fragments in situ, our approach does not use any indirect semi-empirical relationships to evaluate χ, relying directly on the fundamental relationship, eq 1, instead.

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Mechanism-Based Inactivation of *Escherichia coli* β-Hydroxydecanoyl Thiol Ester Dehydrase: Assignment of the Imidazole ^{15}N NMR Resonances and Determination of the Structure of the Alkylated Histidine

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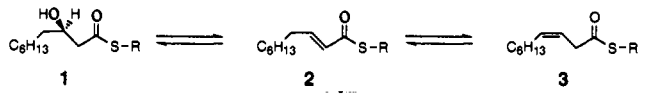
Abstract: Nitrogen-15 nuclear magnetic resonance spectroscopy was used to determine the structure of the active-site histidine-70 adduct formed when β-hydroxydecanoyl thiol ester dehydrase from *Escherichia coli* reacts with the mechanism-based inactivator *S*-(3-decynoyl)-*N*-acetylcysteamine (3-decynoyl-NAC). In order to obtain the amount of labeled enzyme necessary for spectral studies, the *fabA* gene, which encodes dehydrase, was overexpressed to give dehydrase as 15–20% of soluble protein. To simplify the interpretation of the NMR spectra, the non-active-site histidine residue His-129 was converted to an asparagine residue using site-directed mutagenesis. The specific activity and response of the mutant to 3-decynoyl-NAC are unaltered. It is known that type β ("pyridine-like") nitrogens in imidazoles resonate 60–80 ppm downfield of type α ("pyrrole-like") nitrogens. To assign the imidazole nitrogen resonances in dehydrase, wild-type and mutant enzymes were labeled with $^{15}NH_4Cl$ or with [$^{15}N^{\delta 1}$]histidine. Analysis of the ^{15}N NMR spectra allowed the assignment of the resonances of the imidazole nitrogens of His-129 and His-70. For His-70, the spectra show that $N^{\delta 1}$ resonates upfield of $N^{\epsilon 2}$ in the native enzyme and is therefore a type α nitrogen. In the inactivated enzyme the signals are reversed, and $N^{\epsilon 2}$ is a type α nitrogen. These results demonstrate that $N^{\epsilon 2}$ of His-70 becomes alkylated upon inactivation of dehydrase with 3-decynoyl-NAC and thus is the probable locus of active-site basicity in the normal reactions catalyzed by dehydrase. In addition, the imidazole nitrogen chemical shifts suggest that $N^{\delta 1}$ may be involved in a hydrogen bond in native dehydrase but that $N^{\epsilon 2}$ is not. The mechanistic implications of this are discussed.

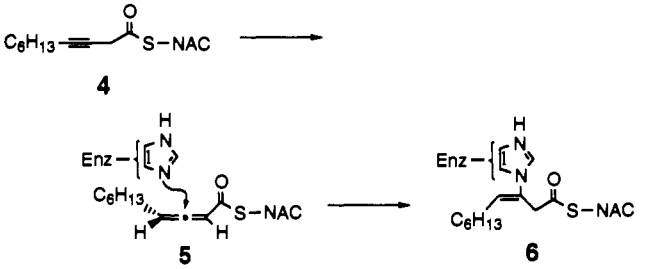
Introduction

β-Hydroxydecanoyl thiol ester dehydrase catalyzes reactions at a critical branch point in the biosynthetic pathway for unsaturated fatty acids in *Escherichia coli* and (presumably) other strictly or facultatively anaerobic bacteria.[1] Dehydrase catalyzes the interconversion of 3-hydroxydecanoyl, (*E*)-2-decenoyl, and (*Z*)-3-decenoyl thiol esters (**1**, **2**, and **3**, respectively; Scheme I). In vivo, (*E*)-2-decenoyl-ACP[2] is reduced and then elongated to saturated fatty acids, while direct elongation of (*Z*)-3-decenoyl-ACP leads to the common monounsaturated fatty acids. Extensive mechanistic studies have shown that the allylic rearrangement[3] and dehydration reactions catalyzed by dehydrase are suprafacial[4] and syn[5] processes, respectively. Thus, a single active-site acid/base group is implied.[6,7]

Scheme I



Scheme II



Chemical modification studies as well as examination of the pH–rate profile of dehydrase (inflection points at pH 7 and 10)[8] provide compelling evidence for the involvement of a histidine residue in the catalytic mechanism as well as equivocal evidence

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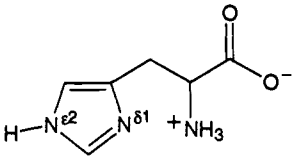
for the involvement of a tyrosine residue.[8,9] The primary structure of dehydrase has been deduced from the nucleotide sequence of the structural gene for dehydrase, *fabA*, as well as by limited protein sequencing, and His-70 has been identified as a key active-site amino acid.[10] (The sequence of the C-terminal region has recently been revised, however, as described below.) A homodimer with a protomer molecular weight of 18 838, wild-type dehydrase has two histidine residues per subunit.

Dehydrase is of particular interest because it is rapidly and completely inactivated by 1 equiv of *S*-(3-decynoyl)-*N*-acetylcysteamine (3-decynoyl-NAC, **4**) per enzyme subunit,[8,11-13] thus providing the earliest recognized example[14] of mechanism-based or "suicide" enzyme inactivation.[15] The mechanism of inactivation (Scheme II) has been carefully studied.[8,9,11-13,16-19] The acetylenic inhibitor first undergoes propargylic rearrangement to 2,3-decadienoyl-NAC (**5**) in a manner completely analogous to the normal allylic rearrangement catalyzed by dehydrase. The electrophilic allenic thiol ester then alkylates His-70.[10] While the fate of 3-decynoyl-NAC during inactivation had been completely characterized,[9,13] prior to this study the structure of the alkylated imidazole moiety of **6** (i.e., whether alkylation occurs at $N^{\delta 1}$ or $N^{\epsilon 2}$) remained to be determined.



