The Reaction of Pyridoxal 5'-Phosphate with an Essential Lysine Residue of Saccharopine Dehydrogenase (1-Lysine-forming)∗

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Saccharopine dehydrogenase (EC 1.5.1.7) from bakers' yeast was reversibly inactivated by pyridoxal and pyridoxal 5'-phosphate. The inactivation by pyridoxal 5'-phosphate was accompanied by the appearance of a peak at 425 nm, which was shifted to 325 nm upon reduction. The inactivation was due to Schiff base formation with a lysyl residue of the enzyme. Correlation between the loss of enzyme activity and the amount of pyridoxal 5'-phosphate incorporated into the enzyme showed that the modification of 1 lysyl residue per molecule of enzyme completely inactivated the enzyme. The inactivation was fully protected by α-ketoglutarate in the presence of a saturating concentration of NADH. Since α-ketoglutarate is bound only to the enzyme-NADH complex, the result strongly suggests that the lysine residue essential for activity is located in the vicinity of the substrate-binding site. The kinetics of inactivation by pyridoxal 5'-phosphate and reactivation of the inactivated enzyme, as well as the relationship between the residual activity at equilibrium and pyridoxal 5'-phosphate concentration, indicated that no noncovalent enzyme-pyridoxal 5'-phosphate complex was formed prior to Schiff base formation. In contrast to many enzymes that have a lysine residue at the coenzyme- and substrate-binding sites, Saccharopine dehydrogenase (EC 1.5.1.7) contains one binding site for L-lysine and α-ketoglutarate in the presence of a pyridine nucleotide coenzyme. The enzyme from bakers' yeast is a monomeric protein with a molecular weight of 39,000 (Ogawa and Fujioka, 1978) and contains one binding site for reactants (Ogawa et al., 1979). In an effort to identify the amino acid residues at the active site which are involved in substrate and coenzyme binding and catalysis, we have carried out chemical modification studies on the enzyme. Previous investigations have shown that the enzyme possesses 1 cysteine and 1 histidine residue at the coenzyme- and substrate-binding sites, respectively, both of which appear to be essential for activity (Ogawa et al., 1979; Fujioka et al., 1980).

In addition to these residues, a number of pyridine nucleotide-dependent dehydrogenases are known to have a lysine residue essential for activity, and its role in coenzyme binding, substrate binding, and in catalysis has been implicated (Ronchi et al., 1969; Piszksiewicz and Smith, 1971a and b; McKinley-McKee and Morris, 1972; Brown et al., 1973; Chen and Engel, 1975a, b, c, and d; Wimmer and Harrison, 1975; Oshima et al., 1978; Blaner and Churchich, 1979). The present investigation was undertaken to examine the possible involvement of a lysine residue in the function of saccharopine dehydrogenase using pyridoxal-P as a selective chemical reagent for the modification of the lysyl residue. This paper reports that pyridoxal-P reversibly inactivates saccharopine dehydrogenase by forming a Schiff base with a lysyl residue, and describes the results of kinetic and equilibrium analyses of the reaction of the enzyme with pyridoxal-P.

EXPERIMENTAL PROCEDURES

Materials—Saccharopine dehydrogenase was prepared from bakers' yeast (Oriental Yeast Co., Tokyo) as described previously (Ogawa and Fujioka, 1978). Pyridoxal hydrochloride, pyridoxal-P, and pyridoxamine dihydrochloride were purchased from Nakara Chemicals, Kyoto, and NaBH₄ was from Wako Pure Chemicals, Osaka. Na[H]BH₄ (100 mCi/mmol) was obtained from New England Nuclear. e-N-Pyridoxyllysine was a gift from Dr. T. Hase, Faculty of Science, Osaka University. All other chemicals were of the highest grade available from commercial sources and were used without further purification.

