Source of Catalysis in the Lactate Dehydrogenase System. Ground-State Interactions in the Enzyme·Substrate Complex†

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Received May 14, 1993; Revised Manuscript Received October 7, 1993⊗

ABSTRACT: The Raman spectra of both the NAD–pyruvate and the pyridine aldehyde adenine dinucleotide (PAAD)–pyruvate bound to pig heart, pig muscle, and *Bacillus stearothermophilus* lactate dehydrogenases were measured and are nearly the same, which is consistent with the conserved shell of residues surrounding the active-site cavity in these enzymes. The symmetrical stretching mode of the pyruvate carboxylate group, found at 1398 cm^{-1}, is shifted only slightly when complexed to these enzymes, which shows that the group remains ionized in the ion pair complex with Arg-171 on the enzyme. The vibrational mode for the carbonyl stretch of the bound pyruvate moiety is shifted about 35 cm^{-1} to a lower frequency than observed for the carbonyl of unliganded pyruvate in the bacterial enzyme because of polarization of the carbonyl bond. Thus, the bacterial enzyme shows the same substrate activation because of the C^+–O^- charge separation that was seen previously with the mammalian enzymes. On the basis of an empirical Badger–Bauer relationship between frequency shift and interaction enthalpy, this shift in frequency is equivalent to an approximately −14 to −17 kcal/mol interaction between the enzyme and the adduct C=O coordinate, a substantial part of which is an electrostatic interaction (hydrogen bond) between the C=O and the protonated His-195. Thus, while the C=O bond is polarized on the enzyme (which requires energy), the overall ground-state enthalpy of the carbonyl imidazolium part of the reaction coordinate is stabilized substantially relative to its value in solution, and this is the dominant enthalpic effect on the entire reaction coordinate since the other internal coordinates for the hydride transfer are not much affected during formation of the ternary complex. The total enthalpy of binding for pyruvate analogs to lactate dehydrogenase is nearly the same as the sum of *local* enthalpies for interactions between pyruvate's C=O and the protein. Thus, even though ligand binding may cause any number of protein conformational changes, the net binding enthalpy of these changes must reflect the sum of a number of large, mostly compensating effects. The Raman spectra of the PAAD–pyruvate adduct bound to two different sets of mutant forms of the bacterial enzyme also were measured. Mutation of Arg-109, which normally hydrogen bonds to the pyruvate C=O, to Gln-109, reduces the extent of C^+–O^- charge separation by about 4 kcal/mol. Similarly, mutation of Asp-168, which normally forms an ion pair with His-195 in the presence of a carbonyl-containing substrate to either Asn-168 or Ala-168 also reduces charge separation but to a somewhat greater degree, 4 and 10 kcal/mol, respectively. The ground state for the carbonyl imidazolium interactions of the mutant complexes is thus destabilized relative to the wild-type enzyme, and yet, the height of the transition-state barrier must increase, which clearly indicates the height of the barrier must increase faster than the ground state is destabilized. This view is analyzed from a plot of the log of the hydride transfer step versus the change in frequency of the C=O stretch (or ground-state interaction enthalpy).

Lactate dehydrogenase (LDH;[1] EC 1.1.1.27) accelerates the oxidation of lactate by NAD^+ to pyruvate and NADH by about 10^{14}-fold relative to a corresponding model reaction (Burgner & Ray, 1984c). This reversible reaction apparently involves the direct transfer of a hydride ion, H^-, to the *pro-R, re face* of the nicotinamide moiety of NAD^+ from the C2 carbon of L-lactate forming NADH and pyruvate with a high degree of stereochemical fidelity (Anderson & LaReau, 1988). Clearly, differences in noncovalent ground-state interactions as well as transition-state interactions between substrates and enzyme must be responsible for the catalytic and stereochemical fidelity observed with LDH; so, it is of obvious interest to determine the nature of these interactions at a fundamental level. The direct interactions between substrates and enzymes determine why some molecules bind more tightly than others and, hence, why the enzymes catalyze a generally small set of chemical reactions. Also, a knowledge of how the bond orders of the bonds of the reactants vary as the Michaelis complex is formed provides important information about the catalytic mechanism. The aim of this report is to determine these properties by vibrational spectroscopy and so yield a

† This work was supported by grants from the National Institutes of Health, GM35183 to R.C. and GM42668 to J.W.B., by a SERC (U.K.) grant to J.J.H., and with travel funded by a NATO grant.

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⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1994.

[1] Abbreviations: NAD^+, oxidized β-nicotinamide adenine dinucleotide; $PAAD^+$, oxidized 3-pyridinealdehyde adenine dinucleotide; LDH, lactate dehydrogenase; BS LDH, LDH from *Bacillus stearothermophilus*; H4 LDH, H4 pig heart isozyme of LDH; M4 LDH, M4 isozyme of LDH; FBP, fructose bisphosphate; OMA, optical multichannel analyzer; NAD–pyr and PAAD–pyr, adducts formed in the presence of LDH from pyruvate and NAD^+ and $PAAD^+$.

0006-2960/94/0433-2297$04.50/0

basis whereby mechanisms based on either ground-state destabilization or transition-state stabilization can be directly or indirectly probed, respectively.

The nature of the LDH·NADH·pyruvate complex was originally studied by examining an adduct complex, E·NAD–pyr, that is formed by the addition of the C3 carbon of pyruvate enol to the C4 position of the nicotinamide ring of NAD^+ in the presence of LDH (Burgner & Ray, 1978). Like its normal reaction, LDH also accelerates this reaction at a significant rate (10^{11}-fold). The dissociation constant for the adduct complex is about 10^{-10} M, which is only slightly smaller than the product of the dissociation constants for E·NADH and E·NADH·Pyr—about 10^{-9} M^2 for H_4 LDH (Burgner & Ray, 1984c). In addition, the pyruvate moiety of this adduct seems to interact with the same residues that interact with oxamate in the E·NADH·oxamate ternary complex and that are proposed to interact with pyruvate in its central complex with LDH based on X-ray structural evidence (Grau et al., 1981; Holbrook et al., 1975; White et al., 1976; Wigley et al., 1992) and chemical studies (Burgner & Ray, 1984a–c). Thus, it seems reasonable to propose that the strength of the interactions between the enzyme and pyruvate in the E·NADH·Pyr central complex are largely maintained in the adduct complex in spite of the additional covalent linkage present in the adduct complex.

In this paper, we have extended our previous Raman studies on the adduct complex in a number of directions (Deng et al., 1989). We compare how LDH isolated from several different species, human heart, pig heart, and *Bacillus stearothermophilus*, interacts with the adduct. Furthermore, we examine changes in the vibrational spectrum of the bound adduct produced by site-directed mutations of specific residues at LDH's active site using the *Bacillus stearothermophilus* enzyme. The Raman spectrum of the bound adduct is obtained by subtracting the spectrum of the apoenzyme from that of the enzyme·adduct complex. Because of a preresonance effect produced by two different electronic transitions (see Results), the intensity of the major adduct Raman bands is comparable to that of the protein's spectrum at least with wild-type enzymes. Thus, accurate Raman difference spectra of the bound adduct are easily obtained.

