position s^2U has been found to be a triplet rather than a two-base recognition,[40] and the third base discrimination is conferred primarily by thiolation.[9] Studies in vivo and in vitro[9] have shown that tRNAs with anticodon wobble position thiolated uridine-34 will select codons ending in adenosine and exhibit only a minimal amount of wobble to guanine. Wobble position-34 uridines that are modified only at C(5) recognize G just as well as A (Yokoyama, S., personal communication).

Conformational constraints imposed on particular tRNA anticodons through position-34 modifications would facilitate correct codon recognition. This is particularly important for the pyrimidine-rich anticodons of tRNAs for glutamine, glutamic acid, and lysine. It is just these tRNAs in which the s^2U derivatives are found.[1-4] The anticodon loops of glutamic acid and lysine tRNAs are composed of $U_{33}PyPyPy$, tetrapyrimidine stretches, and those of glutamine tRNAs are composed of $U_{33}PyPyG/A$, tripyrimidine stretches. In contrast to purine-rich anticodons, such as that of $tRNA^{Phe}$,[41] the pyrimidine-rich anticodons probably lack the effective stacking interactions which would facilitate codon recognition and Watson-Crick base pairing with mRNA. In the absence of effective stacking interactions, thiolation of the position-34 uridine may be an evolved mechanism of constraining the anticodon conformation in a 3′ direction for correct codon recognition and base pairing.

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Raman Spectroscopic Studies of the Effects of Substrate Binding on Coenzymes Bound to Lactate Dehydrogenase†

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Abstract: We have studied how known changes in protein structure, i.e., "loop closure" in lactate dehydrogenase brought about by the binding of substrate, affect the Raman difference spectra of both bound NADH coenzyme and other catalytically active analogues. The Raman spectrum of the NADH coenzyme in binary enzyme·NADH complex is compared to its spectrum in ternary enzyme·NADH·substrate analogue complex. As expected, we find that the protein conformational change does not modify the binding patterns of the adenosine moiety of NADH. On the other hand, there are a number of changes in the Raman spectra of the dihydronicotinamide ring of NADH and its dihydropyridine analogue, especially the vibrational modes related with the amide $-NH_2$ of NADH and C=O of PAADH motions. On the basis of our preliminary normal mode analysis of the Raman data and other studies, we suggest that the amide C=O bond of NADH changes from a loose cisoid (to N1 nitrogen) conformation in solution to a tightly hydrogen bonded transoid conformation in LDH. Furthermore, a decrease in the number of accessible conformational states available to NADH is observed when a substrate analogue oxamate binds to the binary complex as judged by the narrowing of certain Raman bands, particularly the C4-H stretch mode of NADH. From this data, we calculate that an entropy loss corresponding to ~0.7 kcal/mol of free energy is associated with this constraint of NADH nicotinamide ring, and another ~0.7 kcal/mol is associated with the constraint of the carbonyl of pyruvate. On the basis of this, we suggest that, of the 4.2 kcal/mol decrease of the transition state barrier for hydride transfer caused by the loop closure,[1] at least 1.4 kcal/mol arises from elimination of various nonproductive conformations of nicotinamide ring and pyruvate upon binding of the substrate.

Introduction

Pyridine nucleotide dependent dehydrogenases catalyze the oxidation of alcohols by the direct transfer of a hydride ion to $NAD(P)^+$. For instance, lactate dehydrogenase (LDH^2), the subject of this paper, catalyzes the direct transfer of the hydrogen from *l*-lactate to the re face of the nicotinamide ring of NAD^+ with a stereospecificity of >99.999999%; thus, *pro*-4R transfer is favored over *pro*-4S transfer by <−10 kcal/mol.[3] The catalytic efficiency of LDH is high, since this enzyme accelerates the hydride transfer step during the oxidation of lactate by NAD^+ by at least 10^{14}-fold relative to the uncatalyzed reaction.[4] This represents a reduction in the height of the transition state barrier of at least 19 kcal/mol. The catalytic mechanism of LDH is predominantly ordered with the cofactor first on and last off (cf. Adams[5]). The presence of the cofactor drives (induces) a subtle conformational change in the active site of the enzyme (cf. Holbrook et al.[6]) that increases the affinity of the enzyme for substrate by about −2 to −3 kcal.[7]

Binding of either the substrate or a substrate analogue induces a conformational change in LDH.[1,8,9] A "loop" of the protein involving residues 98-110 closes over the active site, which moves

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§ City College of the City University of New York.
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(1) Clarke, A. R.; Wigley, D. B.; Chia, W. N.; Holbrook, J. J. *Nature* **1986**, *324*, 699-702.
(2) Abbreviations: NADH, reduced β-nicotinamide adenine dinucleotide; NAD^+, oxidized β-nicotinamide adenine dinucleotide; [4,4-D2]NADH, NADH doubly deuterated at the C4 position of the nicotinamide ring; LDH, lactate dehydrogenase; $PAAD^+$, oxidized 3-pyridinealdehyde adenine dinucleotide; PAADH, reduced 3-pyridinealdehyde adenine dinucleotide; OMA, optical multichannel analyzer.
(3) LaReau, R. D.; Wan, W.; Anderson, V. E. *Biochemistry* **1989**, *28*, 3619-3624.
(4) Burgner II, J. W.; Ray, W. J. *Biochemistry* **1984**, *23*, 3636-3648.
(5) Adams, M. J. In *Enzyme Mechanisms*; Page, M. I., Williams, A., Eds.; Royal Society of Chemistry: London, 1987; pp 477-505.
(6) Holbrook, J. J.; Liljas, A.; Steindel, S. J.; Rossmann, M. G. In *The Enzymes*, 3rd ed.; Boyer, P. D. Ed.; Academic Press: New York, 1975; pp 191-293.
(7) Burgner II, J. W.; Ray, W. J. *Biochemistry* **1984**, *23*, 3626-3635.
(8) Grau, U. M.; Trommer, W. E.; Rossmann, M. G. *J. Mol. Biol.* **1981**, *151*, 289-307.
(9) Parker, D. M.; Holbrook, J. J. In *Pyridine Nucleotide-Dependent Dehydrogenases*; Sund, H., Ed.; de Gruyter: Berlin, 1977; pp 485-502.

