Wagner, P. D., & Weeds, A. G. (1979) *Biochemistry 18*, 2260–2266.
Wagner, P. D., Slater, C. S., Pope, B., & Weeds, A. G. (1979) *Eur. J. Biochem. 99*, 385–394.
Webb, M. R., & Trentham, D. R. (1981) *J. Biol. Chem. 256*, 10910–10916.
Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54–56.
West, J. J., Nagy, B., & Gergely, J. (1967) *Biochem. Biophys. Res. Commun. 29*, 611–616.
White, H. D., & Taylor, E. W. (1976) *Biochemistry 15*, 5818–5826.

A Covalent Nicotinamide Adenine Dinucleotide Intermediate in the Urocanase Reaction†

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ABSTRACT: When imidazolepropionate, a competitive inhibitor of the nicotinamide adenine dinucleotide (NAD) dependent urocanase from *Pseudomonas putida*, binds to the enzyme, a chromophore is produced that exhibits an absorption maximum near 330 nm. In order to establish the identity of this chromophore, and particularly to determine whether this material might be enzyme-bound reduced NAD (NADH) formed by a hydride ion transfer from imidazolepropionate, fluorescence studies of the imidazolepropionate–enzyme complex were undertaken, along with isolation of the pyridine nucleotide coenzyme from the enzyme. The chromophoric material could be readily distinguished from NADH by its fluorescence emission spectrum and by its failure to react with pyruvate and lactate dehydrogenase after extraction of the coenzyme from urocanase. [^{14}C]Imidazolepropionate or urocanase containing [^{14}C]NAD^+ was used to generate a labeled enzyme–analogue complex. In both cases detergent treatment of the complex released a ^{14}C-labeled coenzyme that was then oxidized with phenazine methosulfate and purified by gel filtration and ion-exchange chromatography, whereupon it was established to be an addition product of NAD^+ and imidazolepropionate. Proton nuclear magnetic resonance spectroscopy of the oxidized pyridine nucleotide derivative revealed an attachment of the τ nitrogen of the imidazole ring of the analogue to the 4 position of the nicotinamide portion of NAD^+. The isolation of an oxidized NAD–imidazolepropionate addition complex provides support for a mechanism suggested earlier [Egan, R. M., Matherly, L. H., & Phillips, A. T. (1981) *Biochemistry 20*, 132–137] in which NAD^+ functions in urocanase catalysis through an intermediate involving the reversible addition of NAD^+ to a nucleophilic site on the imidazole ring of the substrate.

The recent identification of a very tightly bound NAD^+ (nicotinamide adenine dinucleotide) on the urocanases (EC 4.2.1.49) from *Pseudomonas putida* (Egan & Phillips, 1977; Keul et al., 1979) and bovine liver (Keul et al., 1979) has provided an opportunity for clarification of the chemistry presented by this unusual hydration reaction. During the urocanase-catalyzed addition of water across the conjugated system of urocanate to produce oxoimidazolepropionic acid (Figure 1), the hydrogen atom on carbon 5 of urocanate (I) is lost and hydrogen additions are made to the side chain α and β carbons plus carbon 4 in giving rise to the keto tautomeric form, oxoimidazolepropionic acid (II). Although hydride transfer involving transient NADH formation could be used to explain some of these hydrogen movements subsequent to water addition, such mechanisms are generally incompatible with the solvent origin of the hydrogens added to the side chain α and β carbons (Egan et al., 1981; Kaeppeli & Retey, 1971) and with the identification of the enol tautomer of oxoimidazolepropionic acid (III) as the true reaction product rather than the more stable keto form (Kaeppeli & Retey, 1971; Matherly & Phillips, 1981).

Hug et al. (1978) observed that the binding of imidazolepropionate, a substrate analogue, to urocanase gave rise to an increased UV absorbance with a maximum at 331 nm. The spectrum of the chromophore resembles that for borohydride-generated, enzyme-bound NADH (reduced NAD) [λ_{max} = 335 nm (Egan & Phillips, 1977)], but Hug et al. (1978) attributed this instead to an addition compound of imidazolepropionate with NAD. Also, incubation of enzyme with imidazolepropionate did not lead to irreversible inactivation, whereas treatment of urocanase with $NaBH_4$ did. Although imidazolepropionate is not hydrated by urocanase, both the analogue and urocanate undergo an enzyme-catalyzed exchange of the imidazole 5 hydrogen with solvent (Egan et al., 1981; Gerlinger & Retey, 1980). This would imply a similar mode of binding for the two compounds and suggests that a study of the chromophore produced upon imidazolepropionate binding might provide new mechanistic information regarding urocanase catalysis.