^{15}N NMR spectroscopy is ideal for characterizing the structure of histidine residues in proteins since the imidazole nitrogen resonances are well resolved from other protein nitrogen resonances.[20] A number of studies both of model compounds and of proteins show that ^{15}N NMR can reveal a wealth of information about the structure of the imidazole ring and the environments of the individual nitrogen atoms.[21-25] For instance, Bachovchin has used ^{15}N NMR to characterize the state of histidine-57 in the catalytic triad of the serine proteinase α-lytic protease.[26] Recently, Lodi and Knowles have used ^{15}N NMR to determine the role of histidine-95 in the catalytic mechanism of triosephosphate isomerase.[27] In the present study, ^{15}N NMR has been used to determine the tautomeric form of His-70 in the native and inactivated enzyme and, from this, the structure of the alkylated active-site histidine residue.

Experimental Section

All materials were of reagent grade unless otherwise indicated. $^{15}NH_4Cl$ (91% or 99% enriched) was from Cambridge Isotope Labs. [$^{15}N^{\delta 1}$]Histidine hydrochloride (>95% enriched) was provided by the NIH-sponsored Stable Isotope Resource at Los Alamos National Laboratory. Oligodeoxynucleotides were synthesized at the Laboratory for Macromolecular Structure at Purdue University and purified by PAGE.[28] [^{35}S]ATP (>1000 Ci/mmol) was obtained from Amersham.

General microbiological and molecular biological methods used have been described.[28]

DNA Sequencing. Dideoxynucleotide sequencing[29] was performed using the Sequenase kit (United States Biochemical) with [^{35}S]ATP and substituting 7-deaza-dGTP for dGTP.[30]

Overexpression of Dehydrase. A 620-bp fragment of DNA containing the *fabA* gene was amplified from the genomic DNA of *E. coli* W3110[31] by the polymerase chain reaction (PCR) using the primers 5′-GGGAATTCCATGGTAGATAAACGCGAATCC-3′ and 5′-GGCTGCAGACAGGGTAGTAATGGCCTG-3′.[32] The reaction mixture (100 μL) contained primers (1.0 μM each), template DNA (1 ng), dNTPs (0.2 mM each), $MgCl_2$ (1.5 mM), Tris·HCl, pH 8.3 (20 mM), KCl (50 mM), gelatin (0.1 mg/mL), and 2 U of *Taq* polymerase (Promega) and was overlaid with 100 μL of mineral oil. After the mixture had been incubated an initial 5 min at 94 °C, 30 cycles of annealing (2 min, 54 °C), extension (2 min, 72 °C), and denaturation (2 min, 94 °C) were performed, followed by a final extension at 72 °C for 15 min. The product was cloned into the *Eco*RI and *Sma*I sites of pBluescript II SK(+) (Stratagene), giving pRA198. DNA sequencing of pRA198 confirmed the sequence of the PCR product. A 612-bp *Eco*RI-*Pst*I fragment containing *fabA* was subcloned into pJF119EH,[33] giving pRA224. This vector construct contains *fabA*, under the control of a hybrid *tac* promoter, followed by the strong *rrnB* transcription terminator. It also contains the *bla* gene for ampicillin resistance and the *lacI*^{Q} gene for the *lac* repressor.

Mutagenesis. Uracil-containing ssDNA was isolated by the method of Katayama[34] from *E. coli* strain CJ236[35] that had been transformed with pRA198. Site-directed mutagenesis was performed on this template, using T4 DNA polymerase, by the method of Kunkel,[35,36] with one minor modification: the phosphorylation reaction on the primer (5′-TAC-CGTATTAACTTTAAAC-3′; the underlined base designates the change from wild-type) was stopped by heating for 15 min at 75 °C with *no* added EDTA, since it was found that EDTA inhibits the annealing of primer to the ssDNA. DNA sequencing verified the mutation. The modified *fabA* gene was subcloned into pJF119EH as above, giving pRA340.

Purification of Dehydrase. Unlabeled dehydrase was isolated from *E. coli* XL1-Blue[37] (Stratagene) that had been transformed with pRA224 (for wild-type dehydrase) or with pRA340 (for H129N dehydrase). The bacteria were grown on 2xYT medium supplemented with ampicillin (50 μg/mL) and tetracycline (12 μg/mL) and induced with IPTG (1 mM) when the optical density had reached 1-2 (600 nm). After an additional 6-8-h growth at 37 °C, cells were harvested by centrifugation and stored at -80 °C. Uniformly ^{15}N-labeled dehydrase was isolated from *E. coli* XL1-Blue, transformed with the appropriate expression vector. The bacteria were grown on a MOPS-buffered minimal medium[38] made to 5 mM in $^{15}NH_4Cl$ (91% enriched) and supplemented with glucose (10 mM), citric acid (0.1 mM), ampicillin (50 μg/mL), tetracycline (12 μg/mL), thiamine hydrochloride (20 μM), calcium pantothenate (20 μM), 4-hydroxybenzoic acid (20 μM), and 2,3-dihydroxybenzoic acid (20

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μM).[39] Cells were grown as above, induced with IPTG (1 mM), supplemented with glucose (an additional 5 mM) and $^{15}NH_4Cl$ (an additional 5 mM, 99% enriched), and harvested as above. [$^{15}N^{\delta 1}$]-Histidine-labeled H129N dehydrase was isolated from the histidine auxotroph *E. coli* FB1[40,41] (*ΔhisGDCBHAFIE*) that had been transformed with pRA340. The bacteria were grown on the same minimal medium, with the addition of 10 mM unlabeled NH_4Cl, as well as the 19 non-histidine amino acids plus adenine, guanine, cytosine, and uracil.[39] [$^{15}N^{\delta 1}$]Histidine (75 μM) was added, and the cells were grown to an o.d. (600 nm) of 1.5. The culture was then induced with IPTG (1 mM), glucose (an additional 5 mM) and [$^{15}N^{\delta 1}$]histidine (an additional 75 μM) were added, and the cells were harvested after an additional 6-h incubation. All bacterial cultures for protein purification were grown in 2.8-L Fernbach flasks (1 L of culture in each) at 37 °C with shaking at 250 rpm.