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Arg-109 0.8 nm from a position in the solvent to one in the active site in the enzyme/cofactor binary complex. Site-directed mutagenesis experiments have suggested that this arginine, which is conserved in all sequenced lactate dehydrogenases and homologous malate dehydrogenases,[10] is important to catalysis. When Arg-109 is mutated to glutamine,[1] k_{cat} for hydride transfer is slowed by over 1200-fold and the K_m for pyruvate (or K_d for oxamate) is increased by 17-fold. Therefore, Arg-109 reduces the barrier of hydride transfer between NADH and substrate by more than 4.2 kcal/mol and stabilizes the binding of pyruvate by some 1.6–1.9 kcal/mol. Fersht and co-workers recently proposed that these large structural changes, which occur upon formation of the central complex, are important to catalysis in that they allow the enzyme to present charged groups in an optimal geometry to the substrate as the transition state is approached, to dehydrate the substrate and yet allow free access of the bound substrate to the solvent.[11] Certainly, part of this conformational change in LDH serves the purpose of moving the guanidium group of Arg-109 to a position near the substrate in the ternary complex.[6] Dehydration of the active site has not been demonstrated but probably occurs as well. These protein conformational changes also may serve to restrict the number of conformers available to the cofactors and substrate. In this report, we assess the effects of loop closure on cofactor binding by difference Raman spectroscopy.

Recently, we studied the binding properties of substrates and NAD cofactors to lactate dehydrogenase (LDH) using Raman difference spectroscopy.[12,13] In this technique, the Raman spectrum of the bound molecule is determined by subtracting the spectrum of the enzyme from that of the enzyme·substrate complex. This results in the spectrum of the bound molecule plus protein moieties that have been perturbed by substrate binding. A number of general factors influence the vibrational spectrum of a molecule. One is its geometry. Different molecular geometries often yield quite different vibrational normal modes. Interactions between molecules can also affect normal modes. These interactions, such as hydrogen bonding between a ligand and protein, modify electronic distributions, which yield modified vibrational force constants often along quite well-defined and important coordinates. For example, the frequency of a carbonyl, C=O, stretch decreases in proportion to the strength of the interaction between it and a proton donor because of electrostatic interactions. Both heterogeneous and homogeneous line broadening contribute to vibrational band widths. The heterogeneous broadening of Raman bands is a simple measure of varying environments that a bound substrate may have,[14] each uncorrelated state yields a somewhat different center band frequency with essentially the same Raman cross section (generally a good approximation for close lying resonances). Thus the heterogeneous bandwidths of vibrational bands can be used as a measure of conformational accessibility.

In this paper, we examine how the vibrational spectra of bound cofactors respond to substrate binding by comparing the vibrational spectra of NADH (and NAD^+ and enzymatically active analogues) when bound to LDH as a binary complex to its spectrum when bound as a ternary complex with substrate analogues. For example, we have used oxamate as an analogue of pyruvate. It is isoelectronic and isosteric to pyruvate and triggers loop closure of the enzyme;[9] however, hydride transfer is prevented. A preliminary normal mode calculation has also been carried out in order to understand the changes observed in the Raman spectrum of NADH when it binds to LDH.

Experimental Section

Methods and Materials. Materials. NADH (100%), NAD^+ (100%), and glutamate dehydrogenase were purchased from Boehringer Mannheim Co. (Indianapolis, IN); $PAAD^+$, alcohol, and aldehyde dehydrogenases were purchased from Sigma Chemical Co. (St. Louis, MO), and they were used without further purification.

[4-D]NADH labeled at the *pro*-R position was prepared from NAD^+, using the procedure for PAADH (below), except that deuterated ethanol was used. The monodeuterated NADH was then mixed with 100 units of glutamate dehydrogenase, 1.5-fold α-ketoglutarate, and 20-fold NH_4Cl at pH 7.0 under room temperature to oxidize the [4-D]NADH to [4-D]NAD^+. Finally, [4-D]NAD^+ was rereduced to [4,4-D2]NADH by above procedure for deuterated NADH. The purity of [4,4-D2]NADH was confirmed by NMR and UV spectroscopy to be at least 90% deuterated at C4 positions of NADH.

PAADH was prepared by mixing 100 mg of $PAAD^+$, 5 mg of ethanol, 100 units of aldehyde dehydrogenase, and 50 units of alcohol dehydrogenase in 3 mL of 100 mM Tris buffer at pH 9 under room temperature. The pH of the mixture was periodically adjusted back to pH 9.0 with 1 N KOH. After the reaction was completed, the proteins were removed by votexing the mixture with a few drops of carbon tetrachloride and centrifugation. The supernatant was then loaded on to a 2.5 × 40 cm P2 (Bio-Rad, Richmond, CA) column and eluded with distilled water. The fractions with a 260 nm/360 nm ratio of 1.55 or lower were collected and lyophilized to powder. This powder was stored under argon at −20 °C, and no detectable change in its absorption could be measured within a few months.

Pig H4 LDH and its binary complex for Raman measurements were prepared according to the published procedures.[4,12,13] A greater than stoichiometric amount of substrate analogue is added to LDH·cofactor to insure that all substrate sites in the ternary complex were occupied by analogue.

Spectroscopy. The Raman spectra were measured using an optical multichannel analyzer (OMA) system. The OMA system uses a Triplemate spectrometer (Spex Industries, Metuchen, NJ) with a Model DIDA-1000 reticon detector connected to an ST-100 detector (Princeton Instruments, Trenton, NJ). Details of the system can be found elsewhere.[12,15] Either the 457.9-, 488.0-, or the 514.5-nm line from argon ion laser (Model 165, Spectra Physics, Mountain View, CA) was used to irradiate the sample. Separate spectra for enzyme and either enzyme·cofactor or enzyme·cofactor·inhibitor complexes were measured using a special split cell and a sample holder with a linear translator as previously described.[12,15] A difference spectrum is generated by numerically subtracting the spectra. A typical protein concentration is 1 mM (4 mM in active sites), and a typical sample volume is 30 μL. Resolution of the spectrometer is 6 cm^{-1}. A spectral calibration is done for each measurement using the known Raman lines of toluene, and absolute band positions are accurate to within ±2 cm^{-1}. None of the spectra presented here have been smoothed.

Computational. *N*-(hydroxymethyl)-1,4-dihydronicotinamide was used as the model compound for our preliminary normal mode calculations. The AM1 method,[16] implemented in the QCPE program AMPAC2.1, and the ab initio method (using SCF/3-21G basis set), as implemented in Gaussian 90,[17] were used to optimize the geometry of the molecules and then to calculate their vibrational normal modes.

