Kinetic Mechanism of *Bacillus subtilis* L-Alanine Dehydrogenase†

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ABSTRACT: L-Alanine dehydrogenase from *Bacillus subtilis* has a predominately ordered kinetic mechanism in which NAD adds before L-alanine, and ammonia, pyruvate, and NADH are released in that order. When pyruvate is varied at pH 9.35, levels of ammonia above 50 mM cause uncompetitive substrate inhibition and cause the slope replot to go through the origin. This pattern suggests that iminopyruvate (2% of pyruvate at this pH with 150 mM ammonia) can combine with E–NADH much more tightly than pyruvate does but reacts much more slowly because uptake of the required proton from solution is hindered. Isomerization of the initially formed E–NAD complex to a form which can productively bind L-alanine is the slowest step in the forward direction at pH 7.9, and substrate inhibition by L-alanine largely results from combination of the zwitterion in a nonproductive fashion with this initial E–NAD complex, with the result that the isomerization is prevented. All bimolecular rate constants approach diffusion-limited values at optimal states of protonation of enzyme and substrates except that for ammonia, suggesting that ammonia does not form a complex with E–NADH–pyruvate but reacts directly with it to give a bound carbinolamine.

Bacillus subtilis L-alanine dehydrogenase, isolated in crystalline form by Yoshida & Freese (1964, 1970), catalyzes the reversible oxidative deamination of L-alanine to pyruvate and ammonia:

$$\text{L-alanine} + \text{NAD} + \text{H}_2\text{O} \rightleftharpoons \text{pyruvate} + \text{NH}_4^+ + \text{NADH} + \text{H}^+ \quad (1)$$

The reported pH optimum of 10–10.5 in the forward direction suggested that the reaction might be similar to the monocarboxylic amino acid deamination reaction catalyzed by glutamate dehydrogenase at high pH. However, in contrast to the B-side (*pro-S*) specificity with respect to NAD observed with both glutamate (Levy & Vennesland, 1957) and leucine (Oshima et al., 1978) dehydrogenases, alanine dehydrogenase has been shown to be A-side (*pro-R*) specific by Alizade et al. (1975). The lack of allosteric effects, and the stability of the enzyme over the pH range 5–11, makes reaction 1 amenable to a detailed kinetic investigation over a wide pH range. The present work was undertaken to determine the kinetic and chemical mechanisms of the alanine dehydrogenase reaction and contrast them with those of glutamate dehydrogenase, which have recently been determined in this laboratory by Rife & Cleland (1980a,b). In this paper, we present evidence that the kinetic mechanism is largely an ordered one, that isomerization of E–NAD to a form capable of combining productively with alanine is a major rate-limiting step in the mechanism, and that nonproductive combination of alanine with the first E–NAD complex leads to substrate inhibition. In the following paper (Grimshaw et al., 1981), we will present isotope effect and pH studies which determine the chemical mechanism of the enzyme.

Materials and Methods

Materials. Crystalline *Bacillus subtilis* alanine dehydrogenase, obtained from Sigma as the ammonium sulfate suspension, was centrifuged, redissolved in a buffer composed of 1 mM EDTA, 0.1 mM dithiothreitol, 1 mg/mL bovine serum albumin, and 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes),[1] pH 7.9, and dialyzed 2 times for 12 h against 500 volumes of the same buffer. Enzyme treated in this manner has a specific activity (100 μmol of L-alanine oxidized per min per mg at 25 °C, pH 10, 0.10 M ionic strength) which is about 3-fold higher than that reported by Sigma and is stable to storage at 4 °C for several months. L-Alanine, D-alanine, pyruvate (Na^+ salt), and L- and D-lactate (Li^+ salts) were from Sigma. Oxamate (Na^+ salt) and tetramethylammonium chloride were from Aldrich, while NAD (free acid) and NADH (disodium salt) were from Boehringer Mannheim. L-Alanine-*2,3,3,3-d*$_4$ was prepared by the method of Cooper (1976) and shown by 270-MHz proton NMR to contain 97.3% and 95.4% deuterium label at the 2 and 3 positions, respectively. A-side (*pro-R*) NADD was prepared by the method of Viola et al. (1979). All other chemicals or biochemicals were from Sigma and were used without further purification.

