Enhancement of the Activity of Horse Liver Alcohol Dehydrogenase by Modification of Amino Groups at the Active Sites*

by Bryce V. Papp

From The Rockefeller University, New York, New York 10021

SUMMARY

Reaction of horse liver alcohol dehydrogenase with imidoesters or cyanate at pH 8 significantly increases the activity of the enzyme, as assayed in high concentrations of NAD+ and ethanol at pH 9.0. Methyl picolinimidate activates the enzyme 19-fold and modifies about 50 of its 60 amino groups, as determined by spectral and amino acid analyses. When the active sites are protected with NAD+ and pyrazole (or NADH and isobutyramide) methyl picolinimidate activates only 2-fold, although most of the amino groups still react; after removal of the reagents by gel filtration, the partially substituted enzyme could be activated 11-fold more by methyl picolinimidate or 2-fold more by 14C-cyanate with the modification of about three amino groups per active site. A similar experiment with ethyl acetimidate in the first step and methyl picolinimidate in the second step gave similar results.

Product inhibition studies show that the reactions catalyzed by both the native and picolinimidylated enzymes at pH 9.0 conform to the same mechanism, ordered bi bi. The modified enzyme has 12- to 53-fold larger Michaelis and inhibition constants for NADH and NAD+ and 12- and 30-fold larger turnover numbers. The rate-limiting step in either the forward or the reverse reaction with the native enzyme is the breakdown of the enzyme-coenzyme complex; the picolinimidylated dehydrogenase probably gives higher maximum velocities because the complexes dissociate faster.

Picolinimidylation of the enzyme does not greatly affect the binding of AMP, ADP, or adenosine 5'-diphosphoribose, but markedly decreases the binding of NAD+1, NADH, and 3-acetylpyridine adenine dinucleotide. The reactivities of the essential —SH groups and the zinc ions at the active sites of the enzyme are not affected by picolinimidylation.

These results indicate that the amino groups that can be modified are not required for the catalytic activity of the enzyme and that there is probably at least one amino group near the binding site for the nicotinamide ring of the coenzyme.

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Studies on the amino acid residues at the active sites of horse liver alcohol dehydrogenase (alcohol:NAD+ oxidoreductase, EC 1.1.1.1) have indicated that carboxymethylation of 1 cysteine residue per site inactivates the enzyme (2, 3) even though the modified enzyme can still interact with NADH and ethanol (4). Other amino acids have not been directly implicated, but Kosower has predicted that the ε-amino group of a lysine residue participates in the binding of coenzyme and substrate (5). We have studied the effects on the enzyme of the imidoesters, methyl picolinimidate and ethyl acetimidate, and cyanate, which form stable derivatives with primary amino groups (6, 7) but not with other functional groups of proteins, such as the cysteinyl —SH groups. MPI1 was introduced by Benisek and Richards (8) for attaching metal-chelating groups onto enzymes.

We thought that the zinc ion at the active site of the enzyme (9-12) might bind MPI and facilitate its reaction with a nearby amino group.

Unexpectedly, reaction of the enzyme with the reagents increased the activity of the enzyme. The chemical and kinetic studies reported here allow us to estimate the number of amino groups at the active sites, to explain the enhancement of the enzyme activity, and to implicate amino groups in the activity of alcohol dehydrogenase.

EXPERIMENTAL PROCEDURE

Materials—NAD+ and crystalline horse liver alcohol dehydrogenase were purchased from Boehringer Mannheim. NAD+, NADH, AMP, ADP, adenosine 5'-diphosphoribose, 3-acetylpyridine adenine dinucleotide, and 2,2'-bipyridine were purchased from Sigma. Picolinonitrile, sodium methoxide, and acetald-
zyme was diluted in 1 mg per ml of bovine serum albumin in the final concentrations of the compounds that buffer at the indicated pH.

Methyl picolinimidate was synthesized (13): b.p. 105-106° at 18 mm (lit. 118-122° at 28 mm).

\[ \text{C}_3\text{H}_7\text{ON}_3 (136.2) \]

Calculated: C 61.75, H 5.92, N 20.58

Found: C 61.85, H 5.96, N 20.68

The reagent was stored at -10°. A 0.1 M solution was prepared by the addition of 12.5 μl of reagent (density 1.1 g per ml) to each ml of reaction mixture.

