Activation and Inactivation of Horse Liver Alcohol Dehydrogenase with Pyridoxal Compounds

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SUMMARY

Pyridoxal compounds can either activate or inactivate horse liver alcohol dehydrogenase in differential labeling experiments. Amino groups outside of the active sites were modified with ethyl acetimidate, while the amino groups in the active sites were protected by the formation of the complex with NAD⁺ and pyrazole. After removal of the NAD⁺ and pyrazole, the partially acetimidylated enzyme was reductively alkylated with pyridoxal and NaBH₄, with the incorporation of one pyridoxal group per subunit of the enzyme. The Michaelis and inhibition constants increased 13-fold or more. Pyridoxyl phosphate and NaBH₄, also modified one group per subunit, but the turnover numbers decreased by 10-fold and the kinetic constants were intermediate between those obtained for pyridoxyl alcohol dehydrogenase and the partially acetimidylated enzyme.

With native enzyme, the rates of dissociation of the enzyme-coenzyme complexes are rate-limiting in the catalytic reactions. The pyridoxyl enzyme is activated because the rate of dissociation of the enzyme-coenzyme complexes is increased. The rates of binding of coenzyme to phosphopyridoxyl enzyme have decreased due to the introduction of a negatively charged phosphate. The size of the group is greatly decreased by the incorporation of pyridoxal. For both pyridoxyl and phosphopyridoxyl alcohol dehydrogenases, the interconversion of the ternary complex is at least partially rate-limiting.

Chymotryptic-tryptic digestion of pyridoxyl enzyme produced a major peptide corresponding to residues 219 to 229, in which Lys 228 had reacted with pyridoxal. The same lysine residue reacted with pyridoxal phosphate.

Modification of ε-amino groups at the active sites of horse liver alcohol dehydrogenase (EC 1.1.1.1) with substituents that retain the positive charge increases the maximum velocities of the reactions catalyzed by the enzyme (2-4). The native and modified enzymes have mechanisms consistent with Ordered Bi Bi, but the modified enzymes are activated due to increased rates of dissociation of the enzyme-coenzyme complexes, the rate-limiting steps in the reactions catalyzed by the native enzyme (2-7). Reagents that introduce a net negative charge on the amino group partially inactivate the enzyme (4, 8). Pyridoxal-P inactivates by 80%, from which McKinley-McKee and Morris (8) concluded that there are two mutually exclusive sites for pyridoxal-P at each active center. They proposed that binding at the first site completely inactivates the enzyme, but binding at the second site does not affect activity, although it prevents binding of pyridoxal-P at the first site. Thus, reaction with pyridoxal-P produces a mixture of two enzymatic forms, one active and one inactive (8). The present work supports an alternative explanation that pyridoxal-P reacts at a single site to form a derivative with reduced activity.

EXPERIMENTAL PROCEDURE

Materials—NAD⁺ and NADH used for kinetics were purchased from Boehringer Mannheim; NAD⁺, pyridoxal-P, pyridoxal, and ribonolactone from Sigma; arginylphenylalanine from Cyclo Chemicals; acetaldehyde, pyrazole, and ethyl acetimidate from Eastman Organic Chemicals; methyl picolinimidate from Aldrich; NaBH₄ from Gallery; hexadeuteroethanol from International Chemical and Nuclear Corp.; and ethylenimine and all sequencing reagents from Pierce. Horse liver alcohol dehydrogenase was obtained from Boehringer Mannheim or purified by a modification of the method of Theorell et al. (9). Chymotrypsin, trypsin, and Escherichia coli alkaline phosphatase were obtained from Worthington Biochemical Corp.

Spectral Measurements—All spectra and kinetics were recorded with a Cary 118 C spectrophotometer. A Hitachi MPF-2A spectrofluorimeter was used for fluorescence measurements. Molar extinction coefficients at 325 nm of 7500 for Pxy-lysine and 10,000 for P-Pxy-lysine were used (10).