Dehydrase was purified by a modification of the method of Kass et al.[42] For a typical preparation, 5.18 g of frozen *E. coli* from 1 L of 2xYT culture was thawed in 31 mL of 20 mM Tris·HCl, pH 8.0, and 50 mM $MgCl_2$ at room temperature over a period of 30 min. All subsequent steps were carried out at 0–4 °C. All centrifugations were performed at 30000*g* for 15 min. After the cells had been disrupted by two passes through a French pressure cell (Aminco) at 20000 psi and the cell debris had been removed by centrifugation, the nucleic acids were precipitated with 6.7 mL of 1% protamine sulfate solution (pH 7.2, 1.3 mL per gram of cells). The nucleic acid-free supernatant (35 mL) was dialyzed against 2 L of Buffer A (10 mM potassium phosphate, pH 7.4) with three changes over 10 h until the conductivity and pH of the dialysate were the same as those of the buffer. The dialysate was loaded onto a 2.5- × 40-cm DE52 (Whatman) column equilibrated in Buffer A and eluted with a 600-mL linear gradient of 0–100 mM KCl in Buffer A. Fractions (5 mL) were collected at 1 mL/min, and those fractions that showed dehydrase activity greater than 10 U/μL were pooled and dialyzed against Buffer A. The dialysate was frozen in liquid nitrogen and stored at −80 °C. For preparation of NMR samples, the frozen dialysate was thawed with gentle warming and concentrated to 1–2.5 mM (in active sites) by centrifugal ultrafiltration using Centriprep devices (Amicon).

SDS-Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out in 16% acrylamide gels according to the method of Laemmli.[43] Gels were stained for protein with Coomassie Brilliant Blue R-250.

3-Decen-1-ol.[44] (*Z*)-3-Decen-1-ol was synthesized by a modification of the method of Kass et al.[42] 3-Decyn-1-ol (2.61 g, 16.9 mmol, Farchan) was added to a 25-mL hexane suspension which contained 185 mg of $Pd/CaCO_3$ (5% Pd, Aldrich) and 37 mg of quinoline (0.272 mmol, freshly distilled from Zn powder) and had been previously equilibrated with H_2. The suspension was stirred at room temperature under H_2 at atmospheric pressure until all starting material had been consumed (analysis by capillary GC). After the catalyst had been removed by filtration, the solvent was evaporated under reduced pressure, and the resulting oil was purified by flash chromatography on silica gel (3:1 Et_2O:hexanes) to yield 2.27 g (86%) of 3-decen-1-ol (>98.5% pure by GC). ^1H NMR (500 MHz): δ 0.86 (t, *J* = 7.1 Hz, 3 H, CH_3), 1.21–1.36 (m, 8 H, CH_2), 2.04 (dt, *J* = 7.3 Hz, 7.1 Hz, 2 H, $CH_2CH_2CH_2CH$=), 2.31 (dt, *J* = 7.2 Hz, *J* = 6.5 Hz, 2 H, =$CHCH_2CH_2OH$), 3.62 (dt, *J* = 6.3 Hz, *J* = 6.0 Hz, 2 H, CH_2OH), 5.33 (m, 1 H, $CH_2CH_2CH_2CH$=), 5.55 (m, 1 H, =$CHCH_2CH_2OH$).

Substrates and Inhibitors. 3-Decynoyl-NAC was synthesized from 3-decyn-1-ol (Farchan) by Jones oxidation[45] followed by conversion to the thiol ester.[13] (*Z*)-3-Decenoyl-NAC was synthesized from (*Z*)-3-decen-1-ol by Jones oxidation followed by conversion to the thiol ester using the method of Schwab and Klassen.[4]

Dehydrase Assay. Dehydrase activity was monitored by following the isomerization of (*Z*)-3-decenoyl-NAC to (*E*)-2-decenoyl-NAC at 263 nm, according to the method of Kass.[46] One unit of activity corresponds to the formation of 1.0 nmol of (*E*)-2-decenoyl-NAC per minute at 27 °C.

Protein Determination. The concentration of purified dehydrase was determined by the absorbance at 280 nm, using an extinction coefficient of 1.288 $(g/L)^{-1}$ cm^{-1}.[13] The protein concentration of crude solutions was determined by the method of Bradford[47] using the Bio-Rad kit (Bio-Rad Laboratories) with bovine serum albumin as the standard.

```
                                                    129
                lys val thr tyr arg ile his phe lys
a)              AAA GTG ACC TAC CGT AAT CAC TTT AAA

                                                    170
                val gly leu phe gln asp thr ser ala phe  *
b)              GTC GGT CTG TTC CAG GAT ACG TCT GCC TTC TGA

                                                    170
                val gly leu phe gln asp thr leu phe  *
c)              GTC GGT CTG TTC CAG GAT AGT CTG TTC TGA
```

Figure 1. Sequence of selected regions of *fabA* and implied sequence of dehydrase. (a) Sequence of the region around codon 129 of *fabA* in pRC1 and pRA198. (b) Sequence of the 3′ end of *fabA* in pRA198. (c) Sequence of the 3′ end of *fabA* in pRC1. Data for pRC1 are from Cronan et al.[10] The asterisks denote stop codons.

NMR. All NMR experiments were performed on a Varian VXR-500 spectrometer operating at 499.843 MHz for protons and 50.653 MHz for ^{15}N. ^1H spectra were acquired using a 5-mm broad-band indirect detection probe at ambient temperature. Two-hundred fifty-six transients of 16K data points over a 7000 Hz spectral width were collected using a recycle time of 2.17 s, processed with 1.75 Hz line broadening, and referenced to HDO. ^{15}N spectra were acquired using a 10 mm broad-band probe at 10 °C. Spectra were acquired (16K data points, spectral width 20000 Hz, 0.41-s acquisition time, 1-s pulse delay) using a 10-μs pulse (22.5° flip angle) with a transmitter offset of 3750 Hz and referenced to external 1 M $H^{15}NO_3$. Other acquisition parameters are given in the figure legends for the individual spectra. Dehydrase samples were inactivated by the addition of 1.5 equiv of 3-decynoyl-NAC as a 20 mM stock solution in ethanol, directly to the NMR sample tube.

^1H–^{15}N multiple bond correlation spectroscopy ($^1H\{^{15}N\}$MBC) on 1-vinylimidazole (35 mg) in CD_3OD (0.7 mL) was performed using the pulse sequence described by Bax and Summers,[48] as implemented by Varian. Data were collected in 224 increments of 96 transients at 25 °C using a 5-mm broad-band inverse detection probe. The spectral width was 2000 Hz in the F2 dimension and 14000 Hz in the F1 dimension. A delay (τ_{mb}) of 0.063 s was used to optimize for 8-Hz couplings. TPPI[49] was used for phase-sensitive spectra. The data were zero-filled to 512 points in the F1 dimension and processed using Gaussian weighting.