Enzyme Assays—The enzymes were routinely assayed in 1 ml of 55 mM Na₄P₂O₇, 0.5 mM semicarbazide hydrochloride, 18 mM glycine, 550 mM ethanol, and 1.75 mM NAD⁺, at pH 9.0 and 25° (Boehringer Mannheim, 1968 catalogue). A fresh solution of NAD⁺ was prepared each day and added to a solution of the other compounds. Enzyme solutions were diluted (if necessary) in 1 mg per ml of bovine serum albumin in buffer at pH 7.7 to 9.0, and measured volumes were introduced into the assay mixture on a plastic spoon. The time required for a change of 0.1 A₅₄₀ was determined; 10 μg or less of enzyme were assayed.

Enzymes were also assayed according to Dalsiel (14) in 62 mM sodium glycinate (pH 10.0), 8.2 mM ethanol, and 0.42 mM NAD⁺.

Kinetic Studies—The buffer for the product inhibition studies was 10 mM Na₄P₂O₇ and 20 mM glycine, pH 9.0. Acetaldehyde was redistilled on the day of use. Reagent grade 95% ethanol was also redistilled. NAD⁺ was purified (15) for some of the experiments. Solutions of substrates were prepared daily. Solutions of NAD⁺ and adenine nucleotides were neutralized before use. Concentrations of NAD⁺ and NADII were determined from the absorbances at 260 μm and 340 μm, respectively (16). Enzyme activity was determined in a total volume of 2 ml in 1-cm cuvettes, after the reaction had been initiated by the addition of 20 or 50 μl of enzyme with an adder-mixer (17). Initial velocities of NAD⁺ reduction or NADH oxidation were determined from the tangents to the curves recorded with a Zeiss spectrophotometer PMQ II equipped with a TE converter so that 0.2 A (at 340 μm) could be recorded linearly; the recorder speed was varied from 1 to 4 inches per min. Solutions and reaction mixtures were kept at 25° with circulating water. Enzyme was diluted in 1 mg per ml of bovine serum albumin in 10 mM Na₄P₂O₇ and 20 mM glycine, pH 9.0, with or without 1 mg per ml of reduced glutathione, or in 0.05 M Tris-HCl, pH 8.0, and kept in ice. Dilute solutions of the picolinimidylated enzyme lost about 20% of their activity (in the routine assay) during the 2 to 3 hours required for the kinetic studies; therefore, each assay point was corrected for the actual activity at that time. The normality of the enzyme was calculated on the basis of an equivalent weight of 40,000 (18).

Analysis of Kinetic Data—The approach and computer programs of Wratten and Cleland (19) and Cleland (20) and a CDC 160 G computer were used. For each concentration of inhibitor, data were fitted to the equation²

\[ v = \frac{v_S}{K + S} \]

by means of a least squares method and on the assumption of equal variance for the velocities. Slopes (K/V) and intercepts (1/V) were plotted against inhibitor concentration; weighted least squares were fitted to a line, a parabola, and a “two-one” function as a means of determining which type of inhibition the data fitted best. In every case but one a line gave the best fit. We distinguished between competitive and noncompetitive inhibition by applying t tests to the intercepts (21). If the probability was greater than 5% that the two intercepts differing most were equal, competitive inhibition was assumed. If the probability was less than 1% that two intercepts were equal, noncompetitive inhibition was assumed. Final values for the kinetic constants and their standard errors were obtained by fitting all of the data for the experiment to the equation² describing the type of inhibition: linear competitive for the inhibitions by NAD⁺ and NADH,

\[ v = \frac{v_S}{K(1 + I/K_d) + S} \]

and linear noncompetitive for the inhibitions by ethanol and acetaldehyde:

\[ v = \frac{v_S}{K(1 + I/K_d) + S(1 + I/K_d)} \]

Analyzes of Proteins—Protein concentration was determined from the A₂₆₀ of 0.455 for 1 mg per ml (22) or by amino acid analysis. The molarities of the buffers refer to the final concentrations of the compounds that buffer at the indicated pH.

² Throughout this work, the molarities of the buffers refer to the final concentrations of the compounds that buffer at the indicated pH.
analysis on the assumption that the enzyme has a molecular weight of 80,000 (18) and 4 zinc ions (12). The composition determined in our laboratory by analyses of 22- and 72-hour hydrolysates (compare Reference 23) was aspartic acid 52, threonine 48, serine 52, glutamic acid 60, glycine 78, alanine 58, cysteine 26, valine 80, methionine 16, isoleucine 46, leucine 52, phenylalanine 38, tyrosine 8, and histidine 14, arginine 24, and tryptophan 4. The concentration was also determined by alkaline hydrolysis and ninhydrin analysis (24, 25) with the experimentally determined color yield equivalent to 6.2 nmoles of leucine per mg of enzyme. The concentration of the PI-enzyme was determined with the latter two methods.

