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Use of Isotope Effects and pH Studies To Determine the Chemical Mechanism of *Bacillus subtilis* L-Alanine Dehydrogenase†

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ABSTRACT: Analysis of deuterium isotope effects with L-alanine-d_4 and L-serine-d_3, and of pH profiles with the same substrates, shows that L-alanine is sticky (that is, reacts to give products 1–7 times as fast as it dissociates) while L-serine is not. The pH profiles show the following: (1) NH_3 and monoanionic amino acids are the substrates; (2) a cationic acid group on the enzyme (probably lysine) with a pK of 9.0–9.6 in E–NAD, but a pK well above 10 in E–NADH, must be protonated for activity and good binding of inhibitors and is probably important for maintaining the proper conformation of the enzyme; (3) a cationic acid group on the enzyme (probably histidine) with a pK around 7 in both E–NAD and E–NADH must be unprotonated for oxidation of amino acids but protonated for binding and reaction of pyruvate. This latter group is the acid–base catalyst for the chemical reaction. In E–NAD, it is so positioned that it can hydrogen bond to (and thus when protonated enhance the binding of) a D-hydroxy or a carbonyl group of an inhibitor, but its state of protonation does not affect the binding of L-lactate or propionate. In E–NADH, it is so placed that it can hydrogen bond to both D- and L-hydroxy groups, as well as carbonyl groups. A chemical mechanism is postulated in which the dehydrogenation of L-alanine by NAD to produce iminopyruvate is followed by attack of water from the same side from which the hydride was removed. The catalytic histidine transfers a proton from the attacking water to the amino group of the resulting carbinolamine and then removes a proton from the hydroxyl group of the carbinolamine as ammonia is eliminated to give pyruvate.

In the previous paper (Grimshaw & Cleland, 1981), we have shown that alanine dehydrogenase from *Bacillus subtilis* has a predominantly ordered kinetic mechanism and that isomerization of E–NAD to a form capable of productive combination with alanine is a major rate-limiting step in the forward direction. The unusual substrate inhibition by ammonia in the reverse reaction at high pH also suggests that iminopyruvate is a substrate with a very low K_m, but with a low maximum velocity because of the necessity for becoming protonated prior to reduction. In this paper, we present pH profiles for the various kinetic constants, including isotope effects with deuterated substrates, and use these data to determine the chemical mechanism. We will show that the reaction chemically resembles the glutamate dehydrogenase mechanism, but shows opposite sterochemistry for both the 4 position of the nucleotide and the intermediate carbinolamine, and has different groups acting as acid–base catalysts. Reaction of the monoanion, rather than the zwitterionic form of the amino acid, and bonding of the 1-carboxyl to a positively charged group on the enzyme are other differences from glutamate dehydrogenase.

Materials and Methods

Materials. Crystalline *Bacillus subtilis* alanine dehydrogenase from Sigma was prepared for kinetic studies as described in the previous paper. L-Alanine, D-alanine, pyruvate (Na^+ salt), L- and D-lactate (Li^+ salts), and *N*-methylglycine hydrochloride were from Sigma. Oxamate (Na^+ salt), L-2-amino-1-propanol, and tetramethylammonium chloride were from Aldrich, while NAD (free acid) and NADH (disodium salt) were from Boehringer Mannheim. DL-Serine-*2,3,3,*-d_3 was from Merck. L-Alanine-*2,3,3,3*-d_4 (97.3% label at C2) was prepared by the method of Cooper (1976). All other chemicals or biochemicals were from Sigma and were used without further purification.

Assay Conditions. General assay conditions are described in the previous paper. Buffers normally used for pH profiles were 2-(*N*-morpholino)ethanesulfonic acid (Mes)[1] (pH 5–6),

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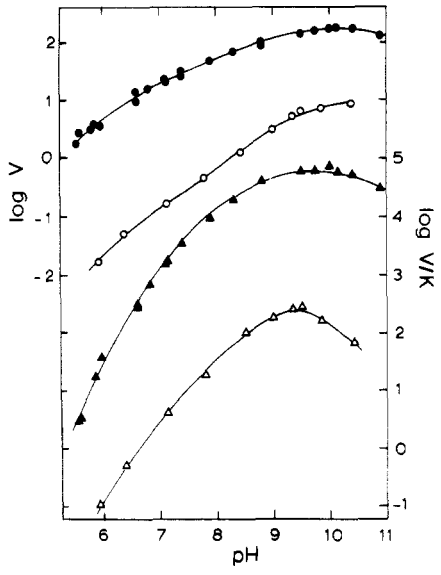


FIGURE 1: pH profiles for the forward direction with L-alanine or L-serine as the variable substrate. (●) L-Alanine, log (V/E_t); (○) L-serine, log (V/E_t); (▲) L-alanine, log [$V/(KE_t)$]; (△) L-serine, log [$V/(KE_t)$]. The units of V/E_t are s^{-1}, and those of $V/(KE_t)$ are M^{-1} s^{-1}.

Mops (pH 6–7), Tes (pH 7–8), glycylglycine (pH 8–9), Caps, and Ches (pH 9–10). Overlaps were used in all cases, and checks were made to ensure that no buffers were inhibitory. For certain experiments in the presence of dimethyl sulfoxide, the cationic acid buffers were replaced with a neutral acid buffer consisting of 50 mM cacodylate, 40 mM phosphate, and 10 mM pyrophosphate. All solutions were adjusted to pH with KOH.