In this report, we present evidence that the chromophore generated on urocanase in the presence of imidazolepropionate is definitely not NADH but rather is a reversible covalent addition complex between the nicotinamide ring of the active site NAD and imidazolepropionate. A preliminary account of these findings has previously appeared (Phillips & Matherly, 1980). The following paper (Matherly et al., 1982) provides kinetic evidence that an analogous complex involving urocanate itself forms during catalysis.

Experimental Procedures

Chemicals. Deuterium oxide (100 atom %), urocanic acid, Sephadex G-15, beef heart lactate dehydrogenase, potassium

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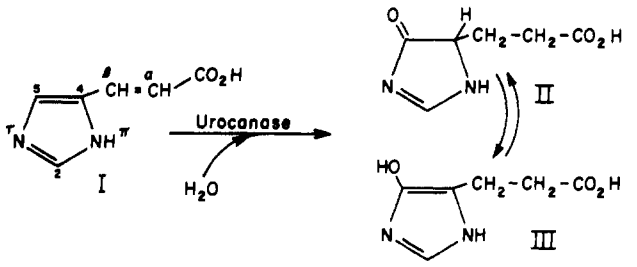

FIGURE 1: Urocanase reaction. The structures shown are urocanic acid (I) and the keto (II) and enol (III) forms of oxoimidazolepropionic acid.

pyruvate, NADH, and NAD^+ were purchased from Sigma Chemical Co. [^{14}C]Urocanic acid was prepared from L-[U-^{14}C]histidine (New England Nuclear) according to the procedure of Mehler et al. (1955). Imidazolepropionate, both ^{14}C labeled and unlabeled, was made from urocanic acid as described by Phillips et al. (1977). DE-52 was purchased from Whatman Biochemicals, Ltd. All other chemicals were obtained from various commercial sources.

Urocanase Preparation and Assay. Urocanase was isolated from *P. putida*, ATCC 12633, as described by George & Phillips (1970) and stored frozen in liquid N_2 until used. For urocanase containing [^{14}C]NAD^+, a nicotinic acid auxotroph of *P. putida* grown on [^{14}C]nicotinic acid was the source of the enzyme (Egan & Phillips, 1977). The purified enzyme preparations used in this work had specific activities between 1.8 and 2.2 μmol of urocanate consumed min^{-1} (mg of protein)$^{-1}$, equivalent to an active enzyme content of 85–100%, although when initially purified each was deemed to be at least 95% pure on the basis of specific activity and gel electrophoresis.

Urocanase was assayed spectrophotometrically as described by George & Phillips (1970). Protein determinations were made by the method of Groves et al. (1968) with bovine serum albumin as the standard.

Dithionite Reduction of NAD on Urocanase. Urocanase (8.0 mg) was treated with 16 μmol of sodium dithionite in 0.2 M Tris-HCl,[1] pH 8.0, under nitrogen for 2 h (final volume, 0.35 mL). The excess reductant was removed by dialysis against 1 L of 50 mM Tris-HCl, pH 7.5, for 10 h. Less than 1% of the original enzyme activity remained after these procedures.

For determination of bound NADH, a 0.28-mL portion of reduced urocanase (6.2 mg, 56 nmol) was treated with boiling methanol to 80% final concentration (v/v); after being allowed to cool, the precipitated protein was removed by centrifugation and discarded. The methanol was evaporated under a stream of nitrogen, and the free NADH was redissolved in 1 mL of 50 mM Tris-HCl, pH 7.5. The NADH was assayed fluorometrically with beef heart lactate dehydrogenase as described by Williamson & Corkey (1969). The same procedure was also performed on native urocanase (6.2 mg) treated with 20 μmol of imidazolepropionate in 0.28 mL of 50 mM Tris-HCl, pH 7.5, for 2 h and on a control treated similarly but without imidazolepropionate. In these latter two samples, methanol treatment was performed directly on the incubation mixtures.

Spectral Measurements. Fluorescence emission spectra were recorded on a Perkin-Elmer MPF-44B fluorescence spectrophotometer. Samples were excited at 330 nm while obtaining emission spectra; slits were set at 5-nm half-bandwidth. Absorption spectra were recorded on a Cary Model 17 spectrophotometer. All fluorescence and absorbance measurements were at 20 °C.

Radioactivity Measurements. Radioactivity measurements were conducted in a Beckman LS255B liquid scintillation counter by using Tritosol scintillation fluid (Fricke, 1975). Efficiencies were corrected by internal standardization with [^{14}C]toluene.