Reaction of Saccharopine Dehydrogenase with Pyridoxal and Pyridoxal-P—The reaction of saccharopine dehydrogenase with pyridoxal and pyridoxal-P was carried out in 0.1 M potassium phosphate buffer in the dark as much as possible. The concentrations of pyridoxal and pyridoxal-P were determined spectrophotometrically in 0.1 N NaOH using molar extinction coefficients of 8,800 M⁻¹ cm⁻¹ at 300 nm and 6,600 M⁻¹ cm⁻¹ at 388 nm, respectively (Sober, 1970). The extent of inactivation was determined by measuring the residual enzymic activity on an aliquot removed from the incubation mixture. Since the inactivation was reversible on dilution, the aliquot was treated with NaBH₄ to reduce the Schiff base before activity measurement. The reduction was carried out at 0°C by the addition of NaBH₄ in a slight excess over pyridoxal or pyridoxal-P, and the mixture was allowed to stand for about 30 min at 0°C before the enzyme assay at 24°C. NaBH₄ had no effect on the activity of the native enzyme. The enzyme activity was determined by the rate of decrease of absorbance of NADH at 340 nm in the direction of the reaction of the enzyme with pyridoxal-P.

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∗ The abbreviation used is: pyridoxal-P, pyridoxal 5'-phosphate.
concentrate was again chromatographed with the authentic \( \epsilon \)-N-pyridoxyllysine in pyridine/acetate acid/butanol/water (20:6:30:24) (Dempsey and Christensen, 1962).

Other Determinations—Protein was determined by the method of Lowry et al. (1951). The concentration of saccharopine dehydrogenase was calculated using a molecular weight of 38,000 (Ogawa and Fujikawa, 1978). Radioactivity was determined in an Aloka liquid scintillation spectrometer model LSC-903, and fluorescence spectra were taken in a Shimadzu model RF-501 spectrofluorometer. Absorption spectra were recorded with a Beckman model 24 spectrophotometer equipped with a JEOL recorder U-125, and absorbance determinations were made with a Hitachi model 200-10 spectrophotometer.

RESULTS

Inactivation of Saccharopine Dehydrogenase by Pyridoxal and Pyridoxal-P—Incubation of saccharopine dehydrogenase with pyridoxal or pyridoxal-P in 0.1 m potassium phosphate buffer, pH 6.8, and at 24°C caused a time-dependent decrease of activity which reached a steady value in about 5 min. Prolonged incubation resulted in no further loss of activity. Pyridoxamine was without effect. With pyridoxal and pyridoxal-P, each at a concentration of 0.5 mM, the remaining activities were 90 and 33% of the initial value, respectively. The inactivation by these reagents was reversible; the enzymic activity was almost fully restored by dialysis or dilution with the buffer. Treatment with NaBH₄, however, rendered the inactivation irreversible. The residual activity found after prolonged incubation is not a property of the modified enzyme, but is due to an equilibrium established between the free and modified enzymes, as shown by the following experiment. The enzyme that had been inactivated to a maximal degree by the reaction with a given amount of pyridoxal-P was reduced with NaBH₄ and dialyzed. Treatment of the reduced enzyme with the same concentration of pyridoxal-P resulted in a further inactivation exactly to the same extent as in the first treatment (Table 1). The residual activity at equilibrium decreased with increased concentration of pyridoxal-P; an almost complete inactivation was obtained at high concentrations of the reagent.

