, 3-bromopyruvate; **4**, 2-oxo-1,6-hexanedioate; CP-A, carboxypeptidase-A; MALDI, matrix assisted laser desorption−ionization; H(C)N, proton to nitrogen correlation via carbon; HSQC, heteronuclear single-quantum correlation; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; NOE, nuclear Overhauser effect; DMSO, dimethyl sulfoxide.

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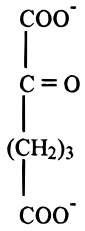
phate isomerase (Lodi & Knowles, 1993), general acid−base catalysis of this process may be anticipated.

In preliminary studies[2] it was observed that 4-OT catalyzes H → D exchange of the C-3 protons of the α-keto acids pyruvate and 2-oxo-1,6-hexanedioate (**4**). This observation



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suggested that 3-bromopyruvate might act as an active site directed irreversible inhibitor of the enzyme by covalent modification of the active site base. In support of this hypothesis, a proton NMR study (Stivers *et al.*, 1994) showed, by transferred NOEs, that the partial substrate **4** is bound near Phe-50 indicating that this residue is in the active site. 3-Bromopyruvate irreversibly displaced **4** from the active site of 4-OT, abolished the transferred NOE, and changed the proton chemical shifts of Phe-50.

A recent high-resolution X-ray structure of an isozyme of 4-OT isolated from *Pseudomonas* CF600 showed that the reactive species generated from the mechanism-based inactivator 2-oxo-4-pentynoate covalently modified the amino-terminal proline, implicating this residue as the general base (Subramanya et al., 1996; Whitman *et al.*, 1995). However, the reactive species, presumably an allene, is highly electrophilic and could modify residues other than those directly involved in catalysis (Penning & Talalay, 1981; Kuliopulos *et al.*, 1989). The data presented here and in the following paper provide evidence establishing Pro-1 to be the general base by showing that it is the sole target of modification by the substrate-based affinity label 3-BP and that the pK_a value for Pro-1 is consistent with the pH dependence of 3-BP inactivation and the pH-rate profile of 4-OT. The phenomenon of half-of-the-sites inhibition by 3-BP is observed and discussed in relation to the quaternary structure of 4-OT. A preliminary abstract of this work has been published (Stivers *et al.*, 1995).

MATERIALS AND METHODS

Materials

Crystalline 3-bromopyruvic acid was obtained from Sigma. 2-^{14}C- and 3-^{13}C-labeled sodium pyruvate were obtained from New England Nuclear and Isotec, respectively. Bromine (99.99+%) was purchased from Aldrich and redistilled immediately before use. Lactate dehydrogenase, sequencing grade endoproteinase glu-C (protease V8), and buffers were obtained from Sigma. All other solvents and reagents were of the highest quality commercially available. The synthesis of **2a** has been described (Whitman *et al.*, 1991). Centricon (10 000 MW cutoff) centrifugal microconcentrators and ultrafiltration membranes were purchased from Amicon. Dialysis tubing (6000−8000 MW cutoff) was obtained from Spectrum Medical Industries, Inc. The construction of the plasmid pBAot1 which contains the gene for 4-OT (*xylH*) under the control of the alkaline phosphatase promoter has been described (Chen *et al.*, 1992). 4-Oxalocrotonate tautomerase was purified to homogeneity as assessed by SDS−PAGE and specific activity according to published procedures (Chen *et al.*, 1992).

Methods

Protein Concentration. Identical determinations of 4-OT (±4%) were obtained by extensive lyophilization of enzyme solutions in tared vials and weighing, correcting for salts, and by the method of Waddell (Waddell, 1956; Wolf, 1983) in which the concentration (in μg/mL) is determined from the difference in absorption measurements at 215 and 225 nm multiplied by the factor 144. The latter method is as accurate a measure of total protein as determinations by nitrogen analysis, with little influence by variation in protein structure (Wolf, 1983). This assay is linear in the range 30−110 μg/mL of 4-OT.

Overexpression and ^{15}N-Labeling of 4-OT. Techniques for restriction enzyme digestions, ligation, and transformation were based on methods described elsewhere (Sambrook *et al.*, 1989). Our strategy for the overexpression of isotopically labeled 4-OT was to subclone the gene for 4-OT into the T7 expression system (pET system, Novagen) which is amenable to ^{15}N-labeling in a MOPS-buffered medium (Neidhardt *et al.*, 1974) containing 50 μg/mL of kanamycin and 0.8 g/L of 99% enriched $^{15}NH_4Cl$. Accordingly, the gene for 4-OT was obtained by the digestion of pBAot1 with the restriction enzymes *Nde*I and *Sal*I. The insert was eluted and purified from 1.5% agarose gel using the commercially available MERmaid DNA kit (Bio 101, La Jolla, CA). The commercially available plasmid pET24a was used as the expression vector. Upon complete digestion of pET24a with *Nde*I and *Sal*I, the linearized vector was isolated from an agarose gel and ligated with the similarly digested *xy1I* gene. The newly constructed plasmid (pETOT) was transformed into the commercially available strain *Escherichia coli* BL21 (DE3) according to the supplier's directions. ^{15}N-labeling was accomplished by growing the bacteria at 37 °C for 2.5 h in Luria Broth medium. Subsequently, 1 mL of this culture was used to innoculate 100 mL of the MOPS-buffered medium which was then incubated in a 2 L Erlenmeyer flask for 16 h at 28 °C with vigorous shaking. This culture was diluted to 350 mL with fresh MOPS-buffered medium and divided into two 175 mL portions. These cultures were grown at 37 °C to an A_{600} = 1.8, induced by the addition of 1 mM (final concentration) of isopropyl thiogalactoside, grown for an additional 4 h at 37 °C, and harvested. A yield of ∼40 mg/L of purified enzyme was typically obtained using the purification procedure of Chen *et al.* (1992).

Kinetics of Irreversible Inhibition. Kinetic data for the irreversible inhibition were obtained on a Hewlett Packard 8452A Diode Array spectrophotometer. The inactivation of 4-OT by 3-BP was determined by the incubation of varying amounts of inhibitor (0.45−4.5 mM) with enzyme (1.7 μM) in 20 mM sodium phosphate buffer (pH 6.75) at 23 °C. The incubation mixtures (total volume: 200 μL) were made up in 1.5 mL eppendorf micro test tubes. Aliquots (5 μL) from these solutions were removed at various time intervals, diluted into 1 mL of 20 mM sodium phosphate buffer (pH

[2] C. P. Whitman, unpublished observations, 1993.

7.25), and assayed for residual 4-OT activity. Enzyme activity was monitored by following the formation of **3a** at 236 nm (Whitman *et al.*, 1991). The assay was initiated by the addition of **2a** to give a final concentration of 0.14 mM. Stock solutions of 3-BP were made fresh daily in 20 mM sodium phosphate buffer and the pH adjusted to 6.70. Stock solutions of **2a** were made fresh daily in ethanol.

The observed rate constant for inactivation (k_{obsd}) at each inhibitor concentration was determined from a nonlinear least-squares fit of the data for loss in enzyme activity as a function of incubation time to the equation for a first-order decay. At all concentrations of 3-BP used, the decrease in activity was pseudo-first-order in enzymatic activity for at least three half-lives. The reciprocals of the k_{obsd} values for inactivation were initially plotted against the reciprocals of the inhibitor concentrations and fitted to a straight line by unweighted regression analysis to yield K_I, k_{inact}, and k_{inact}/K_I (Meloche, 1967). Additionally, the data were fitted directly to a rectangular hyperbola by nonlinear least-squares analysis with comparable results, within experimental error.

Protection against inactivation of 4-OT by an equilibrium mixture of **1**−**3a** was carried out as described above with the following modifications. The enzyme (4.4 μM) was incubated with varying concentrations of **2a** (0−4.5 mM) in 20 mM sodium phosphate buffer (pH 6.80) at 23 °C. After a 5 min interval in which **2a** is converted into an equilibrium mixture of **1**−**3a** (Whitman *et al.*, 1991), a fixed concentration of 3-BP (3.75 mM) was added to the mixture, and aliquots (5 μL) were removed at various time intervals over a 3 min period and assayed for residual activity (see above). The data were plotted and analyzed as described above.