Preparation of an Oxidized NAD–Imidazolepropionate Adduct. To an incubation mixture of 20 mg (0.18 μmol) of purified urocanase and 22 μmol of [^{14}C]imidazolepropionate (6.1×10^6 dpm) in 0.9 mL of 50 mM Tris-HCl, pH 7.5, was added 0.1 mL of a solution containing 2.5% phenazine methosulfate and 2% sodium dodecyl sulfate. After 90 min at 30 °C in the dark, the solution was deproteinized by perchloric acid treatment (3.5% final concentration) for 20 min at 0 °C, followed by neutralization (0.28 mL of 2 N KOH) and centrifugation to remove the precipitated material. The supernatant was removed and lyophilized. A similar procedure was used to prepare the complex from unlabeled imidazolepropionate and urocanase containing [^{14}C]NAD. The method was also scaled up to accommodate 220 mg (2 μmol) of urocanase and 250 μmol of [^{14}C]imidazolepropionate.

For all preparations, the lyophilized residue containing oxidized NAD–imidazolepropionate was dissolved in 0.5 mL of H_2O and purified on a column of Sephadex G-15 (0.9×150 cm) equilibrated with 10 mM ammonium bicarbonate, pH 8.0, with a flow rate of 5 mL h^{-1} cm^{-2} (fraction volume 1.1 mL). Alternatively, purification could be achieved by chromatography on a column (1.5×30 cm) of DE-52, using an 800-mL linear gradient from 0 to 0.2 M ammonium bicarbonate, pH 8.0, or high-voltage paper electrophoresis with 0.3 M *N*-ethylmorpholineacetate, pH 8.0, and 2.5 mM dithiothreitol (Egan & Phillips, 1977).

NMR Spectroscopy Measurements. ^{1}H NMR spectra were obtained with a Brucker Instruments, Inc., WM-360 spectrometer operating at 360.13 MHz in the Fourier-transform mode. The samples were prepared by lyophilizing 3 times from D_2O, then dissolved in 0.4 mL of D_2O buffered to pD 7.4 with 20 mM potassium phosphate, and placed in 5-mm NMR tubes. The spectra were acquired with an inversion-recovery pulse sequence ($180°$–τ–$90°$; $\tau = 4$ s) to suppress the residual HOD signal. A spectral width of 5000 Hz was measured by using 32K data points. The spectra were referenced to external sodium 4,4-dimethyl-4-silapentanesulfonate. The signal to noise ratios were enhanced by using 0.2-Hz line broadening. So that better resolution of the aromatic proton signals in the adduct could be obtained, a small crystal of ethylenediaminetetraacetate (disodium salt) was dissolved in the sample prior to the acquisition of the data.

Results

Spectral Properties for the Imidazolepropionate–Urocanase Complex. The competitive inhibitor imidazolepropionate binds to urocanase readily and exhibits a K_i of 0.9 mM at pH 7.5 (Phillips et al., 1977). In Figure 2, the difference absorption spectrum for imidazolepropionate bound to urocanase vs. untreated urocanase is presented, along with the difference spectrum for urocanase containing an active site 1,4-NADH prepared by dithionite reduction. The difference spectra were quite similar, with the maxima centered at 328 nm. Upon acidification to pH 1, both maxima shifted to 290–295 nm (data not shown), probably attributable to the acid degradation products of dihydropyridine-like nucleotides (Burton & Kaplan, 1954).

The spectrum for dithionite-reduced urocanase was only

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; NaDodSO$_4$, sodium dodecyl sulfate; ^{1}H NMR, proton nuclear magnetic resonance.

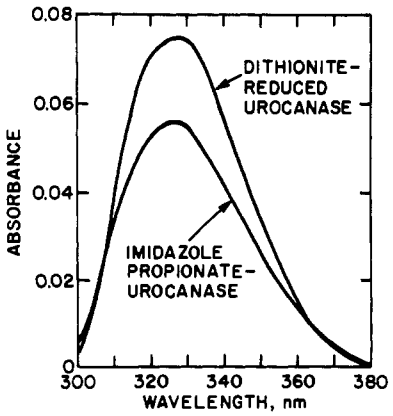

FIGURE 2: Difference absorption spectra for dithionite-reduced urocanase and the imidazolepropionate–urocanase complex. The spectra were recorded at protein concentrations of 2.7 and 2.5 mg/mL, respectively, in 50 mM Tris-HCl, pH 7.5 (1-mL total volume), vs. a corresponding amount of untreated urocanase in the same buffer. The concentration of imidazolepropionate was 10 mM.