Assay Conditions. Unless otherwise noted, initial velocity and inhibition patterns were determined in 50 mM buffer at constant ionic strength (0.10 M maintained with KCl in the forward reaction and 0.25 M by using tetramethylammonium chloride in the reverse reaction).[2] Buffers normally used were Mops (pH 6–7), Tes (pH 7–8), Caps, and Ches (pH 9–10). 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.

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[1] Abbreviations used: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic 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, $^D(V/K)$, and ^Dk are deuterium isotope effects (that is, values for the hydrogen-containing molecule divided by values for the deuterated molecule) on the respective parameters.

[2] Activity was not strongly affected by ionic strength; an increase from 0.1 to 0.3 M using KCl caused a 20% increase in V, a 2-fold decrease in $V/K_{alanine}$, and no change in K_I for alanine substrate inhibition at pH 7.9. The major inhibitory effect of these salts probably results from the cation, rather than from ionic strength effects, since KCl was more inhibitory (K_i = 80 mM) than tetramethylammonium chloride (K_i = 350 mM) vs. alanine at pH 7.9. In calculating ionic strength, we have assumed no contribution from the zwitterionic forms of alanine, serine, and the buffers.

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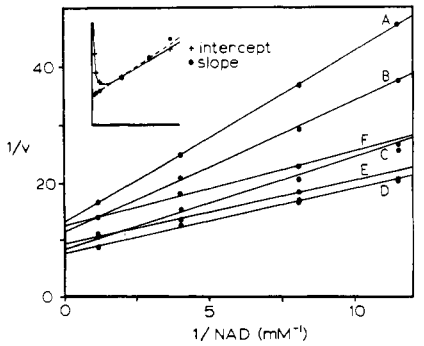


FIGURE 1: Initial velocity pattern for the oxidation of L-alanine at pH 7.9. The insert shows replots of slopes or intercepts vs. 1/[alanine] and demonstrates that substrate inhibition by L-alanine is uncompetitive. L-Alanine concentrations (millimolar) are the following: (A) 2; (B) 2.75; (C) 5.5; (D) 20; (E) 50; (F) 100.

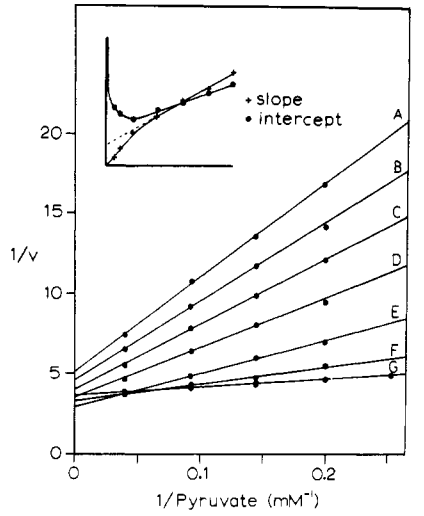


FIGURE 2: Initial velocity pattern at pH 9.35 in the reverse direction for the reductive amination of pyruvate. NADH was 200 μM, and ammonia concentrations (millimolar) were the following: (A) 10; (B) 12.5; (C) 16.5; (D) 25; (E) 50; (F) 100; (G) 150. The insert shows replots of slopes and intercepts vs. 1/[ammonia] to illustrate the uncompetitive substrate inhibition by ammonia and the curvature of the slope replot which causes it to go through the origin.

Table I: Kinetic Constants at pH 7.9[a]

$(V_{alanine})/E_t$ (s^{-1})	48 ± 3
K_{NAD} (mM)	0.22 ± 0.02
$K_{i\,NAD}$ (mM)	0.33 ± 0.07
$K_{alanine}$ (mM)	4.4 ± 0.7
$(V_{pyruvate})/E_t$ (s^{-1})	1000 ± 90
K_{NH_3} (mM)	160 ± 16
$K_{pyruvate}$ (mM)	0.44 ± 0.06
$K_{i\,pyruvate}$ (mM)	0.30 ± 0.02
K_{NADH} (μM)	38 ± 5
$K_{i\,NADH}$ (μM)	2.7 ± 0.1

[a] Tes (50 mM), 0.25 M ionic strength, 25 °C.