The irreversibility of inactivation was established as follows. The enzyme (0.04 μmol) was incubated with a large excess of 3-BP (17 μmol) in 10 mL of 20 mM sodium phosphate buffer (pH 6.80) for 20 min at 4 °C. In a separate control experiment, the same quantity of enzyme was incubated without 3-BP under identical conditions. Both samples were dialyzed exhaustively against 20 mM sodium phosphate buffer (pH 6.80) for several days. The samples were monitored periodically for activity by the removal of an aliquot (5 μL) and its dilution into 200 mL of 20 mM sodium phosphate buffer (pH 6.80). An aliquot (5 μL) was removed from the resulting solution and assayed as described above. After 6 days, the inactivated enzyme regained ∼3% of its original activity.

pH Dependence of Inactivation. The pH dependence of the kinetic parameters for inactivation by 3-BP was determined in 100 mM sodium phosphate buffer over a pH range of 5.45−8.00. Higher buffer concentrations and other buffers significantly accelerate the nonenzymatic reaction (Whitman *et al.*, 1991). The inactivation at each pH was determined as described above using five concentrations of inhibitor. The pH of each solution was checked before and after reaction with 3-BP. In control experiments it was shown that the enzyme retains ≥95% of its initial activity when preincubated at pH 5.4 or 8.00 prior to assaying for activity at pH 7.3. The kinetic parameters (k_{inact}/K_I, k_{inact}, and K_I) were obtained as described above. The acid dissociation constants of the free enzyme (K_E) and EI complex (K_{EI}) were determined from a nonlinear least-squares fit of the pH dependence of these parameters using eq 2−4 (see Results).

Synthesis of Isotopically Labeled 3-BP. 2-^{14}C- and 3-^{13}C-labeled 3-BP were synthesized by the method of Barnett *et al.* (1971) with the following modifications. Briefly, 1.6 μmol of 2-^{14}C-labeled sodium pyruvate (31 mCi/mmol) and 7.8 μmol of unlabeled sodium pyruvate were dissolved in 250 μL of glacial acetic acid in a 1.5 mL eppendorf tube. Freshly distilled bromine (20 μL) was diluted to 2 mL with glacial acetic acid, and 28 μL of this solution was added to the pyruvate−acetic acid solution. Concentrated H_2SO_4 (1 μL) was then added, and the parafilmed tube was incubated at 65 °C for 2 h. Analysis of the reaction products by silica gel thin layer chromatography (acetonitrile/acetic acid/H_2O, 14:2:5) with visualization by iodine staining indicated a yield of ≥70% of crude bromopyruvate. The pyruvic acid, acetic acid, and residual bromine were removed by evaporation *in vacuo* at 40 °C for 2 h. The resulting brownish-yellow oil was suspended in 100 μL of acetone and centrifuged in a table-top microfuge for 5 min to pellet insoluble salts (mostly NaBr). The supernatant was carefully removed and concentrated by evaporation *in vacuo* to give bromopyruvate (60% yield) as a brownish-yellow oil which was stored at −80 °C. Before use, the oil was dissolved in 400 μL of 0.2 M potassium phosphate buffer, pH 7.3. The radioactivity of the resulting solution was measured and the concentration of 2-^{14}C-labeled bromopyruvate was determined using the specific activity of the sodium pyruvate starting material. 3-^{13}C-labeled bromopyruvate was synthesized from 10 mg of 3-^{13}C-labeled sodium pyruvate in a similar manner. The concentration of 3-^{13}C-labeled bromopyruvate was determined spectrophotometrically at 340 nm by following the oxidation of NADH in the presence of lactate dehydrogenase (Barnett *et al.*, 1971). The concentrations of solutions prepared from crystalline unlabeled 3-BP (0.5 mol H_2O/mol) were determined gravimetrically and enzymatically; both methods gave equivalent results (±4%).

Stoichiometry of Inactivation by 3-BP. For the determination of the number of essential residues modified by 3-BP, various concentrations (0−2.7 mM) of 2-^{14}C-labeled or unlabeled 3-BP were added to samples of 4-OT (0.5−0.69 mM in monomer concentration) in 20 mM sodium phosphate buffer, pH 7.3, to a final volume of 40−160 μL. After the samples were incubated for 2 h at 30 °C, 10 μL portions were removed and serially diluted 6700-fold. The remaining enzymatic activity was then determined as described above. The fractional activity remaining at each concentration of 3-BP was determined by dividing the residual activity in the modified samples by the activity of the unmodified enzyme sample. After determination of the residual activities, the remaining portions of the radiolabeled enzyme samples were reduced by the addition of 10−40 μL of a freshly prepared solution of sodium borohydride (19 mg/mL in H_2O) to prevent loss of the labile pyruvyl moiety. After incubation for 10 min at room temperature, the samples were diluted to a final volume of 1 mL and freed from nonprotein bound radioactivity by binding the protein to a reverse-phase C_{18} resin (Waters, Sep-Pac Classic cartridges), washing with 20 mL of water and eluting the labeled protein with 2 mL of acetonitrile.

The fractions containing protein were concentrated to dryness *in vacuo* and redissolved in 150 μL of H_2O. The protein concentrations of the samples were determined by absorbance measurements (Waddell, 1956), and the radioactivity was determined by liquid scintillation spectrometry. The stoichiometry of labeling for each sample was determined from the known specific activity of the 2-^{14}C-labeled

3-BP and the measured protein concentrations. The stoichiometry of affinity labeling by unlabeled 3-BP was taken to be the ratio of 3-BP added to the enzyme hexamer on the basis of the following results: the quantitative stoichiometric labeling obtained by modification with radiolabeled 3-BP, the stoichiometric blockage of the amino terminus of 4-OT by unlabeled 3-BP as determined by Edman degradation, and the loss of the ^{15}N NMR resonance of the amino nitrogen of proline-1 at $\delta^{15}N = 49.2$ ppm upon addition of 1.1 equiv of 3-BP (see Results). The fraction of the activity remaining (a) after partial modification is given by $a^{1/i} = n$, where i is the number of essential residues modified and n is the molar ratio of bound affinity label to enzyme hexamer. The integer value of i which gave the best straight line was determined from plots of $a^{1/i}$ versus n (Tsou, 1962; Paterson & Knowles, 1972; Horiike & McCormick, 1979, 1980).

Peptide Mapping. An 0.5 mg sample of [2-^{14}C]bromopyruvate-modified and reduced 4-OT (containing six covalently bound lactyl groups per hexamer) prepared as described above was lyophilized and digested with protease V8 from *Staphylococcus aureus* [V8/4-OT = 1/20 (w/w)] in 1.0 mL of 40 mM ammonium acetate (pH 4) for 18 h at 37 °C (Smith, 1988). The digested sample was lyophilized to remove volatile buffer salts and redissolved in 500 μL of water/0.06% TFA. The radioactive peptide was then purified on a C_{18} reverse-phase HPLC column (30 cm × 7.8 mm), washing first with H_2O/0.06% TFA for 10 min, eluting with a linear gradient (0−100% acetonitrile/0.06% aqueous TFA) over the next 45 min, followed by a 100% acetonitrile/0.06% aqueous TFA wash for 15 min at a flow rate of 0.6 mL/min. Fractions (1 mL) were collected, and the radioactivity in a 25 μL aliquot of each fraction was determined by liquid scintillation spectrometry. To ensure purity, the radioactive peak was pooled, lyophilized, redissolved in 200 μL of aqueous 0.06% TFA, and rechromatographed in an identical manner. The rechromatographed material was lyophilized and redissolved in 0.06% aqueous TFA to give a final concentration of 8 pmol/μL peptide as determined from the specific activity. The sample was then analyzed by MALDI mass spectrometry and Edman degradation.