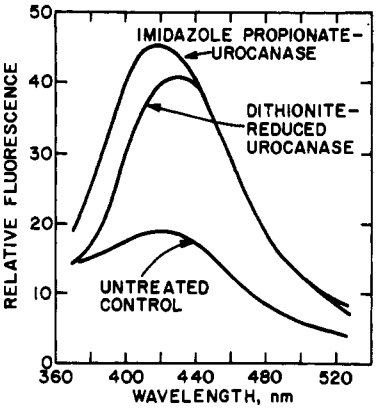

FIGURE 3: Fluorescence emission spectra for dithionite-reduced and imidazolepropionate-treated urocanase. Emission spectra were determined as described under Experimental Procedures on enzyme reduced with sodium dithionite, enzyme incubated in the presence of 4 mM imidazolepropionate, and untreated (control) enzyme. Protein concentration was 2 mg/mL in a total volume of 0.4 mL of 50 mM Tris-HCl, pH 7.5.

slightly different from that reported for the enzyme treated with sodium borohydride (Egan & Phillips, 1977). The latter treatment generates a mixture of NADH isomers, including the longer wavelength absorbing 1,2-NADH isomer (Chaykin & Meissner, 1964), while dithionite reduction is reported to produce only the 1,4 isomer of NADH (Pullman et al., 1954).

The NADH remaining bound to dithionite-treated urocanase following dialysis was estimated from difference absorbance measurements at 328 nm. Assuming a millimolar extinction coefficient of 6.2 cm^{-1} for the enzyme-bound NADH, we calculated that 30 nmol of NADH was present per 56 nmol of enzyme reduced. Direct measurement of this NADH after its removal from urocanase by methanol extraction gave values of 28 ± 1 nmol when analyzed with pyruvate and lactate dehydrogenase. Thus the two methods produced agreement for the absolute quantity of NADH formed, and furthermore, the latter assay confirmed its identity as the reactive 1,4 isomer. Egan & Phillips (1977) have previously established that 1 mol of NAD^+ is bound per mol of enzyme. Therefore, only half of the expected amount of 1,4-NADH was recovered from dithionite-treated, dialyzed enzyme even though activity measurements revealed complete inactivation upon reduction. We attribute this difference to a loss of NADH upon dialysis, resulting from a somewhat weaker binding between NADH and urocanase than is found with NAD^+ and urocanase; dialysis of native (NAD^+-containing) enzyme under comparable conditions gave essentially complete recovery of active enzyme.

Urocanase treated with imidazolepropionate was not irreversibly inactivated (as determined by dilution and subsequent assay), but nevertheless an amount of material equivalent to 0.4 mol/mol of enzyme could be detected spectrally in the presence of imidazolepropionate (Figure 2) by assuming an extinction coefficient at 328 nm of 6.2 mM^{-1} cm^{-1}. Significantly, this chromophoric material was found to show no reaction when tested for NADH content with pyruvate and lactate dehydrogenase under the same conditions as had been used in analyzing the NADH content of dithionite-reduced enzyme.

The fluorescence emission spectra for the imidazolepropionate–urocanase complex and dithionite-reduced urocanase are compared to that for untreated (control) enzyme in Figure 3. Both spectra show enhanced fluorescence relative to the control, but they are clearly not identical. The maxima are observed at 420 and 430 nm for imidazolepropionate and dithionite-treated urocanase, respectively. These data, along with the results of the assays for NADH discussed above, indicate that a modification of the urocanase NAD^+ by imidazolepropionate has occurred, to form a product that is distinct from NADH.

Isolation of the Modified NAD. Unlike the modifications to the urocanase NAD^+ introduced by a number of nucleophiles such as hydroxylamine and sulfite (Phillips et al., 1977; Hug et al., 1978), that arising from incubation with imidazolepropionate results in no loss of catalytic activity upon direct assay, consistent with a rapid dissociation of the addition complex. Therefore, to stabilize the modified pyridine nucleotide and permit its isolation, we treated the imidazolepropionate–urocanase complex with phenazine methosulfate so as to oxidize the dihydropyridine-like nucleotide to a substituted NAD^+ derivative. A similar procedure but with ferricyanide as the oxidant was employed by van Eys (1958) in the preparation of various imidazole derivatives of NAD^+. Oxidation of the addition products formed between nucleophiles and NAD produces stable derivatives of NAD^+ in which the nucleophilic group has been prevented from leaving.

Preliminary experiments revealed that oxidation was much more efficient if $NaDodSO_4$ was added along with phenazine methosulfate. This mixture, after deproteinization and lyophilization, was chromatographed on a column of Sephadex G-15 (Figure 4). The experiments were performed with urocanase containing [^{14}C]NAD with unlabeled imidazolepropionate or, alternatively, unlabeled urocanase treated with [^{14}C]imidazolepropionate. In both cases a labeled material was observed to migrate just after the void volume and separate from imidazolepropionate or NAD^+. Total radioactivity recoveries in the experiments with urocanase containing [^{14}C]NAD urocanase were generally 90–95%, and on the basis of the specific radioactivity for the labeled NAD, the proportion of the total pyridine nucleotide in the earliest eluting fraction was reproducibly 35–45%. Significantly, no similar labeled material was observed from experiments in which one component (urocanase or imidazolepropionate) was absent or from incubations of free NAD and imidazolepropionate. Analogous results were obtained when the mixtures were chromatographed on DE-52 (Figure 5) or subjected to high-voltage paper electrophoresis at 2000 V and pH 8.0 (data not shown).