Spectra of the Modified Enzyme—The difference spectrum between the pyridoxal-P-treated enzyme and pyridoxal-P showed a positive peak at 428 nm and a negative one at 378 nm (Fig. 1), characteristic of an imine linkage between the formyl group of pyridoxal-P and an amine. Similar spectra have been observed in reactions of pyridoxal-P with many enzymes. Treatment with NaBH₄ caused the appearance of a new peak at 325 nm with the disappearance of the 428 nm band (Fig. 2A). The native saccharopine dehydrogenase has a fluorescence with an emission maximum at 340 nm when excited at 280 nm. When the reduced, modified enzyme was examined fluorometrically, it showed a shoulder in the 390 nm region and a decreased fluorescence at 340 nm (Fig. 2B). The decreased protein fluorescence may result from radiationless energy transfer from the aromatic residues of the protein to the bound pyridoxyl group (Churchich, 1965). The spectral data obtained indicate the formation of a Schiff base between the amino acid residue involved in the Schiff base formation, NaBH₄-reduced enzymes. The enzyme (17.9 nmol) was incubated with 0.5 mM pyridoxal-P in 1.0 ml of 0.1 m potassium phosphate buffer, pH 6.8, for 30 min at 24°C. After the addition of NaBH₄ (4 \( \mu \)mol), the mixture was allowed to stand at 0°C for 30 min, and then dialyzed against 0.1 m potassium phosphate buffer, pH 6.8, in the dark. Ultraviolet absorption spectra and fluorescence emission spectra of the native (\( \cdots \cdots \)) and the reduced (\( \cdots \cdots \)) enzymes were recorded at the same protein concentration. The fluorescence intensity is expressed in arbitrary units. Excitation wavelength, 280 nm.

TABLE I

<table>
<thead>
<tr>
<th>Treatment with pyridoxal-P</th>
<th>Percentage of initial activity</th>
<th>Percentage of previous treatment</th>
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<tr>
<td>1</td>
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<td></td>
</tr>
<tr>
<td>2</td>
<td>25.0</td>
<td>48.4</td>
</tr>
<tr>
<td>3</td>
<td>12.0</td>
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Identification of \( \epsilon \)-N-Pyridoxyllysine—In order to identify the amino acid residue involved in the Schiff base formation, an acid hydrolysate of pyridoxal-P-treated, NaBH₄-reduced enzyme was analyzed as described under "Experimental Procedures." Paper chromatography revealed the presence in the acid hydrolysate of a single fluorescent material indistinguishable from \( \epsilon \)-N-pyridoxyllysine, indicating that the Schiff base was with lysine residues of the enzyme.

Correlation between Enzymic Activity and the Amount of Pyridoxal-P Bound per Mol of Enzyme—The stoichiometry between the loss of enzymic activity and the amount of pyridoxal-P bound per mol of enzyme was determined by measuring the residual enzymic activity and the absorbance.
at 325 nm after reduction with NaBH₄. When the linear portion of a plot of residual activity versus absorbance at 325 nm was extrapolated to zero activity, a value of 1 ml of pyridoxal-P bound/mol of enzyme was obtained by assuming a molar absorbance of 10⁴ M⁻¹ cm⁻¹ for e-N-phosphopyridoxal-lysine (Fisher et al., 1963) (data not shown). Although this value of molar absorbance has generally been used for this purpose, some examples have been published recently in which this is not applicable (Blackburn and Schachman, 1976; Paech and Tolbert, 1978). Therefore, in order to ascertain the stoichiometry, the Schiff base was reduced with Na[³H]BH₄ and the radioactivity fixed to the enzyme was determined. Fig. 3 shows a plot of activity against the number of ³H atoms incorporated. Extrapolation to zero enzymic activity showed the incorporation of 1 atom of ³H/mol of enzyme, indicating that the modification of 1 lysyl residue caused complete inactivation.

The existence of a 1:1 stoichiometry between the loss of enzyme activity and the number of residues modified enables us to estimate the molar absorbance of the Schiff base of saccharopine dehydrogenase with pyridoxal-P. To this end, saccharopine dehydrogenase was reacted with various amounts of pyridoxal-P in 0.1 M potassium phosphate buffer, pH 6.8, for 30 min at 24°C. Extrapolation of a plot of residual activity versus A₄₃₀ to zero activity yielded a value of 5,600 ± 300 for the molar absorbance (not shown).