Vibrational spectroscopy is a useful tool for exploring the interactions between proteins and bound ligands on a quantitative basis. The observed vibrational frequencies arise from interatomic force constants, and these are a measure of the distribution of the electrons within the molecule. This interatomic electronic distribution is disturbed, in some cases, by protein–ligand interactions in direct proportion to the degree of interaction. For example, the strengths of hydrogen bonds in model systems can be determined directly from linear free energy correlations between frequency measurements and the ΔH of formation of the H-bonds. Previously (Deng et al., 1989), we were able to assign several of the bands in the difference spectrum to normal modes associated with the pyruvate group of the bound adduct based on isotope labeling studies, while others were assigned to the dehydro moiety of the adduct. This study showed that the frequency of the carbonyl stretch of pyruvate shifts downward about 35 cm^{-1} relative to its solution value upon forming a complex with pig heart LDH. This downward shift in frequency is associated with a strong bond polarization where a significant single bond, $^+C–O^-$, resonance form is mixed into the mostly double bond of the carbonyl upon binding. The net interaction energy between the protein and the carbonyl moiety that produces this bond polarization is about −14 to −17 kcal/mol; we will discuss this value further below. Another mode of special interest is the symmetric stretch of the carboxylate group of pyruvate, which is located at 1398 cm^{-1} in solution and shifts to 1390 cm^{-1} upon binding to LDH. This shift in frequency also suggests that the binding interaction energy between the pyruvate's COO^- group is stronger in LDH than in water.

The active site of LDH contains a number of charged and uncharged residues that are probably important in its catalysis. For instance, the dyad of His-195 and Asp-168 that forms a structural arrangement found in several enzymes such as malate dehydrogenase, serine proteases, and phospholipase A_2 is an intriguing feature of the active site. This is particularly interesting when lactate is bound at the active site, since the geometry of the carboxylate, imidazole, and alcohol is quite similar to that triad in chymotrypsin. In the adduct complex, the N3 of the imidazole (His-195) approaches the carbonyl oxygen of the pyruvate moiety at an angle optimal for proton transfer to occur. This interaction is clearly essential in the redox reaction, since both the H195E and H195Q mutant forms of the *Bacillus* enzyme are relatively inactive (k_{cat}/K_M is reduced by (5×10^7)-fold; J. Porter and J. J. Holbrook, University of Bristol, unpublished results). During either the reduction of pyruvate or the decomposition of the adduct, this imidazole ring is protonated and almost certainly stabilizes the developing negative charge on the carbonyl oxygen during hydride transfer. During the oxidation of lactate, the imidazole ring assists in removing the proton from the alcohol moiety, which should increase the electron density on the C2 of lactate and, thus, ease hydride transfer. The role of the aspartate is of special interest, particularly as the His–Asp pair is a structural motif found in so many enzymes. It has been suggested that this group raises the pK_a of His-195 to near neutrality, enhancing its ability to carry out both general acid and general base catalysis under physiological conditions, and fixing the orientation of His-195 in the most favorable position (Fersht & Sperling, 1973). More recently, a theoretical approach has been used to suggest that the aspartate carboxylate stabilizes the electrostatic interactions that define the charge distribution in a transition state proposed for trypsin, principally, by stabilizing positive charge on the histidine (Soman et al., 1989; Warshel et al., 1989). Finally, the binding of substrate to LDH "drives" a protein conformation change where a protein "loop" (residues 98–110) folds over the active site, bringing the positively charged guanidino group of Arg-109 close to the oxygen of the carbonyl moiety. It has been postulated that Arg-109 plays a substantial role in polarizing pyruvate's carbonyl, in addition to His-195 (Clarke et al., 1986).

In this paper, we investigate the role of these residues in producing the large frequency shift noted above for the carbonyl moiety of the bound adduct. The Raman spectrum of the pyruvate adduct bound to the mutated proteins is presented and compared to the wild-type spectrum. The active site Asp-168 has been mutated either to a neutral but polar asparagine, D168N, or to alanine, D168A. These particular mutations were chosen because the D168N mutant is capable of forming a hydrogen bond that might still orient His-195 while the D168A mutant cannot. In addition, we have studied a mutant enzyme where the Arg-109 residue has been changed to glutamine, R109Q. This retains the hydrophilic nature of the wild-type residue but removes the positive charge. All three of these mutant proteins have been characterized kinetically (Clarke et al., 1986, 1988), and each shows substantial reductions in catalytic activity (cf. Table 1). We find, consistent with the poorer catalytic activity of these

mutants, that the bound NAD–Pyr adduct forms either slowly and incompletely or not at all in the presence of the mutants. To overcome this lack of reactivity, we have used the NAD analog PAAD in these studies since it is a more powerful oxidizing agent than NAD. The use of PAAD has the additional benefit that its aldehyde carbonyl is easily observable by Raman spectroscopy.

EXPERIMENTAL PROCEDURES

Materials. NAD^+ (100%) was purchased from Boehringer Mannheim Co. (Indianapolis, IN); sodium pyruvate (type II, 99%), $PAAD^+$, and FBP were purchased from Sigma Chemical Co. (St. Louis, MO); sodium pyruvate-*1*-^{13}C, -*2*-^{13}C, and -*3*-^{13}C (99% ^{13}C) were purchased from MSD Isotopes (Montreal, Canada); $H_2{}^{18}O$ (95%) was purchased from Monsanto Research Co. (Miamisburg, OH); and all were used without further purification.

Wild type and mutants of the lactate hydrogenase gene from *B. stearothermophilus* (Barstow et al., 1986) were generated by the oligonucleotide mismatch procedure in M13 with the mutagenic oligonucleotide as the primer for in vitro chain extensions. Wild-type and mutant LDHs (D168N, D168A, and R109Q) were expressed in the pKK223-3 plasmid in *Escherichia coli* as previously described (Barstow et al., 1986; Clarke et al., 1986, 1988). The wild-type protein was purified by affinity chromatography on a oxamate-Sepharose column, while the mutants were purified on a Blue Sepharose-F3GA and DEAE-Sepharose columns owing to their poor binding to oxamate. Dog fish M4 LDH (Burgner & Ray, 1974) and human heart LDH (J. Burgner, unpublished) are prepared. Just before Raman experiment, the LDH, which was stored in 3 M ammonium sulfate, was dialyzed against 20 mM triethanolamine at pH 7.4 and then concentrated to about 1.5 mM by Centricon 30.

Concentrations of enzyme and coenzymes were determined by UV–vis absorption spectroscopy, using $\epsilon_{280} = 120\,000\ M^{-1}\ cm^{-1}$ for BS LDH and $\epsilon_{259} = 18\,000\ M^{-1}\ cm^{-1}$ for NAD^+ or $PAAD^+$. Since LDH contains four independent active sites, LDH·NAD–pyr and LDH·PAAD–pyr complexes were prepared by mixing a 1:3:5 molar ratio of LDH to NAD^+ or $PAAD^+$ to pyruvate. The typical concentration of LDH was 1.5 mM. Under these conditions, virtually all of the NAD^+ or $PAAD^+$ will be bound to the enzyme (Burgner & Ray, 1974, 1978). Unbound pyruvate is not observed in the Raman difference spectra reported below due to its low Raman cross section relative to the preresonance enhancement found in the pyruvate adduct (Deng et al., 1989). FBP was also added to the concentrated BS LDH to prevent the aggregation of the apoenzyme. *B. stearothermophilus* LDH·PAAD–pyr complex can be prepared either with or without FBP; no aggregation was observed in either case. No detectable NAD–pyr adduct could be formed in the mutant BS LDH within a reasonable time even under forcing conditions (concentration of pyruvate of about 0.2 M). Therefore, most of the complexes used in this study are prepared with $PAAD^+$.