Data Processing. Reciprocal initial velocities were plotted vs. reciprocal substrate concentrations, and the experimental data were fitted to eq 2–12

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

$$v = VA/(K + A + A^2/K_I) \quad (3)$$

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

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB + AB^2/K_I) \quad (5)$$

$$\log v = \log \{VABC/[(\text{constant}) + (\text{coef } A)A + (\text{coef } B)B + (\text{coef } C)C + K_aBC + K_bAC + K_cAB + ABC]\} \quad (6)$$

$$v = v_0/(1 + I/K_i + J/K_j + IJ/\beta K_iK_j) \quad (7)$$

$$v = VA/[K(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (8)$$

$$v = VAB/[K_aB(1 + F_iE_{V/K_a}) + (1 + F_iE_V)AB + K_b(A + K_{ia})(1 + F_iE_{V/K_b})] \quad (9)$$

$$v = VA/[K + A(1 + I/K_{ii})] \quad (10)$$

$$v = VA/[K(1 + I/K_{is}) + A] \quad (11)$$

$$v = VA/[K(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (12)$$

by the least-squares method, assuming equal variances for the v or log v 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 2, and eq 3 was used when substrate inhibition was observed. Equations 4 and 5 describe intersecting initial velocity patterns, with the latter including uncompetitive substrate inhibition by B. Initial velocity data corresponding to a terreactant kinetic mechanism were fitted to eq 6 and to the corresponding equation lacking the B term. Data for the double inhibition experiment were fitted to eq 7. Data for kinetic deuterium isotope effects when one substrate was varied were fitted to eq 8 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. Equation 9 was used when isotope effects were seen on V, V/K_a, and V/K_b in a sequential mechanism where full initial velocity patterns were run with deuterated and unlabeled substrates. Data conforming to linear uncompetitive, competitive, and noncompetitive inhibitions were fitted to eq 10–12.

Results

Initial Velocity Patterns at pH 7.9. The forward reaction shows normal sequential kinetics (intersecting pattern) with uncompetitive substrate inhibition by L-alanine (Figure 1). However, above pH 10, the value for $K_{i\,NAD}$ increases, while K_{NAD} remains constant, until at pH 10.9 the reaction appears equilibrium ordered (NAD adding first). In the reverse direction, initial velocity patterns ($1/v$ vs. 1/[NADH] at four fixed levels of pyruvate) were determined at ammonia levels of 20, 30, 50, and 200 mM. Preliminary graphical analysis of these data suggested that the B (pyruvate concentration) term in eq 6 might be missing. The data were thus fitted to eq 6, and to a similar equation lacking the B term. The value of coefficient B was 0.23 ± 0.17 mM^2 from the fit to eq 6, while the value of coefficient A was 47 ± 4 mM^2 (A = NADH concentration), and that of coefficient C was 0.007 ± 0.001 mM^2 (C = ammonia concentration). Leaving out the B term only raised the residual least squares from 0.0376 to 0.0378, and thus while A and C terms are definitely present in eq 6, the B term may not be. To check further on the significance of the B term, we ran an initial velocity pattern by varying ammonia concentration at saturating pyruvate concentration (30 mM) and at five fixed levels of NADH concentration. The data were fitted both to eq 4 and to the similar equation lacking the $K_{ia}K_b$ term. The residual least squares was the same for both fits, and the value of the constant term from the fit to eq 4 was 0.36 ± 0.46 mM^2. Since the B term in eq 6 is responsible for the constant term in eq 4 when pyruvate is saturating, we again cannot show that the value of coefficient B in eq 6 is significantly different from zero.

Table II: Dead-End Inhibition at 25 °C vs. L-Alanine or Pyruvate[a]

inhibitor	pH	vs. [L-alanine] K_{is}	vs. [L-alanine] K_{ii}	vs. [pyruvate] K_{is}	vs. [pyruvate] K_{ii}	$(K_{ii}$ vs. [Pyr]$)/(K_{is}$ vs. [Ala])	$(K_{ii}$ vs. [Ala]$)/(K_{is}$ vs. [Pyr])
L-lactate	6.6	8.5	∞	9.2 ± 0.5	∞	∞	∞
	7.9	8.9 ± 0.2	∞	31 ± 2	160 ± 20	18	∞
D-lactate	6.6	29	37	4.7 ± 0.3	∞	∞	7.9
	7.9	130 ± 20	62 ± 6	18 ± 1	∞	∞	3.4
	9.5	426	1000	28 ± 3	1400 ± 400	3.3	36
D-alanine	7.9	70 ± 10	210 ± 50	11 ± 1	∞	∞	19
L-alanine	7.9	3.2 ± 0.4[b]		3.4 ± 0.1	110 ± 20	34	
oxamate	6.6	3.1 ± 0.2	16 ± 14	1.08 ± 0.05	∞	∞	16
	7.9	15 ± 1	31 ± 5	4.1 ± 0.2	∞	∞	7.6