Proteins were hydrolyzed (26) and analyzed for amino acids (27) with accelerayed systems (28) on Beckman-Spinco AA-15 and AA-27 or M-82 and PA-35 resins. Picolinimidyllysine was eluted as a broad peak at about 2.2 times the elution volume of arginine (Fig. 1) and was assumed to have about the same color value as arginine; the sum of the lysines and picolinimidyllysines (calculated on this assumption) for the PI-enzyme equaled the number of lysines in the native enzyme. About 60% of the picolinimidyllysine present in the PI-enzyme is converted to lysine by hydrolysis in 6 M HCl at 110°C in 22 hours. For accurate determination of the picolinimidyllysine content, therefore, the values found after hydrolysis at different times were extrapolated with first order kinetics to zero time.


dose assay Radioassay Radioactivity was measured with a Nuclear-Chicago scintillation counter, model 720. Samples (10 to 200 μl) were added to vials (2.5 × 8 cm) containing 15 ml of scintillation liquid (7 g of 2,5-diphenyloxazole, 50 mg of p-bis(2'-5'-phenylphenoxazoyl)benzene, and 70 g of recrystallized naphthalene made to 1 liter with spectroscopic grade p-dioxane). Counting efficiency was 58% for 14C.

RESULTS

Activation of Alcohol Dehydrogenase by Modification of Amino Groups—As shown in Fig. 2, reaction of the enzyme with MPI for 3 hours increased by 17-fold the activity of the enzyme as assayed with NAD+ and 0.5 M ethanol. MPI (4 mm) in the

![Fig. 2. Activation of liver alcohol dehydrogenase by methyl picolinimidate. Enzyme, 1.1 mg per ml, in 0.5 M N-ethylmorpholine-HCl buffer, pH 8.0, was allowed to react with 0.1 M MPI at 25°C for the times shown. Suitably diluted aliquots were then assayed at 25°C in 20 mm Na2P04 and 40 mm glycine, pH 9.0, with 1.7 mm NAD+ and 0.50 mm ethanol (— ); in 10 mm Na2P04 and 20 mm glycine, pH 9.0, with 0.2 mm NADH and 20 mm acetaldehyde (Δ—Δ); or in 0.1 M sodium glycinate, pH 10.0, with 0.42 mm NAD+ and 8.2 mm ethanol (□—□). The activity is given relative to a control that contained no MPI.](http://www.jbc.org/)

![Fig. 3. Determination of the incorporation of picolinimidyl groups into picolinimidylated enzyme by difference spectrophotometry. Enzyme, 10 mg, in 2 ml of 0.25 M N-ethylmorpholine·HCl buffer, pH 8, 5% ethanol, and 10 mm phosphate was activated 17-fold by reaction with 0.1 M MPI at 27°C for 3 hours. The reagent and buffers were exchanged for 50 mm sodium phosphate and 0.5 mm EDTA, pH 7.0, on a column (0.9 × 31 cm) of Sephadex G-25, fine, at room temperature. From the absorption spectrum (corrected for light scattering due to slight turbidity) and the protein concentration, the molar absorptivities were calculated. The molar absorptivities of native enzyme (LADH) determined in the same buffer were subtracted from those of PI-enzyme (PI-LADH), and the difference is presented (□—□). The number of picolinimidyl groups incorporated was calculated from the molar absorptivities of N-butylpicolinamidine hydrochloride at 362 μm, namely 5790 μM cm-1 (6); the absorption expected for 52 such groups is given (●—●).](http://www.jbc.org/)
 MPI. The α-amino groups of the enzyme are probably acetylated (29) and hence cannot react with MPI. The activation is apparently due to a chemical modification of the enzyme.

**Reaction of Amino Groups at Active Sites**—Fig. 4 shows that the activation of the enzyme by MPI was less when the active sites of the enzyme were protected by the prior formation of binary or ternary complexes of the enzyme. The coenzymes alone protected only partially even though the active sites should have been almost fully occupied by coenzyme, since the dissociation constants for NAD⁺ and NADH are 51 μM and 1 μM, respectively (in buffers of low ionic strength, at pH 8 (30)). The ternary complexes were activated only 2-fold; the concentrations of NADH and isobutyramide, or NAD⁺ and pyrazole, far exceeded the dissociation constants (30–32). After reaction of the enzyme and MPI in the presence of NAD⁺ and pyrazole (as in Fig. 4, but for 4 hours) and removal of the MPI, NAD⁺, and pyrazole by gel filtration, the enzyme was found to have been activated only 2-fold, but nevertheless to have 50 ± 5 picolinimidyl groups per molecule. Further reaction of the coenzyme-free, partially substituted PI-enzyme with MPI gave 11-fold more activation, for an over-all activation of 22-fold (Fig. 5). It appears that the activation is due to the modification of a few amino groups at the active sites of the enzyme.