Results

The Raman Spectra of NADH. Recently, we reported the Raman difference spectra for both the LDH·NADH (Figure 1b) and the LDH·NAD^+ binary complexes minus the apoenzyme.[12,13] In those studies and the current ones, the Raman spectrum of the protein and the complex, which are collected sequentially, are numerically subtracted from each other. In a series of different measurements on various coenzyme analogues and isotopically labeled coenzymes, we showed that these difference spectra almost exclusively arise from vibrations associated with the bound cofactors (either NADH or NAD^+). Moreover, it was also shown[12] that the observed bands generally arise from motions located on smaller portions of the coenzymes, the nicotinamide (N), ribose

(10) Birktoft, J. J.; Fernley, R. T.; Bradshaw, R. A.; Banaszak, L. J. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 6166–6170.
(11) Sali, D.; Bycroft, M.; Fersht, A. R. *Nature* **1988**, *335*, 740–743.
(12) Deng, H.; Zheng, J.; Burgner, J.; Sloan, D.; Callender, R. *Biochemistry* **1989**, *28*, 1525–1533.
(13) Deng, H.; Zheng, J.; Burgner, J.; Callender, R. *J. Phys. Chem.* **1989**, *93*, 4710–4713.
(14) The characteristic time scale associated with vibrational spectroscopy is on the order of the vibrational motion ($<10^{-12}$ s). Thus, large scale dynamic motions of the enzyme can be treated as separate static structures that slowly interchange (on a Raman time scale).
(15) Yue, K. T.; Deng, H.; Callender, R. *J. Raman Spec.* **1989**, *20*, 541–546.
(16) Dewar, M. J. S.; Zoebisch, E. G.; Healy, E. F.; Stewart, J. J. P. *J. Am. Chem. Soc.* **1985**, *107*, 3902–3910.
(17) Frisch, M. J.; Head-Gordon, M.; Trucks, G. W.; Foresman, J. B.; Schlegel, H. B.; Raghavachari, K.; Robb, M.; Binkley, J. S.; Gonzalez, C.; Defrees, D. J.; Fox, D. J.; Whiteside, R. A.; Seeger, R.; Melius, C. F.; Baker, J.; Martin, R. L.; Kahn, L. R.; Stewart, J. J. P.; Topiol, S.; Pople, J. A. *Gaussian 90, Revision J*; Gaussian, Inc.: Pittsburgh, PA, 1990.

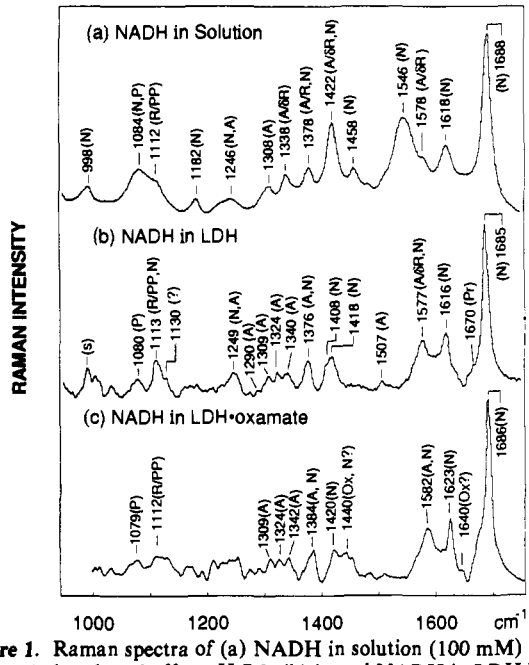

Figure 1. Raman spectra of (a) NADH in solution (100 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2, (b) bound NADH in LDH•NADH (LDH/NADH = 4:6 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2, and (c) bound NADH in LDH•NADH•oxamate (LDH/NADH/oxamate = 1:2.5:10 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2. All spectra in this figure were obtained by using ~100 mW 488.0 nm laser line.

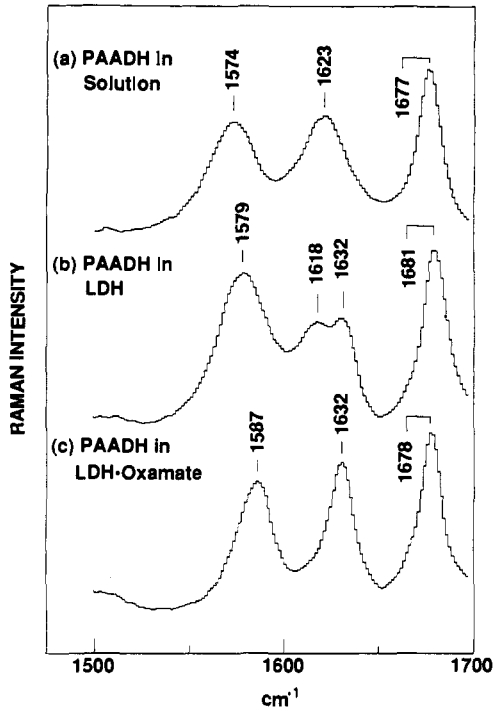

Figure 2. Raman spectra of (a) PAADH in solution (100 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2, (b) bound PAADH in LDH•PAADH (LDH/PAADH = 4:6 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2, and (c) bound PAADH in LDH•PAADH•oxamate (LDH/PAADH/oxamate = 1:2.5:10 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2. All spectra in this figure were obtained by using ~40 mW 457.9 nm laser line.

(R), phosphate (P), pyrophosphate (PP), and adenine moieties (A). Some bands arise from motions located on (unknown) protein group(s), labeled by Pr, and some bands have an unknown origin, labeled "?".

In the present study, we have extended this work by measuring the difference spectra between either the LDH•NADH•oxamate (Figure 1c) or the LDH•NAD^+•oxalate ternary complex (data not shown) and the apoenzyme, where oxamate and oxalate are used as analogs of pyruvate and lactate, respectively. These difference spectra contain signals from the cofactors, the substrate analogue, and contributions from changes in the structure of the protein; however, the spectra are dominated by the vibrational bands of the bound dinucleotide cofactors and they are labeled according to the above conventions. Two bands at 1440 and 1640 cm^{-1}, which probably contain contributions from oxamate vibrations judging from their equivalent solution positions, are labeled by "Ox".