Rate of Inactivation by Pyridoxal-P.—Since the reaction of pyridoxal-P with saccharopine dehydrogenase was too rapid at room temperature to obtain reliable values of kinetic constants, the kinetics of inactivation and reactivation was studied at 0°C. From the foregoing results, the reaction of pyridoxal-P (P) with saccharopine dehydrogenase (E) may be represented most simply as

\[ E + P = E-P \]  

where E-P is a covalent Schiff base. Since pyridoxal-P was present in all cases in great molar excess over the enzyme, the following relationship will be obtained.

\[ \ln \frac{A_0 - A_{eq}}{A_t - A_{eq}} = (k_1 [P] + k_2)t \]  

where A₀, Aₜ, and Aₐₜ denote enzyme activities at zero time, time t, and at equilibrium, respectively. Fig. 4 shows plots of log \([A₀ - A_{eq}]/(A_t - A_{eq})\) versus time at several concentrations of pyridoxal-P. When the apparent first order rate constants \(k_{app} = k_1 [P] + k_2\) were plotted against pyridoxal-P concentrations, a straight line was obtained as predicted by Equation 2. The values of the second order rate constant for the forward reaction \(k_1\) and the first order rate constant for the reverse reaction \(k_2\) obtained from the slope and the ordinate intercept, respectively, were 0.15 min⁻¹ mmol⁻¹ and 0.03 min⁻¹.

Reactivation of the Inactivated Enzyme—Since full enzymic activity is regained by extensive dilution, the recovery of activity from the inactivated enzyme can be regarded as a simple, irreversible, first order process. (The recombination of the free enzyme with pyridoxal-P is negligible under these conditions). Therefore, the first order rate constant, \(k_2\), in Equation 2 may also be estimated by following the time course of reactivation. The reactivation is related with time as

\[ \ln \frac{A_0 - A_{eq}}{A_0 - A_t} = k_2 t \]  

where A₀ is the activity at zero time (the residual activity before reactivation); Aₜ and Aₐₜ are those at time t and at infinite time, respectively. Saccharopine dehydrogenase was incubated with pyridoxal-P, and when the residual activity reached a steady value, aliquots of the reaction mixture were diluted 170-fold with 0.1 M potassium phosphate buffer, pH 6.8. At periodic intervals, NaBH₄ was added to stabilize the Schiff base, and the enzymic activities were determined. An almost full recovery of the enzyme activity occurred within 100 min. Fig. 5 shows a plot of data according to Equation 3. The value of k₂ obtained in this experiment (0.026 min⁻¹) is in close agreement with that found from the ordinate intercept of Fig. 4, inset.

Equilibrium Study—From the findings that the enzyme can be inactivated almost completely by high concentrations...
Reaction of Saccharopine Dehydrogenase with Pyridoxal P

**Fig. 5 (left).** First order plot of the recovery of activity from the enzyme inactivated by pyridoxal-P. The enzyme (2.3 nmol) was incubated with 1.0 mM pyridoxal-P in 0.2 ml of 0.1 M potassium phosphate buffer, pH 6.8, at 0°C. After 30 min, 10-µl aliquots were transferred to test tubes containing 1.7 ml of 0.1 M potassium phosphate buffer, pH 6.8, at 0°C. At the times indicated, 0.2 ml of 100 mM NaBH₄ were added to each tube to fix the Schiff base, and the enzyme activities were determined.

**Fig. 6 (right).** Plot of reciprocal pyridoxal-P concentrations against \( A_{eq}/(A_0 - A_{eq}) \). The enzyme (2 nmol) was incubated in 0.1 M potassium phosphate buffer, pH 6.8, with pyridoxal-P at the concentrations indicated. The residual activities were determined after 30 min at 24°C. See text for details.

**Fig. 7 (left).** Effect of pH on the equilibrium constant for inactivation. The enzyme (2.5 nmol) was incubated at 24°C with 1.0 mM pyridoxal-P in 0.1 ml of 0.1 M potassium phosphate buffer at the pH values indicated. Values of equilibrium constants were calculated from the equation, \( K_{eq} = (A_0 - A_{eq})/A_0 \). The points were determined experimentally, and the line was calculated from Equation 5, with values of \( K, K_{eq}, \) and dissociation constants for pyridoxal-P and Schiff base as described in the text.