Preparation of Modified Proteins—We prepared P-Pxy-alcohol dehydrogenase by incubating partially acetimidylated enzyme (3) for 30 min at 25°C with 10 mM pyridoxal-P in 85 mM NaPO₄ (adjusted to pH 8.4 with HCl). The solution was cooled to 0°C and 2 to 10 µl of octanol were added to prevent foaming. About 0.5 to 1 mol of NaBH₄ per mol of pyridoxal-P were then added (11). The addition of 30 µg of ribonolactone per mg of NaBH₄ destroyed the excess NaBH₄. The reaction mixture was then desalted on a column of Sephadex G-50. The entire procedure was repeated two or three times to completely modify the enzyme. Pxy-alcohol dehydrogenase was prepared in a similar procedure, except that partially acetimidylated enzyme was incubated only

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1 The abbreviations used are: Pxy-, pyridoxyl-; Pxy-lysine, N'-pyridoxyllysine, P-Pxy-, phosphopyridoxyl-.
once for 30 min at 0.1 mM pyridoxal followed by the addition of 0.5 to 1 mol of NaBH₄ per mol of pyridoxal during a 60-min period.

**Sequence Studies**—Partially acetimidylated alcohol dehydrogenase (80 to 130 mg) was reductively alkylated with pyridoxal or pyridoxal P, S aminoethylated (12), and digested simultaneously with trypsin and chymotrypsin (each totaling 1% of the concentration of protein) at pH 8 and 0°C in a Radiometer pH-Stat for 7 hours. The mixture of peptides was freeze-dried and dissolved in 0.1 M acetic acid. The pyridoxyl peptide was degraded by the Edman procedure in a JEOL JAS 47K automatic sequenator using the standard JEOL short peptide program or in a manual procedure developed by Tarr.*

**Amino Acid Analysis**—Amino acid compositions were routinely determined with a Beckman 120 C or a JEOL 6AH automatic amino acid analyzer. The color value used for Pxy-lysine was 0.86 of the value for lysine (10). Samples obtained from the manual sequencing procedure were analyzed on a narrow bore, single column analyzer designed by Liao et al. (13) that used fluorescence sequencing procedure developed by Tarr.*

**RESULTS**

Reaction with Pyridoxal Compounds—Since native alcohol dehydrogenase reacts with six to seven pyridoxal phosphates per subunit (8), it is difficult to determine how many different amino groups at the active site are modified. Therefore we used a differential labeling procedure in which the amino groups outside of the active sites were first acetimidylated, while those amino groups at the active sites were protected by the formation of a complex with NAD⁺ and pyrazole. About 50 of 60 lysine residues per molecule were modified as shown by amino acid analysis (15). After removal of the NAD⁺ and pyrazole, partially acetimidylated alcohol dehydrogenase had a specific activity of 1 unit per mg and one amino group per active site that reacted with methyl picolinimidate (3). When partially acetimidylated alcohol dehydrogenase was incubated with pyridoxal-P and diluted into an assay mixture, the enzyme activity was decreased about 80% (Fig. 1). These results are similar to those of McKinley-McKee and Morris (8). If one assumes that the complex of pyridoxal-P and alcohol dehydrogenase has 18% of the original activity, an equilibrium constant of 1400 M⁻¹ can be calculated for the formation of the complex. A straight line was obtained when the ratio of enzyme-P-Pxy-complex to free enzyme was plotted as a function of pyridoxal-P concentration using the equation:

\[
\frac{[\text{Enzyme}-\text{Pyridoxal-P}]}{[\text{Enzyme}]} = \frac{1 - \text{Fold Activity}}{\text{Fold Activity} - 0.18} = \frac{[\text{Pyridoxal-P}]}{K_{\text{eq}}}
\]

At pH 7.2 a similar value of 3000 M⁻¹ was obtained for isocitrate dehydrogenase (16) and at pH 8.4 a value of 10⁴ M⁻¹ was reported for glutamate dehydrogenase (17).

Although enzyme preincubated with pyridoxal-P has decreased activity, the reagent did not instantaneously inhibit the activity in 50 μM NAD⁺ and 8 mM ethanol at pH 8 and 25°C at concentrations of pyridoxal-P up to 1.5 mM. Thus it seems unlikely that much noncovalent enzyme-inhibitor complex forms before the Schiff base forms. Experiments with completely acetimidylated enzyme gave similar results.

In contrast to pyridoxal-P, pyridoxal increased the specific activity 5-fold when the enzyme was preincubated with 10 mM reagent (Fig. 1). Inasmuch as the pyridoxal group appears to dissociate very quickly from the enzyme, at higher concentrations of reagent the assay for activity was not linear with time and calculation of initial velocities was not possible. When the enzyme was reductively alkylated with pyridoxal and NaBH₄, it was easy to determine that the specific activity increased more than 15-fold over that of the partially acetimidylated enzyme.

