Pyruvate–Glyoxylate Carboligase Activity of the Pyruvate Dehydrogenase Complex of *Escherichia coli*†

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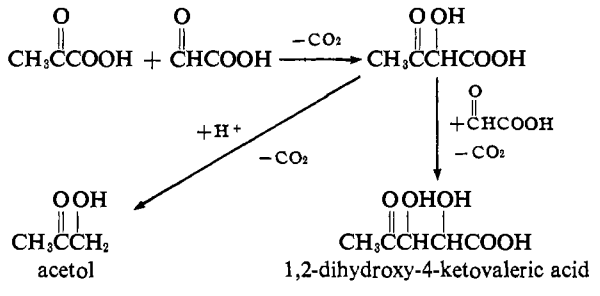
ABSTRACT: Incubation of [2-^{14}C]glyoxylate and nonlabeled pyruvate with a highly purified pyruvate dehydrogenase complex from *Escherichia coli* yielded two labeled products. One of these was identified as acetol and was presumably formed by a nonenzymatic decarboxylation of the initial product of the reaction, 2-hydroxy-3-ketobutyric acid. The second was identified as 2,3-dihydroxy-4-ketovaleric acid. Periodate oxidation of acetol yielded [^{14}C]formaldehyde and unlabeled acetic acid; 2,3-dihydroxy-4-ketovaleric acid yielded unlabeled acetic acid plus labeled formate and glyoxylate. The pyruvate–glyoxylate carboligase activity was associated with the pyruvate dehydrogenase component (E_1) of the complex when the latter was dissociated and separated into its E_1 and E_2E_3 components. As with the beef heart enzyme, the α-ketoglutarate dehydrogenase complex from *E. coli* also catalyzed the condensation of α-ketoglutarate and glyoxylate.

The enzymatic condensation of α-keto acids with various aldehydes is well established. α-Ketoglutarate condenses with acetaldehyde in the presence of the α-ketoglutarate dehydrogenase complex to form 5-hydroxy-4-ketohexanoic acid (Bloom and Westerfeld, 1966). It also condenses with glyoxylate to form two products. The major product is 2-hydroxy-3-ketoadipic acid, which decarboxylates nonenzymatically to yield 5-hydroxy-4-ketovaleric acid (Schlossberg *et al.*, 1968). The second minor product was identified as 2,3-dihydroxy-4-ketopimelic acid (Schlossberg *et al.*, 1970). Pyruvate undergoes similar carboligase condensation reactions. Its reaction with acetaldehyde to form acetoin is well known. It also reacts with succinic semialdehyde to form 5-keto-4-hydroxyhexanoic acid (Shaw and Westerfeld, 1968).

This report describes the condensation reaction between pyruvate and glyoxylate to form acetol as the major product and 1,2-dihydroxy-4-ketovaleric acid as a minor product according to the following proposed sequence of reactions



In addition to the identification of the products of the pyruvate–glyoxylate reaction, this communication also reports that the pyruvate dehydrogenase component (E_1) of the pyruvate dehydrogenase complex (pyruvate–lipoate oxidoreductase E.C.1.2.4.1) of *Escherichia coli* is responsible for the condensation reaction between pyruvate and glyoxylate. As with the mammalian enzyme, the ketoglutarate dehydrogenase complex (α-ketoglutarate–lipoate oxidoreductase, E.C.1.2.4.2) of *Escherichia coli* has also been shown to be responsible for the condensation reaction between α-ketoglutarate and glyoxylate in this organism.

Methods

E. coli λ K-12 strain was purchased as the frozen cell paste, late log phase (General Biochemicals). The two dehydrogenase complexes were isolated, purified, and dissociated as described by Reed and Willms (1966). Protein (Lowry *et al.*, 1951), glyoxylate (Dekker and Maitra, 1962), and acetol (Grant, 1948) were determined as described in the references. The periodate–chromotropic acid procedure of Frisell and MacKenzie (1958) was also used for determining acetol and 5-hydroxy-4-ketovaleric acid. Redistilled [35°(5 mm)] commercial acetol (Aldrich Chemical Co.) and previously prepared 5-hydroxy-4-ketovaleric acid provided standard curves.