A comparison of spectrum b with c shows that the bands that have been assigned to adenine (1290, 1309, 1324, and 1340 cm^{-1}) are not affected by the formation of the LDH•NADH•oxamate ternary complex. Also, the nicotinamide band at 1685 cm^{-1} is not affected by the binding of oxamate. However, there are many bands associated with the nicotinamide group that are affected. Those, at 1376, 1408, 1418, 1577, and 1616 cm^{-1} in Figure 1b, appear to have shifted upwards to 1384, 1420, 1440, 1582, and 1621 cm^{-1}, respectively. The spectral region in the 1377–1420 cm^{-1} contains C–H bending motions, and a detailed analysis of this spectral region will be reported elsewhere. The 1582- and 1621-cm^{-1} modes likely contain C=C and C=O stretching character as seen in our theoretical calculations below. The intensity of the peak at 1112 cm^{-1} is reduced by about two thirds when oxamate binds to the binary complex. There are two normal modes which contribute intensity to this band in the binary complex. One is associated with motions of the pyrophosphate group with some influence from the bonded ribose group;[18] the other mode is the amide $-NH_2$ rocking mode. In solution, this $-NH_2$ mode lies at 1084 cm^{-1} and moves to 1112 cm^{-1} when NADH binds to LDH (or liver alcohol dehydrogenase[19]). We have interpreted this shift in terms of an increased hydrogen bonding between the amide group and the apoprotein relative to water.[19] This interaction is expected to "stiffen" the effective rocking force constant so that the frequency increases with increasing interaction energy. The relative intensity of the pyrophosphate to $-NH_2$ rocking modes is about one to three.[18] It seems doubtful that the pyrophosphate mode at 1112 cm^{-1} is affected by oxamate binding, given the invariance of the adenine modes and that the other phosphate mode at 1080 cm^{-1} (Figure 1a) is unaffected. However, it is unclear where the $-NH_2$ rocking band shifts upon binding of oxamate.

The Raman Spectra of PAADH. The NAD^+ analog $PAAD^+$ oxidizes lactate nearly as efficiently as NAD^+ in the presence of pig heart LDH and differs from NAD^+ in that the amide on the C3 carbon of pyridine ring is replaced by an aldehyde. Figure 2a shows the Raman spectrum of PAADH in the 1500–1700-cm^{-1} range. The replacement of the amino group of the amide with a hydrogen changes the resonance interactions between the C7=O7 of amide group and the rest of the dihydropyridine ring, and an intense band at 1623 cm^{-1} is evident in the spectrum of PAADH. This band shifts 20 to 1603 cm^{-1} when the carbonyl oxygen of PAADH is labeled with ^{18}O (data not shown), which is less than the 40-cm^{-1} shift that normally occurs for a highly localized carbonyl stretch (cf. Deng et al.[19]). In addition, neat benzaldehyde, where little mixing with ring modes is expected, has a carbonyl mode at about 1700 cm^{-1}, while *p*-(dimethylamino)benzaldehyde (DABA) in water (pH 9.6), where considerable mixing of ring modes with C=O occurs,[20,21] has a carbonyl mode at 1640 cm^{-1}. Thus, we can assign the 1623-cm^{-1} band to an extended mode that contains substantial C7=O7 stretching character mixed with vibrations of the dihydropyridine ring. Figure 2b shows the difference spectrum between LDH•PAADH

(18) Yue, K. T.; Yang, J. P.; Charlotte, M.; Lee, S. K.; Sloan, D.; Callender, R. *Biochem.* **1984**, *23*, 6480–6483.

(19) Deng, H.; Zheng, J.; Burgner, J.; Callender, R. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 4484–4488.

(20) Jagodzinski, P. W.; Peticolas, W. L. *J. Am. Chem. Soc.* **1981**, *103*, 234–236.

(21) Callender, R.; Chen, D.; Lugtenburg, J.; Martin, C.; Ree, K. W.; Sloan, D.; VanderSteen, R.; Yue, K. T. *Biochemistry* **1988**, *27*, 3672–3681.

and LDH. Since the λ_{max} of PAADH is at about 360 nm, the preresonance effect enhances the Raman band intensities of the pyridinealdehyde moiety so that the relatively weaker protein or adenine bands are not observed in this region (in contrast to the data of Figure 1). The PAADH solution band at 1623 cm^{-1} now appears as two bands, at 1618 and 1632 cm^{-1}. Both bands shift downwards when PAADH is labeled with ^{18}O (data not shown). For the LDH·PAADH·oxamate complex, spectrum 2c, a single C7=O7 band is observed at 1632 cm^{-1}. The band at 1574 cm^{-1} in solution (Figure 2a) shifts to 1579 and 1587 cm^{-1} in LDH binary (Figure 2b) and ternary (Figure 2c) complexes, respectively. The band at 1677 cm^{-1} in solution changes little in binary complex but shifts to 1681 cm^{-1} in the ternary complex (Figure 2c).

The Raman Spectra of Deuterium Labeled NADH. Figure 3 shows the Raman difference spectrum between [4,4-D2]NADH, where both of NADH's nicotinamide C4 protons are deuterated, and NADH in the 1800–2300-cm^{-1} spectral region. Spectrum 3a is the difference spectrum between [4,4-D2]NADH and NADH when the cofactors are in aqueous environment, spectrum 3b is the difference when the cofactors are bound to LDH, and spectrum 3c is the difference when the cofactors are bound in a ternary complex with oxamate. There are generally no other vibrational bands in the 1800–2300-cm^{-1} region for any of the molecules under study here apart from some weak overtone bands.[22] Replacing hydrogens with deuterons on the C4 carbon brings the C–H stretching frequency, normally found at ca. 3000 cm^{-1}, down to ca. 2100 cm^{-1}. This has two advantages for the present study. First, it is not possible to discern the C–H stretches of the nicotinamide ring from all the other C–H stretches that are present in the protein and cofactor. Thus, the specific deuteration of the cofactor isolates these particular modes from all others in the Raman spectrum. Secondly, the C–H stretching modes of dihydropyridine ring are generally delocalized and involve complicated combinations with the various ring C–H internal coordinates. However, the frequency of a C–D stretch is so far away from those of any other coordinate, at either higher or lower frequencies, that a decoupling occurs. This is both expected intuitively and predicted by normal mode calculations.[22-24] Thus, the bands observed in the 2000–2300-cm^{-1} range are largely localized to motions involving the C4-D stretches.

The lower frequency band at 2085 cm^{-1} in spectrum 3a corresponds to a symmetric combination of the two C4-D internal stretching coordinates, while the 2180-cm^{-1} band corresponds to the asymmetric combination. Upon the binding of [4,4-2D]-NADH to LDH (Figure 3b), the symmetric band shifts up to 2105 cm^{-1}, while the asymmetric band is relatively unaffected. Since the *pro*-S C4-D stretch mode upshifted while the *pro*-R C4-D stretch mode is unchanged when NADH binds to LDH,[22] the symmetric stretching frequency increase is due, at least in part, to a *pro*-S stretch force constant change. Small changes in the frequencies of these two bands also occur when oxamate binds to LDH·[4,4-D2]NADH (compare spectra 3b and 3c). Perhaps more significantly, the widths of the two bands decrease substantially. For instance, the full width at half maximum of the band at 2105 cm^{-1} in spectrum 3b is 48 cm^{-1} compared to 19 cm^{-1} for the 2110-cm^{-1} band in spectrum 3c, which, after corrected for the 6-cm^{-1} spectrometer resolution, is a ratio of 2.5. This band sharpening is unlikely due to enhanced resonance effect of a specific conformation because the absorption band envelopes are essentially the same for the ternary and binary complexes (particularly, no new red shifted oscillator strength is observed), while the λ_{max} of LDH·NADH·oxamate ternary complex is slightly (~2–3 nm) blue shifted relative to the LDH·NADH binary complex. Moreover, the observed band width changes are the same using 514.5- or 488-nm laser excitation. We suggest that these data indicate that the number of conformations accessible to the cofactor is significantly reduced in the ternary complex.