These experiments suggest that urocanase catalyzes the formation of a reversible addition complex between its NAD^+ and the substrate analogue, imidazolepropionate. This in-

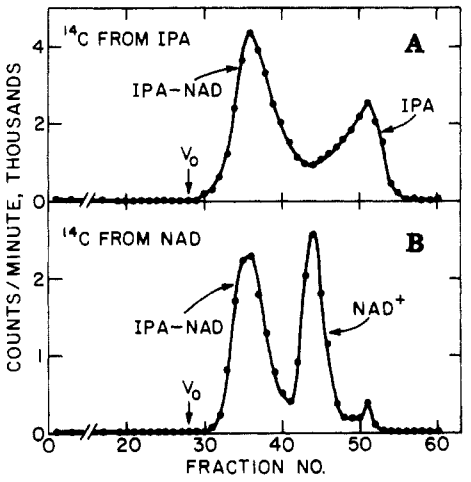

FIGURE 4: Gel filtration of imidazolepropionate-modified NAD. Incubations of (A) 20 mg of urocanase and 22 μmol of [^{14}C]-imidazolepropionate (2.76×10^5 dpm μmol^{-1}) or (B) 10 mg of urocanase containing [^{14}C]NAD (37 000 dpm) and 11 μmol of imidazolepropionate were treated with phenazine methosulfate as described under Experimental Procedures. After lyophilization, the residual material was dissolved in 0.5 mL of H_2O and chromatographed on a column of Sephadex G-15. Because of the large excess of [^{14}C]-imidazolepropionate used in the first incubation, it was necessary to pool fractions 30–40 from an initial column and rechromatograph in order to obtain the separations shown in (A). IPA, imidazolepropionate; IPA–NAD, the oxidized complex of IPA and NAD.

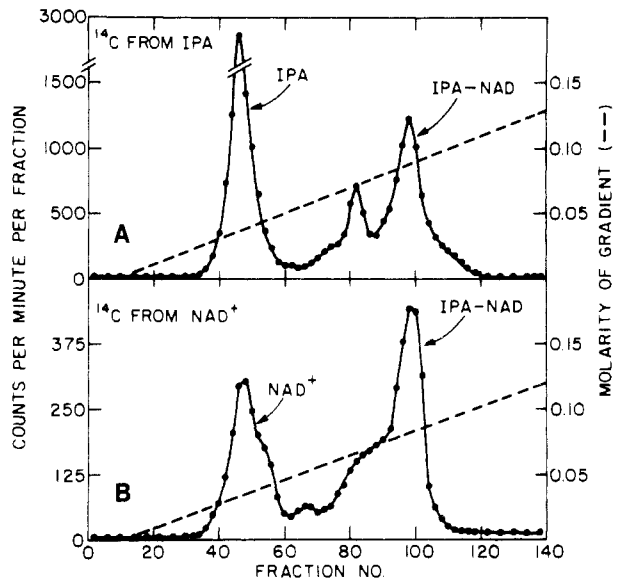

FIGURE 5: DE-52 chromatography of imidazolepropionate-modified NAD. Incubations were performed as described under Experimental Procedures in which (B) urocanase containing bound [^{14}C]NAD or (A) [^{14}C]imidazolepropionate was used. The mixture following oxidation was passed once through a Sephadex G-15 column (Figure 4), and fractions 30–45 were pooled and lyophilized for chromatography on a column of DE-52. The peak designations are the same as those used in Figure 4.

teraction is then stabilized by oxidation with phenazine methosulfate, for when this reagent was omitted, no complex could be isolated upon treatment with $NaDodSO_4$.

Analogous experiments were also performed with unlabeled urocanate, the natural substrate, and urocanase containing [^{14}C]NAD. A component migrating ahead of [^{14}C]NAD$^+$ could be detected on gel filtration (data not shown), although at such low levels (5% of total NAD) so as to preclude its further characterization. This would indicate that the same type of intermediate complex can be formed with urocanate as with the analogue, although at a considerably lower