The overall dehydrogenase activities of the two complexes were measured by determining the rate of reduction of DPN (Mukherjee *et al.*, 1965). Pyruvate and α-ketoglutarate dehydrogenase (E_1), dihydrolipoyl transacetylase (E_2), and lipoamide dehydrogenase (E_3) were measured as described by Reed and Willms (1966). Dihydrolipoyl transsuccinylase (E_2) (Knight and Gunsalus, 1962) and α-ketoglutarate–glyoxylate carboligase (Schlossberg *et al.*, 1970) were assayed as described in the references. Pyruvate–glyoxylate carboligase was assayed by the procedure for α-ketoglutarate–glyoxylate carboligase, except that 50 μmoles of neutralized pyruvate and 20 μmoles of neutralized [1-^{14}C]glyoxylate were used as substrates and the pH was 6.5. The enzyme units were defined as follows: overall complex dehydrogenase, 1 μmole of DPNH/hr at 30°; pyruvate or α-ketoglutarate dehydrogenase (E_1), 2 μmoles of ferrocyanide/hr at 30° (equivalent to 1 μmole of substrate reacted); dihydrolipoyl transacetylase (E_2), 1 μmole of acetyl lipoate/hr at 30°; lipoamide dehydrogenase (E_3), 1 μmole of DPNH/hr at 30°; and both glyoxylate carboligases, 1 μmole of [^{14}C]CO_2/hr at 37°. Specific activities were expressed as units per milligram of protein.

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TABLE I: The Purification of Pyruvate Dehydrogenase Complex and Pyruvate–Glyoxylate Carboligase from *E. coli* λ K-12.

Fraction	E_1[a] (Act.[a])	Pyruvate–Glyoxylate Carboligase (Act.[a])	% Recov (Based on E_1 Act.)
Protamine sulfate eluate	1.20	1.9	100
Ultracentrifuge pellets	3.1	5.6	86
pH 5.0 precipitate	5.0	10.1	63
pH 5.0 precipitate[b] repelleted	11.3	38.2	58

[a] Specific activities are expressed as units/mg of protein (see Methods). [b] Overall complex specific activity was 504.

Results

Identification of Acetol. The following were incubated at 37° with stirring: 70 mmoles of potassium phosphate buffer (pH 6.5), 0.28 mmole of thiamine pyrophosphate, 7 mmoles of $MgCl_2$, 10 g of bovine serum albumin, 20 mmoles of sodium pyruvate, 8 mmoles of sodium [2-^{14}C]glyoxylate (specific activity, 2440 dpm/μmole), and approximately 90,000 units of purified pyruvate dehydrogenase complex in 150 ml of 0.05 M phosphate buffer (pH 7.0) with a total volume of 2 l. Additional aliquots of 20 mmoles of pyruvate and 8 mmoles of glyoxylate were added after 1, 2, and 3 hr of incubation. One hour after the last addition, perchloric acid was added to give a final concentration of 5%, and the mixture was incubated an additional 2 hr with stirring to complete the decarboxylation of the postulated intermediate. The protein precipitate was centrifuged and washed with 5% perchloric acid. The supernatant and wash were combined, neutralized with KOH to pH 7, chilled overnight, and filtered to remove $KClO_4$. The perchlorate filtrate (2800 ml) was divided and each half was passed through 1 lb of Dowex 1-X10 (formate form) (Busch *et al.*, 1952), followed by 400 ml of water and 1400 ml each of 1 and 2 M formic acid as previously described (Bloom and Westerfeld, 1966). Fractions (100 ml) were collected and assayed for radioactivity by scintillation counting.

The foreruns and water washes (fractions 1–18) from the Dowex columns containing the nonacidic acetol had 57% of the radioactivity originally present in the glyoxylate. The second radioactive product (25% of the original) was eluted with 1 M formic acid (fractions 20–22), and was identified as 2,3-dihydroxy-4-ketovaleric acid. Only trace amounts of glyoxylate were recovered unchanged in fractions 28–30. Colorimetric assay of the combined forerun and water wash showed the presence of 1670 mg of acetol. Based on the specific activity of the original glyoxylate and the radioactivity in this fraction, the calculated yield of acetol was 1755 mg. The acetol in this fraction was distilled by flash evaporation at 40° and collected along with the water in the distillate. Of the acetol 60% was concentrated in the last 30% of the distillate.