If the coenzyme-free, partially substituted PI-enzyme was treated with cyanate instead of with MPI (Fig. 5), a 2-fold activation was observed, for an over-all activation of 4-fold, as assayed in 0.5 M ethanol. (However, as assayed in 17 mM ethanol with 1.7 mM NAD⁺ at pH 8.8 (4) and 25°, cyanate inactivated this PI-enzyme with a half-time of 90 min. Native enzyme was activated slightly by cyanate and then inactivated, with a half-time of 56 min as shown in the routine assay.) Using radioactive cyanate in the experiment of Fig. 5, we could determine the number of amino groups at the active sites of the enzyme. The partially substituted PI-enzyme was treated with 0.2 M ¹⁴C-cyanate (76 ± 0.07 (S.E.) cpm per nmole) for 380 min at 40° (other conditions as in Fig. 5); 5.2 moles of cyanate were incorporated per mole of enzyme (400 ± 4 (S.E.) cpm per nmole), as determined after removal of excess cyanate by gel filtration and dialysis.

![Fig. 4. Protection by coenzymes and ternary complexes against the activation of the enzyme by methyl picolinimidate](image)

![Fig. 5. Reaction of methyl picolinimidate and cyanate with amino groups at the active sites of partially picolinimidylated enzyme](image)

![Fig. 6. Reaction of methyl picolinimidate with the amino groups at the active sites of partially substituted aceticidylated enzyme](image)
tion with MPI. About six (±one) picolinimidyl groups were incorporated into the enzyme, as determined spectrophotometrically; the difference spectrum due to the picolinimidyl groups was qualitatively similar to the one shown in Fig. 3. Amino acid analysis of a 22-hour hydrolysate showed that one molecule of the enzyme had about 8 picolinimidyllysine residues (after correction for 60% loss of this derivative during hydrolysis). This determination is less accurate than the spectrophotometric one because it depends upon integration of a low, broad peak (compare Fig. 1); moreover, extrapolation of values from timed hydrolysates was not made.

Kinetics of Reactions Catalyzed by Native and Picolinimidylated Enzymes—The kinetic basis of the enhanced activity of the PI-enzyme was investigated by means of product inhibition studies. The native and modified dehydrogenases were compared at pH 9.0 since the activation was observed with the routine assay at pH 9.0. Fig. 7 gives the results for the native enzyme. NAD+ and NADH gave linear competitive inhibition

Fig. 7. Product inhibition studies on liver alcohol dehydrogenase at pH 9.0. The experiments were performed as described under "Experimental Procedure." In each lettered figure, the lower part is a Lineweaver-Burk plot of the primary data. $V$ has units of $\Delta A_{440}^{-1}$ per min, and the reciprocal of the concentration of the varied substrate is indicated on the figure. The lines are least square fits of the data to a hyperbola. The upper part of each figure is a replot of secondary data from the lower part. $K/V$ is the slope of the line from the Lineweaver-Burk plot and has units of mM.min $(\Delta A_{440})^{-1}$. $1/V$ is the intercept from the primary plot. The lines in the secondary plots are least squares fits to a line. A, inhibition by NADH with variable concentrations of NAD+. Ethanol, 8.15 mM. Enzyme, 51 nM. NADH: 1, 0 μM; 2, 50 μM; 3, 100 μM; 4, 150 μM. B, inhibition by NADH with variable concentrations of NAD+. Acetaldehyde, 6 mM. Enzyme, 14.3 nM. NAD+: 1, 0 μM; 2, 40 μM; 3, 80 μM; 4, 160 μM. C, inhibition by ethanol with variable concentrations of acetaldehyde. NADH, 166 μM. Enzyme: 1, 0 mM; 2, 6 mM; 3, 12 mM; 4, 18 mM. D, inhibition by acetaldehyde with variable concentrations of ethanol. NAD+, 1.66 mM. Enzyme, 80 nM. Acetaldehyde: 1, 0 μM; 2, 100 μM; 3, 200 μM; 4, 300 μM; 5, 400 μM.