Mass Spectrometry. The mass of the purified radioactive peptide was determined by MALDI mass spectrometry using a Kratos Kompact MALDI III time-of-flight laser desorption instrument equipped with a nitrogen laser at 337 nm. The matrix consisted of saturated α-cyano-4-hydroxycinnamic acid in ethanol/water (1:1). The peptide solution was mixed with the matrix solution in a 1:1 (v/v) ratio. The matrix/sample solution (0.3 μL) was applied to a stainless steel target, air dried, and introduced into the spectrometer. Carboxyl-terminal sequencing of the modified peptide using carboxypeptidase-A was performed essentially as described (Woods *et al.*, 1995) except that 0.3 μL of 1 M ammonium citrate or ammonium bicarbonate buffer was added to the matrix/sample solution.

Amino-Terminal Sequence Analysis. Amino-terminal sequencing of unmodified 4-OT, lactyl modified 4-OT, and the [2-^{14}C]lactyl modified peptide fragment (see above) was done by automated Edman degradation on an Applied Biosystems Model 470A peptide sequencer interfaced with a Model 120A PTH analyzer. While the pyruvyl adduct of 4-OT is stable for at least 24 h in the pH range 6.3−7.3 based on NMR studies (not shown), it decomposes during the acidic steps of the Edman procedure. Hence, reduction to the lactyl form was required to stabilize the adduct.

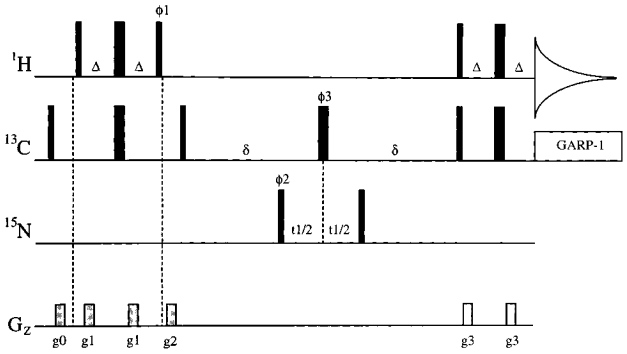


FIGURE 1: Pulse sequence of the H(C)N experiment. The values Δ and δ are set to 1.87 and 18.0 ms, respectively. Thin and thick bars represent 90° and 180° pulses, respectively, and unless otherwise mentioned all pulses are applied along x. The phase cycling used is as follows: $\phi_1 = y, -y$; $\phi_2 = 2(x), 2(-x)$; $\phi_3 = 4(x), 4(y), 4(-x), 4(-y)$; Acq = $x, 2(-x), x, -x, 2(x), -x$. The phase of ϕ_2 is incremented by 90° to generate complex data in the t_1 dimension. Quadrature detection is achieved using the States−TPPI method (Marion *et al.*, 1989). All ^{13}C pulses and GARP-1 decoupling (Shaka *et al.*, 1985) are centered at 60 ppm, using 14.6 and 1.3 kHz RF fields, respectively. The duration and strengths of the gradients are $g_o = 1.0$ ms, 8 G/cm; $g_1 = g_3 = 500$ μs, 8 G/cm; and $g_2 = 500$ μs, 6 G/cm.

One-Dimensional ^{15}N NMR Spectroscopy. NMR experiments were performed at 42 °C on 0.6 mL samples of 6 mM uniformly ^{15}N-labeled 4-OT (monomer concentration) which were dissolved in 90% H_2O/10% D_2O containing 6.9 mM sodium phosphate buffer, pH 6.42. Spectra were obtained on a Varian Unity-Plus 500 NMR spectrometer operating at 50.659 MHz for ^{15}N. The ^{15}N spectra were acquired without proton decoupling using a 5-mm broadband direct detection probe. The acquisition parameters for each experiment are described in the figure legends. Spectra were referenced to external liquid ammonia as described (Weber *et al.*, 1993).

Heteronuclear NMR Spectroscopy. Direct bonding between an ^{15}N atom of 4-OT and the C-3 carbon atom of the bound affinity label [3-^{13}C]bromopyruvate was demonstrated using two 2D NMR experiments. First, the chemical shift of the 3-^{13}C resonance of the enzyme-bound affinity label was correlated with that of its attached methylene protons using the ^{1}H−^{13}C HSQC pulse sequence (Vuister & Bax, 1992). Second, the chemical shift of the methylene proton resonance was correlated with that of the directly bonded ^{15}N atom of uniformly ^{15}N-labeled 4-OT using a novel 2D heteronuclear inverse correlation experiment which we call H(C)N. The H(C)N experiment involves magnetization transfer steps using the large single bond ^{1}H−^{13}C coupling ($J_{CH} = 140$ Hz) and the smaller ^{13}C−^{15}N coupling ($J_{CN} \cong 10$ Hz). Figure 1 shows the pulse scheme used for the 2D H(C)N experiment which is analogous to the original HNCO triple-resonance 3D sequence of Kay *et al.* (1990). A description of the pulse sequence is as follows. In order to ensure that the magnetization originates on ^{1}H and not on ^{13}C, the carbon magnetization is completely randomized at the start of the experiment by the application of a ^{13}C 90° pulse followed by a field gradient (Kay, 1993). Magnetization originating on the ^{13}C-bound protons is then transferred to the directly coupled ^{13}C resonance by an INEPT transfer. During the delay δ, ^{13}C magnetization becomes antiphase with respect to the polarization of the ^{15}N spin. The first

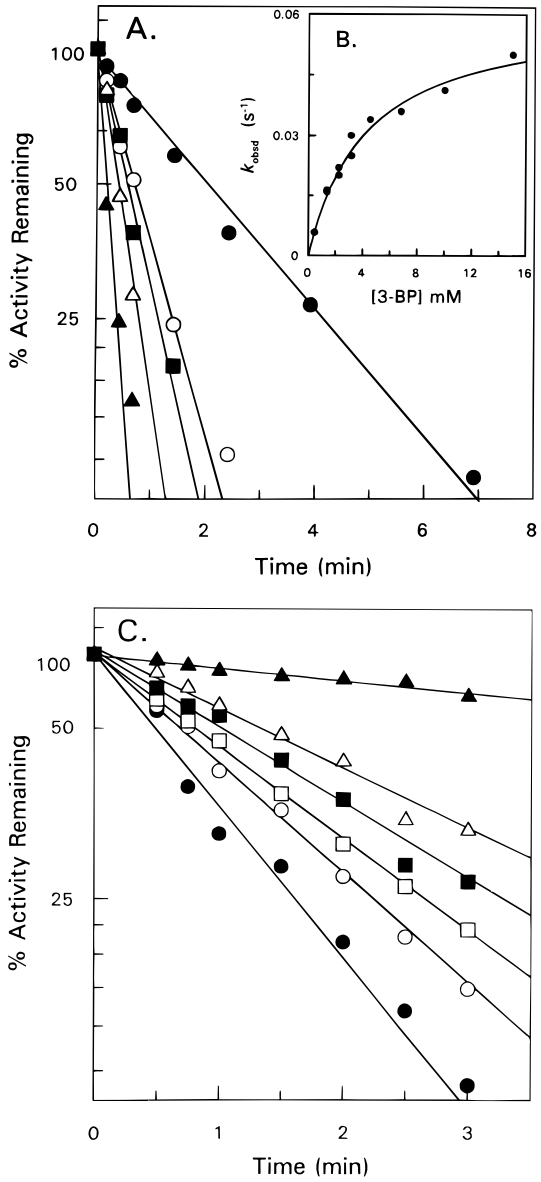


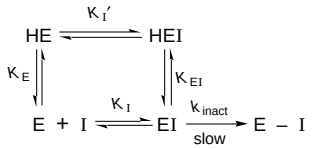
FIGURE 2: Kinetics of 4-OT inactivation by 3-BP and protection by **1**−**3a**. (A) A logarithmic plot of the percent 4-OT activity remaining as a function of the incubation time with varying amounts of 3-BP (filled circles, 0.45 mM; open circles, 1.35 mM; filled squares, 2.24 mM; open triangles, 3.14 mM; filled triangles, 4.5 mM). (B, insert) Plot of k_{obsd} for inactivation against the 3-BP concentration in the preincubation mixtures. The data are fit by a rectangular hyperbola using the $(k_{inact})^{max}$ and $(K_I)^{max}$ values given in the text. (C) Protection against 3-BP inactivation by a mixture of **1**−**3a**. The enzyme (4.4 μM) was preincubated for 5 min with various concentrations of **2a** (filled circles, 0 mM; open circles, 0.71 mM; open squares, 1.43 mM; filled squares, 2.14 mM; open triangles, 2.85 mM; filled triangles, 4.5 mM) prior to the addition of 3-BP (3.75 mM). Aliquots were removed and assayed for residual enzymic activity.