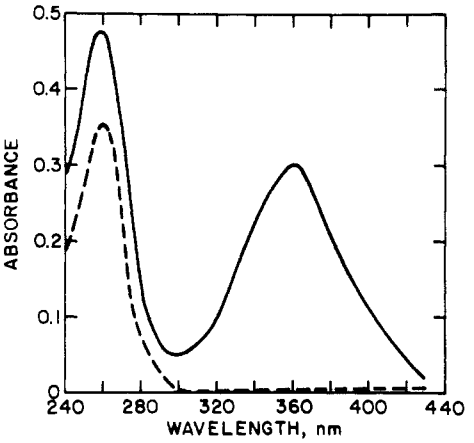

FIGURE 6: Absorption spectrum for oxidized NAD–imidazolepropionate. The spectrum was recorded on a solution of 22 μM NAD–imidazolepropionate (—) in 10 mM ammonium bicarbonate, pH 8.0 (1-mL total volume), for comparison with NAD$^+$ (19 μM; - - -) under identical conditions.

steady-state concentration for the former compound. The parallel experiment using radiolabeled urocanate could not be performed because the products arising from the decomposition of oxoimidazolepropionic acid interfered with the chromatographic separations.

Large-Scale Preparation of Oxidized NAD–Imidazolepropionate and Its Absorption Spectrum. For purposes of structural characterization of the oxidized NAD–imidazolepropionate compound, we prepared the complex from 2 μmol of urocanase. Purification was achieved by sequential application of the techniques described earlier (gel filtration, ion exchange, and paper electrophoresis) and resulted in approximately 160 nmol of the oxidized adduct on the basis of the specific radioactivity of the starting [^{14}C]imidazolepropionate. Its ultraviolet absorption spectrum is shown in Figure 6. The compound exhibited the usual nucleotide absorbance at 260 nm, but in contrast to NAD$^+$, an additional strong maximum was found at 360 nm. The basis for this longer wavelength absorption maximum is considered under Discussion.

^{1}H NMR of Oxidized NAD–Imidazolepropionate. In Figure 7 are presented the 360-MHz ^{1}H NMR spectra for (A) oxidized NAD–imidazolepropionate isolated from urocanase, (B) NAD$^+$, and (C) imidazolepropionate. The assignment of peak signals for NAD$^+$ under similar conditions has been previously reported (Jardetzky & Wade-Jardetzky, 1966). The adduct spectrum (A) clearly reveals features found in the spectra for the individual components, and several of the resonances can be unambiguously assigned on the basis of chemical shift similarities and the observed multiplicities.

The methylene resonances for imidazolepropionate (i.e., the hydrogens attached to carbons α and β; see inset, Figure 7C) form a pair of triplets at δ 2.90 and δ 2.51 (J = 7.3 Hz). In the adduct spectrum, the corresponding triplets are found at δ 3.02 and δ 2.50 (inset, Figure 7A). The resonances for the anomeric N-glycosidic hydrogens of NAD$^+$ are found at δ 6.05 (J = 5.5 Hz) and δ 6.005 (J = 6.1 Hz) while for NAD–imidazolepropionate this pair of doublets occur at δ 5.97 (J = 6.8 Hz) and δ 5.93 (J = 6.0 Hz).

The aromatic region of the adduct spectrum is simpler than the sum of the spectra for the two standards. While signals corresponding to the 2 hydrogen (δ 8.97, s), and the 6 hydrogen (δ 8.87, d; J = 6.8 Hz) of the nicotinamide ring are easily discerned, the doublet of doublets assigned to the nicotinamide 5 hydrogen in NAD$^+$ (δ 8.16; J = 6.4 and 7.7 Hz) has been