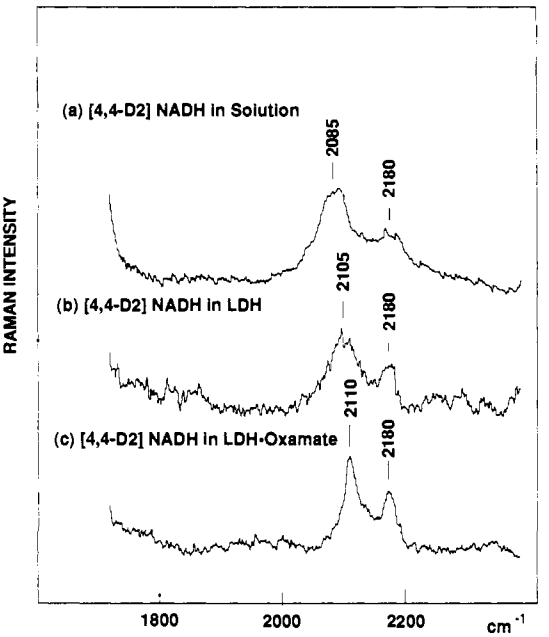

Figure 3. The Raman spectra of NADH where both the C4 protons of the nicotinamide group have been deuterated, [4,4-D2]NADH. Panel (a) is the solution spectrum of 100 mM [4,4-D2]NADH at 4 °C. Panels (b) and (c) are of [4,4-D2]NADH in LDH and in LDH·oxamate, respectively, at 3 mM and 4 °C. All spectra in this figure were obtained by using ~100 mW 514.5 nm laser line.

Theoretical Calculations. In order to understand the changes in the Raman spectrum of NADH when it binds to LDH, especially in the 1500–1700-cm^{-1} region, we have carried out ab initio calculations at the SCF/3-21G level on a model of the reduced nicotinamide moiety of NADH, *N*-(hydroxymethyl)-1,4-dihydronicotinamide. Two stationary states of the model compound were found by geometry optimization starting from C7=O7 cisoid and C7=O7 transoid. Conformer A, with its amide C7=O7 bond cisoid to the N1 nitrogen of the ring, was found to be the most stable form, consistent with the calculations on the similar compounds.[25,26] The dihydronicotinamide head of NADH in solution is likely to have this conformation as determined by NMR studies.[27] Conformer B, with the amide C7=O7 bond transoid to the N1 nitrogen of the ring but tilted by about 20°, was found to lie at a local energy minimum. Its heat of formation was calculated to be about 4.6 kcal/mol higher than that of the conformer A. X-ray crystallographic studies of many NADH dehydrogenase complexes, including the ternary complex of dog fish and *B. stearothermophilius* LDH·NADH·oxamate, suggest that the bound dihydronicotinamide has a tilted C7=O7 transoid conformation.[28,29]

In addition to these energy calculations, we also carried out normal mode frequency calculations on the two geometry optimized conformers the SCF/3-21G basis set. It is well-known that such calculations consistently overestimate C=C and C=O stretch force constants by about 20% and their frequency by about 10%. Normally, this can be corrected for by "scaling" the force constants to fit the experimental data (cf. Pulay et al.[30]). However, in many cases, the relative frequency order of the

(22) Deng, H.; Zheng, J.; Sloan, D.; Burgner, J.; Callender, R. *Biochemistry* **1992**, *31*, 5085–5092.
(23) LaLonde, R. T.; Ding, J.-y.; Tobias, M. *J. Am. Chem. Soc.* **1967**, *89*, 6651–6657.
(24) Rafilipomanana, C.; Cavagnat, D.; Lassegues, J. C. *J. Mol. Struct.* **1985**, *129*, 215–227.
(25) Donkersloot, M. C. A.; Buck, H. M. *J. Am. Chem. Soc.* **1981**, *103*, 6554–6558.
(26) Cummins, P.; Gready, J. *J. Mol. Struct. (Theochem)* **1989**, *183*, 161–174.
(27) Fischer, P.; Fleckenstein, J.; Hones, J. *Photochem. Photobiol.* **1988**, *47*, 193–199.
(28) Griffith, J. P.; Rossmann, M. G. *Brookhaven Data Bank* **1987**, *1LDM*.
(29) Wigley, D. B.; Gamblin, S. J.; Turkenburg, J. P.; Dodson, E. J.; Piontek, K.; Muirhead, H.; Holbrook, J. J. *J. Mol. Biol.* **1992**, *223*, 317–335.
(30) Pulay, P.; Fogarasi, G.; Pongor, G.; Boggs, J. E.; Vargha, A. *J. Am. Chem. Soc.* **1983**, *105*, 7037.

Table I. Comparison between Observed and Calculated C7=O7 and C=C Stretch Modes of Reduced Nicotinamide for Cisoid and Transoid Conformations of the Amide[a]

observed (cm^{-1})			calculated (cm^{-1})		
	change when in LDH				
soln freq	binary	ternary	cisoid conformer (mode)	transoid conformer (mode)	change
1688	−3	−2	1676 (C2=C3 − C5=C6)	1676 (C2=C3 − C5=C6)	0
1618	−2	+5	1638 (C5=C6 + C7=O7)	1651 (C7=O7)	+13
1546	+31	+36	1566 (C2=C3 + C7=O7)	1609 (C2=C3 + C5=C6)	+43

[a] The observed frequencies (cm^{-1}) are from Figure 1. The calculated frequencies are obtained from ab initio calculations at the SCF/3-21G level for a cisoid conformation of the amide group and a tilted transoid conformation. "Mode" indicates the dominant contributions to a particular normal mode with regards to the C2=C3, C5=C6, and C7=O7 internal coordinates.

calculated normal modes is correct. Since we are more concerned with frequency differences between the cisoid and transoid conformers than their absolute frequency in this study, here we just reduce the calculated frequencies by 12%. The calculated normal mode frequencies of the two conformers and their comparison to the three observed reduced nicotinamide Raman bands (from Figure 1) in the spectral region from 1500–1700 cm^{-1} are given in Table I. In addition, the dominant contributions to a particular mode with regards to the C2=C3, C5=C6, and C7=O7 internal coordinates are also given.