[a] Values without standard errors are interpolated from pH profiles, since a value was not obtained at the exact pH shown. Values with standard errors are from fits to eq 11 or 12. K_{is} and K_{ii} values are in millimolar. When L-alanine concentration was varied, NAD was 2 mM (K_{is} values are corrected to saturating NAD concentration), while when pyruvate concentration was varied, 50 mM ammonia and 80 μM NADH was used. [b] Michaelis constant.

Substrate Inhibition by Ammonia in the Reverse Reaction. While the initial velocity patterns in the reverse direction are normal intersecting ones at neutral and low pH (see the following paper for the pH variation of the kinetic parameters), at pH 9.35 the pattern seen in Figure 2 was obtained. It is clear that ammonia levels above 50 mM give uncompetitive substrate inhibition and cause the slope replot to go through the origin. The data at noninhibitory levels of ammonia were fitted to eq 4, while the intercept replot was fitted to eq 3 to give V and the K_i value for ammonia (160 ± 30 mM).

Haldane Relationship. The kinetic constants at pH 7.9 in both directions are listed in Table I (the dissocation constant of NADH was determined from its competitive inhibition vs. NAD at 3 mM alanine). The internal consistency of these kinetic parameters was checked with the kinetic Haldane relationship for an ordered Bi-Ter mechanism (Cleland, 1963):

$$\text{app } K_{eq} = \frac{(V/K_{alanine})K_{i\ pyruvate}K_{i\ NADH}}{(V/K_{ammonia})K_{i\ NAD}} \qquad (13)$$

The kinetic constants in Table I give a value at pH 7.9 of 4.3 ± 0.9 μM for this expression, which agrees with the experimentally observed value of 5.4 μM at this pH, and corresponds to a thermodynamic K_{eq} value of 5.4×10^{-14} M^2 for the expression

$$\frac{[NH_4^+][\text{pyruvate}^-][NADH][H^+]}{[\text{L-alanine}^\pm][NAD^+]}$$

The value reported by Yoshida & Freese (1964) was 3.1×10^{-14} M^2.

Product Inhibition Patterns. Product inhibition by ammonia vs. NAD at 3 mM L-alanine was noncompetitive, with values from a fit to eq 12 of K_{is} = 38 ± 4 mM and K_{ii} = 81 ± 10 mM at pH 7.9. As noted above, NADH was competitive vs. NAD. NADH and substrate inhibition levels of L-alanine were also used in a double inhibition experiment at pH 7.9. Initial velocities were measured at 0.2 mM NAD (=K_{NAD}), 0–22.5 μM NADH, and L-alanine levels from 30 mM to 0.6 M (all saturating with respect to substrate activity). The data fitted eq 7 with β = 4.9 ± 0.8, $K_{i\ alanine}$ = 75 ± 3 mM, and $K_{i\ NADH}$ = 3.3 ± 0.1 μM.

Dead-End Inhibition Patterns. The inhibition constants for a number of dead-end inhibitors which were used in both the forward reaction vs. L-alanine and the reverse reaction vs. pyruvate are shown in Table II. In addition to the values shown, L-lactate gave noncompetitive inhibition vs. NAD (K_{is} = 120 ± 20 mM and K_{ii} = 21 ± 1 mM) at pH 7.0 with L-alanine at a level of $0.5K_m$. To test for the degree of order in the reaction, we determined the inhibition pattern of oxamate vs. NADH at 100 μM pyruvate and 50 mM ammonia. At pH 6.45, the pattern was uncompetitive with K_{ii} = 7.1 ± 0.2 mM (the K_{is} value was 57 ± 75 mM when eq 12 was fitted). At pH 9.5, however, the pattern was noncompetitive, with K_{is} = 90 ± 17 mM and K_{ii} = 39 ± 2 mM.