The reactions were followed by using a Beckman DU monochromator with deuterium lamp, a Gilford optical density converter, and a 10-mV recorder with adjustable zero and multispeed drive. Full-scale sensitivity of 0.025–0.10 OD and a chart speed of 0.2–2 in./min were used. Below pH 9, reactions were started by the addition of enzyme (5–20 μL) via an adder mixer to a 3.0-mL mixture containing all reaction components which had been preequilibrated at 25 °C in a 1.0-cm cuvette. At pH 9 or above, NAD, preequilibrated at 25 °C, was added immediately before enzyme was added to start the reaction. This procedure minimized inhibition by breakdown products of NAD formed in basic solution. Similarly, NADH was added just before enzyme in the back-reaction at or below pH 7, and the observed rate was corrected for the background rate of NADH breakdown. Enzyme stock solution activities were standardized by comparing rates with 50 mM Caps, pH 10, 15 mM L-alanine, and 2 mM NAD (added last before enzyme).

Below pH 6.5, the reaction time course showed pronounced curvature with the velocity gradually slowing down to a steady-state rate. This effect was not due to approach to equilibrium and could be alleviated by starting the reaction with a stock enzyme solution made up in buffer (containing EDTA, dithiothreitol, and bovine serum albumin as above) of the composition and at the pH of the reaction to be measured.

Data Processing. Reciprocal initial velocities were plotted vs. reciprocal substrate concentrations, and the experimental data were fitted to eq 1–14 by the least-squares method, assuming equal variances for the v or log Y values (Wilkinson, 1961), and using a digital computer and the Fortran programs of Cleland (1979). The points in the figures are the experimentally determined values, while the curves are calculated from fits of these data to the appropriate equation. Linear double-reciprocal plots were fitted to eq 1, and eq 2 was used when substrate inhibition was observed. Equations 3 and 4 describe intersecting initial velocity patterns, with the latter including uncompetitive substrate inhibition by B. Equation 5 is for an equilibrium-ordered initial velocity pattern. Data for kinetic deuterium isotope effects when one substrate was varied were fitted to eq 6 where F_i is the fraction of deuterium in the substrate, $E_{V/K}$ and E_V are the isotope effects minus 1 on V/K and V, respectively, and A is the concentration of the deuterated or unlabeled substrate that was used. Data conforming to linear competitive and linear noncompetitive inhibition were fitted to eq 7 and 8.

$$v = VA/(K + A) \quad (1)$$

$$v = VA/(K + A + A^2/K_I) \quad (2)$$

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (3)$$

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB + AB^2/K_I) \quad (4)$$

$$v = VAB/(K_{ia}K_b + K_bA + AB) \quad (5)$$

$$v = VA/[K(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (6)$$

$$v = VA/[K(1 + I/K_{is}) + A] \quad (7)$$

$$v = VA/[K(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (8)$$

$$\log Y = \log [C/(1 + H/K_1)] \quad (9)$$

$$\log Y = \log [C/(1 + K_2/H)] \quad (10)$$

$$\log Y = \log [C/(1 + H/K_1 + K_2/H)] \quad (11)$$

$$\log Y = \log \{C/[1 + H/K_2 + H^2/(K_1K_2) + K_3/H]\} \quad (12)$$

$$\log Y = \log \{C(1 + H/K_2)/[1 + (H/K_3)(1 + H/K_1)]\} \quad (13)$$

$$\log Y = \log \{[YL + YH(K_1/H)]/(1 + K_1/H)\} \quad (14)$$

The pH profiles were fitted to eq 9–14, which describe log Y vs. pH curves which are level above pK_1 but decrease with unit slope below pK_1 (eq 9); are level below pK_2 but decrease with −1 slope above pK_2 (eq 10); decrease both below pK_1 and above pK_2 (eq 11); decrease below pK_2 with unit slope and then below pK_1 with a slope of 2, and decrease above pK_3 (eq 12); decrease at low pH but show a hollow (pK_3 > pK_2 > pK_1; eq 13); and show two plateaus with pK_1 being the point where Y is the average of the two plateau values (eq 14). In eq 9–14, H is [H^+], and C is the pH-independent value of Y at the optimal state of protonation.

Results

pH Profiles of the Kinetic Constants. Initial velocity patterns in the forward reaction were determined over the pH range 5.5–10, and the data were fitted to eq 3, or patterns were determined in the pH range 6.5–10.5, where L-alanine shows

[1] Abbreviations used: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Caps, 3-(*N*-cyclohexylamino)-propanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid. The isotope effect nomenclature is that of Northrop (1977) in which a leading superscript indicates "deuterium isotope effect on". Thus, ^DV, $^D(V/K)$, ^Dk, and $^DK_{eq}$ are deuterium isotope effects (that is, values for the hydrogen-containing molecule divided by values for the deuterated molecule) on the respective parameters. The term "commitment" is used for the ratio of the rate constant for the isotope-sensitive bond-breaking step and the net rate constant for dissociation of the substrate (for the forward commitment, c_f) or of the first product (for the reverse commitment, c_r).