On the basis of these calculations, we can now assign the 1688-cm^{-1} band in the NADH spectrum to the out-of-phase C=C stretch mode. This mode is apparently insensitive to the amide C7=O7 bond orientation. The band at 1546 cm^{-1} in the solution NADH Raman spectrum is a combination of C2=C3 and C7=O7 stretches from these calculations. Its position and composition is very sensitive to the orientation of the amide. It changes to a C=C in-phase mode and shifts up by about 40 cm^{-1} when the C7=O7 orientation changes from cisoid to transoid.[31] On the basis of this, the large upward shifts in frequency of NADH's 1546-cm^{-1} solution band upon binding to LDH and to the various dehydrogenases we have measured to date and the very small shifts of the 1688-cm^{-1} band[12,32] may be understood as resulting from the cisoid to transoid conformational change that accompanies binding. According to the calculations, some upward shift in the solution 1618-cm^{-1} band upon NADH binding is predicted while little is observed. On the other hand, this mode is calculated to contain substantial C7=O7 stretch character especially in the transoid conformer. Its frequency is thus susceptible to bond polarization caused by hydrogen bonding between NADH's amide carbonyl and apoprotein at the coenzyme bindng site. This interaction decreases the bond order of the C7=O7 bond (for example, see refs 12 and 32), hence its stretch frequency, which could easily compensate for the increase in frequency associated with the cisoid to transoid conformational change. The fact that the dielectric constant of the medium is not considered in our preliminary calculations may also contribute to the discrepancy. Such effects may be present in the binding data of PAADH, and this is discussed below.

Discussion

Many of the bands associated with the reduced nicotinamide ring of NADH shift when oxamate binds to LDH·NADH. Three nicotinamide C–H bending modes at 1376, 1408, and 1418 cm^{-1} in LDH·NADH all shift upward by about 3–10 cm^{-1} when loop closure is driven by the presence of substrate. We believe these changes are suggestive of a conformational change of the reduced ring upon substrate analogue binding such as a planar to boat or half boat conformational change when NADH binds to dehydrogenase.[22,33] In addition, the amide $-NH_2$ rocking band at 1112-cm^{-1} shifts to an unknown position. The C=C and C=O stretch bands at 1577 and 1616 cm^{-1}, which contain C7=O7 stretch character by our calculation (Table I), shift upward upon oxamate binding. These changes are likely due to a perturbation of the hydrogen bonds on the amide formed with protein residues or bound water molecules. Recent X-ray crystallographic studies on LDH·NADH and LDH·NADH·oxamate complexes[28,29] suggest that the hydrogen bond between the hydroxyl oxygen of Ser-163 and the NADH amide nitrogen in LDH·NADH complex is likely disrupted upon oxamate binding, while a new hydrogen bond between the backbone carbonyl oxygen of Ala-138 and the NADH amide nitrogen is formed. Such changes in the hydrogen bonding pattern on the amide nitrogen may result in a rotation around the C7–N7 bond. According to a recent ab initio study on the formamide at SCF/6-31G* and MP2/6-31G* levels,[34] the changes observed for the $-NH_2$ rocking band and the C=C and C=O stretches are consistent with a C–N single bond rotation. The rotation will bend the two amino hydrogens out of the O7=C7—N7 plane so that the $-NH_2$ bending motions will surely be affected. The rotation will also shift upward the amide C7=O7 frequency, and this is observed for the 1577- and 1616-cm^{-1} bands which contain C7=O7 stretch. An alternative interpretation for the upward shift of the bands with C7=O7 stretch character is that the binding of oxamate causes a weakened hydrogen bonding between the amide C7=O7 bond and the apoprotein. This possibility is discussed in more detail for complexes formed with PAADH.

PAADH is an interesting analog of NADH in that any interaction between the $-NH_2$ of the amide and the enzyme has been eliminated. Yet, the affinity of pig heart LDH for PAADH is similar to that for NADH,[35] and the stereospecificity of the redox reaction is decreased.[36] In Figure 2 we contrast the spectrum of unliganded PAADH with both that of E·PAADH and E·PAADH·oxamate. The fact that the C7=O7 stretching mode at 1623 cm^{-1} separated into two modes in the binary complex and combined back into a single mode at a slightly higher frequency is a striking example of the subtle changes in structures (and interaction strengths) that can be detected by Raman spectroscopy. The observation of two bands in the binary complex spectrum means that PAADH exists in two separate conformations in situ as a heterogeneous mixture which affect the C7=O7 stretch differently.[14] Several possibilities exist as explanations for the splitting of, and shifts in, the frequency of the C=O band, and we consider two of the most plausible ones here.

One is the strength of hydrogen bonding to the PAADH's carbonyl group. Both extensive experimental[37] and theoretical[38] studies show that the frequency of a carbonyl stretch follows a Badger–Bauer-like rule in that the stretching frequency is directly proportional to the interaction energy of the hydrogen bond over a range of −2 to at least −12 kcal. The effect of hydrogen bonding on a carbonyl depends somewhat on the internal coordinates that make up the specific normal mode and the degree that the rest of the molecule can donate electrons to the C=O moiety. For simple ketones such as acetone, $\Delta\nu/\Delta E \approx -2$ cm^{-1}/kcal.[37,38] The

(31) Similar calculations using AM1 method also suggest that the 1546-cm^{-1} band is very sensitive to the amide C7=O7 bond orientation even though this mode is an in-phase C=C stretch for both the *cisoid* and *transoid* conformations.

(32) Deng, H.; Burgner, J.; Callender, R. *Biochemistry* **1991**, *30*, 8804–8811.

(33) Cook, P. F.; Oppenheimer, N. J.; Cleland, W. W. *Biochemistry* **1981**, *20*, 1817–1825.

(34) Wiberg, K. B.; Breneman, C. M. *J. Am. Chem. Soc.* **1992**, *114*, 831–840.

(35) Kaplan, N. O.; Ciotti, M. M.; Stolzenbach, F. E. *J. Biol. Chem.* **1956**, *221*, 833–844.

(36) LaReau, R. D.; Anderson, V. E. *Biochemistry* **1992**, *31*, 4174–4180.

(37) Thijs, R.; Zeegers-Huyskens, T. *Spectrochemica Acta*, **1984**, *40A*, 307–313.