90° ^{15}N pulse converts the antiphase ^{13}C magnetization into ^{13}C−^{15}N two-spin coherence. The contributions of ^{13}C chemical shift and ^{1}H−^{13}C J coupling are removed during the t_1 period by the application of a 180° ^{13}C pulse. At the end of the ^{15}N evolution period, t_1, magnetization is transferred back to the ^{13}C-bound methylene protons by reversing the INEPT transfer steps described above. As discussed by Bax and Pochapsky (1992), the remaining gradient pulses were applied to eliminate spectral artifacts as well as to attenuate large residual signals resulting from protons other than those directly bonded to ^{13}C.

RESULTS

Time-Dependent Inactivation of 4-OT by 3-BP. Incubation of 4-OT with 3-BP in phosphate buffer resulted in the time-dependent, irreversible inactivation of 4-OT in a pseudo-first-order process for approximately three half-lives (Figure 2A). The k_{obsd} values measured in 10 experiments were plotted vs the inhibitor concentration and fit to a rectangular hyperbola (Figure 2B). The values of $(k_{inact})^{max}$ and $(K_I)^{max}$ obtained from this plot are 0.061 ± 0.004 s^{-1} and 4.2 ± 0.6 mM, respectively. The hyperbolic inactivation suggests that the enzyme and 3-BP form a dissociable complex at the active site prior to covalent bond formation and inactivation. Active-site binding by 3-BP is further indicated by the fact that an equilibrium mixture **1**−**3a** protects the enzyme against inactivation (Figure 2C).[3] Finally, dialysis does not regenerate enzymatic activity, which is consistent with the formation of a covalent bond between 3-BP and 4-OT.

pH Dependence of Inactivation. The inactivation by 3-BP as a function of pH was studied in order to determine the pK_a of the group undergoing modification and to compare this value with the pK_a's determined in the pH rate profiles. The data were analyzed using the model shown in eq 1, where HE and E are the protonated and unprotonated forms of the enzyme, K_E and K_{EI} are the acid dissociation constants for HE and HEI, and K_I and K_I' are the dissociation constants for 3-BP from the HE and HEI complexes, respectively. The data for the pH dependence of (k_{inact}/K_I), k_{inact}, and K_I were fitted to eq 2s−4 using a nonlinear least-squares method.



(1)

$$\frac{k_{inact}}{K_I} = \frac{(k_{inact}/K_I)^{max}}{\left(1 + \frac{[H^+]}{K_E}\right)} \quad (2)$$

$$k_{inact} = \frac{(k_{inact})^{max}}{\left(1 + \frac{[H^+]}{K_{EI}}\right)} \quad (3)$$

$$K_I = K_I^{max} \frac{\left(1 + \frac{[H^+]}{K_E}\right)}{\left(1 + \frac{[H^+]}{K_{EI}}\right)} \quad (4)$$

The rate of inactivation by 3-BP was studied only in the pH range 5.1−8.5 because hydroxide ion dependent decomposition of 3-BP occurs at higher pH values and reversible dissociation of the hexamer occurs at pH values <5.[4]

Evidence that 3-BP modifies the active site base of 4-OT was provided by the pH dependence of k_{inact}/K_I (Figure 3),

[3] Under the conditions of the protection experiment, an equilibrium mixture consisting of 11% **1a**, 8% **2a**, and 81% **3a** exists (Whitman *et al.*, 1991). Thus the apparent $K_{0.5}$ = 2.1 mM for half-maximal protection against inactivation by 3-BP (Figure 2C) is a concentration weighted average of the true dissociation constants for **1a, 2a**, and **3a** from 4-OT.

[4] J. T. Stivers, unpublished observations, 1994.

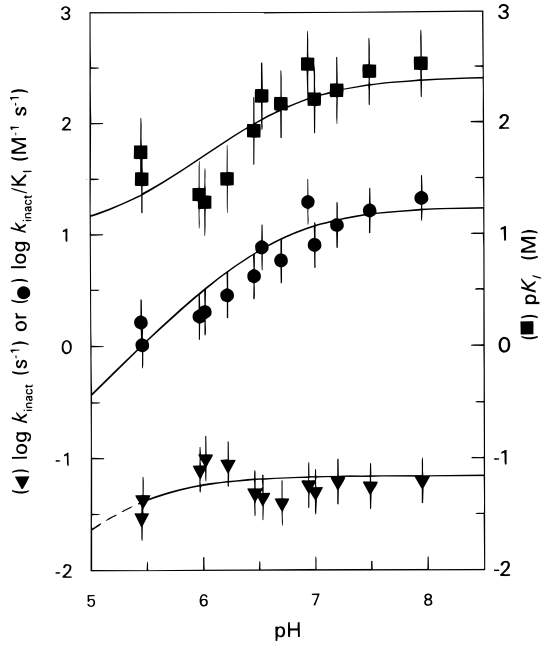


FIGURE 3: pH dependence of inactivation by 3-BP. The pH dependences of log k_{inact} (triangles), log k_{inact}/K_I (circles), and pK_I (= −log K_I, squares) are shown. The curves were obtained from a nonlinear least-squares fit of the data to eqs 2−4 with values for pK_E = 6.7 ± 0.3 and pK_{EI} = 5.3 ± 0.4. While the pH dependence of log k_{inact} reveals a pK_{EI} ≤ 5.7, the dashed curve has been drawn with a pK_{EI} of 5.3, for consistency with the pH dependence of pK_I.

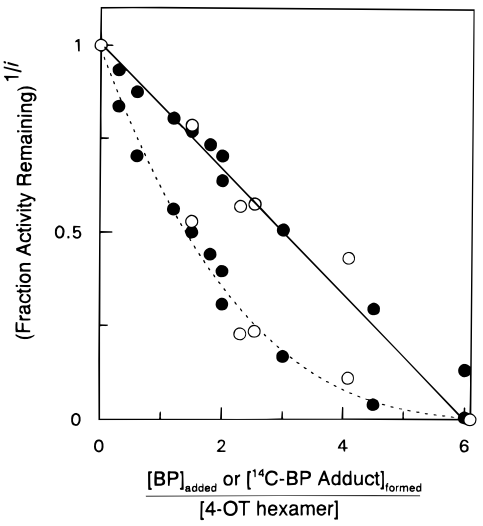


FIGURE 4: Stoichiometry of 3-BP inactivation and determination of the number of essential residues (*i*) modified. Samples of 4-OT (83−115 μM) were incubated with increasing concentrations of 2-^{14}C-labeled (open circles) or unlabeled 3-BP (closed circles) in 20 mM sodium phosphate buffer at 30 °C and pH 7.3 for 2 h. The samples were then diluted and assayed for residual enzymatic activity using **2a**. The raw data (dashed line, i = 1) are well linearized using a value for i of three (solid line) indicating that there are three essential catalytic residues/hexamer. The intercept on the abcissa (= 6 ± 0.1) shows that the final stoichiometry of affinity labeling is six sites/4-OT hexamer.

which gave a pK_E of 6.7 ± 0.3 for the free enzyme. This pK_E is similar to the pK_a obtained from the pH dependence of k_{cat}/K_M for the nonsticky substrate 2-hydroxy-2,4-pentadienoate (**2b**) (6.2 ± 0.3) and to the pK_a of 6.4 ± 0.2 for Pro-1 which was determined directly by ^{15}N NMR titration (see following paper).