Table I: pK Values from pH Profiles of the Kinetic Parameters for Alanine Dehydrogenase

parameter	eq fitted	pK_1	pK_2	pK_3	C^a
			Forward Reaction		
log ($V/K_{alanine}$)	12	6.72 ± 0.06	8.65 ± 0.03	10.8 ± 0.1	100 ± 5 mM^{-1} s^{-1}
log (V/K_{serine})	12	6.2 ± 0.1	9.28 ± 0.04	9.47 ± 0.05	0.52 ± 0.05 mM^{-1} s^{-1}
log ($V_{alanine}$)	13	6.5 ± 0.1	7.6 ± 0.2	8.5 ± 0.1	185 ± 7 s^{-1}
log (V_{serine})	13	6.9 ± 0.2	7.6 ± 0.2	9.3 ± 0.1	8.5 ± 0.3 s^{-1}
log (V/K_{NAD})	9	9.0 ± 0.2			1500 ± 150 mM^{-1} s^{-1}
p$K_{i\,NAD}$	11	6.1 ± 0.1	10.0 ± 0.1		0.21 ± 0.01 mM
p$K_{is\ sarcosine}$	11	9.3 ± 0.1	10.7 ± 0.2		2.6 ± 0.2 mM
p$K_{is\ oxamate}$	14	7.6 ± 0.1			3.4 ± 0.2; 103 ± 9 mM
p$K_{is\ D\text{-}lactate}$	14	7.02 ± 0.05			22 ± 1; 434 ± 17 mM
p$K_{is\ L\text{-}lactate}$	14	9.0 ± 0.1			8.2 ± 0.2; 43 mM
$^D(V/K_{alanine})$	14	8.2 ± 0.1			1.97 ± 0.02; 1.48 ± 0.02
$^D(V_{alanine})$	14	8.4 ± 0.2			1.35 ± 0.02; 1.06 ± 0.02
			Reverse Reaction		
log ($V/K_{pyruvate}$)	10	7.9 ± 0.1			4460 ± 320 mM^{-1} s^{-1}
log ($V/K_{ammonia}$)	9[b]	8.9 ± 0.1			100 ± 14 mM^{-1} s^{-1}
	9[c]	8.8 ± 0.1			66 ± 2 mM^{-1} s^{-1}
p$K_{i\ pyruvate}$	10	7.1 ± 0.1			38 ± 2 μM
p$K_{is\ oxamate}$	10	7.3 ± 0.1			0.98 ± 0.05 mM
p$K_{I\ alanine}$[d]	11	7.5 ± 0.1	9.5 ± 0.1		34 ± 2 mM

[a] For pK_i profiles, eq 9–14 were fitted with $Y = 1/K_i$. The value tabulated for C, however, is K_i, not $1/K_i$. For fits to eq 14, the value of K_i or of the isotope effect at low pH is given first, and the value at high pH second. The pK given is that at which pK_i or the isotope effect begins to fall. [b] From initial velocity patterns in which both pyruvate and ammonia concentrations were varied. [c] From apparent V at low ammonia concentration, variable pyruvate concentration, and saturating NADH concentration. [d] Substrate inhibition constant.

uncompetitive substrate inhibition, and the data were fitted to eq 4. Since above pH 10 the value for $K_{i\,NAD}$ increases (Figure 3), while K_{NAD} remains constant, at pH 10.9 the reaction appears equilibrium ordered with NAD adding first, and the data were fitted to eq 5. In addition, at a number of pH values, L-alanine concentration was varied at saturating (2 mM) NAD concentration, and the resulting data were fitted to eq 1 or 2. The kinetics with L-serine as the variable substrate at 2 mM NAD were also determined from pH 5.95 to 10.4. The pH profiles of V and V/K for both alanine and serine are shown in Figure 1, and the pKs from fits to these profiles are in Table I as well as for profiles for V/K_{NAD} and p$K_{i\,NAD}$.

To gain information on the nature of the groups reponsible for the pKs seen, we redetermined the V/K_{serine} profile in cationic acid buffers from pH 8 to 10.4 in the presence of 30% dimethyl sulfoxide. This level of solvent causes reactants to bind much more tightly and reduces K_{serine} from 20 mM at the pH optimum to 1.5 mM with only a small change in maximum velocity. The V/K_{serine} profile was fitted to eq 11 and gave identical pK values of 9.30 ± 0.02. Although serine, unlike alanine, did not give substrate inhibition in the absence of dimethyl sulfoxide, in the presence of 30% dimethyl sulfoxide substrate inhibition was observed in the pH range 9–9.85 with a K_i from fits to eq 2 of 30–40 mM.

For the reverse reaction, initial velocity patterns were measured at pH 6.6, 8.5, and 9.35 by varying pyruvate concentration at various fixed levels of ammonia concentration (below the substrate inhibition level at pH 9.35) and a saturating level of NADH concentration (200 μM), and the data were fitted to eq 3. The dissociation constant for pyruvate was also determined at pH 5.5–10 as its apparent Michaelis constant at low ammonia (2 mM) and saturating NADH (80 μM) concentrations; the data were fitted to eq 1. The apparent V under these conditions is $V/(1 + K_{ammonia}/[ammonia])$, and since [ammonia] was much less than $K_{ammonia}$, this expression is $(V/K_{ammonia})[ammonia]$ and thus provides an additional measure of $V/K_{ammonia}$.