Spectrometer System. The procedures and controls that we use in obtaining sensitive Raman difference spectra between a protein and a protein·ligand binary complex have been discussed at length previously (Callender et al., 1989; Chen et al., 1987; Yue et al., 1989). Differences as small as 0.1% can be achieved. A specially designed split cell cuvette holds the two samples, one side containing the protein and the other side containing the protein–ligand complex. The cuvette is mounted on a translator stage–stepping motor combination so that one or the other side may be placed in the exciting laser irradiation area. Approximately 50 mW of the 457.9- or 488.0-nm lines from an argon laser or 468.0-nm line from a krypton laser was used to excite Raman scattering. Each side of the split cell has an inside dimenion of 2.5 × 3 mm and is loaded with 30 μL of sample in a typical experiment. Under these conditions, a range of about 1150 cm^{-1} of scattered light can be detected simultaneously. Spectra from one side of the cuvette are collected, and then data from the other side are collected in a sequential fashion. The difference spectrum is computer calculated. The instrument was calibrated against known Raman peaks of the toluene spectrum. Major band positions are accurate to within ±3 cm^{-1} as are the shifts in the bands. The slits were set to achieve a resolution of 8 cm^{-1}. None of the spectra presented here have been smoothed.

RESULTS

LDH catalyzes the nucleophile attack by the C3 of the enol form of pyruvate at the 4-position of the nicotinamide ring of NAD, forming the E–adduct complex [cf., Burgner and Ray (1978)]. We have demonstrated that the Raman bands of the dihydronicotinamide-like and pyruvate moieties of this adduct are easily identified by difference Raman spectroscopy because of a preresonance enhancement effect (Deng et al., 1989). This effect likely involves two different electronic transitions: one with a maximum absorbance at 327 nm that is produced by the $\pi \rightarrow \pi^*$ transition of the dihydronicotinamide-like moiety and the other with a maximum at 390 nm that is only apparent when the NAD–pyr adduct or its analogs are bound to LDH (Burgner & Ray, 1978). The long wavelength transition is likely a charge-transfer band where electrons from the carbonyl oxygen are transferred to the imidazolium of His-195. The importance of the interaction between the histidine and the carbonyl is demonstrated by a substantial blue shift in λ_{max} for this band observed with mutant enzymes that bind the adduct (L. D. Wilsbacher and J. W. Burgner, unpublished observations). Such charge-transfer bands have been observed in "charge-transfer complexes" between iodine and either aldehydes or ketones (Schmulbach & Drago, 1968). The frequency of the C=O stretch in these iodine-containing complexes is 20–30 cm^{-1} lower than for the uncomplexed carbonyl. Thus, some electron redistribution is expected to occur, as observed, in the ground-state C=O bond of the bond adduct as a result of this type of interaction (see below).

The Raman spectrum of either NAD–pyr or PAAD–pyr bound to various enzymes is obtained by subtracting the enzyme spectrum from that of the complex, leaving a difference spectrum that is dominated by bands arising from the bound NAD(PAAD)–pyr adduct. The original study was accomplished with LDH isolated from the porcine heart tissue. We now extend this study to other LDH isozymes of LDH from various sources and on a series of mutant forms of the *B. stearothermophilus* enzyme as well as to the NAD analog PAAD. Figure 1a shows the Raman spectrum of pyruvate in solution. Figure 1b is the spectrum of the NAD–pyr adduct bound to pig heart H4 LDH, which is from the original study and is shown for reference purposes (Deng et al., 1989). Figure 1c shows the difference spectrum of NAD–pyr bound to *B. stearothermophilus* LDH. All the protein studies reported here are performed at pH 7.4. Other studies (not shown) at pH values between 7.5 and 5.5 on the *B. stearothermophilus* enzyme gave the same results as in panel c. No significant change in the difference spectrum of *B. stearothermophilus*

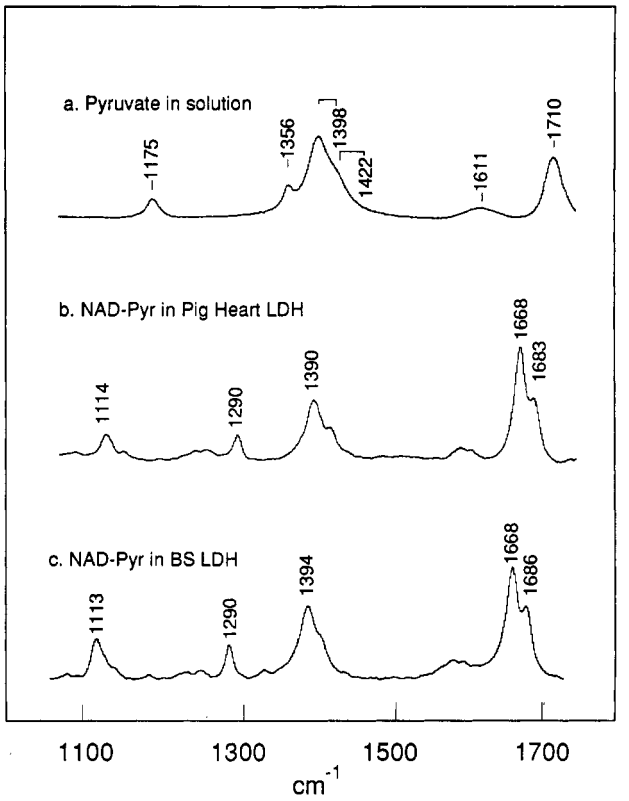

FIGURE 1: Raman spectrum of pyruvate in solution (pH 6.5, 400 mM concentration) (a) and Raman difference spectra of LDH·NAD–pyruvate minus LDH from pig heart LDH (b) and from *B. stearothermophilus* LDH (c). (a) and (b) were obtained by 457.9-nm laser line, and (c) was obtained by 468.0-nm laser line.