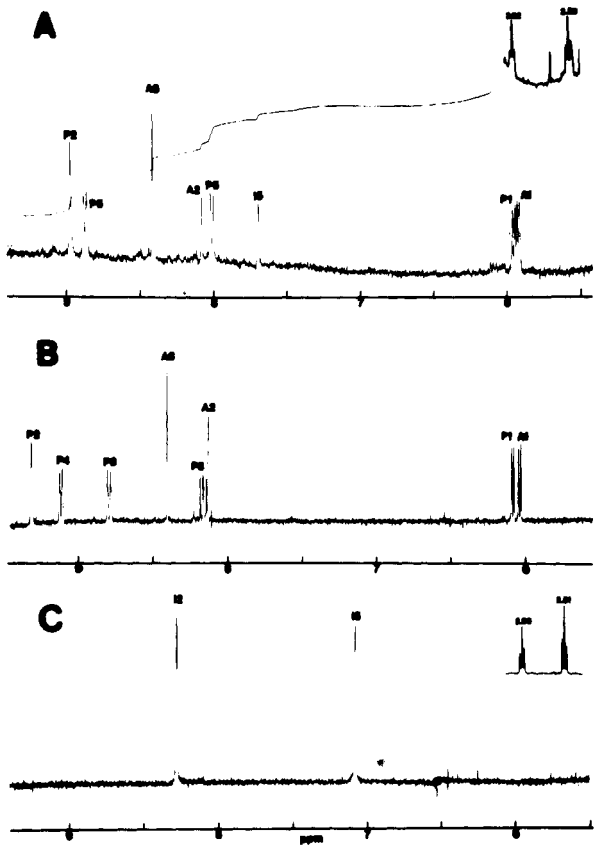

FIGURE 7: ^{1}H NMR spectra were recorded as described under Experimental Procedures for (A) oxidized NAD–imidazolepropionate (160 nmol), (B) NAD^+ (5 μmol), and (C) imidazolepropionate (5 μmol). For the adduct (A), a crystal of disodium ethylenediaminetetraacetate was added prior to accumulating approximately 4000 scans necessary to obtain a usable spectrum. The peak designations are as follows: P2, pyridine 2-H; P4, pyridine 4-H; P5, pyridine 5-H; P6, pyridine 6-H; A2, adenine 2-H; A8, adenine 8-H; A1, adenine ribose 1-H; P1, pyridine ribose 1-H; I2, imidazole 2-H; I5, imidazole 5-H. The insets in (A) and (C) represent the hydrogens attached to carbons α and β for the oxidized NAD–imidazolepropionate compound and imidazolepropionate, respectively.

replaced in the adduct by one doublet (δ 8.01, J = 6.8 Hz), and no signal is observed for the hydrogen attached to carbon 4, consistent with a substitution at this position. The singlets at δ 8.41 and δ 8.08 correspond to the 8 and the 2 hydrogens of the adenine ring, respectively, which in NAD occur at δ 8.40 and δ 8.12. The remaining singlet (δ 7.69) in the adduct spectrum arises from the 5 hydrogen on the imidazole ring of imidazolepropionate. The resonance corresponding to the imidazole 2 hydrogen is not observed for the oxidized NAD–imidazolepropionate adduct. The chemical shifts and coupling constants are summarized in Table I.

The NMR spectra shown in Figure 7 are consistent with an addition complex involving the attachment of a single molecule of imidazolepropionate to the 4 position on the pyridine ring of NAD. While the absence of a singlet corresponding to the imidazole 2 hydrogen might suggest a linkage of the nicotinamide ring of the coenzyme to this carbon, other explanations for the loss of this signal are more plausible and are considered under Discussion.

Discussion

In this report, it was shown that the competitive inhibitor imidazolepropionate bound to urocanase and reversibly formed a covalent addition complex with the active site pyridine nucleotide coenzyme. The complex was detected spectrally and could subequently be isolated from the enzyme after its stabilization by chemical oxidation.

Table I: Chemical Shifts and Coupling Constants for Adenine, Pyridine, and Imidazole Protons of NAD, IPA, and NAD-IPA[a]

peaks[b]	NAD	IPA	NAD-IPA
P2	9.31		8.97
P4	9.12 (J = 6.4)		
P5	8.16 (J = 6.4, 7.7)		8.01 (J = 6.8)
P6	8.80 (J = 7.7)		8.87 (J = 6.8)
A2	8.12		8.08
A8	8.40		8.41
I2		8.28	
I5		7.08	7.69

[a] Abbreviations: IPA, imidazolepropionate; NAD-IPA, oxidized NAD–imidazolepropionate complex. Chemical shifts are given in parts per million and coupling constants in hertz. [b] Peak designations are the same as in Figure 7.

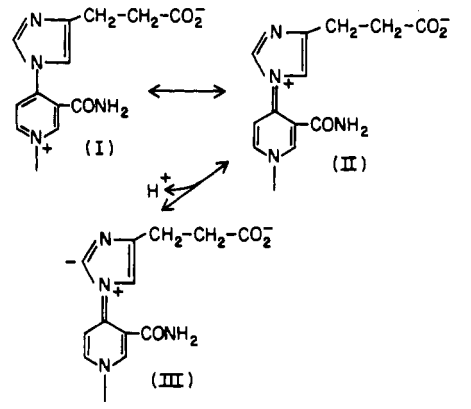

FIGURE 8: Probable structure for oxidized NAD–imidazolepropionate (I), a possible resonance contribution (II), and the ylide intermediate (III) in the hydrogen-exchange reaction at position 2.

The ^{1}H NMR spectrum for the oxidized NAD–imidazolepropionate adduct revealed that the addition product arose from attack on position 4 of the nicotinamide moiety by what we interpret to be the τ nitrogen of imidazolepropionate. This conclusion is based on the following information. The downfield shift of the signal for the imidazole 5 hydrogen in the adduct spectrum relative to the corresponding singlet in the spectrum for imidazolepropionate indicates a reduced electron density at this position in the adduct and suggests a substitution of the adjacent (τ) nitrogen with an electron-withdrawing substituent, namely, NAD (structure I, Figure 8). A similar downfield shift of 0.5 ppm is seen in the proton NMR spectrum of N^τ-acetyl-4-methylimidazole for the resonances of both the 2 and 5 hydrogens relative to those of the unacetylated compound (Reddy et al., 1963). However, the chemical shift for the 4 hydrogen of imidazole is unchanged upon acetylation of the distal (τ) nitrogen, thereby indicating that the τ nitrogen of imidazolepropionate, and not the π nitrogen, is the point of substitution.