The pH profiles for V, $V/K_{pyruvate}$, and $V/K_{ammonia}$ in the reverse reaction are shown in Figure 2, and the pKs are in

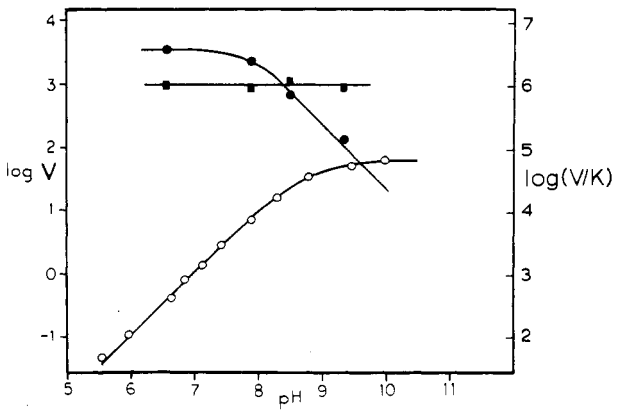


FIGURE 2: pH profiles in the reverse direction for the reductive amination of pyruvate. (■) V/E_t; (●) $V/(K_{pyruvate}E_t)$; (○) $V/(K_{NH_3}E_t)$.

Table I. While V is constant at 1000 ± 90 s^{-1} from pH 6.6 to 9.35, $V/K_{pyruvate}$ and $V/K_{ammonia}$ decrease a factor of 10 per pH unit at high and low pH, respectively.

To determine the nature of the group responsible for the pK in the p$K_{i\,pyruvate}$ profile, we repeated the experiments where pyruvate concentration was varied at low ammonia and saturating NADH concentrations at 16, 25, and 37.5 °C, giving pK values from fits to eq 10 of 7.25 ± 0.02, 7.08 ± 0.06, and 6.74 ± 0.04, respectively. These values correspond to a ΔH_{ion} for the group of 10 ± 1 kcal/mol, and from the temperature variation in the pK_i value below the pK, ΔH for $K_{i\,pyruvate}$ was 5 kcal/mol (the value ranged from 18 μM at 16 °C to 32 μM at 37 °C).

In addition, the effect of 30% dimethyl sulfoxide on the p$K_{i\,pyruvate}$ profile was determined in both cationic and neutral acid buffers. Since the pH values were measured before addition of dimethyl sulfoxide, one expects the pKs (and thus the pHs) of the neutral acid buffers to be elevated by the solvent but that there would be little change for the cationic acid buffers. As noted above, dimethyl sulfoxide enhances the binding of the reactants. As a result, the profiles no longer fitted eq 10, but p$K_{i\,pyruvate}$ leveled out at high pH at a new plateau value, and thus the profiles were fitted to eq 14. The

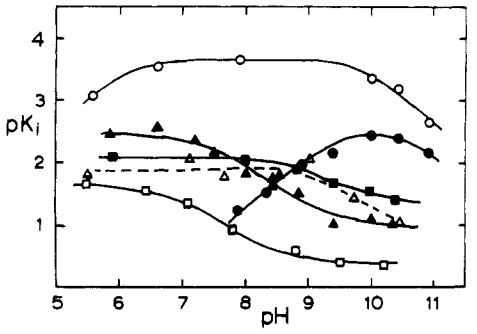


FIGURE 3: pK_i profiles in the forward direction. (○) p$K_{i\,NAD}$ from initial velocity patterns. For the other inhibitors, L-alanine was the varied substrate, and pK_{is} values from fits to eq 7 or 8 at saturating NAD concentration are plotted: (●) Sarcosine; (■) L-lactate; (□) D-lactate; (▲) oxamate; (△) propionate (with dashed line).

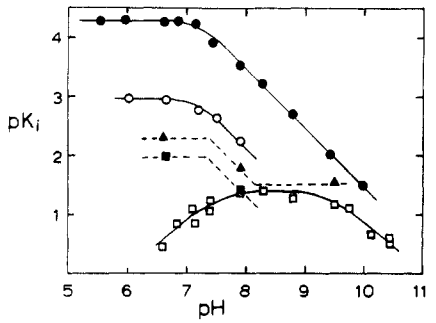


FIGURE 4: pK_i profiles in the reverse direction. (●) p$K_{i\,pyruvate}$ from initial velocity patterns as described in the text. (□) pK_I for L-alanine, where K_I is the substrate inhibition constant. For the other inhibitors, pyruvate was the varied substrate, and pK_{is} values from fits to eq 7 or 8 at saturating NADH concentration and fixed ammonia concentration are plotted: (○) Oxamate; (▲) D-lactate; (■) L-lactate.

pK of the group controlling pyruvate binding was 6.37 ± 0.07 in the cationic acid buffer, and the low-pH $K_{i\,pyruvate}$ value was 4.5 ± 0.5 μM, while the high-pH value was 870 ± 140 μM. With neutral acid buffers, the pK was 5.0 ± 0.2, the low-pH $K_{i\,pyruvate}$ value was 1.0 ± 0.4 μM, and the high-pH value was 850 ± 120 μM.

Inhibition Profiles. The pK_{is} profiles for a number of molecules tested as dead-end inhibitors vs. L-alanine or pyruvate are shown in Figure 3 for the forward reaction and in Figure 4 for the reverse reaction, and the pKs and limiting K_{is} values are in Table I. In the forward reaction, inhibition patterns vs. L-alanine were determined at 2 mM NAD, and the apparent K_{is} values from fits to eq 7 or 8 were corrected to saturation with NAD to give the true dissociation constants. At pH 7.9, L-2-aminopropanol showed a K_{is} value vs. L-alanine of over 250 mM.