Effect of pH on the ^{15}N Spectrum of Dehydrase. The ^{15}N spectrum of H129N dehydrase (38 mg/mL) was obtained at various pH values from approximately pH 4 to 8 as described above, with the application of WALTZ proton decoupling (during acquisition only, offset = 1311 Hz, WALTZ frequency = 10520 Hz). Approximately 8000 transients were acquired using a 20-μs pulse width (45° flip angle) and a 0.5-s pulse delay. Two samples were prepared in 10 mM potassium acetate, 10 mM potassium phosphate buffer containing 10% D_2O (pH 7.7 and 7.3, respectively). After each spectrum was acquired, the pH of the sample was lowered approximately 1 unit by 10-fold dilution into the acetate–phosphate buffer at the appropriate pH, followed by concentration by centrifugal ultrafiltration and addition of D_2O to 10% final concentration before acquisition of the next spectrum. The pH of the dilute sample was measured prior to concentration. (Measurement of the pH of several of the NMR samples after acquisition of spectra showed that the pH values of the dilute and concentrated samples were identical.) A total of approximately 20 h was required for acquisition of a complete series of spectra. Enzymatic activity (measured at pH 7.0) varied less than 10% between samples at the different pH values. The samples were adjusted to neutral pH after the experiment, and spectra were acquired again to verify that the changes were not irreversible.

Results

Cloning, Overexpression, Mutagenesis, and Purification of Dehydrase. The expression system described above, *E. coli* strain RA224, produces dehydrase as approximately 15–20% of soluble protein (estimated from the specific activity), allowing the economical preparation of labeled dehydrase needed for the NMR

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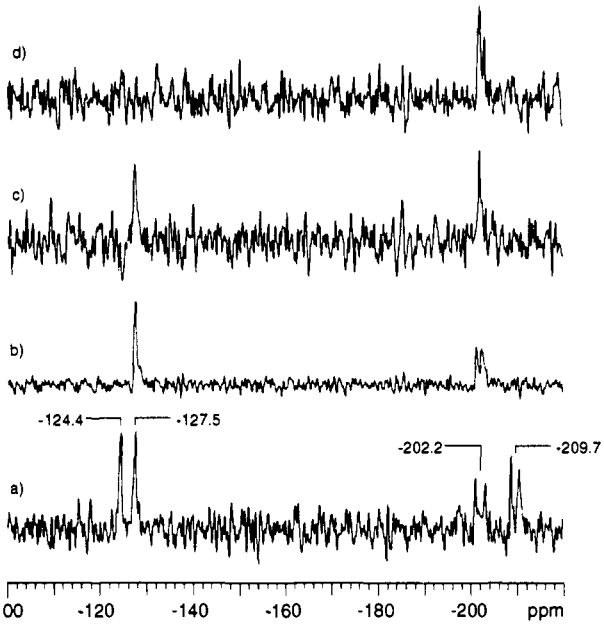


Figure 2. Imidazole region of the ^{15}N NMR spectra of uniformly labeled, active dehydrase at pH 7.4: (a) wild-type dehydrase (14 mg/mL, 25 000 transients); (b) and (c) H129N dehydrase (25 mg/mL, 15 000 transients and 44 mg/mL, 8000 transients, respectively); (d) [$^{15}N^{\delta 1}$]histidine-labeled H129N dehydrase (53 mg/mL, 20 000 transients). Spectra a, b, and d were acquired with incomplete proton decoupling, while spectrum c was acquired with decoupling during acquisition only.

studies. From RA224, 12–15 mg of dehydrase per gram of cells can be purified (25–45 mg L^{-1} of minimal medium culture, up to 100 mg L^{-1} of rich medium culture). This allows the production of substantial quantities (30–100 mg) of labeled protein from only 3–4 L of culture. After one column, dehydrase is approximately 95% pure, as judged by SDS-PAGE.

The DNA sequence of pRA198, confirmed by direct sequencing of the PCR product from genomic DNA, indicated that the *fabA* gene has a slightly different 3′-terminal sequence from that of the *fabA* insert of pRC1, as previously reported by Cronan et al.[10] (Figures 1b and 1c). The sequence of pRC1 contains three fewer bases, resulting in a change in C-terminal sequence from TLF (one-letter codes) in dehydrase from pRC1 to TSAF in dehydrase from pRA224. This observation is supported by the results of Cronan et al.,[10,50] in which carboxypeptidase Y hydrolysis of dehydrase isolated from *E. coli* DM51A released alanine and serine, a finding that could not be explained by their DNA sequence data. The sequence of Cronan was confirmed by direct sequencing of a PCR product derived from pRC1, suggesting that the pRC1 plasmid itself contains the deletion. The revised sequence for the wild-type dehydrase has been confirmed independently by Edman sequencing of a C-terminal peptide obtained by partial digestion of dehydrase from *E. coli* DM51A.[51]

Using site-directed mutagenesis, a non-active-site histidine residue has been changed to asparagine. The H129N mutant has a specific activity of 1.7×10^4 U mg^{-1}, compared to 1.8×10^4 U mg^{-1} for wild-type dehydrase, and it is inactivated rapidly by 3-decynoyl-NAC. The ^1H and ^{15}N NMR spectra of wild-type and H129N dehydrase are very similar (data not shown), suggesting that the mutation causes very little disruption of the tertiary structure of the enzyme.