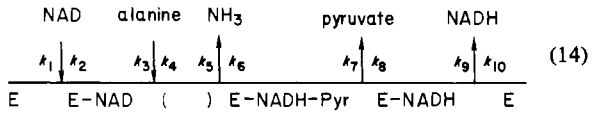
Isotope Effects.[1] Kinetic deuterium isotope effects were measured by direct comparison of the initial velocities obtained with 2 mM NAD and either unlabeled L-alanine or L-alanine-*2,3,3,3-d*$_4$. Isotope effects on V and $V/K_{alanine}$ were obtained from fits to eq 8 or were calculated as the ratios of kinetic parameters for hydrogen- and deuterium-containing substrates from individual fits to eq 3 when substrate inhibition by L-alanine was apparent.[3] At pH 7.9, ^DV was 1.28 and $^D(V/K_{alanine})$ was 1.81. Values at other pH values are reported in the following paper. At pH 10, isotope effects on V/K_{NAD}, $V/K_{alanine}$, and V were obtained from fits of the complete set of initial velocity data for hydrogen- and deuterium-containing L-alanine to eq 9, or to the similar equation in which the logarithm is taken of both sides. (Use of eq 9 assumes equal variance for the velocities, while the log form assumes constant proportional error in velocities.) The values of ^DV and $^D(V/K_{alanine})$ (1.12 ± 0.03 and 1.44 ± 0.05, respectively) were not significantly different from values determined by comparison of reciprocal plots with labeled and unlabeled L-alanine at this pH (see Figure 5 of the following paper), and the values from the log form of eq 9 were the same, with somewhat lower standard errors. The value of $^D(V/K_{NAD})$, however, was 1.02 ± 0.08 from the fit to eq 9, but 1.07 ± 0.03 from the log form.

The effect of substrate inhibition levels of ammonia on the V isotope effect in the reverse direction was measured at pH 9.35 by comparing initial velocities with saturating (200 μM) levels of either NADH or A-side NADD at saturating (25 mM) pyruvate concentration. At 150 mM ammonia, ^DV was 1.08, while the extrapolated value at low ammonia concentration was about 1.5. An ammonia level of 50 mM appeared to reduce the (isotope effect − 1) by half, and infinite ammonia concentration apparently reduced the isotope effect to 1.0.

[3] The observed isotope effects will be the product of the primary and secondary effects resulting from deuterium substitution at C2 and C3 of L-alanine, respectively. The intrinsic primary effect is expected to be close to the value of 6 observed for several other dehydrogenases (Cook & Cleland, 1981a,b; Schimerlik et al., 1977), while the secondary effect should have a maximum value equal to the equilibrium isotope effect of 1.05/deuterium (Cook et al., 1980). Since the secondary effect arises from the effect of deuterium substitution at C3 on the change in hybridization occurring at C2 during hydride transfer, the net result will be only to increase the apparent intrinsic primary effect by a maximum factor of 1.16 without affecting any of the conclusions in the text.

Discussion

Kinetic Mechanism. A number of experiments suggest that the kinetic mechanism of alanine dehydrogenase is ordered as shown in mechanism 14:



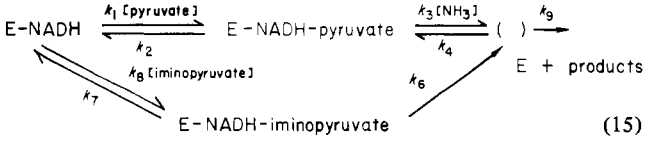
(14)

In the forward direction, the initial velocity pattern becomes equilibrium ordered at pH 10.9 because k_2 has become so much greater than V/E_t that binding of NAD comes to equilibrium, and it is tempting to propose that if alanine can prevent NAD release from the enzyme at high pH, it can also do so at lower pH. There is also a much larger isotope effect on $V/K_{alanine}$ than on V/K_{NAD} at pH 10, which again suggests that NAD does not dissociate rapidly from the ternary complex. The noncompetitive inhibition of dead-end inhibitors vs. pyruvate (Table II) also argues that these molecules combine with E–NAD and prevent NAD release.

On the other hand, L-lactate is noncompetitive vs. NAD at pH 7.9, and thus appears to combine with free enzyme, although more weakly by a factor of 13 than with E–NAD. In addition, a small, but possibly real, isotope effect on V/K_{NAD} is seen at pH 10 when the log form of eq 9 is used to fit the data, and thus, NAD may be released slowly from the central complex (the value of 1.07 seen corresponds to release of NAD at a rate 13-fold slower than reaction to give ammonia, while L-alanine is released from the same complex at nearly the same rate as ammonia is produced; see the following paper). These data argue for some degree of randomness, and the mechanism is thus probably truly random, but with a strongly preferred pathway in which NAD binds before alanine.