In the reverse reaction, the oxamate pK_i profile was measured at 50 mM ammonia and 80 μM NADH. The limited data for L- and D-lactate are similar to those for oxamate in that the K_i value seems to increase with a pK just above 7, but it is clear that for D-lactate the profile levels out at high pH with a K_{is} value of 28 ± 3 mM seen at pH 9.5. The oxamate and L-lactate profiles probably also level out at high pH since propionate shows a K_{is} value vs. pyruvate of 80 mM at both pH 6 and 9, and as noted above, the p$K_{i\,pyruvate}$ profile levels out at high pH in the presence of 30% dimethyl sulfoxide. Also, oxamate shows a K_{is} of 90 mM vs. NADH at pH 9.5 (see the previous paper).

Isotope Effects.[1] Kinetic deuterium isotope effects were measured by direct comparison of the initial velocities obtained with 2 mM NAD (10 mM below pH 6.5) and either unlabeled L-alanine or L-alanine-*2,3,3,3-d*$_4$. Both ^DV and $^D(V/K_{alanine})$

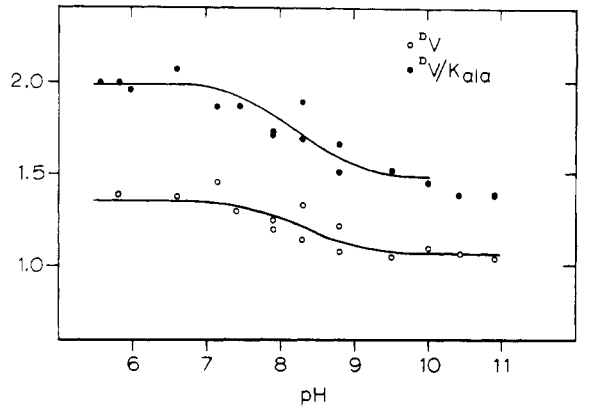


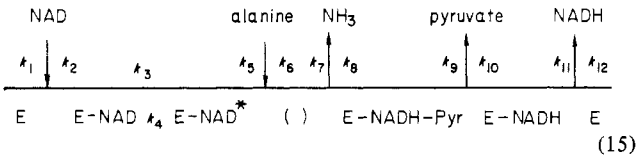
FIGURE 5: pH variation of deuterium isotope effects when L-alanine-d_4 or unlabeled L-alanine were varied at saturating NAD concentration.

(Figure 5) appear to decrease from a constant value at low pH to a lower constant value at high pH, and the pKs and limiting values are in Table I.

Isotope effects were also determined with the alternate substrate L-serine by direct comparison of reciprocal plots for L-serine and DL-serine-d_3 at 2 mM NAD. Since D-serine, like D-alanine and D-lactate, will certainly have some affinity for E–NAD, the apparent values of ^DV are increased by the degree to which D-serine acts as an inhibitor vs. L-serine and thus decreases the apparent V (Grimshaw & Cleland, 1980). However, a competitive inhibitor present in the variable substrate has no effect on V/K, and thus the value of $^D(V/K_{serine})$ obtained by fitting the data to eq 6 should be valid. At pH 7.1, 9.5, and 10.2, $^D(V/K_{serine})$ was 2.06 ± 0.13, 2.18 ± 0.19, and 1.71 ± 0.03, and at pH 9.5, the value in 40% D_2O was 2.09 ± 0.17, and the value in 30% dimethyl sulfoxide was 1.66 ± 0.09. The apparent values of ^DV were 2.7, 3.7, and 2.5 at the three pH values, suggesting that D-serine was in fact causing inhibition and that the true ^DV values may have been similar to the $^D(V/K_{serine})$ ones, as one would expect for a slow alternate substrate.

Discussion

Evidence for the Stickiness of L-*Alanine.* The experiments reported in the previous paper demonstrate that alanine dehydrogenase has an ordered kinetic mechanism with isomerization of the E–NAD complex:



(15)

Several lines of evidence show that L-alanine is sticky at the pH optimum of the forward reaction (that is, reacts to give products as fast or faster than it is released from the enzyme). In terms of mechanism 15, this means that k_7 equals or exceeds k_6 at high pH. First, $^D(V/K_{alanine})$ decreases from 1.97 at low pH to 1.48 at high pH, with a pK of 8.2. The expected isotope effect at the pH optimum in mechanism 15 is

$$^D(V/K_{alanine}) = \frac{^Dk_7 + k_7/k_6}{1 + k_7/k_6} \qquad (16)$$

but Cook & Cleland (1981a) have shown that below the pH optimum in such a mechanism $^D(V/K_{alanine})$ should become equal to $^Dk_7$. With the fitted parameters from eq 14, eq 16 predicts $k_7/k_6 = 1.0$. If the high pH limit is taken as 1.4 (the value actually seen above pH 10.5), $k_7/k_6 = 1.5$.