(38) Latajka, Z.; Scheiner, S. *Chem. Phys. Lett.* **1990**, *174*, 179–184.

carbonyl stretch of PAADH is much more extended than that of acetone so this correlation will not quantitatively apply. For example, we have previously shown that the extended C=O mode of *p*-(dimethylamino)benzaldehyde, whose frequency shifts about as much as does PAADH upon ^{18}O labeling, shifts about 5-fold more than that for a nonextended C=O per kcal/mol of hydrogen bond interaction energy.[19,21] Thus, changes in hydrogen bond patterns between the protein and the CHO moiety could produce the observed spectral patterns. If so, the data show that the interaction energy between the CHO of PAADH and the enzyme in the ternary complex is weaker than that observed in water, since the C=O stretch frequency of the bound molecule is higher than that of the unbound cofactor. Another possible determinant of the C7=O7 stretch frequency is the torsional angle between the pyridine ring and the carbonyl group. The carbonyl moiety may point toward (cisoid) or away (transoid) from pyridine's nitrogen (or in between for that matter). The electronic densities within various bonds of the reduced ring[39] as well as mixing of C7=O7 stretch with internal vibrational mode of the pyridine ring are likely to be a function of this angle, which is also demonstrated in our calculations for the reduced nicotinamide ring (see Results). We currently have no predisposition toward either of these explanations. However, we do think that the 1631-cm^{-1} band represents a structure that is on the normal catalytic pathway, because it is found for both the binary and ternary complexes and because PAADH is nearly as good a cofactor as NADH.

Holbrook and his co-workers[1] found that the binding of pyruvate to LDH/NADH was stabilized by some 1.6–1.9 kcal/mol by loop closure through binding studies comparing native LDH and protein where the loop Arg-109 was replaced by glutamine (R109N mutant). They ascribed this stabilization to the formation of an enhanced electrostatic interaction between pyruvate's carbonyl bond and Arg-109 upon loop closure. The formation of a hydrogen bond between pyruvate's carbonyl and the positively charged guanidinium group of Arg-109 is energetically favorable and polarizes the substrate's carbonyl bond, and this is believed to be important to LDH's catalytic capabilities.[4] Indeed, we have recently measured the stretching frequency of pyruvate's carbonyl in PAAD–pyruvate adduct bound to the active site of native LDH and the R109N mutant. The frequency of the carbonyl is 6 cm^{-1} higher in the mutant enzyme than in the native protein (unpublished data), a direct measure of a weaker hydrogen bond polarization in the mutant.

In Figure 3, we see that the width of the C4-D stretches of [4,4-D2]NADH decreases by a factor of ~2.5 upon the formation of a ternary complex with LDH and oxamate. However, the band widths of the C4-D stretches do not change significantly when [4,4-D2]NADH binds LDH to form a binary complex (Figure 3a,b), despite the dissociation constant of the binary complex being quite low (~1 μM[1]). Such a change in the Raman spectrum of bound NADH from binary to ternary complex suggests that the protein conformational change that accompanies loop closure involves a "stiffening" of the active site in addition to the changes in electrostatic interactions inferred in the discussion above. The width of an isolated band in a spectrum can be a function of not only the spectrometer resolution but also the number of different conformers (and their population distribution) that alter the vibration mode that produces the band. The most likely explanation for the unusual width of the C4-D stretch bands in water and in the binary complex has to do with the possibility that the reduced dihydronicotinamide ring takes on various boat conformations. In solution, such puckering of the dihydronicotinamide ring may be influenced by the interaction between adenine and nicotinamide when NADH changes between folded and open conformations. However, this interaction cannot be responsible for the band broadening when NADH is bound because X-ray crystallographic studies have shown that NADH is in an open conformation in the LDH·NADH binary complex. It is likely that the angle characterizing the ring boat form takes on a range of angles, since quantum mechanical calculations suggest there is little energy difference between various possible boat conformations and the planar conformation.[22,25,26,40] Since the frequency of the C-D stretch is sensitive to this angle,[22-24] a heterogeneous mixture of various boat forms would result in the observed broad bands. Thus, we suggest that the band narrowing found in Figure 3 arises from the selection of a particular ring conformation during formation of the E·NADH·oxamate complex.

The data thus suggest that the protein conformational change driven by substrate binding decreases the number of nicotinamide ring conformations that are energetically available to coenzyme in the ternary E·NADH·substrate complex compared to the E·NADH binary complex and presumably aligns the C4-H (*pro*-R) bond along the direction of the reaction coordinate in the transition state. This suggests that entropic effects are involved in substrate binding and probably influence catalytic activity. Indeed, Burgner and Ray[4] estimated that the immobilization of reactants at the active site of LDH contributes at least 1000-fold (ca. 4.2 kcal/mol) to the lowering of the transition state barrier for hydride transfer to and from NAD to substrate on the basis of a series of reactions catalyzed by LDH.

We can calculate the approximate change in entropy between the binary LDH·NADH and the ternary LDH·NADH·oxamate complexes as monitored by the changes observed on the C4–H bond by assuming that the number of available states, Ω, is proportional to the heterogeneous bandwidth of the observed Raman band. For example, taking the bandwidth of the symmetric C4-D stretch at 2110 cm^{-1} in Figure 3c as arising from an essentially homogeneous line broadening processes and using this to fit the profile of this normal mode in the binary data of Figure 3b, we find that three bands of essentially equal strength are needed. Thus, there are three states available to the ring in the binary complex as opposed to one in the ternary complex. Since $S = R \ln \Omega$, $T\Delta S = RT \ln 3$ or 0.7 kcal/mol.[41] Of course, this just involves the entropy change associated with the available states of the coenzyme that affect the C-D stretch at the C4 position, and the total entropic change is likely to be larger. For example, we have also found a similar sharpening in pyruvate's C=O stretch bandwidth (as PAAD–pyruvate adduct) at the active site of native LDH compared to unbound pyruvate and to the pyruvate adduct bound to the R109N mutant, where loop closure does not occur (unpublished data). Performing the same analysis, we calculate an additional 0.7 kcal/mol change in $T\Delta S$ for this case as well.

Entropic effects may play a role in enzymatic catalysis by lowering the $T\Delta S$ component of ΔG between the ground state and the transition state along the reaction coordinate for the enzyme catalyzed reaction relative to the reaction pathway in solution (cf. Jencks[42]). This may come about either by lowering the number

(39) Maggiora, G.; Johansen, H.; Ingraham, L. L. *Arch. Biochem. Biophys.* **1969**, *131*, 352–358.

(40) Wu, Y.; Houk, K. N. *J. Am. Chem. Soc.* **1991**, *113*, 2353–2358.