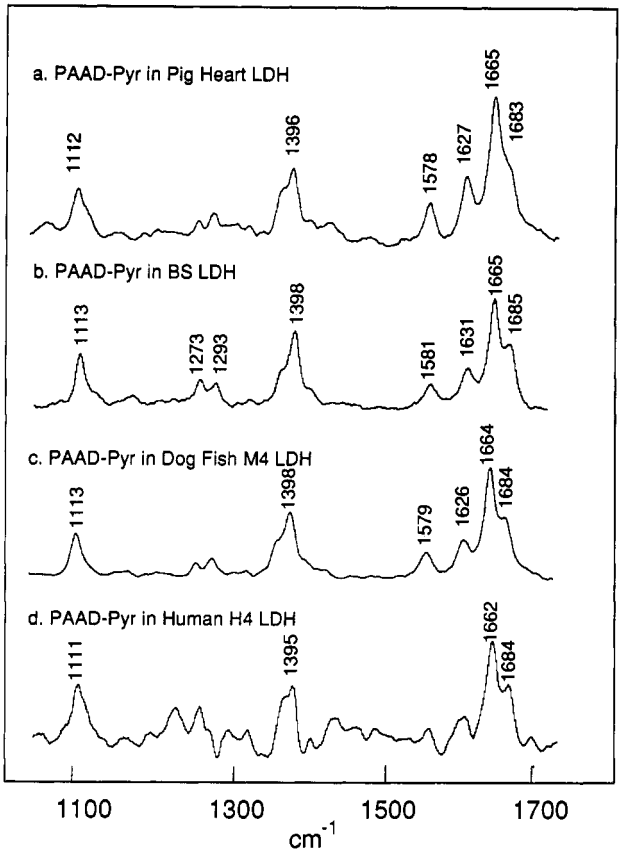

FIGURE 2: Raman difference spectra of LDH·PAAD–pyruvate minus LDH from (a) pig heart LDH, (b) *B. stearothermophilus* LDH, (c) dog fish LDH, and (d) human heart LDH. (a), (c), and (d) were obtained by 488.0-nm laser line, and (b) was obtained by 468.0-nm laser line.

is observed in either the presence or the absence of the allosteric affector, FBP, of this enzyme. Finally, while most spectra are obtained at 4 °C, one spectrum similar to that in Figure 1c for *B. stearothermophilus* was also measured at 35 °C, since the thermophilic enzyme is quite stable at this temperature. Virtually no difference was observed between the 4 °C and the 35 °C spectra.

The band at 1356 cm^{-1} in the solution spectrum (spectrum 1a) of pyruvate was assigned previously to the methyl δCH_3 bending mode on the basis of its 8-cm^{-1} decrease in frequency upon ^{13}C-labeling on the C3 position of pyruvate (Deng et al., 1989). The disappearance of this band in all spectra of all the different adduct complexes is consistent with the formation of a covalent bond between the C3 carbon of pyruvate and the C4 carbon of the pyridine ring of the cofactor. The 1175-cm^{-1} band in the solution pyruvate spectrum (Figure 1a), which contains a significant C2 motion, shifts down to ca. 1114 cm^{-1} in the various LDH complexes. This shift is also characteristic of NAD (or PAAD)–pyr adduct formation (Deng et al., 1989).

In this study, we shall concentrate on two bands found in the difference spectra. The band at 1398 cm^{-1} in the pyruvate solution spectrum was assigned to the COO^- symmetric stretch mode (spectrum 1a) on the basis of its shift in ^{13}C-labeled pyruvate [see Deng et al. (1989) and Figure 3]. The observation of this mode in the bound pyruvate adduct data proves that the carboxylate moiety of pyruvate remains unprotonated while bound to these enzymes. The frequency of the carbonyl C=O stretch at 1710 cm^{-1} in the solution spectrum of pyruvate (Figure 1a) decreased significantly upon formation of the bound NAD–pyr adduct, as shown by difference spectra of panels b and c, Figure 1, to near 1668 cm^{-1}. However, the overlap between the band from the ring C=C double-bond stretching mode (at about 1688 cm^{-1}) and the carbonyl prevents us from assigning the position of the bound pruvate C=O stretching mode accurately (Deng et al., 1989). This overlap can be removed by isotopically labeling the carbonyl carbon and oxygen of pyruvate [see Deng et al. (1989) and Figure 3].

We find, as expected from the binding and kinetic studies of Burgner and Ray (1984b,c), that the pyruvate–NAD adduct forms slowly and incompletely in the presence of the mutants that bind pyruvate poorly. However, it is possible to form the bound adduct in nearly stoichiometric amounts with these mutants by using the NAD analog PAAD. PAAD is a reasonable cofactor for LDH that more readily forms the adduct, since it is at least a 100-fold better oxidizing agent than NAD^+. Figure 2 shows the difference spectra of the PAAD–pyr adduct bound to various LDH isozymes: panel a is the spectrum of the PAAD–pyr adduct bound to pig heart LDH, panel b is the spectrum bound to *B. stearothermophilus* LDH, panel c is the spectrum bound to dog fish M4 LDH, and panel d is the spectrum bound to H4 human heart LDH. The bands at 1578, 1627, and 1665 cm^{-1} arise from motions associated with the dihydropyridine ring (1518 and 1665) and –CHO (1627) of the adduct, since bands at or near these positions and intensities are found in the Raman spectrum of PAADH [cf. Deng et al. (1992)]. As with the NAD–pyr adducts, the 1112-cm^{-1} band is associated with adduct formation, while the bands at 1396 and 1683 cm^{-1} are assigned to the COO^- symmetric stretch and C=O stretch of the pyruvate moiety, as in the NAD–pyr adducts. To isolate modes in the difference spectra of these two key bands and also to shift the frequency of the C=O band frequency to lower

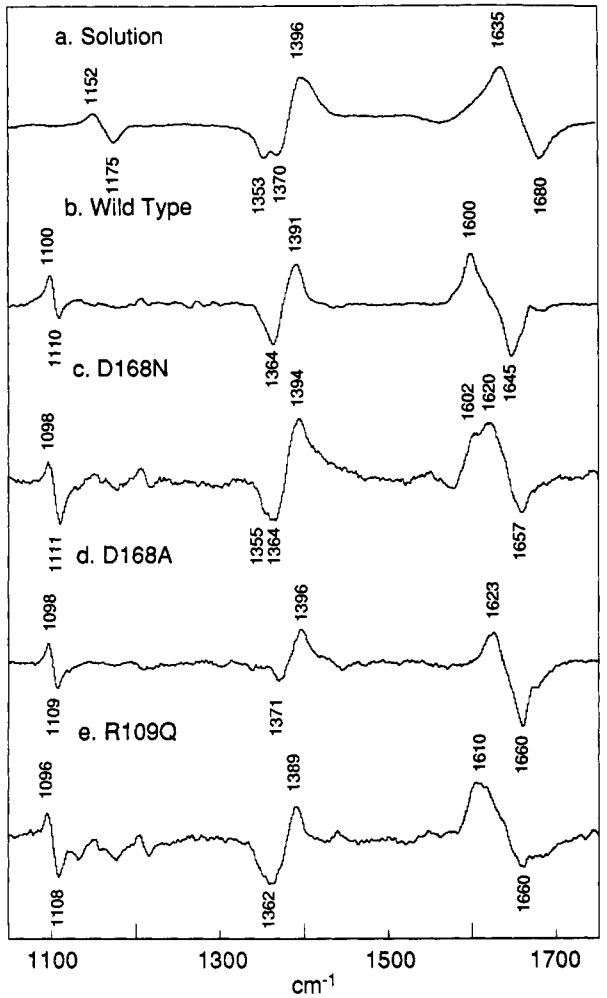

FIGURE 3: Raman difference spectrum of [2-^{13}C]pyruvate minus [1-^{13}C]pyruvate in $H_2{}^{18}O$ (a) and Raman difference spectra of LDH·PAAD–[2-^{13}C]pyruvate minus LDH·PAAD–[1-^{13}C]pyruvate in $H_2{}^{18}O$: (b) in WT BSLDH, (c) in D168N mutant BSLDH, (d) in D168A mutant BSLDH, and (e) in R109Q mutant BSLDH. All spectra in this figure were obtained from 457.9-nm laser line.

frequencies so as to eliminate any possible vibronic coupling between it and the pyridine ring mode at 1665 cm^{-1} as found in our earlier study, we performed a series of double difference experiments. Samples were prepared that contained either [^{13}C2=^{18}O]PAAD–pyr or [^{13}C1,^{12}C2=^{18}O]PAAD–pyr, and the difference spectrum was obtained between these two isotopic forms.