In the reverse direction, the initial velocity pattern at pH 7.9 fail to show the presence of a significant [pyruvate] term in the denominator of the rate equation, suggesting that pyruvate may not form a binary complex with free enzyme, and thus, the mechanism may be ordered as shown in mechanism 14. The noncompetitive inhibition of ammonia vs. NAD and the competitive inhibition of NADH vs. NAD are consistent with such a mechanism. The noncompetitive inhibitions by dead-end inhibitors vs. L-alanine suggest that these molecules combine with E–NADH and prevent release of NADH from the resulting complex. The inhibition of oxamate vs. NADH was uncompetitive at pH 6.45, as expected for mechanism 14, but at pH 9.5, the pattern was noncompetitive, and it is clear that oxamate can combine with free enzyme at this pH.

Evidence for Substrate Activity of Iminopyruvate. One interesting variant of the kinetic mechanism is seen at pH 9.35, where high levels of ammonia cause the slope replot to go through the origin but cause the intercept replot to show uncompetitive substrate inhibition (Figure 2). This unusual pattern probably results from the productive combination of nonenzymatically formed iminopyruvate with E–NADH, as in mechanism 15:



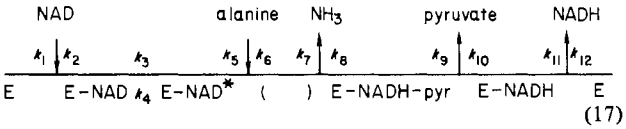
(15)

In order to explain the uncompetitive substrate inhibition by ammonia, k_6 must be less than k_9, and the dissociation constant of iminopyruvate to NH_3 and pyruvate must exceed $(k_2 + k_6)k_4k_6k_8/[k_1k_3k_9(k_6 + k_7)]$. Under these conditions, the slope replot is concave downward and goes through the origin, as observed.[4] Iminopyruvate at pH 9.35 will not have a protonated =NH group, and thus, the step represented by k_6 probably involves protonation of iminopyruvate on the enzyme to give the $=NH_2^+$ form which can be reduced by NADH. This protonation step will be slow at high pH, because the pK of iminopyruvate is near neutrality, and the concentration of protons in solution is low. Since the V isotope effect becomes 1.0 at infinite substrate inhibition levels of ammonia, while it appears to be around 1.5 when data from below the substrate inhibition region are used, the slow step corresponding to k_6 must precede and not include hydride transfer, which must then occur as part of k_9. If the slow step followed, rather than preceded, hydride transfer, some portion of the inverse equilibrium isotope effect of 0.87 (Cook et al., 1980) would be seen at infinite ammonia concentration. The isotope effects thus support the assignment of k_6 to the protonation of iminopyruvate on the enzyme. For this mechanism to be viable, there must be sufficient iminopyruvate formed at levels of ammonia above 50 mM where the effects are seen. The measured dissociation constant of iminopyruvate to free NH_3 and pyruvate is 4 M (Zuman, 1950), and thus at pH 9.35 and 150 mM ammonia, about 2% of pyruvate is in the imino form. Iminopyruvate must be bound much more tightly to E–NADH than is pyruvate to give the kinetic patterns seen in Figure 2, but in view of the probable chemical mechanism (described in the following paper) in which protonated iminopyruvate is a tightly held intermediate, this requirement is not unreasonable.

Isomerization of the E–NAD Complex and Distribution among Enzyme Forms. The availability of the kinetic constants in Table I allows calculation of k_2 in mechanism 14 from the maximum velocity in the forward reaction at pH 7.9 as

$$k_2 = (V/E_t)K_{i\,NAD}/K_{NAD} = 72\ s^{-1} \qquad (16)$$

However, V/E_t in the reverse direction is 1000 s^{-1} at this pH, and k_2 must be larger than this number. Cleland (1963) has shown that if the E–NAD complex isomerizes:



(17)

the above combination of kinetic constants is not k_2 but

$$\frac{k_2k_3k_4}{(k_2 + k_3)(k_3 + k_4)}$$

Since k_2 and k_4 must both exceed 1000 s^{-1} (and as we will show below, actually are much larger than this), the above expression equals k_3, which is then 72 s^{-1}. Since $k_3 \ll k_4$, most of the E–NAD complex at equilibrium is not in the form which combines with L-alanine, but rather in the form from which NAD dissociates. Further, if k_3 is truly 72 s^{-1}, while V/E_t is 48 s^{-1} in the forward direction, k_3 is the major rate-limiting step for V in this direction. At saturating levels of L-alanine and NAD, 67% of the enzyme will be the initial E–NAD complex.