$^{D}k_7$ is not the intrinsic isotope effect on the hydride transfer step but will be smaller as the result of internal commitments (c_f and c_r in eq 18 below[1]). If part of mechanism 15 is made more complex (we will discuss the chemical mechanism in more detail below)

$$\begin{array}{l} \text{E NAD}^*\text{- alanine} \underset{k_{16}}{\overset{k_{15}}{\rightleftharpoons}} \text{E-NAD}^*\text{- alanine}^* \underset{k_{18}}{\overset{k_{17}}{\rightleftharpoons}} \\ \text{E-NADH-iminopyruvate} \underset{k_{20}}{\overset{k_{19}}{\rightleftharpoons}} \text{E-NADH-carbinolamine} \xrightarrow{k_{21}} \\ \qquad\qquad \text{E-NADH-pyruvate} + \text{NH}_3 \end{array} \quad (17)$$

where k_{17} and k_{18} are now the isotope sensitive steps:

$$^{D}k_7 = \frac{^{D}k_{17} + c_f + c_r(^{D}K_{eq})}{1 + c_f + c_r} \quad (18)$$

where

$$c_f = k_{17}/k_{16} \quad (19)$$

$$c_r = (k_{18}/k_{19})(1 + k_{20}/k_{21}) \quad (20)$$

$$^{D}K_{eq} = 1.15 = {}^{D}k_{17}/{}^{D}k_{18} \quad (21)$$

If either c_f or c_r is finite, $^{D}k_7$ will be less than $^{D}k_{17}$, which will probably be similar to the value of 6 observed for liver alcohol dehydrogenase by Cook & Cleland (1981b). Because the partition ratios which make up c_f and c_r are usually pH independent in enzymatic mechanisms, $^{D}k_7$ is not expected to vary with pH. We have no way of knowing which partition ratio reduces $^{D}k_{17}$ to the observed value of $^{D}k_7$ of about 2, but whichever it is, it has a similar value for L-serine, which shows a $^{D}(V/K_{serine})$ value of about 2 which is pH independent, as expected for a slow, and thus nonsticky, substrate. Since the internal commitments are the same in 40% D_2O as in H_2O, and differ only slightly, if at all, in 30% dimethyl sulfoxide, it seems unlikely that steps involving proton transfers or addition of water (such as k_{19}) are involved, unless their rates are determined by the rates of the accompanying conformation changes in the enzyme (a distinct possibility). The reason for the slow reaction of L-serine is probably that a large part of the binding of serine to E–NAD* is nonproductive, so that k_{15} is smaller for L-serine than for L-alanine. Rate constants k_{16}–k_{21} could then be essentially unchanged.[2]

The second piece of evidence for the stickiness of L-alanine is the shape of the pH profile of $V/K_{alanine}$ compared to that of L-serine. When a substrate is not sticky, its V/K profile is sensitive to the state of protonation of those groups on the enzyme and substrate which must be correctly protonated for binding and reaction to occur, and the pKs are observed at their correct values. The pK_i profiles of competitive inhibitors also show the correct pK values of groups whose protonation state affects binding of the inhibitor. On the other hand, Cleland (1977) has shown that in the V/K profile of a sticky substrate, the pK values are displaced outward on the profile by a factor which for L-alanine in mechanism 15 will be log $(1 + k_7/k_6)$.[3]

The $V/K_{alanine}$ profiles shows 2 pKs of 6.7 and 8.7 on the acid side, and a pK of 10.8 on the basic side, while the corresponding pKs for the L-serine profile are 6.2, 9.3, and 9.5. The pKs of L-alanine and L-serine are 9.7 and 9.2, respectively, and certainly represent one of the high pKs in the profiles. When two pKs occur close together, as is the case here at high pH, the least-squares fits determine rather accurately the mean value of the pKs, but ΔpK is quite sensitive to the shape of the curve at the maximum, so that the exact values are more uncertain. If the substrate pK and one from the enzyme are involved, however, the average of the two pKs should be higher for L-alanine by half the difference in pKs of L-alanine and L-serine. The average is actually 0.35 higher, compared to an expected value of 0.25. If the true pKs of the amino acids are compared with the averages of the two observed pKs, the pK of the enzyme group calculates to be 9.6–9.8, with the lower value from the serine profile being better determined.

While the substrate and enzyme pKs are seen at essentially their correct values in the V/K_{serine} profile, they appear to be displaced outward in the $V/K_{alanine}$ profile by nearly a pH unit. If we take 0.9 pH unit as the displacement on the low pH side (which is better defined than on the high pH side), k_7/k_6 = 7. This is higher than the estimate from the isotope effects (1.0–1.5), but in agreement with the expected ratio of k_7/k_6 of 5.9 if we assume that k_6 is pH independent (it largely determines V in the reverse reaction, which is not pH dependent) and extrapolate our value of k_7 at pH 7.9 (see the previous paper) to above the pK of 9.6 for the enzyme. If the pK of the enzyme is only 9.0 in the central complex, however, k_7/k_6 would be 1.5 at the pH optimum. In view of the sensitivity of both the isotope effect and pH profile calculations to small perturbations in the data, we can simply conclude that L-alanine is somewhat sticky, with the pH variation of both $^{D}(V/K_{alanine})$ and log $(V/K_{alanine})$ showing clearly the existence of the stickiness.

Interpretation of pH Profiles. Three groups are visible in the various pH profiles reported here. First, a group with a pK around 7 must be unprotonated for reaction of L-alanine or L-serine and protonated for binding and reaction of pyruvate (although pyruvate does bind weakly to the unprotonated form of E–NADH in the presence of 30% dimethyl sulfoxide). The pK is apparently the same in E–NAD and E–NADH, and thus, the group is not located sufficiently close to be perturbed by the positive charge on N1 of NAD. The group is a cationic acid, since the apparent pK is much lower after addition of 30% dimethyl sulfoxide to neutral acid buffers, where the true pH will be elevated by the solvent, than after addition to cationic acid buffers, where the pH will not change appreciably. The ΔH_{ion} of 10 kcal/mol is consistent with a histidine residue, but a lysine whose pK is perturbed downward by neighboring positive charges cannot be ruled out. This group is presumably the acid–base catalyst for the chemical reaction, and we will discuss its role further below.