2,4-Dinitrophenylosazone. Distillate (200 ml), which contained an estimated 101 mg of acetol by colorimetric analysis, was boiled with 800 mg of 2,4-dintrophenylhydrazine in 100 ml of 2 N HCl for 30 min; yield of precipitate, 79% of theory. Recrystallization from nitrobenzene gave short orange-red rods, mp 305–306°. No depression in melting point upon mixing with the derivative prepared from commercial acetol or methylglyoxal. *Anal.* Calcd for $C_{15}H_{12}N_8O_8$: C, 41.67; H, 2.78; N, 25.93. Found: C, 41.96; H, 2.84; N, 25.53.

Semicarbazone. Distillate (170 ml, 75 mg of acetol) was boiled for 30 min with 75 mg of semicarbazide hydrochloride plus 150 mg of sodium acetate, concentrated to 3 ml, and cooled; yield of precipitate, 40% of theory; recrystallization from water; colorless crystals; mp 196–198°; no depression in melting point upon mixing with the derivative of commercial acetol. *Anal.* Calculated for $C_4H_9N_3O_2$: C, 36.14; H, 6.87; N, 32.06. Found: C, 36.50; H, 7.11; N, 32.27.

Periodate Oxidation. After 66 ml of distillate (43 mg of acetol) plus 344 mg of periodic acid stood for 20 min at room temperature, the pH was adjusted to 5.0 with sodium hydroxide, and the newly formed formaldehyde was precipitated as the dimedon derivative (Frisell and Mackenzie, 1958); yield, 91% of theory; mp 192.5–193.5°; no depression of melting point upon mixing with an authentic derivative. The dimedon derivative contained 85% of the radioactivity originally present in the labeled acetol.

After removing the dimedon precipitate, the filtrate was distilled in order to recover any volatile acids. The distillate was neutralized, evaporated to dryness, and chromatographed on a Celite column (Ueno *et al.*, 1960). The only acid detected by titration with NaOH appeared in tubes 6–14 (peak at 9) and this was equivalent to 424 μmoles of acetate (74% of theory). No radioactivity was present in the acetic acid. The acid was further identified by preparing the *p*-phenylphenacyl ester of fractions 6–14; mp 110°; no depression on mixing with an authentic acetic acid derivative.

Identification of 2,3-Dihydroxy-4-ketovaleric Acid. The eluate was evaporated and rechromatographed on Dowex 1-X10 (formate form; 22 × 1.5 cm). By developing the column with 0.1 M formic acid, two major radioactive peaks of equal size were obtained at tubes 158 (140–173) and 207 (186–240) when 5-ml fractions were collected. Upon evaporation, both fractions yielded white crystalline compounds with indistinct melting points between 90 and 95°. Except for this chromatographic behavior, both compounds were identical in all respects (analysis, nmr, ir, mass spectrograph), and were considered to be stereoisomers. In this respect, the 2,3-dihydroxy-4-ketovaleric acid obtained in the pyruvate–glyoxylate reaction behaved like the 2,3-dihydroxy-4-ketopimelic acid previously obtained from the α-ketoglutarate–glyoxylate reactions (Schlossberg *et al.*, 1970).

Anal. Calculated for $C_5H_8O_5$: C, 40.54; H, 5.44. Found for compound in tube 158: C, 39.87; H, 5.28. Found for compound in tube 207: C, 40.17; H, 5.26. Neither compound gave a good phenyloszaone or 2,4-dinitrophenylosazone for derivative.

Ir. The infrared spectra (KBr pellets) for both compounds showed absorption between 3600 and 3300 cm^{-1} for the OH and acid OH groups, 3000 and 2700 cm^{-1} for the CH and CH_3 groups, and 1730 cm^{-1} for the acid carbonyl.

Nmr. The nmr spectra of both compounds in deuterated acetic acid with 2% Me_4Si standard gave similar absorption near τ 0.38 for the acidic H and OH, which shifted on addition of D_2O to τ 0.88. The following peaks did not shift with D_2O: a pair of doublets at τ 4.83 and 4.62 (for the adjacent HC-CH); and a peak at τ 7.72 for the CH_3 group. The respective integral ratios were 3:1:1:3.