Fig. 8. Product inhibition studies on picolinimidylated alcohol dehydrogenase at pH 9.0. The data are plotted as described in the legend to Fig. 7. A, inhibition by NADH with variable concentrations of NAD+. Ethanol, 1 M. Enzyme, 20 nM. NADH: 1, 0 μM; 2, 75 μM; 3, 150 μM; 4, 225 μM. B, inhibition by NADH with variable concentrations of acetaldehyde. NAD+, 6 mM. Enzyme, 8.6 nM. NAD+: 1, 0 mM; 2, 0.6 mM; 3, 1.2 mM; 4, 1.8 mM; 5, 3.6 mM. C, inhibition by ethanol with variable concentrations of acetaldehyde. NADH, 204 μM. Enzyme, 9.34 nM. Ethanol: 1, 0 mM; 2, 25 mM; 3, 50 mM; 4, 75 mM; 5, 100 mM; 6, 200 mM. D, inhibition by acetaldehyde with variable concentrations of ethanol. NAD+, 1.75 mM. Enzyme, 8.6 nM. Acetaldehyde: 1, 0 mM; 2, 4 mM; 3, 8 mM; 4, 12 mM. $K/V$ has units of mM.min $(\Delta A_{440})^{-1}$.
in Figs. 7 and 8 as follows: Michaelis constants (K)-Kg, K_b, K_p, and K_i; slope inhibition constants (K_a)-K_{ia}, K_{ia}K_p/K_q, and K_qK_p/K_i; intercept inhibition constants (K_i)-K_a and K_q.

The letters a, b, p, and q represent NAD+, ethanol, acetaldehyde, and NADH, respectively. V_i represents the maximum velocity in the reaction of NAD+ and ethanol and V_s is for the reverse reaction. E_i is the normality of the enzyme.

### Table I

| Constant | Native enzyme | PI-enzyme | Ratio |
|----------|---------------|-----------|-------|
| K_a      | 36.3 ± 4.7    | 557 ± 25  | 15    |
| K_b      | 778 ± 44      | 4100 ± 410* | 5.3   |
| K_p      | 520 ± 43      | 6190 ± 340* | 12    |
| K_q      | 0.65 ± 0.76   | 244 ± 130  | 37    |
| K_{ia}   | 40.3 ± 4.1    | 2150 ± 110 | 53    |
| K_{ib}   | 11100 ± 780   | 20300 ± 2700* | 1.8   |
| K_{ib}   | 644 ± 70      | 19400 ± 2400* | 30    |
| K_{ib}   | 6.67 ± 0.75   | 82.6 ± 3.2  | 12    |
| K_{ia}K_p/K_q | 3340 ± 400 | 31500 ± 1400* | 6.4   |
| K_qK_p/K_i | 75.3 ± 5.3   | 2030 ± 240  | 2.7   |

V_i/E_i = 4.9 ± 1* 61 ± 5 12
V_s/E_s = 12 2* 360 ± 10 30

### Table II

| NAD+ analogue | Native enzyme | PI-enzyme | Ratio |
|---------------|---------------|-----------|-------|
| AMP           | 380 ± 35      | 415 ± 40  | 1.1   |
| ADP           | 3190 ± 300    | 3080 ± 110 | 0.97  |
| Adenosine 5'-diphosphoribose | 117 ± 8.9 | 419 ± 12 | 3.6  |
| NADH          | 6.67 ± 0.75   | 82.6 ± 3.2  | 12    |
| NAD+          | 40.3 ± 4.1    | 2150 ± 110 | 53    |
| 3-Acetylpyridine adenine dinucleotide | 128 ± 10 | 1990 ± 140 | 1.5  |

The dissociation constants (K_a) were determined from experiments similar to those shown in Figs. 7 and 8 with the NAD+ analogue as the inhibitor. Conditions were 20 mm glycine, 10 mm Na_2HPO_4, pH 9.0, and 25°C.

#### Effect of Picolinimidylation on Reactivity of Groups at Active Sites

The increased V_i/E_i and K_{ia} of the PI-enzyme are apparently due to modification of amino groups at the active sites, for enzyme that was picolinimidyalted in the presence of NAD+ and pyrazole (Fig. 4) had about the same values for V_i/E_i, K_{ia} and K_a as the native enzyme.

### References

For inhibition by acetaldehyde (Fig. 7D), the closest intercepts for NADH and NAD+ (Ki, and Ki, 12 and 53 times and also the turnover numbers (V/E_i) 12 and 30 times.

The increased V_i/E_i and K_{ia} of the PI-enzyme are apparently due to modification of amino groups at the active sites, for enzyme that was picolinimidyalted in the presence of NAD+ and pyrazole (Fig. 4) had about the same values for V_i/E_i, K_{ia} and K_a as the native enzyme.