Figure 3a shows the spectrum of [^{13}C2=^{18}O]pyruvate minus [^{13}C1,^{12}C2=^{18}O]pyruvate in an aqueous environment. The negative peak at 1680 cm^{-1} is caused by the C=^{18}O stretch while the positive peak at 1635 cm^{-1} is the ^{13}C2=^{18}O stretch. The 45-cm^{-1} downshift upon ^{13}C-labeling is close to that (41 cm^{-1}) predicted from a reduced mass calculation for a simple diatomic oscillator; thus, this mode is quite isolated to motions involving the C=O stretch internal coordinate. The COO$^-$ symmetric stretch is unaffected by ^{18}O-labeling of C=O and shifts down to 1370 cm^{-1} upon ^{13}C-labeling at C1. The 1353-cm^{-1} band likely arises from motion on the C3 methyl group which is affected, somewhat, by ^{13}C2- and/or ^{13}C1-labeling.

To provide a foundation for considering acetone and other simple carbonyls as model systems for estimating the strength of hydrogen bond interactions for pyruvate on the basis of frequency shifts, we have compared the normal mode for these carbonyls. The ^{13}C shifts for acetone, pyruvate, and acetophenone are 43, 40, and 40 cm^{-1}, respectively. These are all consistent with an isolated carbonyl normal mode, since these frequency shifts are essentially those calculated assuming a diatomic oscillator model for C=O, i.e., 39 cm^{-1}. This indicates that these normal modes are not extended over multiple bonds, since the isotopic shifts would have been smaller than 39 cm^{-1} if they were. The peak positions of the C=O bond stretch for acetone, acetophenone, and benzophenone in aqueous solution at 1709, 1683, and 1658 cm^{-1} are clearly consistent with the relative ability of the phenyl ring to stabilize the C$^+$–O$^-$ form over the C=O of the carbonyl. This has the effect of lowering the bond order (hence the bond force constant and stretch frequency) of the C=O bond.

Table 1: Effects of Mutations on Catalytic Constants[a] and Ligand Binding for *B. stearothermophilus* LDH

	Asp-168 (wild type)	Asn-168 (mutant)	Ala-168 (mutant)	Gln-109 (mutant)
K_m (pyruvate) (mM)	0.06	10	3.3	1.0
k_{cat} (pyruvate) (s^{-1})	250[b]	20	5.5	0.6
k_{cat}/K_m	>4200	2	0.6	0.6
K_d (oxamate) (mM)	0.06	11	9	1
$\Delta\nu_{COO^-}$ (soln-bound)[d] (cm^{-1})	5[c]	2[c]	0[c]	7[c]
$\Delta\nu_{C=O}$ (pyruvate) (soln-bound)[d] (cm^{-1})	35[c]	24[c]	12[c]	25[c]
$\Delta E_{interaction}$ to pyruvate's C=O bond (soln-bound) (kcal/mol)[e]	15	11	5	11

[a] Clarke et al. (1986, 1988). [b] The value of the rate constant for the hydride transfer step is 750 s^{-1} (Clarke et al., 1988). [c] For the PAAD–pyr adduct. [d] The accuracy of the wavenumber shifts is ±3 cm^{-1}. [e] As approximated using relevant Badger–Bauer relationships discussed in the text.

Figure 3b shows the difference spectrum resulting from the double difference experiment with PAAD–pyr adduct bound to *B. stearothermophilus* LDH. In this form, all bands not affected with the labeling, especially the two relatively strong bands at 1581 and 1631 cm^{-1} from the pyridine aldehyde ring (spectrum 2b), are removed, and the assignment of the pyruvate C=O stretch mode and the COO$^-$ symmetric stretch is evident. Figure 3, panels c–e, shows the double difference Raman spectra of the PAAD–pyr adduct in D168N (panel c), D168A (panel d), and R109Q (panel e). The ^{13}C2=^{18}O stretch mode of pyruvate in solution (Figure 3a) at 1635 cm^{-1} shifts down to 1600 cm^{-1} in wild-type LDH (Figure 2b). This 35-cm^{-1} shift is not significantly different from the 37-cm^{-1} shift observed in the NAD–pyr adduct in pig heart LDH that we reported previously. Band shifts calculated from these data for the C=O stretch and the COO$^-$ symmetric stretch in the various mutants from their solution values are tabulated in Table 1, along with relevant catalytic constants from other studies. Note that the ^{13}C2=^{18}O stretch band is quite broad in the D168N and R109Q spectra. We have used the average band position in calculating the $\Delta\nu_{C=O}$ band shifts in Table 1. We will discuss these results further below.

DISCUSSION

A number of the essential elements of the reaction scheme for LDH are reasonably well understood. The catalytic binding site involves two domains, one binds the cofactor, either NADH or NAD$^+$, and the other binds the substrate, either pyruvate or lactate. The imidazole ring of His-195 interacts with substrate and performs a number of important functions. The N3 nitrogen of the imidazolium cation donates a proton to the carbonyl of pyruvate and accepts a proton from the alcohol of lactate, thus acting as a general acid–base catalyst. Its interaction with substrate, certainly with the

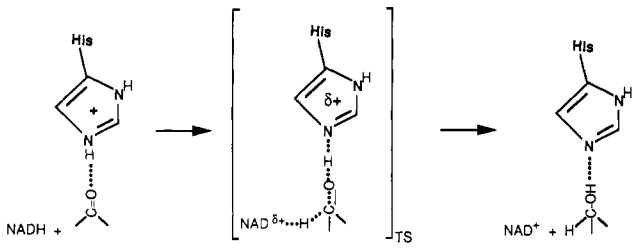

FIGURE 4: Simplified schematic of the reaction coordinate for the LDH-catalyzed reaction.

carbonyl group of pyruvate, helps position the substrate properly for its interaction with the C4 hydrogen of NADH. As we shall show below, the enthalpy of this interaction is large and quite sufficient to tightly orient the substrate. These results also show that the interaction is not simply that of the N3 nitrogen with the carbonyl group, but also includes the extended structure of the substrate binding site (Dunn et al., 1991). It has been previously estimated that the loss of entropy at the reaction site (i.e., by realizing a proper orientation of the reactants) contributes as much as 10^4 to the rate enhancement that LDH brings about over the uncatalyzed reaction (Burgner & Ray, 1984c).