The maximal rate constant for inactivation by 3-BP (k_{inact}) was found to be largely pH-independent over the range 5.4−8.0 (triangles, Figure 3), indicating that the acid dissociation constant for the EI complex (K_{EI}, eq 1) is lowered from that of the free enzyme (K_E). Although the precision and range of the data for the pH dependence of k_{inact} are not sufficient to allow accurate measurement of this pK_{EI} value, we estimate an upper limit value of pK_{EI} ≤ 5.7 on the basis of the maximum decrease in k_{inact} that was observed. In a separate analysis, the pH dependence of K_I (squares, Figure 3) yields values for pK_E = 6.7 ± 0.4 and pK_{EI} = 5.3 ± 0.4 which are consistent with the results above. Accordingly, the dashed curve in Figure 3 for the pH dependence of log k_{inact} has been drawn with a pK_{EI} of 5.3. Figure 3 also shows that K_I', the dissociation constant of 3-BP from HEI, is ≈15-fold greater than K_I, indicating that protonation of the residue with the pK_E of 6.7 ± 0.3 profoundly lowers the affinity of 4-OT for 3-BP.

Stoichiometry of Inactivation by 3-BP. The pseudo-first-order inactivation of 4-OT by 3-BP is consistent with the modification of either a single site or multiple sites which are kinetically indistinguishable. In a probability-based approach initially proposed by Tsou (1962) and subsequently extended and utilized by others (Paterson & Knowles, 1972; Horiike & McCormick, 1979, 1980), the number of essential residues modified by an irreversible active-site-directed reagent may be simply determined from measurements of the number of residues modified and the remaining enzymatic activity in partially modified samples of enzyme. As described in detail elsewhere (Tsou, 1962), if the modification of any one of the essential residues in the enzyme leads to complete loss of activity, then the fraction of activity remaining (a) after partial modification is given by $a^{1/i} = n$, where i is the number of essential residues modified and n is the total number of modified groups. The best integer value of i linearizes the plot of $a^{1/i}$ versus n. An important limitation to this method is that the best value of i may be only a minimum number because the modification of one essential group may prevent modification of another essential residue either by direct steric hindrance or by an induced conformational change in the enzyme.

A plot of $a^{1/i}$ against the molar ratio of 3-BP to 4-OT hexamer is shown in Figure 4. The closed circles show inactivation data by unlabeled 3-BP, and the open circles show inactivation data for 2-^{14}C-labeled bromopyruvate. The data for the ^{14}C-labeled and unlabeled bromopyruvate are indistinguishable, indicating that the molar stoichiometry determined by the specific activity of the ^{14}C-labeled enzyme and the gravimetrically prepared solutions of crystalline 3-BP (both of which were also assayed with lactate dehydrogenase) are in good agreement. Figure 4 shows that a total of six groups per enzyme hexamer are modified when activity is fully lost, and that the raw inactivation data (dashed line, i = 1) is not linear with respect to the molar stoichiometry.

The inactivation data are best linearized using a value for i = 3 (solid line, Figure 4), indicating that there are three essential basic residues per hexamer. Alternative fits of the data, using i values of 2 and 4, were unsatisfactory by three objective criteria: (1) lower linear correlation coefficients as compared to i = 3, (2) the presence of systematic curvature in the plots for i = 2 and 4 (but not for i = 3) resulting in a 2.2-fold (i = 2) and 0.35-fold (i = 4) improvement in the fit of the data when a binomial expression was used instead of a simple linear expression, and (3) a 33% deviation from

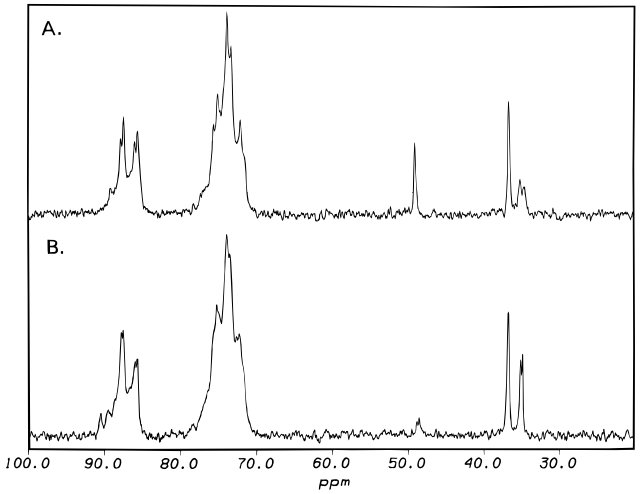


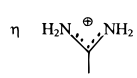
FIGURE 5: Upfield region of the proton coupled 50.66 MHz ^{15}N NMR spectra of uniformly ^{15}N-labeled 4-OT in the absence (A) and presence (B) of bromopyruvate at pH 6.4, 42 °C. In these spectra the $^{15}N_\eta$ and $^{15}N_\epsilon$ resonances of the six arginine residues ($\delta^{15}N \approx 70-90$ ppm), the $^{15}N_\epsilon$ resonances of the three lysine residues ($\delta^{15}N \cong 34-36$ ppm), and the secondary amino nitrogen resonance of proline-1 ($\delta^{15}N = 49.2$ ppm) are observed. Upon addition of 1.1 equiv of [3-^{13}C]bromopyruvate per monomer, the intensity of the nitrogen resonance of proline-1 is significantly diminished. The acquisition parameters were as follows: spectral width, 20 000 Hz; acquisition time, 0.302 s; relaxation delay, 0.7 s; total transients, 7200. A tip angle of 60° for nitrogen was used.

the measured 1:1 stoichiometry of modification at the end point when $i = 4$. Thus, the inactivation of 4-OT by 3-BP exhibits half-of-the-sites stoichiometry, i.e., the rapid modification of three kinetically indistinguishable active sites per hexamer (Figure 2A) appears to abolish catalytic activity at the remaining three active sites as well, although the ultimate stoichiometry of modification is six sites per hexamer. While it is not possible to determine from the stoichiometry alone that modification occurs at a single residue in each monomeric subunit, the results of Edman degradation of the enzyme and NMR spectroscopy, as well as mass spectral analysis of the peptides generated by V8 protease digests of bromopyruvate modified 4-OT, indicate that Pro-1 is the sole target of modification by 3-BP (see below).

Identification of the Modified Residue by ^{15}N NMR and by $^{13}C-^{15}N$ Coupling. Because 4-OT consists of identical subunits of only 62 residues, the upfield region of the one-dimensional ^{15}N NMR spectrum of uniformly ^{15}N-labeled 4-OT is fairly well resolved. Therefore, a comparison of the spectra for native and pyruvyl modified enzyme samples would be expected to show detectable differences if a side chain nitrogen atom were the site of modification. Although changes in chemical shift alone cannot prove that a particular nitrogen atom has been modified, the observation of a change in chemical shift is consistent with modification of a residue, if other unambiguous chemical evidence for the putative site of modification is obtained.

The upfield region of the one-dimensional proton-coupled ^{15}N NMR spectrum of uniformly ^{15}N-labeled 4-OT in the absence and presence of 1.1 equivalent of 3-^{13}C-labeled bromopyruvate per 4-OT monomer is shown in Figure 5, spectra A and B, respectively. The most obvious difference between the two spectra is the large reduction in the intensity of the ^{15}N resonance at 49.2 ppm after the addition of 3-BP. Although not clearly discernible in the one-dimensional ^{15}N spectrum of Figure 5B, a shoulder appears after modification at an ^{15}N chemical shift of 88.6 ppm which moves to 86.5 ppm after reduction of the pyruvyl adduct to a lactyl group (not shown). On the basis of the heteronuclear correlation experiment described below, this shoulder peak is attributed to the nitrogen resonance of Pro-1 in the lactyl-modified enzyme. The ^{15}N resonances at 34.8 and 35.3 ppm narrow upon modification by 3-BP but do not change in their integrated intensities, using the resonance at 36.0 ppm as an integration standard. These resonances are identified below and in Table 1.