Note that in E–NAD, oxamate and D-lactate bind tighter when this group is protonated, while L-lactate and propionate binding is not sensitive to the state of protonation of this group. However, in E–NADH, oxamate and both D- and L-lactate bind well only with this group protonated, while propionate binding again is insensitive. We interpret these data to mean that in E–NAD this group can hydrogen bond to a planar molecule such as oxamate or one with a D-hydroxy group, but not to one with an L-hydroxy group (or presumably an L-amino group). In E–NADH, however, it can hydrogen bond to molecules with either D- or L-hydroxy groups. We will comment more on these differences in site geometry below.

The second group in the pH profiles is seen only in the forward direction and has a pK of 9.0–9.6. This group is also a cationic acid (probably a lysine), since the average of its pK

[2] Note that k_{15} does not affect the internal commitments in eq 19 and 20 but only the external commitment, or stickiness (that is, the ratio of k_6 to k_{15}).

[3] However, if a second group is involved on one side of the pH profile (that is, the profile changes from a slope of 1 or −1 to a slope of 2 or −2), its pK will not be displaced. This will be the case here for the pK seen around 6.5 in the V/K profiles.

and that of L-serine is not elevated by 30% dimethyl sulfoxide in cationic acid buffers. This pK is not seen in the pH profiles of the reverse reaction, and thus the pK appears to be sensitive to the plus charge at N1 of NAD, and to be well above 10 in E–NADH, as the pK of a lysine normally will be in the absence of positive charges in the near vicinity.

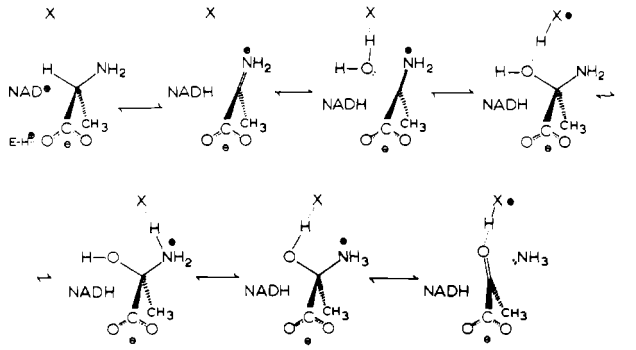
This group must be protonated for tight binding of L-lactate or propionate to E–NAD, and we believe it also must be protonated for binding of sarcosine. The optimal binding of sarcosine between this pK and that of its methylamino group results from the requirement for reverse protonation (that is, for the group with pK 9 to be protonated, and for the methylamino group of sarcosine to be unprotonated). We also interpret the V/K profiles for the substrates to indicate that this group must be protonated and the amino group of the substrate unprotonated, and will comment on this point further below. It seems likely that this group does not play a catalytic role, but rather controls the conformation of the enzyme in some way.

Finally, the pK of the amino group shows up in the V/K profiles for substrates and the pK_i profiles of sarcosine and of L-alanine as a substrate inhibitor. As noted in the previous paper, L-alanine appears to bind as a substrate inhibitor in its zwitterionic form, but the binding of sarcosine, and of L-alanine and L-serine in a protonation state that allows them to act as substrates, is probably as the monoanion with an unprotonated methylamino or amino group.

Explanation of the Shape of the V Profiles. While the V/K profiles for L-alanine for L-serine have shapes that indicate two pKs on the acid side, and one on the basic side, the V profiles for both substrates show a distinct hollow on the acid pH side and approach a slope of 1 rather than 2 at low pH. In addition, any decrease on the basic pH side has a pK of at least 11. Cleland (1977) has shown that a hollow of this sort can result when the substrate is sticky, but since the hollow is seen at the same place (after the initial drop, the curve in both profiles levels out with a pK of 7.6, and then drops again with a pK of about 6.6) in the profiles of L-alanine, which is sticky, and L-serine, which is not, this cannot be the explanation. Since a similar hollow is not seen in the V/K profiles,[4] we must conclude that some conformation change is occurring in the protein which is elevating both K_m and V, but not altering their ratio. Thus, $K_{alanine}$ rises from a nearly pH-independent value of 2 mM above pH 8 to over 100 mM below pH 6, while a similar increase is seen in K_{serine} from about 20 mM at pH 9 to almost 200 mM at pH 6.

It should be noted that the pK values at which the V profiles first begin to drop as the pH is lowered reflect the different rate-limiting steps for the two substrates. With L-serine, where the chemical reaction of the E–NAD*–serine complex is rate limiting, we see a pK of 9.3, corresponding closely to that of the substrate, but the profile has not started to drop on the high side at pH 10.4. Thus, L-serine binds preferentially to enzyme with the group of pK 9–9.6 protonated, so that this pK is displaced to at least 11 in the V profile, but the affinity of the enzyme for L-serine is the same for the zwitterion and monoanion forms because the substrate pK is not altered in the profile. With L-alanine, NADH release is largely rate limiting at the pH optimum, and thus the substrate pK is displaced downward to 8.5. This is approximately the pK predicted from the rate constants we have derived in the previous paper, if the E–NAD* complex has equal affinity for the zwitterion and monoanion forms of L-alanine.