Nmr spectra in deuterated acetone were similar to the above, but the acid H and OH peak appeared at τ 4.28 and

TABLE II: The Enzymatic Activities of the 8.8S Fraction (E_1) and the 32.7S Fraction (E_2E_3).

Fraction	E_1 (Act.[a])	Pyruvate–Glyoxylate Carboligase (Act.[a])	E_2 (Act.[a])	E_3 (Act.[a])	Overall Complex (Act.[a])
Pyruvate dehydrogenase complex predissociation	7.8	54.2	33	639	367
8.8S fraction[b]	25.6	77.0	0	0	0
32.7S fraction[b]	0	0.2	94	1612	0

[a] Specific activities are expressed as units/mg of protein (see Methods). [b] Data taken from a separate experiment; 2.33 mg of the slightly yellow fraction (see text) was centrifuged in the fixed partition cell, and 1.84 mg was recovered in the 8.8S fraction.

were shifted to 5.82 with the addition of D_2O. The HC-CH peaks were located at τ 5.44 and 5.28 and the CH_3 peak at 7.71. These peaks did not shift with D_2O. The respective integral ratios were 3:1:1:3.

Mass Spectrometry. While no parent peak was observed by mass spectrometry, a parent peak minus 18 (*m/e* 130) was observed; in addition a series of peaks were obtained which resulted from the cleavage at each carbon to carbon bond with retention of charges on either the oxygen containing or alkyl fragment (*m/e* ranges, 17–18, 27–30, 41–45, 54–60, 70–77, 86–88, 102–105).

Periodate Oxidation. The oxidation of 30 mg of 2,3-dihydroxy-4-ketovaleric acid with periodate as well as the quantitation and identification of the split products were carried out as previously described for the identification of 2,3-dihydroxy-4-ketopimelic acid (Schlossberg *et al.*, 1970). It reacted with 2 moles of periodate and yielded 0.95 mole of unlabeled acetate, 0.98 mole of labeled formate, and 0.91 mole of radioactive glyoxylate. The acetate was quantitated by titration and was further identified as its *p*-phenylphenacyl ester (mp 110–111°). Formic acid was titrated, analyzed colorimetrically by two procedures, and reduced to formaldehyde, and the latter was precipitated as the dimedon derivative (mp 194°). The glyoxylate was quantitated by titration and colorimetric analysis and was further identified as its 2,4-dinitrophenylhydrazone derivative (mp 193–194°) and by paper chromatography.

Purification and Dissociation of the Pyruvate Dehydrogenase Complex. A highly purified preparation of this complex from *E. coli* gave an ultracentrifuge pattern similar to that described by Koike *et al.* (1960) (one major component at 59 S in 0.02 M phosphate buffer, pH 7.4). Table I shows the association of E_1 and carboligase activities during the purification. The purified complex was then dissociated at pH 9.5 and separated into two fractions by a calcium phosphate gel-cellulose column (Koike *et al.*, 1963). The elution of the calcium phosphate gel–cellulose column gave two main protein peaks; (a) a slightly yellow fraction containing mainly pyruvate dehydrogenase (E_1), and (b) a yellow fraction containing mainly the subcomplex of dihydrolipoyl transacetylase and lipoamide dehydrogenase (E_2E_3). Following ammonium sulfate precipitation and recentrifugation of the slightly yellow fraction (a) in the Spinco Model E ultracentrifuge using a fixed partition cell, a colorless fraction (E_1) was obtained. The colorless fraction had a single peak, 8.8 S in 0.05 M phosphate buffer, pH 7.0, in agreement with the sedimentation coefficient ($s_{20,w}$) of 9.2 S for E_1, as reported by Koike *et al.* (1963). The yellow fraction (b) was also recentrifuged in a Spinco Model L ultracentrifuge to pellet and remove the trace amount of undissociated complex that was still present. The E_2E_3 subcomplex in the supernatant also gave a single peak with a sedimentation coefficient of 32.7 S in 0.05 M phosphate buffer, pH 7.0. Koike *et al.* (1963) reported the $s_{20,w}$ of the E_2E_3 subcomplex to be 26–32 S, depending upon how much E_3 was lost during the experimental procedures. Table II presents the enzymatic activities present in the 8.8S (E_1) and 32.7S (E_2E_3) peaks after dissociation and separation. The carboligase activity of the pyruvate dehydrogenase complex was clearly associated with the thiamine pyrophosphate containing component (E_1). Neither the flavine nor the lipoic acid subunits (E_2E_3) were required for the catalysis of the pyruvate–glyoxylate condensation.