**Incorporation of Pyridoxal Compounds**—For kinetic and sequence analysis we used partially acetimidylated alcohol dehydrogenase that was reductively alkylated with either pyridoxal-P or pyridoxal. P-Pxy-alcohol dehydrogenase had 1 ± 0.1 molecule of pyridoxal-P incorporated per subunit and one-fifth the activity of partially acetimidylated enzyme. Similarly, Pxy alcohol dehydrogenase had 1 ± 0.1 molecule of pyridoxal incorporated and was 20 times more active than partially acetimidylated alcohol dehydrogenase when assayed as described in Fig. 1. The absorption spectra of P-Pxy- or Pxy alcohol dehydrogenase showed a maximum at 325 nm at pH 8 and pH dependencies similar to Pxy-lysine or P-Pxy-lysine (10). The pyridoxal ring did not appear to be buried in the interior of the protein as has been suggested for phosphorylase treated with NaBH₄ (18-20).

The amount of unmodified enzyme present in any of the Pxy- and P-Pxy-enzyme preparations could be determined sensitively from the fold increase in their activities upon treatment with methyl picolinimidate. This imidoceter will activate partially acetimidylated alcohol dehydrogenase 45-fold (3). If the P-Pxy-alcohol dehydrogenase were completely inactive, acetimidylated enzyme that was not completely modified by pyridoxal-P could also be activated 45-fold. Alternatively, if the P-Pxy-enzyme were partially active and mixed with contaminating, partially acetimidylated enzyme, we should observe less than a 45-fold activation. Ideally there should be no activation; however, the preparations used for kinetic experiments were activated by methyl picolinimidate 3- to 8-fold, which corresponds to the presence of less than 4% unmodified enzyme on the basis of protein mass as calculated from the following equation:

\[
\text{Fraction of Partially Acetimidylated Enzyme} = \frac{\text{Fold Increase in Activity} - 1}{A_{0}}
\]

where \(A_{0}\) is the specific activity of the preparation of P-Pxy-enzyme. These results indicate that P-Pxy-alcohol dehydrogenase

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*G. Tarr, manuscript submitted to Anal. Biochem.
has intrinsic activity. The pyridoxyl enzyme was not activated by methyl picolinimidate and therefore seemed to be completely modified by pyridoxal.

**Kinetics of Pyridoxal Derivatives**—To establish that the partially acetimidylated, Pxy-, and P-Pxy-alcohol dehydrogenases have characteristic activities, we determined, using product inhibition studies (21, 22), the kinetic parameters for all three derivatives. The inhibition patterns obtained were consistent with an Ordered Bi Bi mechanism, which is the same as the native enzyme (22). NADH and NAD⁺ are linear competitive inhibitors against one another, and ethanol and acetaldehyde were linear noncompetitive against one another for partially acetimidylated and P-Pxy-enzyme. However, the same four patterns for Pxy-enzyme appeared to be competitive, which would be consistent with rapid equilibrium random or Theorell-Chance mechanisms (5, 21). The kinetic constants obtained for partially acetimidylated alcohol dehydrogenase (Table I) were about the same as for native enzyme. Except for the turnover numbers, the values for P-Pxy-alcohol dehydrogenase were larger than the values for partially acetimidylated alcohol dehydrogenase. The different kinetic constants of P-Pxy alcohol dehydrogenase indicate that the activity is not due to the presence of partially acetimidylated alcohol dehydrogenase that was not modified with pyridoxal-P. All of the kinetic constants for Pxy-alcohol dehydrogenase were larger than those for either pyridoxal-P or partially acetimidylated alcohol dehydrogenase. Pxy-alcohol dehydrogenase had somewhat higher, but similar, kinetic constants as compared to picolinimidylated enzyme (4).

The turnover numbers for the P-Pxy-alcohol dehydrogenase are increased 10-fold for reaction with NAD⁺ and ethanol and 33-fold for reaction with NADH and acetaldehyde. These kinetic results show that all three modified enzymes have characteristic activities and that modification of a single amino group can either activate or inactivate alcohol dehydrogenase.

For picolinimidylated alcohol dehydrogenase the rate-limiting step in the mechanism has changed from the rate of dissociation of the coenzyme to the rate of interconversion of the ternary complex (3). If the interconversion of the ternary complex were at least partially rate-limiting for the pyridoxal derivatives, an isotope effect on V₁ should be found. We compared the V₁ values obtained for the three enzymatic derivatives using H₂ethanol and H₂NADH. The partially acetimidylated enzyme had a low ratio of V₁d:V₁h of 1.2; however, pyridoxal-P alcohol dehydrogenase had an isotope effect of 2.4, and pyridoxal enzyme had an isotope effect of 2.8. Values from 2 to 15 indicate that the rate-limiting steps probably include the breaking of a carbon-hydrogen bond (23).

