Mechanism of Urocanase As Studied by Deuterium Isotope Effects and Labeling Patterns†

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ABSTRACT: Nicotinamide adenine dinucleotide (NAD) dependent urocanase (4′-imidazolone-5′-propionate hydro-lyase, EC 4.2.1.49) from *Pseudomonas putida* was found to catalyze an exchange reaction between solvent and the 4′-hydrogen of urocanate or imidazolepropionate at a rate faster than that of overall catalysis. When urocanate labeled at the 4′ position with deuterium was compared to unlabeled urocanate as a substrate, no isotope rate effect was noted. For examination of the possibility of an NAD^+-mediated intramolecular hydride transfer of the 4′-hydrogen to a position on the side chain of oxoimidazolepropionate, the origins of hydrogen at positions 2 and 3 in the propionate chain were studied as a function of reaction time and extent of exchange of the 4′-hydrogen. No transfer of hydrogen from the 4′ position to the side chain was observed, thereby eliminating mechanisms requiring hydride transfer via NADH between these positions. Catalytic rates in ^1H_2O vs. ^2H_2O revealed a 3-fold difference which was ascribed to a rate-limiting proton addition step. Similarly, a 5-fold decrease in V_{max} was found for the reverse reaction when oxoimidazole[2,3-$^2H_2$]propionate was compared to unlabeled oxoimidazolepropionate. These data support a mechanism involving water addition across the conjugated double bond system of urocanate, rather than an internal oxidation–reduction process, yet NAD^+ is required. A mechanism is proposed which uses electron delocalization in the imidazole nucleus, via an imidazole–NAD adduct, to facilitate water attack and subsequent formation of oxoimidazolepropionate.

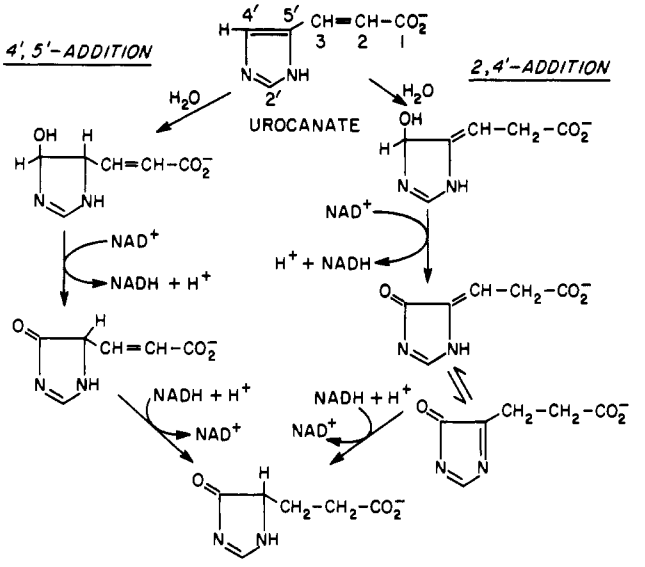
A satisfactory mechanism for the enzymatic hydration of urocanic acid, catalyzed by urocanase (4′-imidazolone-5′-propionate hydro-lyase, EC 4.2.1.49), has proven elusive because there are no obviously related biological reactions from which to draw parallels. With the discovery that urocanase from *Pseudomonas putida* (Egan & Phillips, 1977; Keul et al., 1979) contains a tightly bound NAD^+ which is essential for catalytic activity, most earlier mechanistic proposals, such as that put forth by George & Phillips (1970), were called into question because no role for an oxidation-reduction coenzyme had been envisioned in these.

Unfortunately, simple reaction schemes which depict NAD^+-mediated internal oxidation–reduction following water addition, typified by the two possibilities illustrated in Scheme I, are not easily reconciled with existing data. Kaeppeli & Retey (1971) demonstrated that two hydrogens from solvent are stereospecifically introduced into the side chain of urocanic acid at C-2 and C-3. The usual type of oxidation–reduction process mediated by NAD^+ involves hydride ion transfer, shown in Scheme I as coming from position C-4′ of the imidazole ring, and would lead to formation of NADH. Subsequently, NADH would be reoxidized and the hydride ion transferred to either position 2 or position 3 of the side chain or to position 5′ on the oxoimidazole ring. Because NADH does not exchange with solvent hydrogens (Ludowieg & Levy, 1963), hydride ion transfer from C-4′ to C-2 or C-3 via NADH would conflict with the labeling data of Kaeppeli & Retey (1971), leaving only the possibility for transfer to position C-5′.

The mechanism (Scheme I, 4′,5′ addition) involving addition of water between positions 4′ and 5′ of the urocanate imidazole ring, with a subsequent hydride ion transfer from 4′ to a side-chain carbon, is further regarded as unlikely since it would predict that the substrate analogue imidazolepropionate (dihydrourocanate) should be a substrate for addition and oxidation, yielding oxoimidazolepropionate directly and NADH (instead of NAD^+). There is no evidence, however, that imidazolepropionate serves as a dead-end inhibitor (Phillips et al., 1977).