The distribution of the remaining enzyme can be estimated for mechanism 17 as follows. At pH 7.9, ^DV is 1.28, and since $^Dk_7$ appears to be 1.97 [from $^D(V/K_{alanine})$ at low pH (see the following paper)], we have (Schimerlik et al., 1977)

[4] The slope replot has the algebraic form:

$$\text{slope} = (a/[NH_3] + b/[NH_3]^2)/(1 + c/[NH_3])$$

while the intercept replot also has a constant term in the numerator.

$$^{D}V = 1.28 = (1.97 + c)/(1 + c) \quad (18)$$

where

$$c = k_7(1/k_3 + 1/k_9 + 1/k_{11}) = 2.46 \quad (19)$$

Since

$$E_t/V = 1/48 \text{ s} = 1/k_3 + 1/k_7 + 1/k_9 + 1/k_{11} \quad (20)$$

and $k_3 = 72\ s^{-1}$, we can solve these equations to give $k_7 = 166\ s^{-1}$ and $1/(1/k_9 + 1/k_{11}) = 1080\ s^{-1}$. Since inhibitors that are competitive vs. pyruvate do give noncompetitive inhibition vs. L-alanine, E–NADH is present, and it is probable that k_{11} is $1080\ s^{-1}$, and k_9 is at least an order of magnitude larger than this. As it is, the above values suggest that only 4.5% of the enzyme at pH 7.9 is E–NADH when L-alanine and NAD are saturating, and 29% is central complexes. One would thus expect the (K_{ii} vs. [L-alanine])/(K_{is} vs. [pyruvate]) ratio should be around 22 for the inhibitors in Table II that combine with E–NADH. This is true for D-alanine, but the ratio is lower for oxamate and D-lactate, suggesting that there may be somewhat more E–NADH present than we have estimated (the 4.5% figure results from small differences between large numbers).[5]

In the reverse directions, $^{D}k_6$ will be $^{D}k_7$ divided by the equilibrium isotope effect (1.15; Cleland, 1980), or 1.97/1.15 = 1.71. Since ^{D}V from this direction is about 1.5, c in the equation analogous to eq 19 will be about 0.4 and equals $k_6(1/k_2 + 1/k_4)$. V/E_t in the reverse direction ($1000\ s^{-1}$) is the reciprocal of the sums of reciprocals of k_2, k_4, and k_6, and from the data in Table II, 3–5% of the enzyme appears to be present as E–NAD* (the form which combines with L-alanine or inhibitors) at saturating levels of NADH, pyruvate, and ammonia. Since the level of E–NAD* will be $1/k_4$ times V/E_t, we have three equations which can be solved for k_2, k_4, and k_6 at pH 7.9. The resulting value of k_6 is $1400\ s^{-1}$, and central complexes make up 71% of the enzyme. For 3% E–NAD*, $k_2 = 3900\ s^{-1}$ and $k_4 = 32\,000\ s^{-1}$, while for 5% E–NAD*, $k_2 = 4300\ s^{-1}$ and $k_4 = 19\,000\ s^{-1}$. E–NAD makes up 23–26% of the enzyme. Note the very low value of k_3/k_4 (0.002–0.004)! The changes in enzyme distribution which occur with pH will be discussed in the following paper.