Table 1: Comparison of ^{15}N-Chemical Shifts for Upfield Resonances of Uniformly ^{15}N-Labeled 4-OT with Model Compounds

Model Compound	δ ^{15}N (ppm) [a]	δ ^{15}N (4-OT) (ppm)	Assignment
Lysine methyl ester [b]			
ε - $NH_3^{\oplus}$	36.2	34.8, 35.3	KI (ε-$NH_3^{\oplus}$) [c]
ε - NH_2	20-26	36.0	KII, KIII (ε-$NH_3^{\oplus}$) [d]
Proline amide [e]			
	47.8	47.3 (pH 8.5)	Pro-1 ()
	54.6	55.7 (pH 4.9)	Pro-1 ()
Arginine [b]			
ε 	85.7 - 87.2	≈85 - 90	RI-VI (ε-NH)
η 	73.2 - 74.7	≈70 - 78	RI-VI (η-NH_2)

[a] ppm from liquid NH_3. [b] Values were obtained from Martin *et al.* (1981) and referenced to liquid NH_3 by adding 380.2 ppm to the CH_3NO_2 referenced shifts (Srinivasan & Lichter, 1977). The ^{15}N-chemical shift values for the neutral ϵ-amino nitrogen of lysine methyl ester represent upper and lower limit estimates based on $\Delta\delta^{15}N$ for deprotonation of primary amines (Martin *et al.*, 1981). [c] These two resonances are attributed to a single lysine residue on the basis of their integrated intensities. These signals, which move toward coalescence in the 3-BP modified enzyme, most likely result from slow exchange between two environments. [d] This resonance is attributed to two lysine residues on the basis of its integrated intensity. Two distinct chemical shifts for these residues are observed in the lactyl-modified enzyme (not shown). [e] From this work.

Inspection of the primary amino acid sequence of 4-OT reveals six arginine residues, three lysine residues, and an amino-terminal proline residue, the ^{15}N resonances of which should be observable in the upfield chemical shift range shown in Figure 5. The spectrum for the unmodified enzyme does indeed show that the ^{15}N resonances are within the expected chemical shift ranges for these residue types based on appropriate model compounds (Table 1). On the basis of this analysis, the three most upfield resonances in Figure 5A ($\delta^{15}N \approx 34-36$ ppm) are reasonably assigned to the ϵ-nitrogen atoms of the three lysine residues of 4-OT. The multiple, unresolved ^{15}N resonances in the chemical shift ranges ≈70−78 ppm and ≈85−90 ppm are assigned to the η- and ϵ-nitrogen resonances, respectively, of the six arginine

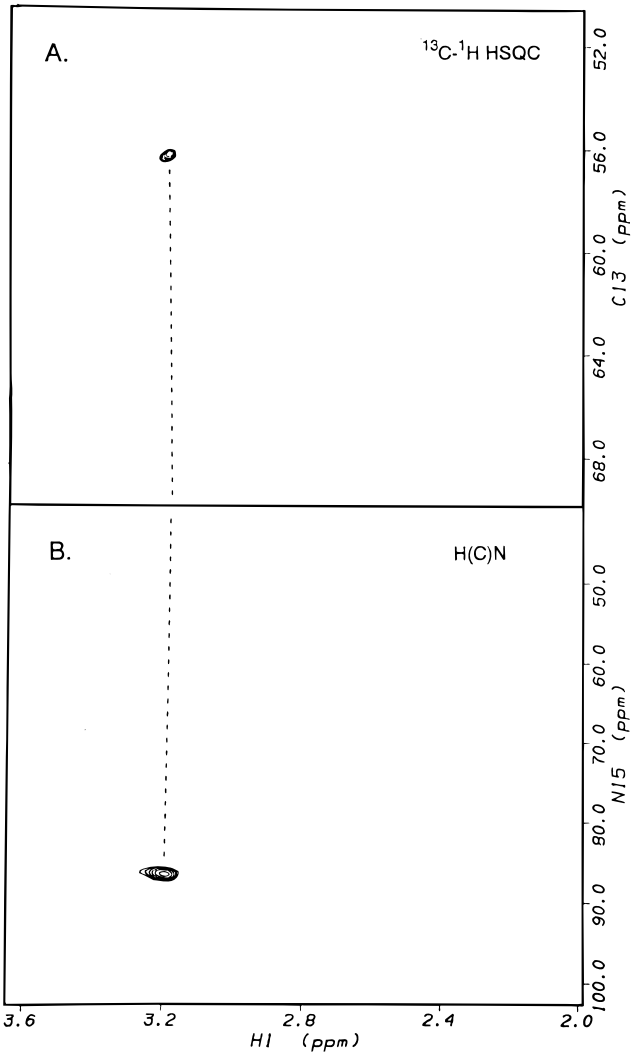


FIGURE 6: Heteronuclear NMR correlation spectra of [3-^{13}C]lactyl-modified, uniformly ^{15}N-labeled 4-OT. (A) ^{1}H−^{13}C HSQC spectrum correlating the methylene proton and carbon chemical shifts of the covalent lactyl adduct (δ^{1}H = 3.19 ppm, δ^{13}C = 56.2 ppm). (B) H(C)N spectrum which correlates the methylene protons of the covalently bound lactyl adduct to a ^{15}N resonance of 4-OT (δ^{15}N = 86.5 ppm). The nitrogen chemical shift is consistent with alkylation and protonation of proline-1 (see text and footnote 5).

residues of 4-OT. In the downfield region of the ^{15}N NMR spectrum (not shown) the δ and ϵ imidazole nitrogens of the two histidines of 4-OT are two broad resonances (120 Hz) at 172 and 238 ppm, consistent with α and β nitrogens of neutral histidines (Pelton *et al.*, 1993), and the backbone and side chain amide nitrogens are within the chemical shift range 105−140 ppm. The histidine ^{15}N resonances narrow but do not shift or lose intensity in the 3-BP modified enzyme (not shown). Similarly, no changes in chemical shift, but some narrowing of the imidazole proton resonances at 600 MHz of His-6 and His-49, are observed in the 3-BP modified enzyme (Stivers *et al.*, 1994). It is therefore reasonable to conclude on the basis of this process of elimination, and the appropriate chemical shift value for the ^{15}N resonance at 49.2 ppm, that this resonance is that of the secondary amino nitrogen of the N-terminal proline of 4-OT, and that this residue undergoes a marked chemical change when the enzyme is modified by 3-BP.

In heteronuclear NMR experiments (Figure 6) the ^{15}N-labeled enzyme was modified with the 3-^{13}C-labeled lactyl group and dialyzed to remove small molecules. A heteronuclear ^{1}H−^{13}C HSQC spectrum of this sample (Figure 6A) showed a correlation between protons at 3.19 ppm and a coupled ^{13}C at 56.2 ppm, reflecting the chemical shifts of the protons and carbon of the 3-methylene group of the lactyl adduct. A heteronuclear H(C)N experiment was then used to correlate the methylene protons of the lactyl group at 3.19 ppm with an ^{15}N resonance of the enzyme at 86.5 ppm, thereby demonstrating ^{13}C−^{15}N coupling and direct bonding of the lactyl group to this enzymic nitrogen (Figure 6B). The ^{15}N chemical shift of 86.5 ppm in the lactyl-modified enzyme indicates a downfield shift of 37 ppm of the Pro-1 nitrogen, consistent with shifts observed upon both alkylation and protonation of cyclic secondary amines.[5]

Table 2: Extent of Modification of Pro-1 by 3-Bromopyruvate As Determined by Edman Degradation

species analyzed	3-BP added / 4-OT monomer	remaining activity (% control)[a]	borohydride reduction	proline-PTH determined (% control)[b]
bromopyruvate modified 4-OT	0.6	43	no	72
bromopyruvate modified 4-OT	10	<0.1	no	53
bromopyruvate modified 4-OT	1.2	<0.1	yes	<4[c]
peptide V1[d]	1.0[e]	<0.1	yes	<4[c]

[a] The enzyme activity remaining after modification is normalized to a control sample of unmodified 4-OT. The activity was determined prior to $NaBH_4$ reduction and protease digestion. [b] The results are normalized to the pmols of proline-PTH recovered in a control sample of unmodified 4-OT which was processed identically. The sequencing of unmodified 4-OT was continued for three cycles to give the expected N-terminal sequence PIA. [c] Estimated detection limit. [d]Peptide V1 (see Table 3) was generated by V8 protease digestion of lactyl modified 4-OT. The mass spectrum of peptide V1 and its carboxypeptidase-A digest (Table 3) gives the expected molecular weight and sequence of the first nine residues of the amino terminus of covalently modified 4-OT. [e] This value (±0.07) was calculated from the specific activity of the 2-^{14}C-labeled bromopyruvate used to covalently modify the enzyme and therefore gives the stoichiometry of covalently bound label.