**Effect of Picolinimidylation on Binding of Parts of NAD+ Molecule** - The weaker binding of the PI-enzyme to NAD+ or NADH could be due to the disruption of one or more of the interactions between the enzyme and the dinucleotide. To locate the binding region affected, we studied the binding of portions of the NAD+ molecule and NAD+ analogues (Table II). AMP, ADP, and adenosine 5'-diphosphoribose had essentially the same dissociation constants with the native enzyme as with PI-enzyme, whereas NAD+, NADH, and 3-acetylpyridine adenine dinucleotide bound much less tightly to PI-enzyme than to native enzyme. It appears that a picolinimidyl group interferes with the binding of the nicotinamide portion of the NAD+ molecule to the PI-enzyme.

**Effects of Picolinimidylation on Reactivity of Groups at Active Sites** - The PI-enzyme could be activated because picolinimidyl groups have increased the reactivity of the —SH groups that may be involved in the catalytic mechanism. Imidazole, for instance, increases the rate of inactivation of the native enzyme by iodoacetate and iodoacetamide which react with the —SH groups (35). But the native and picolinimidyalted enzymes were inactivated by iodoacetate at the same rate (2.4 μM·min⁻¹) and by iodoacetamide at the same rate (0.41 μM·min⁻¹) in 0.5 M N-ethylmorpholine-HCl, pH 8.0, at 25°C.

Zinc ions are also involved in the activity of the enzyme...
(9-12), and picolinimidyl groups of PI-enzyme could chelate the zinc ions (8) and alter their properties. If this were so, the picolinimidyl groups should compete with the metal ion chelator, 2,2'-bipyridine (11), for the two or three available coordination positions of the zinc ions bound to the enzyme (10, 36), and the modified and unmodified enzymes should bind the chelator differently. For this study, we used enzyme that was acetimidylated in the presence of NAD+ and pyrazole and then picolinimidylated (Fig. 6). This derivative contained about five picolinimidyl groups and, unlike PI-enzyme, did not form the slightly turbid solutions that interfere with sensitive spectral studies. The dissociation constants of 2,2'-bipyridine and the enzymes and the extinction coefficients of the complexes were determined by the procedure used by Sigman (11), except that a 0.05 M sodium phosphate and 0.5 mM EDTA buffer, pH 7.5, was used. The native and picolinimidylated enzymes had the same dissociation constants, about 0.3 mM, and the same extinction coefficients at 308 nm, about 10^3 M^-1 cm^-1. Apparently, the picolinimidyl groups do not affect the accessibility of the zinc ions at the active sites of PI-enzyme. Since acetimidyla-
dylation and carbamylation also activate the native enzyme, chelation of the zinc ions is not responsible for the activation.

DISCUSSION

Amino Groups at Active Sites—The increase in the activity (Fig. 2) of the enzyme after modification of amino groups (Fig. 3) and the protection against this activation furnished by NAD+ and pyrazole or by NADH and isobutyramide (Fig. 4) are most simply explained by the hypothesis that there are amino groups at or very near the active sites of the enzyme. From the differential labeling experiments (Figs. 5 and 6) we conclude that there are about six amino groups at the active sites of the dimeric enzyme (18), or about three per active site. This interpretation should be qualified by some limitations in the data. Incorporation of reagents can be determined with an accuracy of perhaps ±10 to 20%. Incomplete substitution of amino groups not at the active sites while the active sites are blocked with NAD+ and pyrazole could leave a small fraction of the large number of amino groups unreacted; these would then react when NAD+ and pyrazole were removed to give some extrinsic incorporation. For example, if 30 amino groups outside the active sites reacted to the average extent of 95%, 2.5 eq of amino groups would still be free to react in the next step. Since one reagent was used to block the amino groups not at the active sites and another was used for those at the active sites (MPI and cyanate, or ethyl acetimidate and MPI), differential reactivity of amino groups could raise or lower the number apparently at the active sites. This possibility is not very likely since the use of two different pairs of reagents gave the same number of amino groups. Changes in conformation of the enzyme when it binds NAD+ and pyrazole could, of course, be invoked. X-ray diffraction (37) and optical rotatory dispersion studies (38, 39) indicate that the enzyme may change conformation when it forms ternary complexes, but the interpretations are still tentative, and we do not know whether the exposure of amino groups would be affected if a conformational change does occur.