NMR. Analysis of labeled wild-type and H129N dehydrase allows the imidazole ^{15}N resonances to be assigned (Figure 2). In active wild-type dehydrase, there are resonances at −124.4, −127.5, −202.2, and −209.7 ppm (relative to $H^{15}NO_3$). The spectrum of active H129N dehydrase shows that the signals at −124.4 and −209.7 ppm come from His-129, while those at −127.5 and −202.2 ppm come from His-70. Analysis of the spectrum of

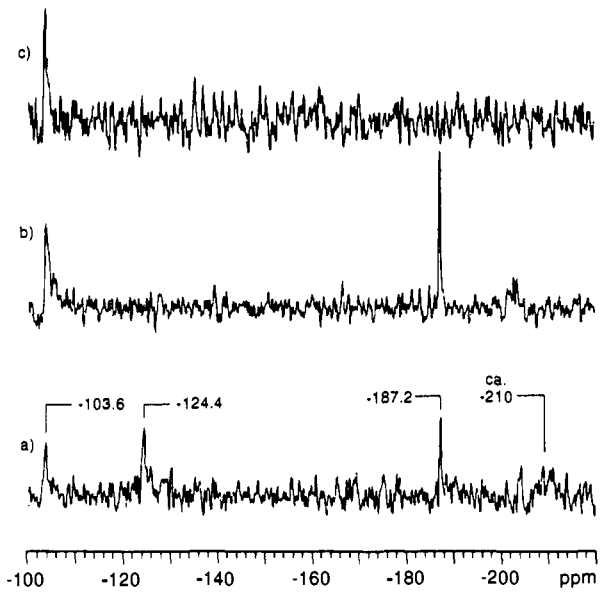


Figure 3. Imidazole region of the ^{15}N NMR spectra of uniformly labeled, inactivated dehydrase at pH 7.4: (a) wild-type dehydrase (25 000 transients); (b) H129N dehydrase (20 000 transients); (c) [$^{15}N^{\delta 1}$]histidine-labeled, inactivated H129N dehydrase (20 736 transients). Protein concentrations are given in the legend to Figure 2.

specifically [$^{15}N^{\delta 1}$]histidine-labeled H129N dehydrase (Figure 2d) allows the assignment of the resonance at −202.2 ppm to $N^{\delta 1}$ of His-70 and, therefore, that at −127.5 ppm to $N^{\epsilon 2}$.

Several differences appear between the ^{15}N NMR spectra of active and inactivated dehydrase. There are minor changes in the amide, guanidine, and amine regions of the spectrum and small shifts in the resonances of the indole nitrogens upon inactivation (data not shown), the significance of which is not known. The major differences occur in the imidazole region of the spectrum (Figure 3). Signals at −127.5 and −202.2 ppm disappear, and new signals appear at −103.6 and −187.2 ppm. In addition, the signal at ca. −210 ppm broadens, so that (at best) it is only barely detectable above the noise. Analysis of the spectrum of inactivated, [$^{15}N^{\delta 1}$]histidine-labeled H129N dehydrase allows the assignment of the signal at −103.6 ppm to $N^{\delta 1}$ of His-70 and therefore that at −187.2 ppm to $N^{\epsilon 2}$.

The pH dependence of the chemical shifts of the His-70 imidazole nitrogens of H129N dehydrase was examined (data not shown). Above pH 6, the positions of the signals do not vary with changing pH, suggesting that the pK_a of the imidazole group of His-70 is less than 5. At pH 5.7, the downfield signal disappears, while the upfield signal is still detectable. Below pH 5.7, neither signal can be detected above the background noise.[52] Thus, the value of the pK_a could not be determined accurately.

The pH dependence of the ^{15}N spectrum of wild-type dehydrase was examined in a similar manner, and the same behavior was observed for the signals assigned to His-70. The positions of the signals assigned to His-129 did not vary until the pH was lowered to 4.2, when a slight shift toward the position of protonated imidazoles (−190 to −200 ppm) was observed (data not shown). In addition, increasing the pH to 9.8 caused no effect on the positions of the ^{15}N resonances from either histidine, although upfield shifts with increasing pH were noted in the resonance positions of several amino group resonances (data not shown).

Discussion

Schwab et al. have observed that incubation of dehydrase with an excess of 3-[2-^{13}C]decynoyl-NAC leads to the formation of a small but significant amount of a second adduct between the protein and the inactivator (in addition to the one involving active

(50) Li, W.-b. Ph.D. Thesis, The Catholic University of America, 1986.
(51) Cronan, J. E., Jr., personal communication.

(52) When the pH of a sample was raised and the ^{15}N NMR spectrum reexamined at pH 6.9 or 6.3, the imidazole ^{15}N signals were again detectable in their original positions, indicating that the pH-dependent changes in the protein's structure are reversible.

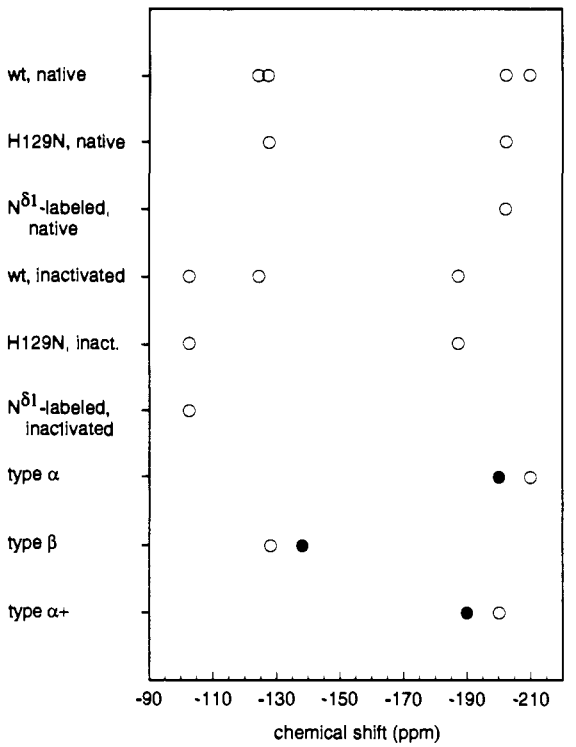
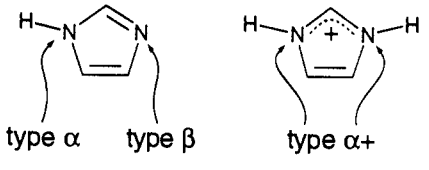


Figure 4. Summary of imidazole nitrogen chemical shifts. Typical values for type α, type β, and type α+ nitrogens are from Bachovchin.[26] Closed symbols show the values for nitrogens involved in hydrogen bonds.

site His-70).[12] Since the chemical shift of the ^{13}C-enriched carbon atom of the minor adduct is almost identical to that of the active-site adduct, both were assigned vinylimidazole structures (cf. **6**). The comparatively narrow line width of the signal associated with the minor adduct suggests that the second histidine (subsequently identified as His-129[10]) is relatively mobile and therefore that it may lie on the surface of the protein molecule, remote from the active site. Indeed, examination of the sequence of wild-type dehydrase in the vicinity of His-129 reveals the alternation of hydrophobic and hydrophilic amino acid residues (Figure 1a). Such patterns are characteristic of strands of β-sheet that lie on protein surfaces.[53] The current results are consistent with these observations. Thus, when His-129 is changed to asparagine, no significant change in the structure or function of dehydrase can be detected. This suggests that His-129 is not involved, directly or indirectly, in catalysis or the maintenance of an enzymatically-active conformation. Furthermore, substitution of asparagine for His-129 simplifies NMR studies of His-70.