An additional complication for the internal redox schemes is seen in the conclusion put forth by Kaeppeli & Retey (1971) that the enol form of oxoimidazolepropionate is the enzymic product, spontaneously tautomerizing to the keto form. The reaction sequences depicted in Scheme I require the keto

Scheme I: Two Hypothetical Reaction Pathways for the Urocanase-Catalyzed Conversion of Urocanate to Oxoimidazolepropionate, Based on an NAD^+-Linked Internal Oxidation-Reduction Mechanism[a]



[a] The left side illustrates a route involving water addition to positions 4′ and 5′ on urocanate, while the right side depicts water addition to positions 4′ and 2. Throughout the text, imidazolone propionate is referred to as oxoimidazolepropionate, a terminology recommended by the Journal.

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tautomer to be the initial product.

The present report is an investigation of deuterium isotope kinetics and exchange reactions, aimed at clarifying the role of NAD^+ in urocanase catalysis. A preliminary account of some of the data herein has appeared earlier (Egan & Phillips, 1976; Egan, 1977), and a recent brief report by Gerlinger & Retey (1980) describes similar findings of exchangeability at the 4′ position of urocanate.

Experimental Procedures

Special Reagents. Deuterium oxide (99.9%) and ion-exchange resins were obtained from Bio-Rad Laboratories. Supelco, Inc., supplied bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 3% SP2250 (100–120 mesh Supelcoport). Urocanic acid was purchased from Sigma Chemical Co. Imidazolepropionic acid was prepared by catalytic hydrogenation of urocanic acid (Phillips et al., 1977).

Enzyme Preparation and Assay. Urocanase was isolated from *Pseudomonas putida* ATCC 12633 essentially as described by George & Phillips (1970). The purified enzyme had a specific activity of 1.9–2.2 μmol of urocanate consumed per min per mg of protein, equivalent to a minimum purity of 95%.

The urocanase assay was based on the spectrophotometric measurement of urocanate disappearance at 277 nm, as described by George & Phillips (1970). Protein determinations were performed according to the method of Groves et al. (1968), with bovine serum albumin as standard.

Exchange of the 4′-Hydrogen on Urocanate and Imidazolepropionate. The reaction mixtures (3 mL) contained 4 mM potassium urocanate or 5 mM potassium imidazolepropionate in 5 mM triethanolamine deuteriochloride, pD 7.5; pD values for deuterium-containing solutions were calculated from measured pH + 0.4 (Lumry et al., 1951). All components except enzyme were previously exchanged three times by lyophilization and resuspension in 3 mL of ^2H_2O to remove labile hydrogens. The reaction was initiated by the addition of 0.4 mg of urocanase in 0.02 mL of nondeuterated buffer. Incubation was performed at 25 °C. For the urocanate reaction, the decrease in substrate levels was monitored by continuous spectrophotometric measurement at 317 nm where the molar extinction coefficient for urocanate was determined to be 610 M^{-1} cm^{-1}. In this instance, reactions were conducted in 1-cm path length cuvettes flushed with N_2 and stoppered with rubber serum caps. At various intervals, 0.1 mL was removed from the solution and acidified with an equal volume of 0.1 M HCl. The samples were then quickly frozen and lyophilized in 1-mL ampules. Analyses for deuterium content via combined gas chromatography–mass spectrometry were as described below. The same general procedure was followed in the experiment on imidazolepropionate exchange except that spectrophotometric monitoring was omitted.

For the experiment using urocanase inactivated with *O*-methylhydroxylamine, the enzyme (4.0 mg) was first treated with 0.2 M *O*-methylhydroxylamine in 0.1 M Tris-HCl, pH 8.5 (total volume, 0.2 mL). For the actual exchange experiment, the incubation was performed in the manner described in the previous paragraph, but with 0.02 mL of this inactivated urocanase solution substituted for active enzyme. Also the incubation mixture contained 1.3 mM *O*-methylhydroxylamine in addition to the components described above.

Relationship of 4′-Hydrogen Exchange on Urocanate to Solvent Hydrogen Incorporation into the Side Chain of Oxoimidazolepropionate. The reaction mixture contained 450 μmol of urocanate and 900 μmol of 2,6-dichlorophenolindophenol in 90 mL of 0.05 M tris(hydroxymethyl)aminomethane deuteriochloride in ^2H_2O, pD 8.4. Reagents were previously exchanged with ^2H_2O as described above. The reaction was begun by the addition of 1.5 mg of urocanase in 0.1 mL of 50 mM potassium phosphate buffer, pH 7.5; incubation was at 25 °C. At various times, samples (3–30 mL) were removed and acidified with HCl to pH 2 or below. From each sample, a portion corresponding to approximately 1 μmol of urocanate was removed for determination of deuterium content as described in later sections. The remainder was subjected to permanganate oxidation according to the method of Kaeppeli & Retey (1971). Derivatization and procedures for the determination of the deuterium content of the resulting succinic acid are described in the section on gas chromatography–mass spectrometry. The function of 2,6-dichlorophenolindophenol in the reaction was to facilitate the conversion of oxoimidazolepropionic acid to succinic acid by directing the nonenzymatic decomposition of oxoimidazolepropionic acid toward 4-oxoglutaramic acid (an oxidation) rather than toward *N*-formylisoglutamine (hydrolytic ring opening; Hassal & Greenberg, 1963).