Before proceeding further, we should point out that several broad conclusions are evident from this study. In the first place, results are reported here for several different isozymes of LDH: from human heart, pig heart, dogfish muscle, and *B. stearothermophilus*. The spectra of the NAD(PAAD)–pyruvate adduct bound to these proteins show few differences. This suggests that the interactions between the enzyme and the cofactor–substrate complex must be virtually identical—showing no prokaryotic–eukaryotic or mesophilic–thermophilic differences, for example. In this context, it should be remembered that the position of a vibrational normal mode is quite sensitive to the bond length (or bond order) of the vibrating bond, and variations of as little as a few thousands of an angstrom in intraatomic distances can yield measurable changes. Thus, the geometry as well as the electrostatic fields of the active sites must be virtually identical in spite of the considerable lack of homology in the amino acids that are peripheral to the active site.

The essential reaction coordinate from pyruvate and NADH to the transition state and then to lactate and NAD^+ may be sketched to a reasonable approximation as shown in Figure 4. During the reduction of pyruvate, the reaction scheme involves three important internal coordinates: the C4–H bond of the NADH coenzyme, the C=O bond of pyruvate, and the hydrogen bond between the C=O group and the imidazole ring. We have shown recently that the frequency of the *pro-R* C4–H bond stretch of NADH is not changed during formation of a E·NADH complex with LDH (Deng et al., 1992). This frequency is also not affected much when oxamate, a substrate analog, binds to form a ternary (enzyme·coenzyme·substrate) complex apart from band narrowing. We have suggested that this band narrowing results from a "stiffening" of the binding site; this stiffening decreases the number of accessible states available to NADH, which is essentially an entropic effect. Thus, the dominating enthalpic interaction in the reaction coordinate is the hydrogen bond between C=O and the imidazole ring. By using the C=O group as a probe of this interaction, we previously estimated that this enthalpic interaction on the enzyme relative to the interaction of the C=O with water must be quite large on the basis that the 35-cm^{-1} shift, which was observed, is a significant fraction of the 100-cm^{-1} decrease observed when the C=O of acetone is fully protonated (Deng et al., 1989). This enthalpic interaction is clearly a ground-state effect that polarizes the C=O bond and makes the carbon more susceptible to (nucleophilic) attack by a hydride ion. Placing some limit on the size of this enthalpic effect is an important aspect of our attempts at understanding how LDH accelerates chemical reactions [cf. Burgner and Ray (1984c)].

It is well-known that when a molecule A–H forms a hydrogen bond with an acceptor B, such as a carbonyl, there is a shift in the vibrational modes of A–H, where ν_{A-H} can shift hundreds of wavenumbers in the case of enthalpically strong hydrogen bonds. Good correlations between the stretching frequency for A–H and the interaction enthalpy (ΔH) are observed in the condensed phase with O–H and N–H groups as well as with systems involving hydrogen bonds to molecular ions [cf. Joesten and Schaad (1974)]. What is less well known is that the normal mode force constants for acceptor bonds like the C=O are also sensitive to the electrostatic effects of hydrogen bond donors and other charged groups because of changes in the electron density of the C=O bond. Thus, the frequency of the C=O mode acts as a quantitative *local* reporter of the interaction between the carbonyl and its surroundings.

Ketones, amides, and esters all show linear correlations between $\nu_{C=O}$ and ΔH upon formation of hydrogen bond complexes between the C=O bond and various proton acceptors in "inert" solvents (Thijs & Zeegers-Huyskens, 1984). In general, the empirical linear free energy relationships obtained (so-called Badger–Bauer relationships) are found to extend over the moderate range of available solution models. For acetone and several other carbonyl-containing compounds, linear relationships between both ν_{A-H} and ΔH as well as $\nu_{C=O}$ and ΔH were found to hold experimentally over a range of 4–8 kcal/mol (Thijs & Zeegers-Huyskens, (1984). The slopes of such plots for $\nu_{C=O}$ range from 0.47 (±0.05) for acetone to 0.3 (±0.1) for acetophenone (that is for a in $\Delta\Delta H = a\Delta\nu_{C=O}$, where frequency is given in cm^{-1} and enthalpy in kcal/mol).[2] At energies greater than 10 kcal/mol, there are no available solution-based model systems, although much stronger hydrogen bonds are known to exist in the gas phase and in intramolecular hydrogen bond systems (Hibbert & Emsley, 1990). On the other hand, recent *ab initio* calculations of the change in frequency of the C=O stretch of formamide versus hydrogen bond interaction energy also yield a linear relationship with a slope (0.5 kcal mol^{-1} cm^{-1}) similar to that experimentally determined for acetone. These calculations also indicate that the linear relationship should hold at much larger values of ΔH, in excess of 30 kcal/mol (Latajka & Scheiner, 1990). Hence, it seems reasonable to employ these empirical relationships between $\Delta\Delta H$ and $\Delta\nu$ even in situations where the calculated interaction energies between enzymes and their ligands are much larger than those observed in the experimental set that forms the basis of the relationship.

The work of Thijs and Zeegers-Huyskens (1984) shows that the slope term for aldehydes depends somewhat on substituent groups bonded to C=O. This is true particularly for resonance interactions which may influence the "mobility" of electrons in the C=O bond, but inductive effects also play a role. Unfortunately, neither the NAD–pyr adduct nor pyruvate is particularly amenable for study in hydrogen bond systems involving "noninteracting solvents" because of their

[2] The errors in the slope term present here are obtained from a recalculation of the data in Table 1 of Thijs and Zeegers-Huyskens (1984).

limited solubility, even as the *tert*-butylammonium salts. However, the linear frequency–ΔH correlations of Thijs and Zeegers-Huyskens provide us with a range of values for the slope term. For example, the largest slope term, that of acetone, yields a value of $\Delta\Delta H = -17$ kcal/mol for the transfer of pyruvate's C=O bond from aqueous solution to the binding site in LDH based on the 35-cm^{-1} shift in frequency, while the smallest, that for acetophenone, yields a $\Delta\Delta H$ of −11 kcal/mol.