Through the use of ^{15}N NMR spectroscopy, we have investigated several significant aspects of the structure and function of dehydrase. We have assigned the resonances of both imidazole nitrogens of the active-site histidine residue His-70 by analyzing the spectra of uniformly and specifically ^{15}N-labeled H129N dehydrase.

Bachovchin,[26] expanding upon the nomenclature system proposed by Witanowski et al.,[54] has identified three distinct types of imidazole nitrogens, referred to as "type α", "type β", and "type α+". Figure 4 summarizes typical chemical shifts for these three



types of imidazole nitrogens[26] as well as the results that we obtained for the histidine residues in dehydrase. Comparison of the typical values with the observed chemical shifts of the nitrogens in His-70 of dehydrase (Figure 2) indicates that in active dehydrase, $N^{\delta 1}$ of His-70 is a type α nitrogen, and therefore His-70 is in the $N^{\delta 1}$–H tautomeric form. The residual ^1H–^{15}N coupling that is observed (Figure 2a,b) is also consistent with the suggestion that $N^{\delta 1}$ is a type α nitrogen.

The chemical shift of $N^{\delta 1}$ (−202.2 ppm) is approximately 8 ppm downfield from the expected position. A shift of the signal in the downfield direction could be caused either by partial protonation of the imidazole ring or by the presence of a hydrogen bond involving $N^{\delta 1}$. Two factors argue against partial protonation. First, the position of the $N^{\epsilon 2}$ resonance (−127.5 ppm) is exactly as expected for a type β imidazole nitrogen. Second, a titration study shows that the positions of the His-70 ^{15}N resonances are unchanged between pH 8 and 6. If the histidine ring were experiencing partial protonation at pH 7.4, a pH-dependent variation in the chemical shifts of the two nitrogen nuclei would be expected.

Such a downfield shift is consistent with a hydrogen bond involving $N^{\delta 1}$, however. Indeed, in model compounds, hydrogen bonds have led to changes of up to 10 ppm in imidazole nitrogen chemical shifts.[25,26] As the $N^{\epsilon 2}$–H tautomer is the favored tautomer for 4-substituted imidazoles,[21,55] a hydrogen bond involving $N^{\delta 1}$ could serve to lock the imidazole into the catalytically active but less stable $N^{\delta 1}$–H tautomer. A similar argument has been put forth by Bachovchin to explain a role for Asp-102 in α-lytic protease.[26,56]

Examination of the ^{15}N spectra of inactivated dehydrase (Figure 3) allows the assignment of the resonances in the inactivated enzyme as well. $N^{\delta 1}$ of His-70 resonates approximately 80 ppm downfield from $N^{\epsilon 2}$, suggesting that $N^{\delta 1}$ is a type β nitrogen. To confirm this assignment, we have also examined the $^1H\{^{15}N\}$MBC spectrum of 1-vinylimidazole in methanol (data not shown), which reveals chemical shifts of −124 and −189 ppm for the imidazole nitrogens. Furthermore, a strong correlation to the trans vinyl proton shows that the signal at −189 ppm owes to N-1 (the alkylated nitrogen), thus indicating that in 1-vinylimidazoles as well as in simple alkyl imidazoles, type α nitrogens resonate upfield relative to type β nitrogens.[26]

That the position of the type β nitrogen resonance in 1-vinylimidazole lies some 20 ppm upfield from the position of the analogous resonance in inactivated dehydrase probably reflects the different environments of the two nitrogens. In 1-vinylimidazole in methanol, N-3 (the type β nitrogen) is subject to hydrogen-bonding interactions with the solvent, causing an upfield shift in the position of its resonance. (N-1 is not subject to such effects, since its lone pair is part of the aromatic sextet, and so it cannot accept hydrogen bonds.) In the active site of inactivated dehydrase, the imidazole side chain of His-70 may be moved from its optimal catalytic position, in which it is involved in a hydrogen bond as described above. It is hypothesized that in this new position $N^{\delta 1}$ cannot interact with its hydrogen-bonding partner, and as a result, its ^{15}N NMR signal is not subject to an upfield, hydrogen-bonding-derived shift.

Scheme III depicts a working hypothesis for the events that occur in the active site during inactivation of dehydrase by 3-decynoyl-NAC. The imidazole ring is held in the correct tautomer for catalytic activity by a hydrogen bond to $N^{\delta 1}$, making $N^{\epsilon 2}$ the basic nitrogen, and it is this nitrogen that is modified during the inactivation of dehydrase by 3-decynoyl-NAC. Upon alkylation of $N^{\epsilon 2}$, hydrogen bonding between $N^{\delta 1}$ and X–H is disrupted.