Measurement of pH (pD) vs. V_{max}. Incubation mixtures contained urocanase and 0.05–0.5 mM potassium urocanate in constant ionic strength tris(hydroxymethyl)aminomethane–cacodylate (0.05 M) at pH (pD) values ranging from 5.5 to 9.0. Cuvettes of either 1-cm or 0.2-cm path length were used to accommodate the molar extinction coefficient of urocanate (18 800 M^{-1} cm^{-1} at 277 nm and pH 7.5; Tabor & Mehler, 1955). Kinetic constants were determined by the least-squares program of Cleland (1967).

Preparation and Kinetic Measurements of [4′-^{2}H]Urocanate and Oxoimidazole[2,3-$^2H_2$]propionate. The syntheses of [4′-^{2}H]urocanate and oxoimidazole[2,3-$^2H_2$]propionate were performed enzymatically according to a modification of the method of Brown & Kies (1959). The reaction mixture in both cases contained 440 μmol of potassium urocanate and 2.5 mmol of potassium phosphate in 50 mL of ^2H_2O (pD 7.5). All components had previously been exchanged with ^2H_2O as described earlier. The mixture was made anaerobic by flushing with nitrogen for 15 min in a serum-stoppered flask at 37 °C in the dark. The reaction was started by the addition of urocanase (0.1 mL, 2 mg). For the synthesis of [4′-^{2}H]urocanate, the reaction was allowed to proceed until the A_{277} (1-cm path) was one-third of the original. For oxoimidazole[2,3-$^2H_2$]propionate, the reaction was conducted until the A_{260}:A_{277} ratio equaled 1; this corresponded to a molar ratio of oxoimidazolepropionate:urocanate of 6.5:1 (Cohn et al., 1975).

The flasks were chilled to 0 °C and 1 mL of 4 M HCl was added to each to stop the reactions. Protein was precipitated by the addition of trichloroacetic acid to 5%, the precipitate removed by filtration, and the excess trichloroacetic acid extracted into ether. The filtrate was dried in vacuo, resuspended in 0.1 M HCl, and fractionated on a column (1 × 30 cm) of AG 1-X8 (H^+ form, 200–400 mesh) with 2 M HCl at 4 °C. During the column separation, care was taken to shield the sample from light. Unlabeled oxoimidazolepropionate was prepared as above, except with ^1H_2O as solvent.

The kinetic constants for [4′-^{2}H]urocanate and [4′-^{1}H]-urocanate were determined under standard assay conditions at concentrations ranging from 0.02 to 0.5 mM; at higher concentrations, 0.2-cm path-length cuvettes were used.

For oxoimidazole[2,3-$^2H_2$]propionate and unlabeled oxoimidazolepropionate, assays were performed in Thunberg cuvettes. Each cuvette contained 10 μg of urocanase, 150 μmol of potassium phosphate, pH 7.5, and in the side arm an ap-

propriate amount of oxoimidazolepropionate in 0.01 M HCl such that the final concentration upon mixing ranged from 0.6 to 3 mM in a total volume of 1.5 mL. Prior to mixing, the cuvettes were made anaerobic by repeated evacuation and replacement with purified N_2. The reactions were monitored at 277 nm for the appearance of urocanate.

The ^1H NMR spectrum of the purified [4′-^{2}H]urocanate in ^2H_2O (buffered with 50 mM potassium phosphate, pD 7.2), taken on a 60-MHz Varian A60 NMR spectrometer, gave no singlet at δ 7.35, the assignment for the 4′-H of urocanate, indicating purity in excess of 95%; this purity was confirmed by gas chromatography–mass spectrometry, as outlined below. The NMR spectrum of oxoimidazole[2,3-$^2H_2$]propionate at pD 7.2 indicated approximately 90% purity since the ratio upon integration of the downfield peak at δ 8.14 (the 2′-H) to the upfield multiplet at δ 2.2 (the side-chain hydrogens) was 0.50 ± 0.05 (SD, n = 6).

Gas Chromatography–Mass Spectrometry. Analyses were performed on the trimethylsilyl derivatives of succinate, urocanate, and imidazolepropionate; a stable derivative of oxoimidazolepropionate could not be prepared. Derivatization was performed in flame-sealable ampules by adding 100 μL of BSTFA–acetonitrile (1:1 volume ratio) per 0.5 μmol of material. The ampules were sealed and heated at 150 °C for 15 min. Electron-impact mass spectra were determined at 70 eV with a Varian 1400 gas chromatograph fitted to a Du Pont 21–490 mass spectrometer. Gas chromatographic separations were performed on a 2% SP2250 (100–120 Supelcoport) column (0.25 in. × 6 ft). For urocanate and imidazolepropionate, the column temperature was 210 °C; for succinate, a temperature gradient from 100 to 150 °C at 2°/min was employed.

The ratio of deuterated to unlabeled urocanate (or imidazolepropionate) was calculated from peak heights of the parent ion (P) (m/e 282 for urocanate, 284 for imidazolepropionate) and the P + 1 peaks, correcting for natural isotope abundances. Actual concentrations were calculated from the total urocanate or imidazolepropionate in the cuvette at the time of sampling.