Of possible model systems, we think that acetone and methyl acetate (a = 0.4 kcal mol^{-1} cm^{-1}) are the best choices, and so we would place the actual value of $\Delta\Delta H$ to be at the upper part of this range (in absolute value), i.e., from −14 to −17 kcal/mol and possibly higher. The ^{13}C isotope produced shifts in $\nu_{C=O}$ (see Results) indicate that neither pyruvate, acetone, nor acetophenone involves extended C=O modes. However, the frequencies of pyruvate and acetone are very close but much higher than that of acetophenone (see Results). It is almost certain that resonance effects with the phenyl group, which result in enhanced C^+–O^- character in the C=O bond, are responsible for this (inductive effects would have resulted in stabilization of C=O relative to C^+–O^-). The fact that larger shifts in $\Delta\nu_{C=O}$ are observed to a given hydrogen bond strength in acetophenone than in acetone is also consistent with the bond order of C=O being influenced by resonance effects in acetophenone that are absent in acetone and methyl acetate because of enhanced polarizability [see also Deng et al. (1989)]. The placement of a methylene bridge between the C=O and the phenyl group in acetophenone largely wipes out the interaction between the phenyl group and the C=O, since $\nu_{C=O}$ for the bridged compound is substantially higher than that of acetophenone and is, in fact, about that of acetone. Thus, one does not expect that the presence of a dihydronicotinamide-like moiety bonded via a methylene bridge will have a significnat effect on the $\nu_{C=O}$ of the NAD–pyr adduct or its response to hydrogen bonding. On the other hand, the carboxylate group of both pyruvate and the adduct is neither a particularly good electron acceptor nor a donor (Menger & Ladika, 1988). The modest inductive effect of the pyruvate carboxylate should stabilize the C^+–O^- form relative to C=O because of its slight electron-donating effect relative to that of a methyl group, but field effects may reverse this. The approximately 10 cm^{-1} higher $\nu_{C=O}$ for pyruvate compared to acetone is consistent with this reversal. The electron-withdrawing effect of the O–CH_3 group of methyl acetate should stabilize the C=O species, and its $\nu_{C=O}$ is slightly higher than either acetone or pyruvate in water. It is also important to note that the chemistry of the enol and keto forms of pyruvate is quite similar to that of acetone [cf. Kresge (1991)].

The $\Delta\Delta H$ for the transfer of the carbonyl moiety of the pyruvate from water to the active site of LDH of about 14–17 kcal/mol is a large value, substantially larger than that found generally for hydrogen bonds in proteins (Jeffrey & Saenger, 1991) or for most simple hydrogen-bonding interactions found in non-hydrogen-bonding solvents, although Karplus and coworkers (Gao et al., 1989) have recently calculated that the interaction energy between acetate and a water molecule is about −11 to −13 kcal/mol. It is also important to recognize that this interaction energy reported by the C=O frequency change is not just a function of the electrostatic interaction between the C=O and the active site histidine, but it is a function of all of the electrostatic interactions between the C=O and nearby polar groups. However, the interaction between the imidazolium ring of H195 and the carbonyl moiety must be the lynch-pin of this effect since, for a H195N mutant form of human heart LDH, little interaction (<1.4 kcal) between the pyruvate moiety of the adduct and the enzyme is observed (Wilsbacher and Burgner, unpublished observations).

We also have argued previously that the decrease in frequency of the COO^- symmetric stretch of pyruvate occurs because of ion pair formation in the protein between this group and the NH_2^+ groups of Arg-171; such a pairing is suggested by X-ray crystallographic studies in different LDH proteins. The NH_2^+ group of Arg-171 is believed to act as the recognition center [cf. Holbrook et al. (1975)]. There is no measured correlation between frequency shift of the COO^- symmetric stretch and interaction energy. Nevertheless, in analogy with the results on the C=O stretch, a downshift in frequency for $\Delta\nu_{COO^-}$ (Table 1) indicates that this group interacts with the protein more tightly than with water.

The *net* enthalpy change when pyruvate binds to the active site of LDH·NADH should be produced primarily by the shift of pyruvate from water to enzyme, differences in coenzyme interactions, and by changes in the internal interaction on enzymes produced by complex formation. The contribution to the net enthalpy of interaction by either pyruvate or the pyruvate moiety of NAD–pyr should arise primarily from differences in interactions involving the carboxylate and carbonyl moieties. Our analysis here yields an approximate value of the carbonyl interaction. Oxamate, which is a nonreacting analog of pyruvate and the pyruvate moiety of NAD–pyr, is the only ligand bound to the reduced binary complex, E·NADH, that has been well-characterized thermodynamically. A comparison between oxamate and pyruvate on an enthalpic basis must be somewhat tenuous because of expected differences in solvation between an amide and an acetyl, which should *increase* the affinity between pyruvate and enzyme relative to oxamate. Nevertheless, the ΔH for formation of the oxamate ternary complex is about −15 kcal/mol of bound ligand to pig heart LDH (Schmid et al., 1976). This value for the overall enthalpy change for ligand binding of oxamate and our approximate value of about −14 to −17 kcal/mol for the carbonyl of NAD–pyr is remarkably close. This result suggests that while significant changes in the structure of the enzyme do occur during formation of the ternary complex (Wigley et al., 1992), their total is not enthalpically significant. Thus, even though ligand binding may cause any number of protein conformational changes, the net binding enthalpy of these changes seems to reflect the sum of a number of large, mostly compensating effects, which is consistent with the suggestion of Gao et al. (1989).

As indicated earlier, the closest charged residue to the C=O moiety in bound NAD–pyr is His-195, and this should be the key residue that defines pyruvate's interaction with LDH. However, our results clearly show that the strength of the interaction between C=O and the moiety of the enzyme is not totally dependent on this key residue, but rather on the active site as an extended unit. This, of course, is not a surprising result given the highly interacting nature of the residues found in active sites, often involving extensive hydrogen bond networks. In Table 1, we have summarized the changes in frequencies of the carboxylate and carbonyl as a function of the three mutants we have examined so far. Previous kinetic studies indicate that the charged guanidino group of Arg-109 affects both k_{cat} and K_m, and it has been suggested that this residue interacts with the C=O group directly and, thus, helps to polarize this bond (Clarke et al.,

1986). The existence of this interaction is borne out by our study, since the frequency of the carbonyl shifts to the blue by 10 cm^{-1}. Approximating the slope term for $\Delta\Delta H/\Delta\nu_{C=O}$ to be 0.44 (halfway between acetone and methyl acetate), this shift represents a difference in $\Delta\Delta H$ of about 4 kcal/mol in the presence of the polar but uncharged Gln-109 mutant relative to the wild type. Normally, a "loop" of residues, 98–110, closes over the active site and positions Arg-109 near to the C=O group (Holbrook et al., 1975). This process, which occurs during formation of the ternary complex, probably facilitates both dehydration and stiffening of the active site (Deng et al., 1992) in the native enzyme (Clarke et al., 1986). In addition, not only the frequency but the width of the C=O stretch in Figure 3e is affected as well; its bandwidth is about twice as wide as that of the wild-type protein. Since the characteristic time scale of a Raman experiment is fast, on the order of a vibrational frequency ($<10^{-12}$ s), large-scale motions of the enzyme may then be treated as static separate structures that comparatively slowly interchange. Thus, such "inhomogeneous" band broadening observed for the R109Q (and D168N) mutant indicates that multiple substrate-binding modes exist. One explanation of such band broadening involves a gain of entropy as the number of active-site interactions are reduced, which results in a loss of active-site stiffening (Deng et al., 1992) or a change in the number of frozen water molecules at the active site (Dunn et al., 1991). From Table 1, it is also seen that C=O bond polarization of NAD–pyr is strongly affected by the carboxylate of Asp-168 although this group is eight bonds from the C=O group in the active site. Interestingly, a mutation of this group involving the polar but uncharged Asn residue results in a more polarized carbonyl than the neutral alanine mutant (blue shifts relative to native enzyme of 11 and 23 cm^{-1}, respectively), which is an unexpected result on the basis of "pure" electrostatic considerations. As stated above, the charged His–Asp pair motif is present in many different enzyme systems as well as LDH. The order of the structural arrangement is C=O···$(His)^{+}$···$(Asp)^{-}$. If the structure of the active site is completely unaffected by the mutations apart from the change in charge or polar character of the aspartate, the positive charge observed by the carbonyl must increase in the polar Asn-168 mutant and increase still further for the nonpolar Ala-168 mutant. As the positive charge on the histidine increases, additional electrons should move into the H-bond, and thus, the C=O bond polarization should change in the opposite direction as observed. It seems clear that the Asp-168 group is present in the active site for reasons having to do with the structure of the protein and particularly that of the active site. Note in this regard the increased bandwidth (obviously a doublet) of the C=O stretch in the D168N mutant (Figure 3c), indicating multiple substrate-binding patterns. A number of protein structural changes may accompany the Asp-168 mutation. For instance, the imidazolium ring might rotate away from the optimal angle for forming a hydrogen bond. Also, electrostatic considerations are likely to be quite important for the structure of the binding site since an unbalanced positive charge located on the active site histidine would bring about a high degree of destabilization of the active site. These qualitative considerations are consistent with the polar D168N mutant showing a higher degree of C=O bond polarization, hence greater interaction energy than the nonpolar D168A mutant.