Scheme I



Chemical Mechanism of Alanine Dehydrogenase. In Scheme I is shown a chemical mechanism which is consistent with all of the data reported here. L-Alanine is shown reacting as a monoanion, as deduced from the pH profiles. The carboxyl is shown hydrogen bonded to lysine or arginine on the enzyme, since L-2-aminopropanol is bound more than 2 orders of magnitude more weakly than L-alanine. This mode of binding can be contrasted with that shown by glutamate dehydrogenase, where C1 of glutamate is not bonded to positively charged groups, but C5 is hydrogen bonded to a lysine (Rife & Cleland, 1980a,b). A further difference with the glutamate dehydrogenase mechanism is that glutamate appears to bind with its amino group protonated and hydrogen bonded to a group with a pK about 5. This group is thought to remove the proton from the amino group to permit dehydrogenation (Rife & Cleland, 1980a,b). With alanine dehydrogenase, however, both of the groups seen in the pH profiles with pKs of 7 and 9–9.6 are cationic acids. Thus, if one of these groups were to remove a proton from the protonated amino group of L-alanine, it would then be positively charged, and dehydrogenation to produce iminopyruvate would result in two neighboring positive charges in the active site.

The X group shown in Scheme I is a cationic acid with a pK of 7 (probably histidine). It is shown in the E–NAD*–alanine complex in a position which allows hydrogen bonding to planar molecules such as oxamate, or molecules with D-amino or -hydroxy groups, but not to L-amino or -hydroxy groups. This location is reasonable, since X plays no role in the initial dehydrogenation, and the enzyme shows equal affinity for the zwitterion and monoanion forms of L-alanine (if X could hydrogen bond to the amino group, nonproductive binding of the zwitterion would be increased). This hypothesis also offers a reasonable explanation for the proposed nonproductive binding to E–NAD (the initially formed complex prior to isomerization to E–NAD*) of L-alanine as the zwitterion with X not protonated. This is the expected binding specificity if X is in a position in E–NAD in which it can hydrogen bond to the L-amino group of alanine (that is, a position similar to the one it has in E–NADH). However, in this location, X is prevented by the same strong interaction between the protonated amino group of L-alanine and unprotonated X from moving to the position it must occupy in E–NAD* for the chemical reaction to occur. Thus, binding of L-alanine to E–NAD* not only is productive but also the preference for the zwitterionic over the monoanionic form is lost, and both bind equally well.

After dehydrogenation to give protonated iminopyruvate, a conformation change must occur which allows access of a water molecule to the active site in place of C4 of NADH.

[4] There is a nonrandom distribution of residuals in the least-squares fit to the $V/K_{alanine}$ profile, with points from pH 6 to 7.5 lying above the fitted curve and ones from pH 7.9 to 8.3 lying below it, but the maximum deviations are less than the average residual least square, so the hollow, if it is real, is very shallow.

This water molecule may have been trapped in the active site by the binding of L-alanine, since the experiments with substrate-inhibiting levels of ammonia reported in the previous paper suggest that once unprotonated iminopyruvate combines with E–NADH the enzyme assumes a closed conformation in which proton access to the iminopyruvate is severely restricted. The conformation change which brings water into a position to react with iminopyruvate presumably alters the position of X as shown so that it now is capable of hydrogen bonding to groups in both the D and L positions. X acts as a general base to facilitate reaction of water with iminopyruvate to give a carbinolamine, which we show with L-amino and D-hydroxy groups, since D-lactate binds almost 3-fold better than L-lactate, while L-alanine binds 3-fold better than D-alanine to E–NADH. By contrast, glutamate dehydrogenase binds L-2-hydroxyglutarate 3-fold better than the D isomer (Rife & Cleland, 1980a,b), and thus the stereochemistry of the carbinolamine may be the opposite of that with alanine dehydrogenase. A ramification of this difference is that with glutamate dehydrogenase it is ammonia, while with alanine dehydrogenase it is water, that attacks the planar keto or imino molecule on the same side as the nucleotide reacts.

The initially formed carbinolamine will have an unprotonated amino group, but transfer of a proton from protonated X gives a protonated amino group which can leave as ammonia while X acts as a general base to accept the proton from the hydroxy gorup of the carbinolamine. The V/K profile for ammonia shows clearly that free ammonia, and not NH_4^+, is the substrate. This is also true for glutamate dehydrogenase (Rife & Cleland, 1980a,b).

Alanine dehydrogenase shows A-side specificity for the pyridine nucleotide (Alizade et al., 1975), which is similar to lactate dehydrogenase but the opposite of glutamate dehydrogenase. Lactate dehydrogenase also has a histidine as its acid–base catalyst, while glutamate dehydrogenase appears to employ lysine (Rife & Cleland, 1980a,b). It appears that alanine dehydrogenase may be a modified lactate dehydrogenase in which NADH is initially too far from the carbonyl carbon to permit reduction of pyruvate, but where there is sufficient room on the opposite side of pyruvate for ammonia to attack the carbonyl carbon. Only after water is eliminated from the carbinolamine does NADH approach closely enough to react with iminopyruvate. In the forward reaction, the location of X so that it cannot hydrogen bond to an L-hydroxy or L-amino group prevents it from acting as an acid–base catalyst and thus giving lactate dehydrogenase activity. The initial dehydrogenation step for an amino acid, however, does not require acid–base catalysis so that L-alanine is oxidized. Thus, although alanine and glutamate dehydrogenases will both catalyze the same reaction (glutamate dehydrogenase has a very high $K_{alanine}$, however), and both appear to involve imine and carbinolamine intermediates, they have different stereochemistry and use different acid–base catalysts, and apparently do not have a common evolutionary origin.

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