For succinate, the proportion of dideuterated material present was calculated with authentic monodeuterated succinic acid (prepared enzymatically; Englard & Hanson, 1969) and unlabeled succinic acid as standards. Calculations were based on the peak heights of m/e 248 for monodeuterated and m/e 249 for dideuterated succinate, corresponding to the loss of a methyl group from bis(trimethylsilyl) succinate.

Results

Back-Incorporation of Deuterium into Urocanate during Catalysis in ^2H_2O. In the urocanase reaction, the 4′-hydrogen of urocanate is the only atom removed, and therefore its possible exchangeability with solvent in a partial reaction would clarify the steps involved in its removal. Figure 1 illustrates the kinetics of loss of the 4′-H of urocanate and its replacement by ^{2}H compared to the rate of overall disappearance of urocanate. The conversion of [4′-^{1}H]urocanate to [4′-^{2}H]urocanate exceeded the rate of urocanate destruction approximately 4-fold. By the time that 50% of the urocanate had been consumed, 85% of the urocanate remaining was [4′-^{2}H]urocanate, yet the rate of urocanate disappearance was not appreciably reduced compared to that seen initially when all urocanate was nondeuterated. These observations indicate that the 4′-hydrogen of urocanate undergoes a rapid exchange with solvent protons, leading in the case of reaction conducted in ^2H_2O to nearly complete deuteration at position 4′. Moreover, the incorporation of ^{2}H at the C-4′ position of urocanate appears to have no profound effect on overall catalysis; this point is expanded upon in a later section.

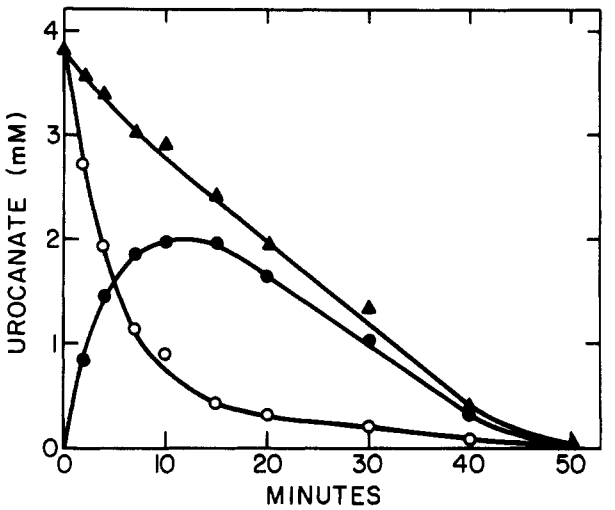


FIGURE 1: Hydrogen exchange at position C-4′ of urocanate: (▲) total urocanate concentration; (○) [4′-^{1}H]urocanate concentration; (●) [4′-^{2}H]urocanate concentration.

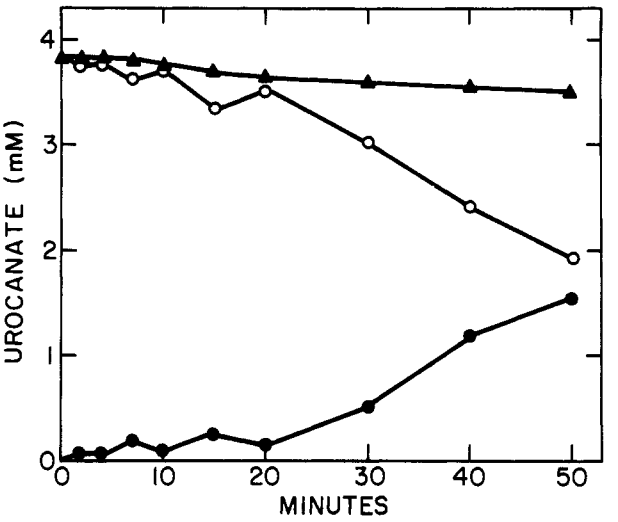


FIGURE 2: Exchangeability of hydrogen on urocanate studied with *O*-methylhydroxylamine-inhibited urocanase: (▲) total urocanate concentration; (○) [4′-^{1}H]urocanate concentration; (●) [4′-^{2}H]-urocanate concentration.

Exchange at the 4′ position of urocanate was definitely enzyme catalyzed, as no formation of [4′-^{2}H]urocanate was observed when urocanase was omitted from a reaction mixture similar to that used for the experiment illustrated in Figure 1. If urocanase was first inactivated with *O*-methylhydroxylamine, which reversibly modifies the active site NAD^+ at high pH (Phillips et al., 1977), and then this modified enzyme was used in a similar exchange experiment, the appearance of [4′-^{2}H]urocanate was delayed (Figure 2). The onset of catalysis after approximately 20 min, arising from a slow dissociation of the NAD–$NHOCH_3$ complex at neutral pH (Phillips et al., 1977), was accompanied by hydrogen exchange at the 4′ position. After 50 min, 44% of the remaining urocanate was [4′-^{2}H]urocanate, but only 10% of the urocanate had been consumed.