Edman Degradation of 3-BP Modified 4-OT. Further direct evidence that the N-terminal proline is the site of modification by 3-BP was provided by Edman degradation of modified and unmodified 4-OT, the results of which are summarized in Table 2. The data clearly show that incubation of the enzyme with a slight molar excess of 3-BP, followed by treatment with sodium borohydride to reduce the less stable pyruvyl to a lactyl adduct, blocks the amino terminus to Edman degradation such that no proline-phenylthiohydantoin (proline-PTH) is detected (<4% of control). In contrast, the unmodified enzyme, treated in an identical manner, gave the expected amino-terminal sequence (PIA) in good yield.

[5] The 37 ppm downfield shift for the ^{15}N resonance of Pro-1 upon lactyl modification is comparable to the $\Delta\delta^{15}$N values ranging from 27.9 to 32.8 ppm which can be estimated on the basis of the measured $\Delta\delta^{15}$N = 14.6 ppm for the addition of a N-propyl group to pyrrolidine and the range of downfield shifts observed upon protonation of a variety of tertiary amines ($\Delta\delta^{15}$N = 13.3−18.2 ppm) (Martin *et al.*, 1981). In addition, nitrogen resonances can be downfield shifted by ~5−9 ppm when the dielectric constant of the medium is decreased from DMSO to CCl_4 (Martin *et al.*, 1981). Interestingly, the UV absorbance spectrum of the active site residue Phe-50 is red-shifted 0.4 nm upon modification of the enzyme with 3-BP, consistent with a decrease in the dielectric constant of the active site from that of 2-propanol in the free enzyme to that of chloroform in the pyruvyl enzyme (J. T. Stivers, unpublished results).

Table 3: Identification of Radiolabeled Peptide from Protease V8 Digest of [2-^{14}C]Lactyl-Modified 4-OT by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

digest and peptide	calculated mass (MH^+)	measured mass
V8[a]		
V1 ($C_3H_5O_3$ − PIAQIHILE)	1124.4	1124.7
V1′ (V1 − H_2O)[b]	1106.4	1106.2
V1* (PIAQIHIL)[c]	906.3	906.9
CP-A[d]		
V1C1 ($C_3H_5O_3$ − PIAQIHIL)	995.5	994.3
V1C2 ($C_3H_5O_3$ − PIAQIHI)	882.1	882.3
V1C3 ($C_3H_5O_3$ − PIAQIH)	768.9	768.1
V1C4 ($C_3H_5O_3$ − PIAQI)	631.7	631.4

[a] V8, endoproteinase glu-C. Peptide V1 corresponds to the [2-^{14}C]lactyl-modified amino-terminal peptide generated from V8 cleavage at residue E9. [b] Peptide V1′ is presumably generated by the loss of covalently bound water from peptide V1 during desorption from the matrix. [c] Peptide V1* is a minor species (see Figure 7) which may result from loss of the C-terminal glutamic acid residue and the lactyl group during desorption from the matrix. [d] CP-A, carboxypeptidase-A. Peptides V1C1−V1C4 were generated by sequential carboxyl-terminal digestion of peptide V1 by CP-A (see Methods).

Isolation of the Modified Peptide and Mass Spectrometry. To further confirm the unusual site of modification, [2-^{14}C]-lactyl-modified 4-OT was digested with endoproteinase glu-C (protease V-8), and the resulting peptides were separated by C18 reverse-phase HPLC (data not shown). At pH 4.0, in 40 mM ammonium acetate buffer, protease V-8 cleaves exclusively at the carboxyl side of glutamic acid residues (Smith, 1988), of which there are seven in the amino acid sequence of 4-OT. If modification occurs solely at the amino terminus, a nine-residue radiolabeled peptide corresponding to V8 cleavage at residue Glu-9 should result. The radioactivity was found to elute in one major peak with a retention volume of 33 mL. A smaller radioactive peak elutes in the aqueous flow-through at a retention volume of about 5 mL and is a low molecular weight degradation product of the modified enzyme. A broad peak of radioactivity which eluted between 20 and 30 mL resulted from partial digestion products.[6]

The HPLC results are consistent with affinity labeling of a single site in 4-OT, and the measured stoichiometry of one modified site per 4-OT monomer (Figure 4). To identify the radiolabeled peptide, the pooled material was rechromatographed, and the radioactive material was analyzed by MALDI mass spectrometry (Figure 7A). As reported in Table 3, the MALDI mass spectrum revealed two major MH^+ ions. One peak corresponds to the expected molecular mass of the lactyl modified amino-terminal peptide (V1) generated from V8 cleavage at residue Glu-9 of 4-OT. The other major MH^+ ion in Figure 7A (V1′) is 18.5 mass units less than peptide V1 and apparently results from the loss of covalently bound water from peptide V1 during desorption from the matrix. The possibility that this additional peak represents a second peptide species is excluded because

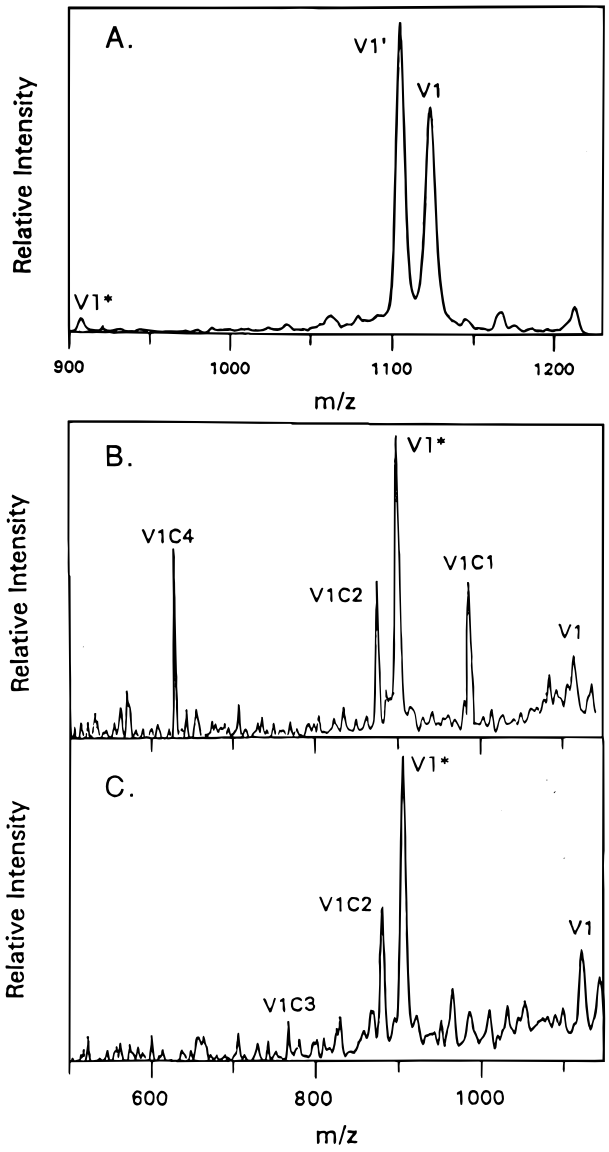


FIGURE 7: Identification and carboxyl-terminal sequencing by MALDI mass spectrometry of the [2-^{14}C]lactyl-modified peptide. The measured masses and identities of the peptides are shown in Table 3. (A) MALDI mass spectrum of the intact purified peptide. The observed mass of peak V1 corresponds to the expected mass of the lactyl-modified amino-terminal peptide generated by protease V8 cleavage at glutamate-9. Peptide V1′ is presumably generated from peptide V1 by the removal of an equivalent of one water molecule during matrix desorption (see text) and peptide V1* is a minor contaminant (see Table 3). (B) Peptides produced from sequential carboxypeptiase-A digestion of the [2-^{14}C]lactyl-modified peptide (see text); ammonium bicarbonate was added to the matrix solution. (C) Same as panel B except ammonium citrate was added to the matrix solution to enhance the desorption of peptide V1C3, which is absent in panel B.