α-Ketoglutarate–Glyoxylate Carboligase Activity. Schlossberg *et al.* (1970) reported that beef heart α-ketoglutarate dehydrogenase complex was responsible for the condensation of α-ketoglutarate and glyoxylate (α-ketoglutarate–glyoxylate carboligase activity); and Saito *et al.* (1971) found an association between the synergistic decarboxylation of α-ketoglutarate and glyoxylate (a carboligase reaction) and the α-ketoglutarate decarboxylase activity of an enzyme from *Rhodopseudomonas spheroides.*

The following studies show that the corresponding enzyme from *E. coli* also catalyzes the carboligase reaction. A purified complex from *E. coli* showed one major component with a sedimentation coefficient of 34.6 S in 0.02 M phosphate buffer, pH 7.0; Mukherjee *et al.* (1965) reported 36 S. Table III shows a parallel increase of carboligase activity along with the E_1 activity of α-ketoglutarate dehydrogenase complex during the purification of the latter. Attempts to dissociate the complex resulted in a loss of E_1 and carboligase activities, but the E_2 and E_3 activities were maintained and were not associated with the carboligase. The major reaction product of the *E. coli* enzyme was also identified as 5-hydroxy-4-ketovaleric acid by the procedures previously described (Schlossberg *et al.*, 1968).

Kinetics. Various kinetic parameters were studied involving the carboligase activities that reside in the highly purified dehydrogenase complexes from *E. coli.* Figure 1 shows the pH optimum for both carboligases to be in the 6.5 range. The reactions were linear over a 1-hr period and were directly proportional to the amount of enzyme present. α-Ketoglutarate dehydrogenase complex and pyruvate dehydrogenase complex yielded 25.5 and 37.5 μmoles of CO_2 per hr per mg of protein, respectively, at substrate concentrations of 20 μmoles

TABLE III: The Parallel Concentration of E_1 and α-Ketoglutarate–Glyoxylate Carboligase Activities during the Purification of α-Ketoglutarate Dehydrogenase Complex from *E. coli* λ K-12.

Fraction	Protein (g)	E_1 (Act.[a])	α-Ketoglutarate–Glyoxylate Carboligase (Act.[a])	E_2 (Act.[a])	E_3 (Act.[a])	Overall Complex (Act.[a])	% Recov (Based on E_1)
Cell-free extract	68.720	1.36	1.12				100
Protamine sulfate eluate	2.781	4.90	6.60				74
Ultracentrifuge pellets	0.684	26.1	26.9			77	49
pH 5.7 precipitate	0.197	42.0	39.0			210	28
pH 5.7 precipitate repelleted	0.121	50.0	48.0	7.2	1541	387	22

[a] Specific activities are expressed as units/mg of protein (see Methods).

of glyoxylate plus 20 μmoles of α-ketoglutarate or 10 μmoles of glyoxylate plus 50 μmoles of pyruvate per vessel, respectively. High concentrations of glyoxylate inhibited the pyruvate–glyoxylate carboligase but not the α-ketoglutarate–glyoxylate carboligase (Figures 2A and 2B). Lineweaver–Burk plots gave the following K_m's: K_m for pyruvate = 5.3 mM at a glyoxylate concentration of 1.7 mM; K_m for α-ketoglutarate = 0.5 mM at a glyoxylate concentration of 7.3 mM.