Finally, we are assuming that the reagents reacted only with amino groups (6, 7). None of the results can eliminate the possibility that the reagents reacted with one group per active site (other than an amino group) with special reactivity. This possibility seems unlikely, however, for several reasons. Both cyanate and the imidoester gave similar results. The difference spectrum of enzyme that was acetimidylated in the presence of NAD+ and pyrazole and then picolinimidylated (Fig. 6) was typical of picolinamidines (e.g., Fig. 3). MPI activated the enzyme and modified amino groups at about the same rates; both reactions were essentially complete in 3 hours (Fig. 2). It would be coincidental if another functional group had the same properties as an ε-amino group.

Kinetic Basis for Activation—The enhanced activity of the PI-enzyme could be due to a change in the mechanism of the enzymic reaction or simply to an increase in the rate of the rate-limiting step. The mechanism for the native enzyme is predominantly ordered bi bi at pH 7.15 (19, 40), although it is probably partly random (41-43). The ordered bi bi mechanism for the forward reaction can be represented by the following scheme (33):

\[
\begin{align*}
NAD^+ & \rightarrow \text{E-NAD}^+ \\
\text{E-NAD}^+ & \rightarrow \text{E-NADH} \\
\text{E-NADH} & \rightarrow \text{E-NADH-CH}_2\text{CHO} \\
\text{E-NADH-CH}_2\text{CHO} & \rightarrow \text{E-NADH} \\
\text{E-NADH} & \rightarrow \text{E-NADH} + \text{CH}_3\text{CHO} \\
\text{E-NADH} + \text{CH}_3\text{CHO} & \rightarrow \text{E-NADH-CH}_2\text{CHO} \\
\text{E-NADH-CH}_2\text{CHO} & \rightarrow \text{E-NADH} + \text{CH}_3\text{CHO} \\
\text{E-NADH} + \text{CH}_3\text{CHO} & \rightarrow \text{E-NADH-CH}_2\text{CHO} \\
\end{align*}
\]

The rate constants on the left of the arrows are for the forward reaction and those on the right are for the reverse reaction. The rate-limiting step in either the forward or the reverse reaction is most probably the breakdown of the enzyme-coenzyme complex (34, 42, 44, 45). In fact, the ternary complexes form, interconvert, and break down so fast over the pH range of 6 to 9 (34) that the reactions were described for many years by the Theorell-Chance mechanism (34, 46, 47).

The product inhibition patterns presented in Figs. 7 and 8 show that the reactions catalyzed by both the native and picolinimidylated enzymes at pH 9.0 conform to the ordered bi bi mechanism. The data are inconsistent with both the simple Theorell-Chance and rapid equilibrium random bi bi mechanisms since ethanol and acetaldehyde are linear noncompetitive inhibitors (19, 33). Moreover, for the PI-enzyme the simple rapid equilibrium random bi bi mechanism is excluded by the observation that, in 8.2 mM or 0.53 M ethanol, K_{in} had about the same value as in 0 M ethanol (experiments analogous to Fig. 8A). If the PI-enzyme had this mechanism, the apparent K_{in} should have increased as the ethanol concentration increased. The simple random bi bi mechanism cannot be excluded, but there is no evidence for it (such as nonlinear reciprocal plots and hyperbolic noncompetitive inhibition (19, 33)). More complicated mechanisms, such as random with dead end complexes, also cannot be excluded. The simplest interpretation of the data is that the basic mechanism is unchanged by picolinimidylation and that the increase in activity is due to the faster breakdown of the enzyme-coenzyme complexes.

Theoretically, the turnover number for the forward reaction, V_f/\epsilon_n, should equal the rate of dissociation of the E-NADH complex, k_f. If there is only one kind of E-NADH complex, k_f can be calculated from the turnover number for the NADH and acetaldehyde reaction, V_f/\epsilon_n, and the Michaelis and inhibition constants for NADH (33). Then the increased activity (V_f/\epsilon_n) of PI-enzyme as compared to the native enzyme should be reflected in an increased dissociation rate, k_f. Table III shows that it is. Furthermore, the increase in the turnover number for the reverse reaction, V_r/\epsilon_n, should equal the increase in k_r, the rate of dissociation of the E-NADH complex.
TABLE III

Correlation of increased turnover numbers and rates of dissociation of enzyme-coenzyme complexes

The data from Table I were used in the calculations.

| Constant | Native enzyme | PI-enzyme | Ratio |
|----------|---------------|-----------|-------|
| $k_1 = V_1K_{s1}/E_1K_a$ | 12 | 120 | 10 |
| $V_1/E_1$ | 4.9 | 61 | 12 |
| $k_2 = V_2K_{s2}/E_2K_a$ | 5.4 | 240 | 44 |
| $V_2/E_2$ | 12 | 300 | 30 |
| $k_3 = V_3/K_{s3}/E_3K_a$ | 1.8 | 1.5 | 0.8 |
| $k_4 = V_4/K_{s4}/E_4K_a$ | 0.14 | 0.11 | 0.8 |

The agreement is good here, also (Table III). The rates of association of the enzyme and coenzymes, $k_3$ and $k_4$, are essentially unchanged.