**Identification of Lysine Residue Modified with Pyridoxal Compounds**—If a unique amino group reacts with pyridoxal compounds, only a single lysine residue should be modified. Pyridoxal dehydrogenase, with 0.85 molecule of pyridoxal incorporated per subunit, was digested with trypsin and chymotrypsin. A single major fluorescent peak due to Pxy-lysine was obtained when the digest was filtered through Sephadex G-25 (Fig. 2A). The material in this peak was applied to a column of Bio-Rad Aminex A-5 resin which was developed at 50° with a 500-ml linear gradient of 0.2 M acetic acid (adjusted to pH 4.9 with acetic acid) at a flow rate of 37 ml per hour. C, the major peak in B was chromatographed on a column (0.9 x 22 cm) of Bio-Rad AG 1-X2 developed at a flow rate of 37 ml per hour with the buffer system described by Schroeder (24). D, the partial acid hydrolysate was chromatographed on Aminex A-5 using the same gradient as in B. Fluorescence at 360 nm as a result of excitation at 330 nm is indicated by —— absorbance at 570 nm due to ninhydrin analysis of alkaline hydrolysates (25, 26) is indicated by ——, and pH by ——.

**Table I**

<table>
<thead>
<tr>
<th>Kinetic constants for partially acetimidylated, P-Pxy-, and Pxy-alcohol dehydrogenases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
</tr>
<tr>
<td>Kₐ</td>
</tr>
<tr>
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<td>Kᵦᵦᵦᵦᵦᵦᵦ</td>
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</tbody>
</table>

**FIG. 2.** Isolation of pyridoxyl peptides. A, the tryptic-chymotryptic peptides from 130 mg of Pxy-alcohol dehydrogenase were filtered through a column (2.4 x 95 cm) of Bio-Gel P-25 developed at room temperature with 0.1 M acetic acid at a flow rate of 50 ml per hour. B, the material from A was applied to a column (0.9 x 16 cm) of Bio-Rad Aminex A-5 resin which was developed at 50° with a 500-ml linear gradient of 0.2 M pyridine (adjusted to pH 3.1 with acetic acid) to 2 M pyridine (adjusted to pH 4.9 with acetic acid) at a flow rate of 37 ml per hour. C, the major peak in B was chromatographed on a column (0.9 x 22 cm) of Bio-Rad AG 1-X2 developed at a flow rate of 37 ml per hour with the buffer system described by Schroeder (24). D, the partial acid hydrolysate was chromatographed on Aminex A-5 using the same gradient as in B. Fluorescence at 360 nm as a result of excitation at 330 nm is indicated by —— absorbance at 570 nm due to ninhydrin analysis of alkaline hydrolysates (25, 26) is indicated by ——, and pH by ——. 
Aminex A-5 (Fig. 2B), where another single major fluorescent peak was detected. Even if the fluorescence is corrected for the quenching due to the high pyridine concentrations, less than 7% of the total fluorescence found in the chromatogram was found in either of the two minor peaks chromatographing after the main peak in Fig. 2B. A single fluorescent peak was detected when the material from the major peak from Dowex 50 was chromatographed on AG 1-X2 (Fig. 2C). The over-all yield of the peptide was 36%, based on an incorporation of 0.85 pyridoxal molecule per subunit. The amino acid composition and the sequence of the material in this peak (Table II) were consistent with the residues 219 to 229 in the complete sequence of the enzyme determined by Jörnvall (27). As indicated in the table, we were not able to determine which of the lysine residues was labeled with pyridoxal. Pxy-lysine was destroyed by HI hydrolysis and acetimidyllysine, which is reasonably stable to HI hydrolysis, did not extract into the chlorobutane.

To obtain smaller peptides, 200 nmol of the undecapeptide were partially hydrolyzed for 20 hours at pH 2 and 110°, under which conditions the peptide bonds on both sides of aspartyl residues should hydrolyze selectively (31, 32). When the hydrolysate was chromatographed on Aminex A-5, two peaks were detected (Fig. 2D). The first peak had 70% and the second peak had 30% of the total Pxy-lysine obtained from the column. The material in each peak contained Pxy-lysine and phenylalanine in equimolar amounts.