**Fig. 8 (right).** Plot of reciprocal apparent equilibrium constants for inactivation against \( \alpha \)-ketoglutarate concentrations. The enzyme (2 nmol) was incubated with 1.0 mM pyridoxal-P in 0.1 ml of 0.1 M potassium phosphate buffer, pH 6.8, containing 0.2 mM NADH and \( \alpha \)-ketoglutarate at the concentrations indicated. The residual activities were determined after 30 min at 24°C. Values of apparent equilibrium constants were calculated as described in the legend to Fig. 7.

of pyridoxal-P and that the degree of inactivation is linearly related to the fractional amount of the residue modified (Fig. 3), it is reasonable to assume that the Schiff base is totally inactivated. If so, the relationship between the residual enzyme activity at equilibrium and the concentration of pyridoxal-P may be given by

\[
\frac{1}{P} = \frac{A_{eq}}{A_0} = \frac{K_{eq}}{A_0 - A_{eq}}
\]  

where \( K_{eq} \) is the equilibrium constant. Therefore, the plot of \( 1/P \) against \( A_{eq}/(A_0 - A_{eq}) \) should give a straight line going through the origin with a slope of \( K_{eq} \). Fig. 6 shows that this is indeed the case. A value of \( 3.3 \times 10^5 \) M⁻¹ was obtained for \( K_{eq} \). Note that Equation 2 holds even when the Schiff base is too slowly formed. In this case, however, \( A_0 \) and \( A_{eq} \) are related as \( 1/P = (A_0 - A_{eq})/A_{eq} \), where \( A_0 \) is the activity of the completely derivatized enzyme, and the plot of \( 1/P \) versus \( A_{eq}/(A_0 - A_{eq}) \) has a negative ordinate intercept.

**Effect of pH on Equilibrium of Inactivation**—The variation of the equilibrium constant with pH is shown in Fig. 7. The inactivation was maximal near pH 8.5. The equilibrium constant is dependent on the dissociation constants of the reactive lysine residue of the enzyme (\( K_p \)), and of various ionic species of pyridoxal-P and the Schiff base. In aqueous solution, both pyridoxal-P and Schiff base are known to exist in four ionic forms (see Metzler, 1957; Piszkieicz and Smith, 1971b; and Marshall and Cohen, 1977). In pyridoxal-P, the dissociation constants for the phenolic group (\( pK_{f1} \)), for the second dissociation of the phosphoryl group (\( pK_{f2} \)), and for the pyridinium nitrogen (\( pK_{f3} \)) are reported to be 4.14, 6.2, and 8.69, respectively (Williams and Neillands, 1954). The reported values for the Schiff base are: \( pK_{f1} = 5.2 \) for the pyridinium nitrogen (Piszkieicz and Smith, 1971b), \( pK_{f2} = 8.0 \) for the second dissociation of the phosphoryl group (Piszkieicz and Smith, 1971b), and \( pK_{f3} = 10.5 \) for the phenolic group (Metzler, 1957). By taking the enzyme with unprotonated lysine residue (\( E_0 \)) and the most anionic forms of pyridoxal-P (\( P^- \)) and the Schiff base (\( S^- \)) as references, and by defining \( K = [S^-]/[E_0][P^-] \), the pH-dependent equilibrium constant \( (K'_{eq}) \) is given by

\[
\log K'_{eq} = \log K + \log \left( \frac{1 + [H^+]^{[K_{f1}]}}{K_{f1} K_{f2} K_{f3} K_{f4} K_{f5}} \right) - \log \left( \frac{1 + [H^+]^{[K_{f1}]}}{K_{f1} K_{f2} K_{f3} K_{f4} K_{f5}} \right)
\]  

according to Metzler (1957) and Auld and Bruce (1967). If we assume that the dissociation constants for various forms of pyridoxal-P and Schiff base are identical with those given in the literature, the values of \( K_f \) and \( K \) may be estimated from the fit of experimental data to Equation 5. The best fit was obtained with \( K_f = 1.58 \times 10^{-9} \) M (\( pK_f = 8.8 \)), and \( K = 2.69 \times 10^6 \) M⁻¹ (Fig. 7).