Exchangeability of the 4′-Hydrogen of Imidazolepropionate. While the urocanase reaction has a rather large equilibrium constant (K_{eq} = 65; Cohn et al., 1975), the possibility existed that the 4′-hydrogen exchange on urocanate could arise from a complete reversal of the reaction. Exchange of the 4′-hydrogen on imidazolepropionate was studied to address this point. Imidazolepropionate is a competitive in-

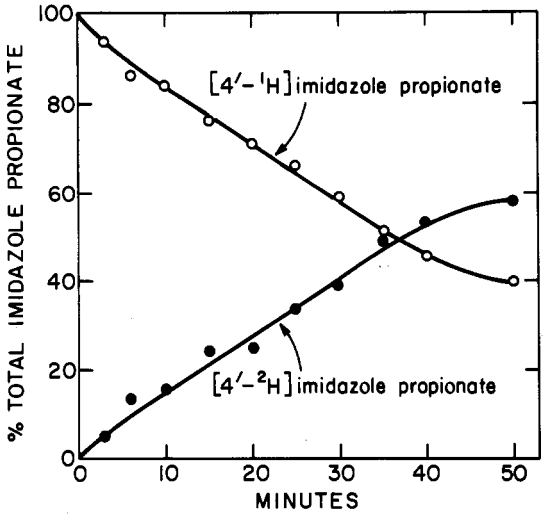


FIGURE 3: Hydrogen exchange at position C-4′ of imidazolepropionate: (○) [4′-¹H]imidazolepropionate; (●) [4′-²H]imidazolepropionate. The total imidazolepropionate concentration was 5.0 mM.

hibitor (K_i = 0.9 mM at pH 7.5) which cannot be converted to a product (Phillips et al., 1977). As shown in Figure 3, imidazolepropionate underwent a similar enzyme-catalyzed hydrogen exchange at the 4′ position, although at a rate approximately one-fifth that for urocanate. Assignment of the position of exchange on imidazolepropionate was by NMR analysis. Since imidazolepropionate cannot add water, this indicates the exchange of the 4′-hydrogen does not require a preliminary water attack.

Reexamination of the Solvent Origin of the Side-Chain Hydrogens of Oxoimidazolepropionate. The demonstration of a rapid hydrogen exchange at the 4′ position of urocanate made it necessary to reexamine the apparent solvent origin of the side-chain hydrogens of oxoimidazolepropionate. It seemed plausible that if NAD^+ functioned as a hydride-transfer agent in catalysis, transferring a hydrogen from position 4′, via NADH formation, to one of the side-chain carbons (C-2 or C-3), such an intramolecular transfer might not have been evident in the earlier studies of Kaeppeli & Retey (1971) because of the rapid exchange of the C-4′ hydrogen. The labeling pattern predicted in oxoimidazolepropionate for a hydride ion transfer mechanism from C-4′ to C-3 or C-2 would initially be monodeuterated oxoimidazolepropionate, later changing to the dideuterated species (^2H at positions 2 and 3) as exchange of the 4′-hydrogen of urocanate became complete.

For closer examination of this possibility, urocanate and enzyme were incubated in ^2H_2O and samples were removed over time, either to be analyzed for [4′-^{2}H]urocanate content or subjected to an oxidative degradation whereby carbons 1, 2, 3, and 5′ of oxoimidazolepropionate were converted to succinic acid. Solvent deuterium incorporation into the C-2 or C-3 positions of oxoimidazolepropionate (but not C-5′) would thus appear as deuterated succinic acid. As seen in Figure 4, dideuterated succinate was found at the earliest sampling times and its content did not vary, even though 60% of the urocanate became deuterated at C-4′ during the incubation. These data support the conclusion of Kaeppeli & Retey (1971) that both hydrogens incorporated into the urocanate side chain to form oxoimidazolepropionate are added as solvent-derived protons and neither comes from C-4′ by hydride transfer.

pH (pD) vs. V_{max}. The rate of urocanase catalysis in ^2H_2O was observed to be markedly reduced relative to that in ^1H_2O. A decrease in the maximal rate of a reaction in ^2H_2O compared to that in ^1H_2O suggests a deuterium isotope effect on the overall reaction. In Figure 5, values of V_{max} for urocanase as a function of pH (pD) in the range of 5.5 to 9.9 are shown. The ratio of V_{max} in ^1H_2O to V_{max} in ^2H_2O was found to be 2 to 3 over the entire pH (pD) range, confirming the presence of a deuterium isotope effect. Because the curves did not intersect at any point, the effect of ^2H_2O could not be attributed to an effect on the pK_a of the substrate or on the ionization of the enzyme or enzyme–substrate complex (Jencks, 1969). Measurement of the initial rate of the urocanase reaction after preincubating the enzyme in ^2H_2O for up to 20 min prior to substrate addition resulted in no change in the observed initial rate (data not shown). Therefore, ^2H_2O does not force the enzyme into an unfavorable conformation which leads to a decrease in V_{max} (Jencks, 1969). The magnitude of the isotope effect is consistent with a proton addition from solvent (or from some exchangeable general acid group on the enzyme) being the rate-limiting step in the forward reaction.