(41) An alternative assumption is that the C4-D stretch mode line broadening is caused by vibrations which involve the C4 carbon out of the ring plane motion. Theoretical calculations and experimental observations show that C4-D stretch mode frequency can be modulated by various ring deformations, including boat or half chair conformational changes.[22-24] Our calculations as well as others also show that such conformational changes require little energy[22,25,26,40] and therefore are quite accessible under our experimental conditions. Our *ab initio* calculations at the SCF/3-21G level on the model compound *N*-hydroxymethyl-1,4-dihydronicotinamide yield a normal mode at ~100-cm^{-1} which involves a C4 carbon puckering motion. Such a vibrational mode can be easily excited by the thermal motion at near room temperature and its amplitude is likely to be large. The cycle time of this vibration is on the order of picosecond, while the Raman process is believed to be much faster (cf. Myers[43]). Therefore, this C4 carbon out of the ring plane vibration can induce a broad C4-D stretch band in the Raman spectrum of NADH in solution or in LDH·NADH. The entropy contribution to the free energy associated with a 100-cm^{-1} vibration mode is about 1 kcal/mole at 300 K and likely to retain about 20% of its value when this vibration is constrained (cf. Page[44]). If we assume the binding of oxamate to the LDH·NADH complex quenches this vibrational motion, the corresponding entropic loss to the free energy would be ~0.8 kcal/mol, practically the same as estimated under the other assumption.

(42) Jencks, W. P. In *Molecular Biology, Biochemistry, and Biophysics*; Chapeville, F., Haenni, A.-L. Ed.; Springer Verlag: New York, 1980; Vol. 32; pp 3–25.

(43) Myers, A. B.; Li, B. *J. Chem. Phys.* **1990**, *92*, 3310–3322.

of available states in the ground state (ground state entropic loss) or by increasing the number of available states at the transition state (transition state entropic gain). In LDH, the hydride transfer reaction follows a fairly precise reaction pathway, and the reaction coordinate involves nicotinamide's C4-H bond and substrate's C=O bond.[40] Entropy must be lost to bring together the C4-H bond of NADH and the C=O bond of pyruvate with the right orientation for the reaction to proceed. If this entropy loss is already realized in the LDH·NADH·pyruvate complex, as evidenced by our Raman results, it need not be done in the transition state, and the catalytic capability of LDH would be enhanced. It seems reasonable to suppose that the ground state entropic loss for the C4-H and C=O coordinates suggested by our data and calculations is expressed as a decrease of the barrier in the reaction pathway. This is the same as assuming that the conformations observed in the binary complex are nonproductive except for the conformation picked out upon the formation of the ternary complex which is structured properly for reaction. Assuming this to be true, this analysis suggests that of the 4.2 kcal/mol lowering of the transition state barrier upon loop closure found by Holbrook and co-workers,[1] some 1.4 kcal/mol arises from a raising of the ground state free energy relative to the transition state from entropic effects.

(44) Page, M. I.; Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1678-1683.

(+)-Hitachimycin: Stereochemistry and Conformational Analysis

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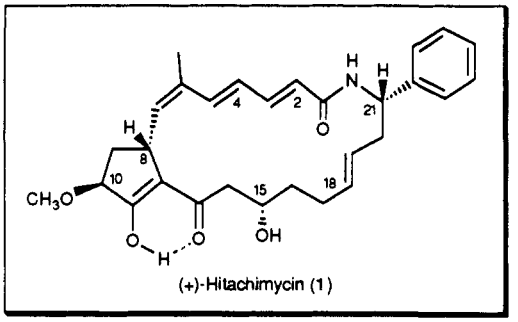
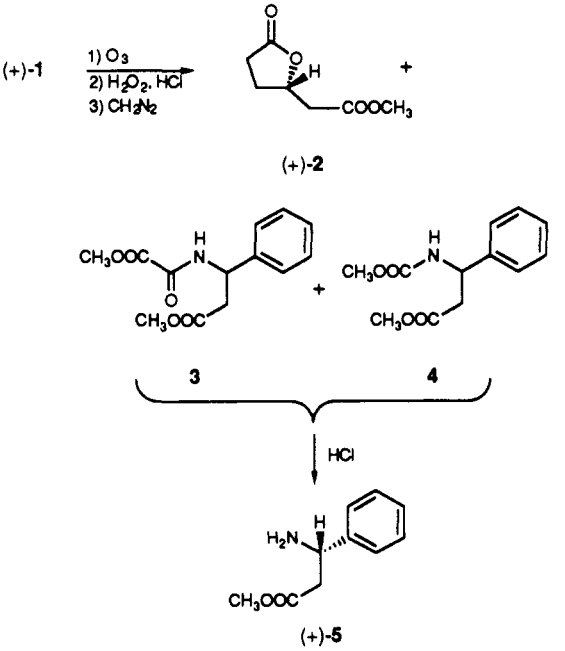
Abstract: The complete relative and absolute stereochemistry and the solid-state and solution conformations of (+)-hitachimycin (a.k.a. stubomycin) (**1**) have been defined via NMR experiments, single-crystal X-ray analysis, and computational methods.

In the early 1980s, Ōmura[1] and Umezawa[2] independently isolated the macrocyclic antitumor antibiotic (+)-hitachimycin (a.k.a. stubomycin) (**1**) from an unidentified *Actinomycetes* strain (MK-4927) and from *Streptomyces* sp. KG-2245, respectively. Whereas the Ōmura group focused primarily on structure elucidation, Umezawa and co-workers demonstrated the cytotoxicity of **1** against Ehrlich ascites carcinoma, P388 lymphocytic leukemia, and HeLa S_3 cells.[1-4] Our interest in (+)-**1** stemmed from both its novel architecture and its reported anticancer activity. Herein we describe the elucidation of the relative and absolute stereochemistry and the solid-state and solution conformations of **1**. In the following article in this issue, we detail the first (and to date only) total synthesis of (+)-**1**.



Ōmura Degradation Studies: Absolute Configurations of C(15) and C(21). Degradation experiments by Ōmura et al. had established the *S* absolute stereochemistry at both C(15) and C(21). Specifically, ozonolysis followed by oxidative workup, acidification, and esterification (CH_2N_2) provided three degradation products: (*S*)-(+)-tetrahydro-5-oxo-2-furanacetic acid methyl ester (**2**) and urethanes **3** and **4**. Hydrolysis of **3** and **4** with aqueous HCl afforded a single compound, which proved to be (*S*)-(+)-β-phenyl-β-alanine (**5**). Importantly, the absolute configurations of (+)-**2** and (+)-**5** had been established via earlier syntheses.[5,6]



X-ray Analysis of (+)-Hitachimycin (1). In an effort to assign the relative and absolute stereochemistry at the remaining centers [i.e., C(8) and C(10)], we collected a complete set of X-ray diffraction data; refinement yielded a structure with an *R* value

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