With the above estimates for the unimolecular rate constants in mecanism 17, it becomes possible to evaluate the bimolecular rate constants at pH 7.9 from the kinetic constants in Table I. The values of k_1 and k_{12} for the two nucleotides (1.3×10^7 and $1.1 \times 10^8\ M^{-1}\ s^{-1}$, respectively) are in the expected range and approach the diffusion-controlled limit, especially for NADH. The value of $3.4 \times 10^7\ M^{-1}\ s^{-1}$ for k_5 will be nearly an order of magnitude higher and near the diffusion-controlled limit when corrected for the proper protonation states of L-alanine and enzyme, and the value of $>2 \times 10^7\ M^{-1}\ s^{-1}$ for pyruvate, which is $>10^8\ M^{-1}\ s^{-1}$ after correction for proper protonation of E–NADH, also is near this limit. On the other hand, k_8 has a calculated value of only $7000\ M^{-1}\ s^{-1}$ at pH 7.9, which is still only $1.5 \times 10^5\ M^{-1}\ s^{-1}$ after correction to the level of free NH_3, which is the substrate. This low value suggests that free NH_3 is not forming a complex with E–NADH–pyruvate but reacting with it chemically.

Analysis of the Double Inhibition by NADH and Substrate Inhibition Levels of L-*Alanine.* At first glance, the intersecting pattern seen in this double inhibition experiment suggests that the substrate inhibition by L-alanine results from combination with E–NADH, but a quantitative analysis of the data shows that alanine also has another effect. When a dead-end inhibitor combines with E–NADH in an ordered mechanism such as 14, β in eq 7 cannot exceed 1.0, and in the present case where NAD was present at its K_m level, β has a maximum value of 0.5 (Northrop & Cleland, 1974). The observed β value of 4.9 thus suggests that L-alanine is inhibiting by combining (1) with the initial E–NAD complex that cannot productively combine with L-alanine, (2) with a central complex, or (3) possibly with E–NADH–pyruvate. These interactions will contribute to the I/K_i term in eq 7 (where I is L-alanine concentration) but not to the $IJ/(\beta K_i K_j)$ term, so that β can now easily exceed 1.0. The high value of β suggests that indeed this combination of L-alanine with an enzyme complex which precedes E–NADH in the mechanism is the major cause of the uncompetitive substrate inhibition. Our previous analysis suggests that there is little E–NADH–pyruvate present with saturating substrates but that 56–67% of the enzyme is the initial E–NAD complex, 5–15% is E–NADH, and 29% is central complexes. Further, the pH variation of ^{D}V discussed in the following paper suggests that the proportion of E–NAD present does not vary appreciably with pH below 9. Therefore, it seems most likely that substrate inhibition results from nonproductive combination of L-alanine with the initial E–NAD complex in a manner that prevents its subsequent conversion to E–NAD*. Moreover, the pH profile for the substrate inhibition constant (see the following paper) suggests that L-alanine is combining in its zwitterionic form and that a group on the enzyme with a pK of 7.5 must be deprotonated for this combination. We will discuss in the following paper the nature of this group and contrast the pH profile of this nonproductive binding of L-alanine to E–NAD with its productive binding to E–NAD*.

Since we have estimates for the rate constants in mechanism 17, we can use the equations for the similar mechanism in which L-alanine combines in dead-end fashion with E–NAD to evaluate from the observed substrate inhibition constant the true dissociation constant of L-alanine from E–NAD–alanine. These calculations suggest a value around 16 mM for the nonproductive ternary complex, which is almost 4 times the K_m of L-alanine for the reaction.

In conclusion, the kinetic mechanism for alanine dehydrogenase appears ordered, with nucleotides binding before the other substrates and pyruvate binding before NH_3. The initial E–NAD complex clearly must isomerize in a step with a very low equilibrium constant before it can productively bind L-alanine for dehydrogenation, while combination of alanine with the initial E–NAD complex in a nonproductive manner prevents the isomerization from taking place, and thus produces substrate inhibition by alanine. In the following paper, we will present pH profiles for various kinetic parameters and use this information to derive a chemical mechanism for the reaction.

[5] A somewhat lower value of k_{11} and a higher proportion of E–NADH at substrate saturation are obtained by considering the pH variation of ^{D}V and the kinetic parameters concerned with k_1, k_2, and k_3, but we will not present the detailed arguments here (they may be obtained from the senior author). This analysis suggests that k_{11} has a value of 310 rather than $1080\ s^{-1}$. This value of k_{11} will fit the observed relationships at pH 7.9 if k_7 remains $166\ s^{-1}$ and k_3 is increased to $86\ s^{-1}$, which is one standard error higher than the observed value. This corresponds to 15% E–NADH at saturating NAD and L-alanine levels at pH 7.9 and predicts a (K_{ii} vs. [alanine])/(K_{is} vs. [pyruvate]) ratio of 6.7, in better agreement with the values in Table II.

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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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