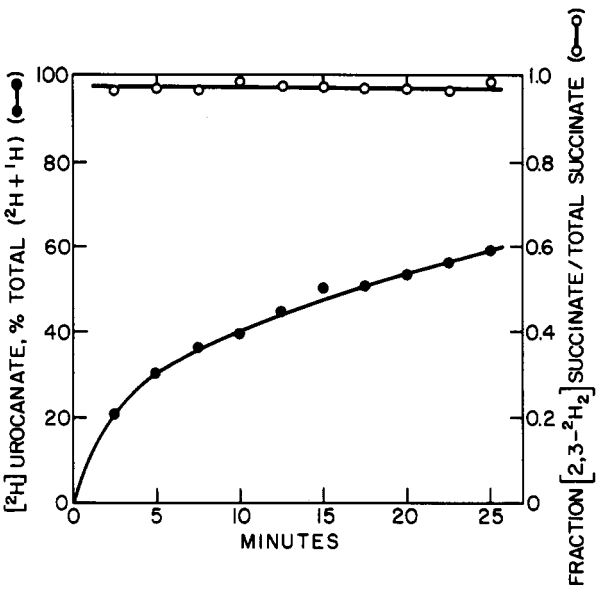


FIGURE 4: Relationship between 4′-hydrogen exchange on urocanate and the incorporation of solvent hydrogens at positions C-2 and C-3 of oxoimidazolepropionate. The extent of solvent hydrogen incorporation was determined after conversion of oxoimidazolepropionate to succinic acid, and analysis of the bis(trimethylsilyl) derivative of succinate by gas chromatography–mass spectrometry. The fraction of doubly deuterated succinate present of the total mono- plus dideuterated succinates was estimated from the peak heights for *m/e* 248 and 249.

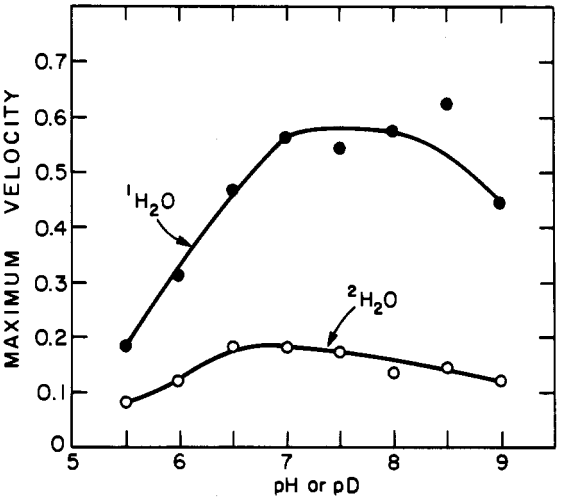


FIGURE 5: Maximum velocity (V_{max}) as a function of pH (pD) for urocanase.

Table I: Kinetic Isotope Effects Observed with [4′-^{2}H]Urocanate in the Forward Reaction and Oxoimidazole[2,3-$^2H_2$]propionate in the Reverse Reaction

substrate	K_m (mM)	V_{max}[a] (nmol min^{-1})	$\frac{V_{max}\ (^1H)}{V_{max}\ (^2H)}$	$\frac{V_{max}/K_m\ (^1H)}{V_{max}/K_m\ (^2H)}$
[4′-^{1}H]urocanate	0.066 ± 0.003	1.04 ± 0.05	1.12	1.22
[4′-^{2}H]urocanate	0.072 ± 0.003	0.93 ± 0.05		
[2,3-$^1H_2$]IOPA[b]	3.2 ± 0.2	1.73 ± 0.25	4.9	1.7
[2,3-$^2H_2$]IOPA	1.1 ± 0.2	0.35 ± 0.11		

[a] Maximum velocity values were obtained by extrapolation to saturating substrate concentration. Because forward and reverse reactions were not performed under identical conditions, V_{max} values should be compared only between substrates for a given direction. All velocities were from initial rates of absorbance change at 277 nm and pH 7.5. Results are given ± standard deviation, based on three determinations. [b] IOPA, oxoimidazolepropionate.

Kinetic Isotope Effects from [4′-^{2}H]Urocanate vs. Unlabeled Urocanate as Substrate. For verification that [4′-^{2}H]urocanate once formed from ^2H_2O was not contributing to the lowered V_{max}, reaction velocities were measured by using unlabeled urocanate and [4′-^{2}H]urocanate as substrates in ^1H_2O. As seen in Table I, deuterium on the 4′ position did not significantly alter either V_{max} or K_m. The 4′-hydrogen is therefore removed in a fast step prior to some rate-determining event.

Kinetic Isotope Effects on the Reverse Reaction. For examination of the consequences on reaction rate of having deuterium rather than protium atoms removed from the side chain of oxoimidazolepropionate, the isotope effect for the reverse reaction was measured by using 4′-oxoimidazole-5′-[2,3-$^2H_2$]propionate. This substrate was prepared enzymatically from urocanate in ^2H_2O and purified in ^1H_2O to remove exchangeable deuterium. As presented in Table I, the ratio of V_{max} for the unlabeled and deuterated oxoimidazolepropionate was 5. This value may include isotope effects from two reaction steps, since one deuterium at C-2 and one at C-3 must be removed; however, the large primary isotope effect indicates that the rate-determining step was breakage of the C–H bond of either the 2- or 3-hydrogen of oxoimidazolepropionate.