Edman degradation of the sample gave no sequence (i.e., a *single* peptide blocked by modification at the amino terminus).

The assignment of peptide V1 as the modified amino-terminal peptide of 4-OT was confirmed by MALDI mass spectral analysis of the peptides generated from a sequential digestion of the carboxyl terminus of peptide V1 with carboxypeptidase-A (CP-A). Shown in Figure 7, panels B and C, are mass spectra of CP-A digests of peptide V1 using either ammonium citrate or ammonium bicarbonate in the matrix/sample solutions. Carboxyl-terminal digestion of peptide V1 yields four new peaks (V1C1, V1C2, V1C3,

[6] The radioactive material which eluted at 5 mL was analyzed by MALDI mass spectrometry and consisted of a mixture of species with molecular weights <170. Therefore, this radioactivity is not peptide bound and most likely results from decomposition of the bound label during the 18 h proteolysis digestion at 37 °C. The radioactivity which eluted between 20 and 30 mL was chased into the major peptide peak eluting at 33 mL upon further digestion of the sample with a fresh aliquot of V8 protease (not shown).

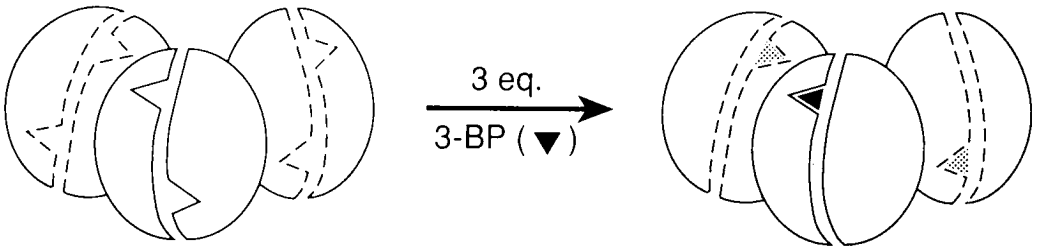


FIGURE 8: Diagram of the "trimer of dimers" quaternary structure of 4-OT showing the half-of-the-sites inactivation by 3-bromopyruvate (3-BP). The affinity label is arbitrarily shown to bind randomly to three of the six active sites thereby inducing a conformational change which disrupts the second active site in the dimer.

V1C4) which have the expected masses of the modified peptides generated from the sequential removal of residues Glu-9, Leu-8, Ile-7, and His-6, respectively, from peptide V1. Two different matrix buffer conditions were used in order to observe peptide V1C3, which is entirely absent in Figure 7B and is a weak peak in Figure 7C. The spectra in Figure 7B,C emphasize how the relative intensities of the observed peaks are dramatically influenced by the buffer composition and pH of the matrix used for ionization as well as the hydrophobicity and charge of the peptide (e.g., note the different relative intensities of peptides V1C1 and V1C4 in Figure 7A,B) (Woods *et al.*, 1995). Edman degradation of peptide V1 gave no sequence, indicating modification of the N-terminus and confirming the result obtained from Edman degradation of the intact enzyme (Table 2).

DISCUSSION

It has been shown here that 3-BP is an active-site-directed irreversible inhibitor of 4-OT which modifies the enzyme with a stoichiometry of one site per monomer. On the basis of peptide mapping studies and sequencing by mass spectrometry, the site of modification was localized to a five-residue amino-terminal peptide of 4-OT. The precise site of modification was then determined by two independent methods. Edman degradation of the lactyl-modified peptide revealed a blocked amino terminus, and heteronuclear NMR spectroscopy of the ^{15}N-labeled enzyme after modification with ^{13}C-labeled 3-BP unambiguously demonstrated that the amino-terminal nitrogen atom of Pro-1 was the site of modification. Modification at other ^{15}N sites (Lys-16, Lys-47, Lys-59, and the two histidine residues His-6 and His-49) was excluded because the chemical shifts of these nitrogen resonances did not change upon affinity labeling. The observed narrowing of these ^{15}N resonances upon affinity labeling does however indicate a change in the environment of these residues. Furthermore, in the following paper (Stivers *et al.*, 1996) it is shown that the lysine and histidine residues all have pK_a values which differ significantly from that of the essential basic group on the free enzyme required for catalysis. The similar pH dependencies of k_{inact}/K_I for 3-BP and k_{cat}/K_M for **2b** (Stivers *et al.*, 1996, following paper), provides evidence that the same enzymic residue is involved both in substrate catalysis and in inactivation. In the following paper we directly demonstrate that Pro-1 has the correct pK_a to act as the general base. These results, and the crystallographic data (Subramanya et al., 1996; Whitman *et al.*, 1995) indicating that Pro-1 is in the active site, strongly implicate this residue as the enzymic general base.

It is of interest to note that an amino-terminal proline residue is conserved among three mechanistically related isomerases. These are the two homologous isozymes of 4-OT from *P. putida mt-2* (Chen *et al.*, 1992) and *Pseudomonas sp.* CF600 (Shingler *et al.*, 1992), which are 73% identical, and the nonhomologous 5-(carboxymethyl)-2-hydroxymuconate isomerase from *E. coli* (Whitman *et al.*, 1992). On the basis of this conservation, it is reasonable to suggest that Pro-1 is the general base catalyst on each of these isomerases.

The heteronuclear H(C)N pulse sequence introduced here to establish covalent binding of the C-3 carbon of the affinity label 3-BP to the nitrogen of Pro-1 should be useful for the identification of nucleophilic nitrogens on enzymes and on DNA that are targets of alkylating agents. A limiting factor is the small coupling constant ^1J (^{13}C$-^{15}$N) of approximately 10 Hz which limits this method to macromolecules with molecular masses of $\leq$50 kilodaltons, unless the ^{13}C$-^{15}$N interaction occurs on a mobile site.

The affinity labeling of 4-OT by 3-BP shows half-of-the-sites stoichiometry (Figure 4), i.e., the rapid modification of three sites per hexamer largely abolishes catalytic activity at the remaining three active sites as well, although the ultimate stoichiometry of modification is six sites per hexamer. Since all six of the modified sites are on the nitrogen of Pro-1, the observed half-of-the-sites stoichiometry requires that three of the Pro-1 residues per hexamer be modified at a slower rate, after most of the enzymatic activity has been lost. The crystal structure of 4-OT, shown schematically in Figure 8, provides a possible mechanism for the observed half-of-the-sites stoichiometry of inactivation. The 4-OT hexamer consists of a trimeric arrangement of strongly interacting pairs of monomers, i.e., a trimer of dimers (Subramanya et al., 1996). In each dimer the two active sites contain residues contributed by both monomers. Binding of 3-BP to one active site may disrupt the second active site in the dimer by a mechanism involving an *intradimer* conformational change. Similar events at two other dimer active sites would account for the observed half-of-the-sites stoichiometry as well as the observed noncooperative binding of 3-BP.

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