**Effect of the Coenzyme and \( \alpha \)-Ketoglutarate on Inactivation**—The effect of the coenzyme and \( \alpha \)-ketoglutarate on inactivation was examined by incubating saccharopine dehydrogenase with pyridoxal-P in the presence of these compounds. Table II shows the residual enzyme activities at equilibrium. NAD⁺ had virtually no effect on the time course as well as on the equilibrium position of inactivation. NADH raised the equilibrium position only slightly even at a saturating concentration. Although \( \alpha \)-ketoglutarate alone had no effect, it gave protection in a concentration-dependent manner when NADH was present. An almost complete protection was observed at saturating levels of NADH and \( \alpha \)-ketoglutarate. It was shown previously that saccharopine dehydrogenase binds reactants in an obligatory order. In the direction of saccharopine synthesis, the order of addition is NADH, \( \alpha \)-ketoglutarate, and lysine (Pujikoka and Nakatani, 1970). Therefore, the lack of effect of \( \alpha \)-ketoglutarate in the absence of NADH obviously results from its inability to combine with the enzyme.

From the finding that NAD⁺ has no effect on inactivation, it is reasonable to assume that a rise in the equilibrium position observed with NADH is due to a different equilibrium of the \( E^- \)-NADH complex and the corresponding Schiff base from that of the free enzyme and its Schiff base. Therefore, if it is assumed that the reaction of pyridoxal-P takes place with the \( E^- \)-NADH complex, but not with the \( E^- \)-NADH-\( \alpha \)-ketoglutarate complex, the apparent equilibrium constant in the presence of \( \alpha \)-ketoglutarate (\( K'_{eq} \)) may be expressed as

\[
K'_{eq} = \frac{[S^-]/[E_0][P^-]}{[S^-]/[E_0][P^-] + [P^-]/[E_0]} = \frac{1}{1 + \frac{[P^-]}{[E_0]}}
\]
The enzyme (2.6 nmol) was incubated in 0.1 ml of 0.1 M potassium phosphate buffer, pH 6.8, with 1.0 mM pyridoxal-P and the compounds listed below at the concentrations indicated in parentheses. After incubation for 30 min at 24°C, the enzyme activities were determined. Residual activity refers to the percentage of activity obtained by comparison with the untreated control. The mM found from the plot is in good agreement with that listed below at the concentrations indicated in parentheses. After phosphate buffer, pH 6.8, with 1.0 mM butyric acid with pyridoxal-P (Olivio et al., 1978; Ogawa et al., 1963). In a number of enzymes that have organophosphates as substrates, including pyridine nucleotide-dependent dehydrogenases (Piszkiecwick and Smith, 1971b; Blumenthal and Smith, 1973; Chen and Engel, 1975a, c, and d; Marshall and Cohen, 1972; Paech and Tolbert, 1978), the formation of a Schiff base between pyridoxal-P and the active site lysine residue is proposed to proceed through a noncovalent enzyme-pyridoxal-P complex (E-P) which is in rapid equilibrium with the free enzyme:

\[
K_1 \leftarrow E + P \rightleftharpoons E \cdot P \rightleftharpoons E \cdot P
\]