**Table II**

**Sequence determination of pyridoxal peptide**

Approximately 350 nmol of the peptide were coupled with 2-amino-5-naphthalenesulfonyl acid (to prevent extraction of the peptide (28)) and sequenced in an automatic sequenator. Acetimidyllysine and Pxy-lysine values for the composition were corrected for destruction during acid hydrolysis by determining the amount of each at 22 and 46 hours of hydrolysis. The content of isoleucine is low due to poor cleavage of the Ile-Ile bond. Amino acids were determined at each step by amino acid analysis after hydrolysis in 6 M HCl for 12 hours at 120° (29). In each step the second highest amino acid was present in an amount less than 50% of the amino acid indicated.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues</th>
<th>Step</th>
<th>Edman degradation</th>
<th>nmol</th>
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<td>Aspartic acid</td>
<td>3.0</td>
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<td>Isoleucine</td>
<td>183</td>
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<tr>
<td>Glycine</td>
<td>1.0</td>
<td>2</td>
<td>Isoleucine</td>
<td>174</td>
</tr>
<tr>
<td>Valine</td>
<td>1.1</td>
<td>3</td>
<td>Glycine</td>
<td>81</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.5</td>
<td>4</td>
<td>Valine</td>
<td>147</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.92</td>
<td>5</td>
<td>Aspartic acid</td>
<td>40</td>
</tr>
<tr>
<td>Pxy-Lysine</td>
<td>0.98</td>
<td>6</td>
<td>Isoleucine</td>
<td>150</td>
</tr>
<tr>
<td>Acetimidyllysine</td>
<td>0.94</td>
<td>7</td>
<td>Asparagine</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>Aspartic acid</td>
<td>47</td>
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<td></td>
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<td>10</td>
<td>NI</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Phenylalanine</td>
<td>150</td>
</tr>
</tbody>
</table>

*a* Acids and amides were identified by thin layer chromatography on silica gel developed with CHCl3, CH3OH (90:10) (30). The low yields of aspartic acid may result from the coupling of the carboxyl groups to the naphthalenedisulfonic acid, thus resulting in poor solubility in the chlorobutane used to extract the thiazolinone amino acids.

About 20 nmol of the material in the second peak were treated with phenylisothiocyanate: excess reagent was extracted, the phenylthiocarbamyl peptide was cyclized with concentrated HCl, and the residue was analyzed directly for the presence of free amino acids. About 5 nmol of free phenylalanine were detected. (Lack of additional material prevented further sequencing of the peptide.)

The first peak of Fig. 2D was also subjected to the manual Edman procedure. After one cycle on 40 nmol of peptide, one-fourth of the organic phase containing phenylthiohydantoins of the amino acids was analyzed on a JEOL gas chromatograph. The phenylthiohydantoin of phenylalanine was not detected. After hydrolysis in 57% HI for 16 hours at 110°, we accounted for 25 nmol of phenylalanine. A small amount of lysine was found, but no Pxy-lysine. When this same organic fraction containing the phenylthiohydantoins of amino acids was hydrolyzed in 6 M HCl for 22 hours at 110°, we accounted for 25 nmol of phenylalanine, 3.4 nmol of lysine, and 16 nmol of Pxy-lysine. After hydrolysis of the aqueous phase in HCl, small amounts of phenylalanine (2.5 nmol) and Pxy-lysine (4 nmol) were found.

We suspected that the first peak in Fig. 2D was the diketopiperazine of phenylalanine and Pxy lysine and the second peak was the simple dipeptide. These results would indicate that Lys 228 was the reactive residue since partial hydrolysis of the parent peptide should yield the dipeptide Pxy-lysylphenylalanine. To determine if such a dipeptide could form the cyclic diketopiperazine, we treated 37 nmol of arginylphenylalanine to the same conditions used for partial acid hydrolysis. When the mixture was chromatographed on Aminex A-5, two peaks containing peptide were obtained again. The material in the first peak, containing 72% of the total material recovered from the column, was ninhydrin-negative and would not react in the manual sequencing system. This peptide was probably the diketopiperazine. The material in the second peak was ninhydrin-positive. Material from the front of the peak had the sequence Phe-Arg and the material from the back side was Arg-Phe; thus, the peak apparently contained dipeptides resulting from random hydrolysis of the diketopiperazine.