Discussion

Although discovery of an exchange reaction involving the C-4′ hydrogen of urocanate reopened the question of the origin of hydrogens added to the side chain of urocanate during formation of oxoimidazolepropionate, it now seems clear that the original conclusion reached by Kaeppeli & Retey (1971) was correct and thus if NAD^+ is involved in urocanase as a hydride transfer coenzyme, it must not involve hydride transfer from C-4′ to either positions C-2 or C-3.

As shown in Scheme I, however, a mechanism depicting addition of water across the conjugated double bond system of urocanate at positions 2 and 4′, followed by oxidation of the secondary alcohol at C-4′ and subsequent reduction at position C-5′, would satisfy the labeling data but falls short of being completely satisfactory on other grounds. The problems raised are the fact that the keto tautomer of the product rather than the enol would have to be formed initially, and the scheme does not account for the rapid exchange of the C-4′ hydrogen with solvent. Also if there were a hydride ion transfer to NAD^+ from urocanate, it was anticipated that this might give rise to an observable isotope effect upon replacement of the 4′-hydrogen of urocanate by deuterium; such was not the case. There was, however, a 2- to 3-fold overall isotope effect as a result of conducting the reaction in ^2H_2O rather than ^1H_2O, suggesting that proton transfer from a group in equilibrium with solvent was rate determining, perhaps the incorporation of hydrogen at positions C-2 or C-3. For the reverse reaction, the evidence is somewhat stronger that breakage of the C–H bond at either C-2 or C-3 is rate limiting.

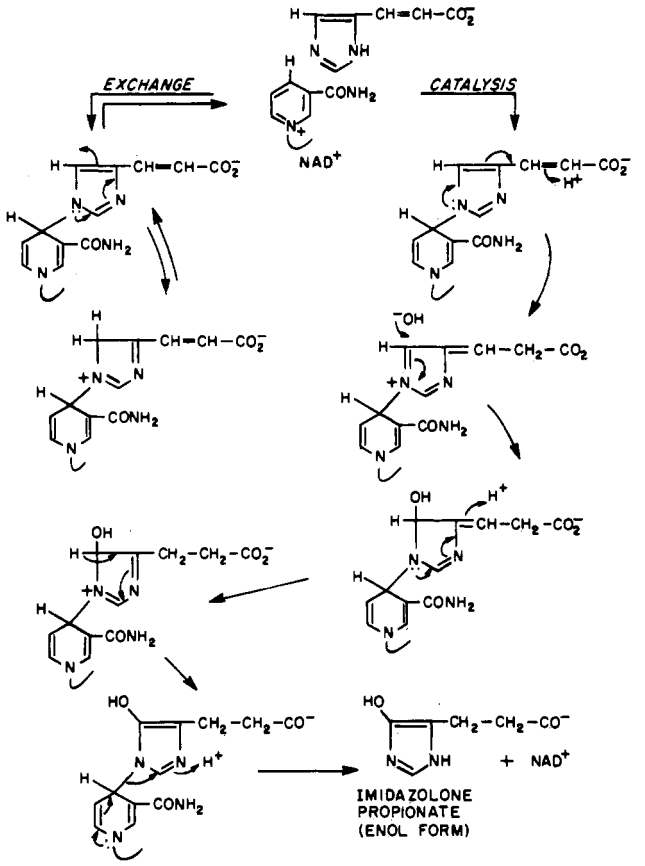
None of these results taken alone are compelling reasons to exclude the 2,4′ addition of water, followed by hydride shift from C-4′ to C-5′, but there are no data providing positive support for this scheme. Our attempts to examine directly the possibility of a hydride ion transfer to C-5′ have been unsatisfactory because enolization of oxoimidazolepropionate cannot be prevented long enough to form a stable derivative of oxoimidazolepropionate in which the C-5′ hydrogen is intact and nonexchangeable (unpublished observations). Obviously, if the enol form of oxoimidazolepropionate is the actual enzyme product, it will not be possible to isolate oxoimidazolepropionate (keto form) without finding solvent incorporation at C-5′.

Earlier results by Phillips et al. (1977) support a role for NAD^+ in catalysis, and the current data indicate that exchange of the 4′-H on urocanate requires an unmodified NAD^+, as judged from the results presented in Figure 2 with *O*-methylhydroxylamine-inhibited enzyme. The exchangeability of the 4′-hydrogen on imidazolepropionate as well as on urocanate indicates that this step is separate from and precedes any addition of water. This conclusion follows from the fact that imidazolepropionate cannot add water and from the data which show an isotope effect on water addition to urocanate but not on exchange of the 4′-hydrogen. Since the exchange reaction itself cannot be visualized in terms of a hydride ion abstraction from substrate and since no data currently point to NAD^+ as a hydride acceptor in this reaction, alternative mechanisms must be considered.