However, the calculations in Table III expose an inconsistency in that the calculated $k_3$ is less than $V_1/E_1$ for both the native and picolinimidylated enzymes. The over-all rate of the reaction cannot be faster than the slowest step. The inconsistency probably is not caused by impurities in the coenzymes (34), for purified NAD$^+$ gave the same inhibition and Michaelis constants as the best grade of commercial NAD$^+$ in our experiments; also, the impurities in commercial grades of NAD$^+$ and NADH did not affect initial rate data at pH 9.0 (34, 48). On the other hand, the presence of inactive (dead end) $E\cdot NAD^+$ or isomeric $E\cdot NAD^+$ complexes in the mechanism could account for the discrepancy (59). For example, consider the effect of isomeric $E\cdot NAD^+$ complexes:

$$E\cdot NAD^+ \xrightarrow{k_{30}} E\cdot NAD^+ \xrightarrow{k_3} E + NAD^+$$

In this case

$$k_2 = k_{30} + k_3 + k_{30}$$

which is less than $k_3$ (33). Although the calculations of $k_2$ given in Table III are not valid, they indicate that changes in the rates of isomerization or dissociation (or both) of the enzyme-coenzyme complexes have occurred after picolinimidylation. From our data, we cannot say whether the $E\cdot NAD^+$ complexes also isomerize (33), but evidence for inactive complexes or isomerization with the dehydrogenase was found at pH 7.0 (10, 49) and pH 8.58 (61). If both enzyme-coenzyme complexes isomerize, none of the rate constants can be calculated (33).

The activity of liver alcohol dehydrogenase may be enhanced without covalent modifications. The enzyme is about 10 times more active with the 3-acetylpyridine analogue of NAD$^+$ than it is with NAD$^+$ in 2.5 M ethanol (52) because reduced 3-acetylpyridine adenine dinucleotide dissociates about 7 times faster from the enzyme than does NADH (53). Imidazole activates the enzyme 5 to 10 times, apparently by destabilizing the $E\cdot NAD^+$ complex (54). Other heterocyclic compounds (55) and cyclohexanol (56) can also activate.

The observation that PI-enzyme is more active than unmodified enzyme in the assay with high NAD$^+$ and ethanol concentrations but has about the same activity as the unmodified enzyme in the assay with low substrate concentrations (Fig. 2) can be understood from the kinetic constants in Table I and the initial velocity equation for the reaction:

$$V = \frac{V}{1 + K_A + K_B + K_{12}K_{23}AB}$$

where $A$ and $B$ represent the concentrations of NAD$^+$ and ethanol, respectively. With high concentrations of substrates, where $v = V$, the PI-enzyme should be 12 times more active than the native enzyme. Actually a 19-fold difference is observed, because the native enzyme is inhibited about 50% in 0.5 M ethanol whereas the PI-enzyme operates at about the maximum velocity. At lower substrate concentrations, the PI-enzyme is less saturated than the unmodified enzyme since the kinetic constants for PI-enzyme are larger than those of native enzyme; thus the velocity of the reaction catalyzed by PI-enzyme can be even less than that catalyzed by the native enzyme.

Role of Amino Groups in Activity of Liver Alcohol Dehydrogenase—The amino groups that can be modified with MPI, ethyl acetimidate, or cyanate are not essential for catalytic activity, but they may participate in coenzyme binding or they may be close enough to the active site so that substitution of them interferes with the binding of coenzymes. Since picolinimidylation does not greatly affect the binding of the adenosine 5'-diphosphoribose portion of the NAD$^+$ molecule but does affect the binding of the nicotinamide part (Table II), there is probably at least one amino group near the binding site for the nicotinamide ring. This amino group is probably very close to the catalytic region since the ternary complexes formed with NAD$^+$ and pyrazole or NADH and isobutryramide protect against the activation by imidoesters (Fig. 4). Thus, at least one amino group, a zinc ion (9-12, 57-59), and an —SH group (2-4, 35) are near the catalytic region of each active site of alcohol dehydrogenase.

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