We also identified the labeled residue for P-Pxy-alcohol dehydrogenase. Aminoethylated P-Pxy alcohol dehydrogenase was digested with trypsin and chymotrypsin, filtered through Sephadex G-25, and chromatographed on AG 1-X2. The major fraction from this column was treated with alkaline phosphatase (33, 34). Rechromatography on Dowex 1 did not alter the position of elution enough to separate the fluorescent peak from the parent ninhydrin peak. Chromatography of the peptide on Aminex A-5 gave a peptide in an over-all yield of 25%. This peptide was also partially hydrolyzed at pH 2 and 110° for 20 hours. This hydrolysate, when chromatographed on Aminex A-5 gave two peaks in positions similar to those shown for the Pxy-peptide in Fig. 2D. Both peaks had the composition Pxy-lysine and phenylalanine in equal molar ratios. The first peak would not react in the Edman reactions as before. Again, it appears that Lys 228 is the reactive residue in the protein.

**Discussion**

Site of Action of Pyridoxal Compounds—Incorporation of 10 or more molecules of pyridoxal-P into liver alcohol dehydrogenase completely inactivates the enzyme (8). The incorporation of a large number of negatively charged groups at what were positively charged lysine residues probably distorts the protein structure. We prevented the additional incorporation of pyr-
idoxal-P by acetylmutidating the amino groups outside of the active sites. Acetimidylation preserves the positive charge without markedly affecting the kinetic constants. The sequencing results with partially acetimidylated enzyme suggest that Lys 228 was the predominant site of reaction with both pyridoxal-P and pyridoxal. We found no evidence for two enzyme sites where binding of pyridoxal-P at one site prevents the other from reacting. The P-Pxy-enzyme had characteristic kinetic constants when compared to other derivatives of the enzyme and was not fully activated by reaction with methyl picolinimidate as would be expected if the partial activity were due to the presence of unmodified partially acetimidylated enzyme. Reduced intrinsic activity was also found by Goldin and Frieden for P-Pxy-glutamate dehydrogenase (35).

Unexpectedly in the sequence work the diketopiperazine of P-Pxy-lysylphenylalanine was formed, when the 11-residue pyridoxyl peptide was heated to 110° at pH 2. Sanger warned of the possibility of inverting sequences of dipeptides as the result of partial acid hydrolysis (30, 37). High temperature is sufficient for the formation of such cyclic structures. Fifty years ago, Abderhalden and Komm converted glycylphenylalanine to its diketopiperazine in 33% yield when the dipeptide was heated at 150° at neutral pH for 6 hours (38). The pyridoxyl or P-pyr-idoxyl peptides containing sequence residues 219 to 229 appeared at 150° at neutral pH for 6 hours (38). The pyridoxyl or P-pyr-

Mechanism of Inhibition and Activation by Pyridoxal Compounds—To explain how pyridoxal-P partially inact. ivates and the dipeptide Pxy-lysylphenylalanine. However, 70% of the peptide was converted similarly. In a control experiment, arginylphenylalanine was converted similarly. The data in Table I were used to calculate the constants (21).

![Scheme 1](image)

For the reaction of NAD⁺ and ethanol catalyzed by native (5-7) and partially acetimidylated enzyme, dissociation of NADH is rate-limiting. However, for enzyme activated with certain imido esters, the interconversion of the ternary complex, k₈, is at least partially rate-limiting (3). Pxy-enzyme is similar to these other activated forms of the enzyme, in that k₈ is increased 60-fold when compared to k₈ for partially acetimidylated alcohol dehydrogenase (Table III). In contrast, k₇ for P-Pxy-enzyme remains the same as partially acetimidylated alcohol dehydrogenase, but the enzyme has a lower turnover number, V₅/E₅. Since k₇ is unchanged, the rate-limiting step must have changed to either dissociation of acetaldehyde, k₈, or the interconversion of the ternary complex, k₉. The introduction of the negatively charged phosphate in the partially active P-Pxy-enzyme may have altered the spatial arrangement of the substrates and catalytic residues so as to decrease the rates of interconversion of the ternary complexes. The isotope effect observed for the derivative is consistent with this suggestion. Although the activity of Pxy-alcohol dehydrogenase is increased due to the increased rate of dissociation of NADH, (k₈), the interconversion of the ternary complexes has also become partially rate-limiting. Thus, both pyridoxal compounds differentially affect the interconversion of the ternary complex and k₈. The rate constants k₁ and k₄ are markedly decreased when the negatively charged phosphate is present but are not changed as much when the pyridoxyl substituent or substituents with a single positive charge are present.

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D C Sogin and B V Plapp


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