Keul et al. (1979) proposed a mechanism based on enzyme-bound NAD^+ as an electrophile. Their scheme did take into account the earlier labeling data and also led to the enol form of oxoimidazolepropionate as initial product. Because it was envisioned that NAD^+ attacked the C-2 position of urocanate, thereby facilitating the addition of water, the scheme does not readily explain the exchange reaction at C-4′ of urocanate and would predict no exchange for imidazolepropionate. Thus its status must be regarded as questionable until further evidence is obtained.

In Scheme II, we present a new mechanism for consideration, one containing a role for NAD^+ wherein it forms a covalent addition complex with urocanate at N-3 of the imidazole ring; the scheme could also be written with NAD^+ addition at an unprotonated N-1 position, and no data currently available allow us to choose between these possibilities. After formation of the substrate–NAD adduct, two reaction courses follow: one is a productive pathway in which C-4′ and C-2 are activated for hydroxyl and proton attack, respectively; the other is a nonproductive rearrangement leading to exchange of the 4′-H with solvent but not to the addition of H_2O. This latter path is available to both imidazolepropionate and urocanate, but only urocanate can participate in the water addition

Scheme II: A New Proposal for the Involvement of NAD^+ in the Urocanase Reaction[a]



[a] The reaction proceeding leftward from urocanate is a representation of the 4'-hydrogen exchange, while the proposed catalytic sequence is shown proceeding rightward from urocanate.

sequence. The scheme accounts for the observed origin of the side chain hydrogens in oxoimidazolepropionate and shows the enol form of oxoimidazolepropionate being formed prior to tautomerization to the keto form.

Although it is documented that NAD^+ can form complexes with substituted imidazoles (van Eys, 1958), their stability under our catalytic conditions has not been fully examined. Nevertheless, we consider this scheme sufficiently attractive that we are presently examining urocanase reactions for evidence that an imidazole–NAD adduct is an actual intermediate in the enzymatic process. Preliminary studies indicate that imidazolepropionate forms an isolatable covalent complex with NAD on urocanase (Phillips & Matherly, 1980). Detailed structural analyses on this stabilized product are under way.

The reaction mechanism illustrated in Scheme II requires six proton transfers, described as follows: proton transfer from N-1 upon formation of the imidazole–NAD adduct; transfer of a proton to C-2; proton removal from H_2O; proton transfer to C-3; proton removal from C-4'; proton addition to N-1 upon cleavage of the oxoimidazole–NAD adduct. We envision at least two bases in the urocanase active site as being necessary to carry out the functions indicated. Identification of active site amino acids capable of accepting and donating protons should prove useful in confirming various details of the mechanism. Two recent studies have each identified a catalytically essential sulfhydryl group in urocanase (O'Donnell et al., 1980; Matherly & Phillips, 1980). It remains to be established precisely what role this sulfhydryl plays in the reaction, but its proximity to the substrate binding site (Matherly & Phillips, 1980) would suggest it as one of the groups involved in proton transfers.

References

Brown, D. D., & Kies, M. W. (1959) *J. Biol. Chem. 234*, 3188–3191.

Cleland, W. W. (1967) *Adv. Enzymol. 29*, 1–32.

Cohn, M. S., Lynch, M. C., & Phillips, A. T. (1975) *Biochim. Biophys. Acta 377*, 444–453.

Egan, R. M. (1977) Ph.D. Thesis, The Pennsylvania State University, University Park, PA.

Egan, R. M., & Phillips, A. T. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, 1749.

Egan, R. M., & Phillips, A. T. (1977) *J. Biol. Chem. 252*, 5701–5707.

Englard, S., & Hanson, K. R. (1969) *Methods Enzymol. 13*, 592–595.

George, D. J., & Phillips, A. T. (1970) *J. Biol. Chem. 245*, 528–537.

Gerlinger, E., & Retey, J. (1980) *FEBS Lett. 110*, 126–128.

Groves, W. E., Davis, F. C., & Sells, B. H. (1968) *Anal. Biochem. 22*, 195–210.

Hassal, H., & Greenberg, D. M. (1963) *J. Biol. Chem. 238*, 1423–1431.

Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp 243–280, McGraw-Hill, New York.

Kaeppeli, F., & Retey, J. (1971) *Eur. J. Biochem. 23*, 198–202.

Keul, V., Kaeppeli, F., Ghosh, C., Krebs, T., Robinson, J. A., & Retey, J. (1979) *J. Biol. Chem. 254*, 843–851.

Ludowieg, J., & Levy, A. (1963) *Biochem. Biophys. Res. Commun. 11*, 19–23.

Lumry, R., Smith, E. L., & Glantz, R. R. (1951) *J. Am. Chem. Soc. 73*, 4330–4340.

Matherly, L. H., & Phillips, A. T. (1980) *Biochemistry 19*, 5814–5818.

O'Donnell, P. S., Hug, D. H., & Hunter, J. K. (1980) *Arch. Biochem. Biophys. 202*, 242–249.

Phillips, A. T., & Matherly, L. H. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1857.

Phillips, A. T., LaJohn, L., & Lewis, B. (1977) *Arch. Biochem. Biophys. 184*, 215–221.

Tabor, H., & Mehler, A. H. (1955) *Methods Enzymol. 2*, 228–233.

van Eys, J. (1958) *J. Biol. Chem. 233*, 1203–1210.