The noncovalent intermediate is assumed to be an ion pair between the phosphoryl group of pyridoxal-P and a cationic site of the enzyme, and this interaction is considered to form a basis for the specificity of pyridoxal-P as a selective modification reagent. In saccharopine dehydrogenase, however, the occurrence of the intermediate prior to Schiff base formation is unlikely, because the apparent first order rate constant for inactivation was a linear function of pyridoxal-P concentration (Fig. 4, inset). The mechanism depicted in Equation 7 requires saturation kinetics with respect to pyridoxal-P concentration. Furthermore, the result of equilibrium analysis of inactivation is also consistent with the nonexistence of a noncovalent intermediate (cf. Equation 4 and Fig. 6). In the mechanism of Equation 7, the relationship between the activity remaining at equilibrium and pyridoxal-P concentration would be given by

\[
1 = A_w K_1 K_2 P = A_w - A_w - K_1
\]

where \(K_1\) and \(K_2\) represent equilibrium constants for the first and second steps, respectively. Thus, the plot of 1/[P] versus \(A_w/(A_w - A_w)\) intersects the ordinate below the abscissa. This type of plot has been obtained with glutamate dehydrogenase (Chen and Engel, 1975d) and alcohol dehydrogenase (Chen and Engel, 1975a), in which the intermediary formation of a noncovalent complex is postulated. Among pyridine nucleotide dehydrogenases studied thus far, saccharopine dehydrogenase appears to be the first example, to our knowledge, in which a noncovalent intermediate is not formed. In this connection, it is of interest to note that in glutamate dehydrogenase, a noncovalent intermediate is found in the formation of a Schiff base with pyridoxal-P but not with pyridoxal (Piszkiecwick and Smith, 1971a and b; Chen and Engel, 1975d).

If the assumed values of proton dissociation constants for various ionic forms of Schiff base are valid, a pH value of about 8.8 is obtained for the essential lysyl residue from the pH dependence of \(K_1\). Although this value is about 1 unit higher than that of the essential lysine residue of glutamate dehydrogenase (Piszkiecwick and Smith, 1971a; Blumenthal and Smith, 1973), it is considerably lower than that expected for a lysine residue in protein. The low pK might result from the hydrophobic environment of the active site or from the presence of cationic groups in the vicinity of the residue.

The inactivation by pyridoxal-P was protected by \(\alpha\)-ketoglutarate but not by the coenzyme. The variation of the apparent equilibrium constant for inactivation with \(\alpha\)-ketoglutarate concentration (Fig. 8) is consistent with the fact that the reaction with pyridoxal-P does not occur when the enzyme forms a complex with NADH and \(\alpha\)-ketoglutarate. This suggests that a lysine residue essential for activity is located at or near the substrate-binding site. In the reductive condensation of L-lysine and \(\alpha\)-ketoglutarate catalyzed by saccharopine dehydrogenase, the intermediary formation of a Schiff base between the substrate lysine and \(\alpha\)-ketoglutarate on the enzyme surface prior to the hydrogen transfer reaction may be postulated (Fujioke and Takata, 1979). As to the formation of

<table>
<thead>
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<th>Compound added (mM)</th>
<th>Residual activity %</th>
</tr>
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<tr>
<td>None</td>
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<tr>
<td>NADH (0.2)</td>
<td>41.2</td>
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<tr>
<td>NADH (1.0)</td>
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<td>NADP (0.5)</td>
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<td>(\alpha)-Ketoglutarate (8.0)</td>
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this intermediate, an attractive hypothesis is that this lysine residue first forms a Schiff base with α-ketoglutarate and a subsequent transimination reaction results in the formation of a Schiff base with the substrate lysine. Although not conclusive, this possibility appears unlikely because we were not able to show any inactivation of the enzyme by NaBH₄ or cyanogen borohydride in the presence of α-ketoglutarate and NADH (NADH being added since α-ketoglutarate combines only with the E.NADH complex). A rather high pK value found for the lysine residue appears to disprove its role as a base catalyst in the proton abstraction reaction. The functional role of this lysine residue in the catalytic mechanism of the dehydrogenase remains to be clarified.

REFERENCES

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