The abnormally low estimate for the pK_a of His-70 obtained in this work could be the result of several features of the dehydrase active site. The fact that pH-dependent shifts in the resonance positions of some amino groups are observed suggests that we are able to detect pH-dependent changes in the protonation state of

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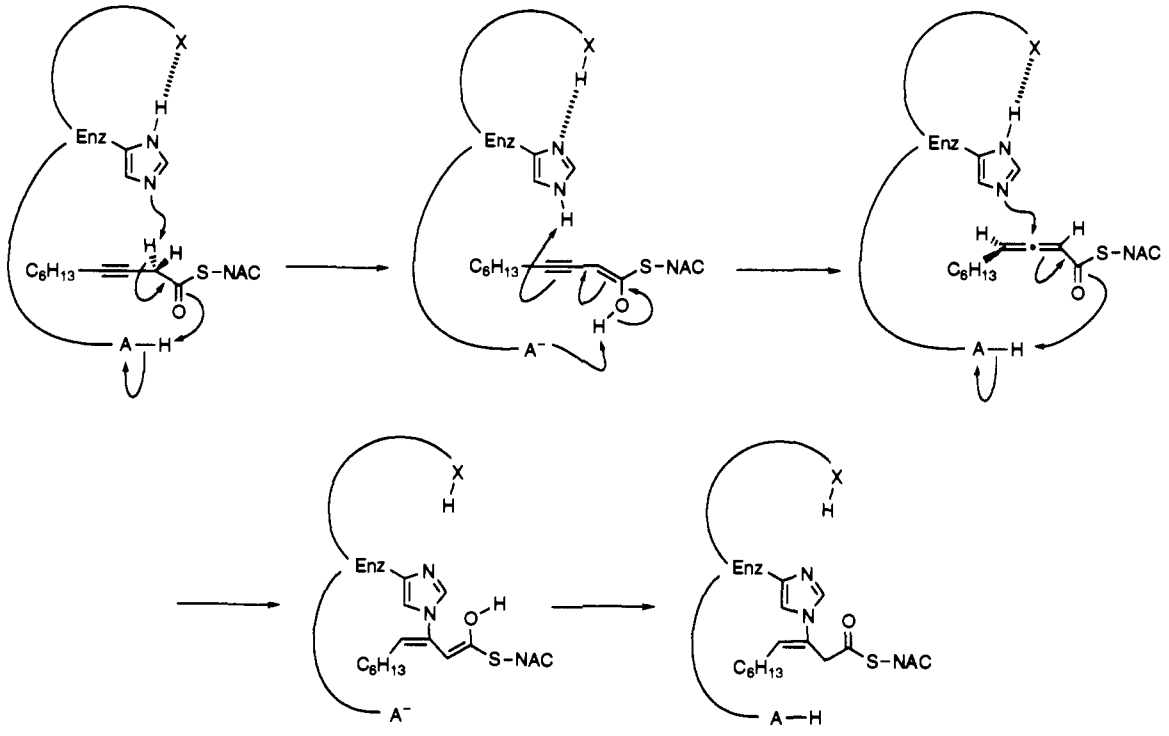
(54) Witanowski, M.; Stefaniak, L.; Januszewski, H.; Grabowski, Z.; Webb, G. A. *Tetrahedron* **1972**, *28*, 637–653.
(55) Gilchrist, T. L. *Heterocyclic Chemistry*; Pitman: London, 1985; p 372.
(56) Factors other than protonation or hydrogen bonding apparently can have substantial effects on imidazole ^{15}N chemical shifts, as evidenced by the fact that both imidazole nitrogens of triosephosphate isomerase His-95 resonate outside the "normal" ranges (cf. Figure 4).[27]

Scheme III



Scheme IV

nitrogenous acid/base groups in dehydrase by ^{15}N NMR. Recent work of Matthews and Dahlquist has shown that the ϵ-ammonium group of a lysine residue which was introduced into the hydrophobic core of lysozyme by site-directed mutagenesis has a pK_a of 6.5.[57] This suggests that a hydrophobic environment in a protein can have a significant effect on the pK_a of a titratable group. The active-site region of dehydrase may be somewhat hydrophobic, and it is possible that this environment would serve to lower the pK_a of His-70. As lowering the pK_a of the catalytic base should hinder the deprotonation of the α-carbon of the substrate, we hypothesize that a hydrogen bond acceptor (cf. X in Scheme III) may be the ultimate proton acceptor. Indeed, this group could be responsible for the pH 7 inflection point seen in the pH–k_{cat} profile for dehydrase.[8] Such a mechanism for dehydrase is illustrated in Scheme IV, which shows that the imidazole ring of His-70 and the substrate may serve as conduits through which the negative charge is shuttled from the hydrogen-bond accepting group X to the conjugate base of another amino acid that serves as an electrophilic catalyst (vide infra). Transfer of charge through an imidazole ring to a hydrogen-bond acceptor is reminiscent of the version of the charge–relay mechanism for serine proteases that was suggested by Hunkapiller et al.[58]

Another explanation for the low apparent pK_a of His-70 would be the failure of protons (or H_3O^+) to penetrate the active site. In this case, the pH of the bulk solution would not accurately reflect the pH at the active site. Since water is a product/reactant in one of the reactions catalyzed by dehydrase, it is hypothesized that water molecules only bind at a specific location in the active site and that they are not generally accessible to all catalytic groups. This brings up the question of why this water binding site would be inaccessible to the imidazole group of His-70 during the titration of the native enzyme but accessible to this imidazole group during normal catalytic turnover. (In the dehydrase-catalyzed dehydration/hydration reaction, a presumed role of the imidazole is to protonate the thiol ester substrate hydroxyl group or to deprotonate the water molecule.[5]) Perhaps protonated water cannot bind at the neutral water binding site. Alternatively, dehydrase could have an ordered kinetic binding mechanism (Scheme V), in which fatty acid thiol ester substrate **2** must bind before the substrate water molecule binds. This model is attractive since substrate **2** is not always released from the enzyme after the dehydration step but can be isomerized immediately to the thiol ester of (*Z*)-3-decenoic acid (**3**).[59] In this model, the pH 7 inflection point in the pH–k_{cat} curve could arise from protonation of the imidazole of His-70. This imidazole could be protonated in the pH–rate studies, in which both substrates are present, but not in the NMR study, where thiol ester substrates are absent.

Scheme V

At low pH, the imidazole ^{15}N signals of His-70 disappear. It is possible that these signals are broadened at low pH due to interaction of the histidine side chain with paramagnetic impurities. This was suggested as the cause of the disappearance of the histidine signal in α-lytic protease observed by Bachovchin and Roberts.[21] Protein denaturation and aggregation (as might occur at pH extremes) can also lead to broadening of NMR resonances

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(58) Hunkapiller, M. W.; Smallcombe, S. H.; Whitaker, D. R.; Richards, J. H. *Biochemistry* **1973**, *12*, 4732–4743.