The absence of a signal for the hydrogen at the imidazole 2 position is not due to NAD substitution at this location but more likely is attributable to its rapid change with solvent D_2O. This hydrogen has been reported to exchange with solvent protons in N-methylimidazoles under neutral or mildly alkaline conditions, at a rate exceeding the hydrogen loss at carbon 4 or 5 by approximately 10^4–10^5-fold (Takeuchi et al., 1978). The mechanism for this exchange has been studied extensively and appears to involve an ylide intermediate, arising from the imidazolium species by direct proton abstraction from carbon 2. The exchange at this position is enhanced with imidazoles substituted at one of the nitrogens by an electron-withdrawing substituent (i.e., p-nitrobenzoate or picrate; Mannschreck et

al., 1963), indicating a possible stabilization of the ylide intermediate. A similar stabilization can be envisioned for the oxidized NAD–imidazolepropionate adduct through the resonance structure (II) depicted in Figure 8. The ylide species is represented by structure III in Figure 8.

The observation of an additional absorbance band at 360 nm for the oxidized NAD–imidazolepropionate adduct provides evidence for a significant contribution by the highly conjugated resonance structure II of Figure 8. Some related types of oxidized NAD–imidazole complexes were synthesized by van Eys (1958), but the materials prepared exhibited no absorbance above 300 nm. An explanation for this discrepancy is not readily apparent if the oxidized NAD–imidazole compounds synthesized were correctly characterized. Our own attempts to prepare a synthetic sample of oxidized NAD–imidazolepropionate, using the procedure of van Eys (1958), have been unsuccessful due to extensive degradation of NAD^+ under the alkaline conditions employed in this method.

The isolation and characterization of a covalent adduct between the NAD from urocanase and imidazolepropionate support a chemistry for this reaction distinct from that for an NADH-mediated transfer of a hydride ion. While novel mechanisms for urocanase catalysis have been previously proposed in which the coenzyme acts as an electrophile, forming a covalent addition complex with the substrate to facilitate electron delocalization (Egan et al., 1981; Keul et al., 1979), the present report constitutes the first direct support for such a possibility. Our results, although derived from the study of a substrate analogue rather than the actual substrate, would seem relevant to the events occurring on the enzyme upon binding urocanate since recent reports indicate that urocanase catalyzes a solvent exchange of the 5 hydrogen of both imidazolepropionate and urocanate (Egan et al., 1981; Gerlinger & Retey, 1980). In addition, the following paper (Matherly et al., 1982) presents kinetic evidence for the existence of a reaction intermediate involving NAD and urocanate or imidazolepropionate.

References

Burton, R. M., & Kaplan, N. O. (1954) *J. Biol. Chem. 206*, 283–297.

Chaykin, S., & Meissner, L. (1964) *Biochem. Biophys. Res. Commun. 14*, 233–240.

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

Egan, R. M., Matherly, L. H., & Phillips, A. T. (1981) *Biochemistry 20*, 132–137.

Fricke, V. (1975) *Anal. Biochem. 63*, 555–558.

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.

Hug, D. H., O'Donnell, P. S., & Hunter, J. K. (1978) *J. Biol. Chem. 253*, 7622–7629.

Jardetzky, O., & Wade-Jardetzky, N. G. (1966) *J. Biol. Chem. 241*, 85–91.

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.

Mannschreck, A., Seitz, W., & Staab, H. A. (1963) *Ber. Bunsenges. Phys. Chem. 67*, 470–475.

Matherly, L. H., & Phillips, A. T. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1865.

Matherly, L. H., Johnson, K. A., & Phillips, A. T. (1982) *Biochemistry* (following paper in this issue).

Mehler, A. H., Tabor, H., & Hayaishi, O. (1955) *Biochem. Prep. 4*, 50–53.

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.

Pullman, M. E., San Pietro, A., & Colowick, S. P. (1954) *J. Biol. Chem. 206*, 129–141.

Reddy, G. S., Mandell, L., & Goldstein, J. H. (1963) *J. Chem. Soc.* 1414–1421.

Takeuchi, Y., Kirk, K. L., & Cohen, L. A. (1978) *J. Org. Chem. 43*, 3565–3570.

van Eys, J. (1958) *J. Biol. Chem. 133*, 1203–1210.

Williamson, J. R., & Corkey, B. E. (1969) *Methods Enzymol. 13*, 485–488.