It is of interest to compare the changes in *local* energy interaction between pyruvate's C=O group, derived from the changes in bond frequency, and an average thermodynamic quantity of substrate affinity as expressed in K_m for pyruvate, or K_d for oxamate, for the various mutants. The K_a of binding is equal to $\exp(-\Delta H/RT + \Delta S/R)$ so that the change of $\Delta\Delta H$ of 5 kcal/mol of the local C=O interaction between the Asp-168 and Asn-168 proteins (Table 1) would be equivalent to a looser binding of substrate of about 6000 if ΔS remains unchanged. The K_d for oxamate changes by close to 200 so that while the agreement is qualitatively correct, it is quantitatively far off. Moreover, it is immediately noted from Table 1 that, while pyruvate and oxamate bind to the Asn-168 and Ala-168 enzymes with about the same affinity, the ΔH for the C=O interaction in the adduct complex differs by 6 kcal/mol, which predicts a binding difference of over 10^4. This suggests that entropic factors play an important role in determining substrate binding by the mutant proteins. This is not surprising given the band broadening observed in the present Raman data (see above). Moreover, it is easily imagined that mutation may result in protein conformational changes away from the C=O group which yield changes in binding enthalpy of a few kcal/mol.

We have also attempted to correlate the changes the enthalpic interaction, as monitored by the shift in frequency of C=O stretch, with the rate of hydride transfer. This can be done after making a number of plausible approximations. The first is that the essential structure of the reacting components is not affected by the mutation except in regard to the H-bonding interaction. This amounts to assuming that the relative positions of the key coordinates of the reacting species are not too disrupted by the mutation. The second is that the "carbonyl" of the transition state obeys a Badger–Bauer relationship in the same way (although not with the same slope term) as the ground-state carbonyl. Application of reaction rate theory then suggests that a plot of k_{cat} versus the shift in carbonyl frequency should yield a straight line. We can write:

$$k_{cat} \sim \exp(-\Delta H^{\ddagger}/RT) \sim \exp(\Delta H/RT) \sim \exp(x\Delta\nu_{C=O}/RT)$$

where x is an unknown numerical factor which depends on the relationship between the transition-state $C^{+}\cdots O^{-}$ stretch and its enthalpic interaction with the imidazole proton. Figure 5 shows a plot of the hydride transfer step rate as a function of shift in carbonyl frequency for the wild-type protein and the mutants (see Table 1). Also plotted is the solution rate of hydride transfer, taken to be the rate found in the enzyme divided by the $10^{5.5}$-fold factor determined for the relative enolization rates (Burgner & Ray, 1984c). It can be seen that reasonable agreement is obtained except for the R109Q mutant. In the R109Q mutant, the hydride transfer rate is substantially below what would be predicted from the observed polarization of the C=O bond. This is not unreasonable. The replacement of arginine by glutamine almost certainly affects loop closure, resulting in an active-site structure that is substantially altered; we have previously reported observations which indicate this is so (Clarke et al., 1986; Deng et al., 1992). The broadness of the C=O stretch band in the R109Q mutant data also suggests that this is true. It is certainly possible that the coenzyme–substrate distance is increased substantially and thus decreases k_{cat}. Of course, it is also possible that some of the problems in the agreement could also arise from our choice of the model system (i.e., the PAAD–pyruvate adduct). In any case, it is apparent from the observed correlation that the force constant (the essential determinant of the C=O frequency) is a key predictor of the ease of hydride transfer.

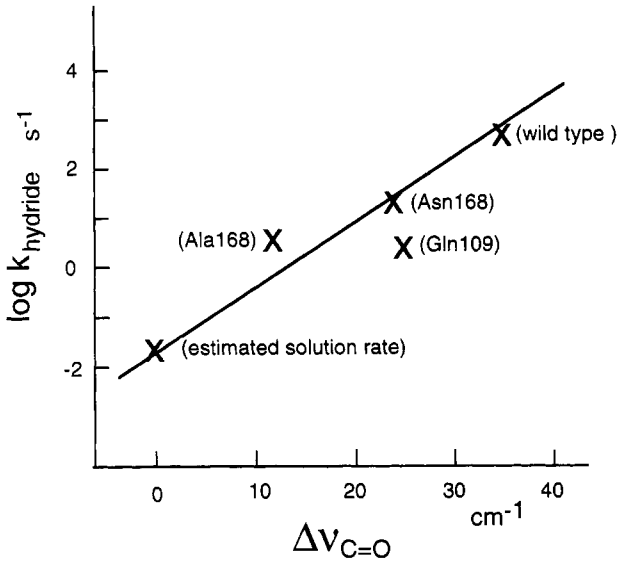

FIGURE 5: Log of the rate of hydride transfer step (in s^{-1}) versus the observed change in C=O stretch frequency of pyruvate upon binding from Table 1. The solution hydride transfer rate, which has not been measured, is estimated from the ratio of enolization rate of pyruvate in solution and in LDH (see text) and taking the transfer rate in LDH to be 750 s^{-1}.

The reasonable agreement observed in Figure 5 can be rationalized by supposing that the enthalpy of the transition state is stabilized to an even greater extent than the ground state so that the net reaction barrier is lowered by the electrostatic interaction. This is true because the carbonyl moiety is substantially more polarized in the transition state, with more negative character on the oxygen. Also, it seems likely that the proton located on the imidazole in the ground state is now closer to the oxygen in the transition state, as in Figure 4. For example, a recent calculation places the proton closer to the oxygen than to the imidazole ring (Wu & Houk, 1991). This analysis is in accordance with a previous study that the equilibrium toward *enol*-pyruvate from pyruvate is increased by $10^{5.5}$-fold at the active site in LDH compared to solution from the stabilization of C–O^- character into pyruvate's C=O. This prompted the suggestion that some $10^{5.5}$-fold of LDH's 10^9-fold rate enhancement arises from the C=O···His-195^+ interaction (Burgner & Ray, 1984c) and may be compared to a decrease in k_{cat}/K_m of (5×10^7)-fold (J. Porter and J. J. Holbrook, unpublished results).

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