(59) Rando, R. R.; Bloch, K. *J. Biol. Chem.* **1968**, *243*, 5627–5634.

and reduction in the signal-to-noise ratio as the molecular weight of the aggregate increases. However, this seems an unlikely explanation for the selective broadening of His-129 signals, since even in an aggregate, His-129 should not be any less mobile than is active-site His-70. In any case, such changes, if they occur, must be reversible, as the low-pH enzyme sample showed a normal spectrum when its pH was raised to 7.4 and then readjusted to 6.3, and the same protein sample exhibited normal enzymatic activity when reassayed at pH 7.0. A third possible explanation is that the signals are broadened due to exchange between protonated and unprotonated forms of the imidazole ring. This is the rationale offered to explain the selective disappearance of the ^{15}N NMR signal for $N^{\delta 1}$ of His-76 in the phosphorylated form of the phosphocarrier protein HPr from *E. coli*.[60] Indeed, Sudmeier et al.[61] have performed theoretical calculations that show that when the exchange rate is intermediate between the limits of slow exchange (i.e., when distinct signals for each state are detectable) and fast exchange (i.e., when one average signal appears), the resonances from the two states can appear as one or two very broad signals. In the ^{15}N spectra of dehydrase, the signal-to-noise ratio is low, and very broad lines might not be detectable above background. This has been suggested as an alternate explanation for the disappearance of the signals of α-lytic protease.[61] It is possible that a combination of these effects is responsible for the disappearance of the His-70 signals in dehydrase.

A major unanswered mechanistic question is how a histidine residue with a pK_a of 5 or less could deprotonate an inhibitor (or substrate) carbon acid with a pK_a of approximately 15–18.[62–64] Indeed, by applying the analysis of Gerlt and Gassman[65] to dehydrase, it can be shown that the maximum value for the difference in pK_a between the active-site base and the substrate acid for which simple acid–base catalysis alone would explain the observed rate of reaction ($k_{cat} = 10\ s^{-1}$)[66] is on the order of 1. This suggests that other modes of catalysis must be operating to effect the reactions catalyzed by dehydrase. Gerlt et al. showed that upon protonation of a thiol ester carbonyl group the pK_a of the α protons is lowered to ca. 4.[67] Hydrogen bonding to (as opposed to protonation of) the carbonyl group of the substrate would also lower the pK_a of the substrate α-protons. Indeed, it has been shown that general acid–general base-catalyzed formation of an enol (as opposed to an enolate) intermediate in β-elimination reactions, such as that catalyzed by dehydrase, can occur at a rate that is thermodynamically and kinetically consistent with the pK_a values of typical protein acids and bases.[65] This suggests a possible role for the tyrosine residue implicated by the work of Helmkamp and Bloch:[8] to serve as a general acid and to participate with the imidazole of His-70, which serves as a general base, in the enolization of the substrate or inhibitor (cf. Schemes III and IV). This is analogous to the role of tyrosine-14 in Δ^5-3-ketosteroid isomerase.[68–71] Alternatively, Bevitt et al. have aligned the amino acid sequences of dehydrase with the dehydratase domains of several fatty acid synthases and polyketide synthases.[72] Relatively few amino acids are conserved; notably, however, a histidine and an aspartic acid, corresponding to His-70 and Asp-63 of dehydrase, are conserved. Bevitt et al. suggest that this aspartic residue, rather than a tyrosine[8] or a cysteine,[3] may be the putative electrophilic catalyst. It is also possible that this aspartic acid residue is the group identified as X in Schemes III and IV.

It is relatively common for the imidazole rings of catalytic histidine residues to be in the $N^{\delta 1}$–H tautomeric form, stabilized by hydrogen bonding. A classic example is the catalytic histidine residue in the serine protease active site. The $N^{\delta 1}$–H tautomer was suggested for His-57 of chymotrypsin by Blow from examination of the X-ray crystal structure[73] and confirmed by Bachovchin and Roberts for His-57 of α-lytic protease using ^{15}N NMR.[21] This pattern is by no means universal, however. By examining the ^{13}C NMR spectra of ribonuclease A at various pH values, Walters and Allerhand determined that His-12 is in the $N^{\delta 1}$–H tautomer, while His-119 is in the $N^{\epsilon 2}$–H tautomer, as had been predicted by examination of the X-ray crystal structure.[74] In a peptide hormone,[75] as well as in myoglobins,[76] non-active-site histidine residues show a preference for the $N^{\epsilon 2}$–H tautomer. The use of hydrogen-bonding interactions to stabilize the normally less stable $N^{\delta 1}$–H tautomer of histidine side chains appears to be a common motif in Nature's design for enzyme active sites.

Conclusions

We have developed a strain of *E. coli*, pRA224, that overproduces dehydrase as at least 15% of soluble protein, greatly simplifying both the purification of the enzyme and labeling with ^{15}N for analysis by NMR spectroscopy. Changing the non-active-site histidine in dehydrase (His-129) to asparagine has little effect on the structure or function of dehydrase. In active dehydrase, the active-site histidine residue His-70 is in the $N^{\delta 1}$–H tautomeric form, held in that form by hydrogen bonding. Upon inactivation, the tautomeric form of His-70 is reversed, and His-70 becomes alkylated on $N^{\epsilon 2}$. A titration study shows no variation in the position of the imidazole nitrogen resonances of His-70 over the range from pH 8 to 6. It is concluded that either His-70 has an abnormally low pK_a, in which case dehydrase might use a charge–relay-type mechanism, or His-70 is not accessible to solvent to become protonated. The latter scenario is consistent with an ordered binding kinetic mechanism. A revised model for events occurring in the active site of dehydrase shows that $N^{\epsilon 2}$ of His-70 is the probable locus of active-site basicity.

Acknowledgment. This work has been funded by Public Health Service Grant GM36286 (to J.M.S.) and Award 6056 from the Showalter Trust Fund (to V.J.D.). R.R.A. is the recipient of a David Ross Fellowship. We express our appreciation to the Stable Isotope Resource at Los Alamos, which is supported by NIH Grant RR02231, for generously providing [$^{15}N^{\delta 1}$]histidine. We would like to thank Barry S. Henderson and John E. Cronan, Jr., for allowing us to describe their results prior to publication.

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