Discussion

The results show that the E_1 component of the pyruvate dehydrogenase complex from *E. coli* was responsible for the condensation reaction between pyruvate and glyoxylate. Acetol was the major product and was presumably formed by the nonenzymatic decarboxylation of the initial product, 2-hydroxy-3-ketobutyric acid. Only 28% as much acetol was found in the incubation vessels without acidification as there was after acidification. The formation of 2,3-dihydroxy-4-ketovaleric acid was analogous to the additional condensation product previously described for the α-ketoglutarate–glyoxylate reaction (Schlossberg *et al.*, 1970).

In thiamine deficiency, pyruvate dehydrogenase complex activity is decreased and both pyruvate (Williams *et al.*, 1943; Peters, 1953) and glyoxylate (Laing, 1962; Buckle, 1963) accumulate; the excess pyruvate and glyoxylate disappear

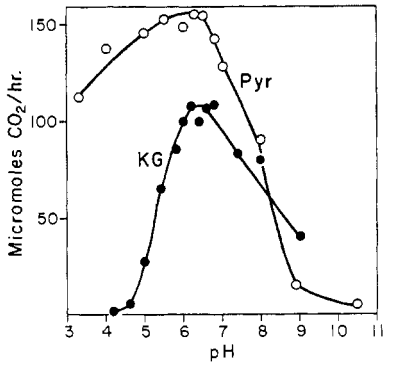

FIGURE 1: Effect of pH on the α-ketoglutarate–glyoxylate carboligase (KG, ●—●) and the pyruvate glyoxylate carboligase (Pyr, ○—○) activities. All flasks contained 0.4 μmole of thiamine pyrophosphate, 10 μmoles of $MgCl_2$, 20 mg of albumin, and 100 μmoles of potassium phosphate. The α-ketoglutarate and pyruvate dehydrogenase complex protein per flask were 50 and 67 μg, respectively. Substrate per flask was for α-ketoglutarate–glyoxylate carboligase, 20 μmoles of [1-^{14}C]glyoxylate, and 20 μmoles of α-ketoglutarate; for pyruvate–glyoxylate carboligase, 10 μmoles of [1-^{14}C]glyoxylate, and 50 μmoles of pyruvate. Tris (0.1 M) and histidine buffers were used for the pH curve of pyruvate–glyoxylate carboligase and phosphate buffer for that of α-ketoglutarate–glyoxylate carboligase. The total volume in each incubation mixture was 3 ml and the incubation time was 15 min at 37°.

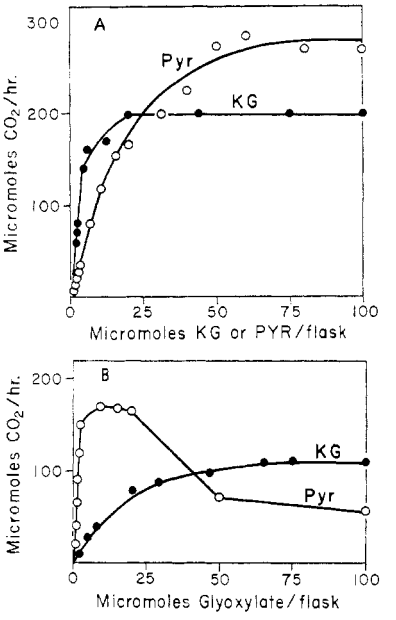

FIGURE 2: (A) The effect of varying substrate concentrations on the pyruvate–glyoxylate (Pyr, ○) and α-ketoglutarate–glyoxylate (KG, ●) carboligases, respectively. [1-^{14}C]Glyoxylate was present at 75 μmoles/flask in the α-ketoglutarate–glyoxylate carboligase assay; α-ketoglutarate dehydrogenase complex protein concentration was 100 μg/flask. [1-^{14}C]Glyoxylate was present at 10 μmoles/flask in the pyruvate–glyoxylate carboligase assay; pyruvate dehydrogenase complex protein concentration was 30 μg/flask. (B) The effect of varying [1-^{14}C]glyoxylate substrate concentration on the pyruvate–glyoxylate and α-ketoglutarate–glyoxylate carboligases. Pyruvate was present at 50 μmoles/flask in the pyruvate–glyoxylate carboligase assay; pyruvate dehydrogenase complex protein concentration was 20 μg/flask. α-Ketoglutarate was present at 20 μmoles/flask in the α-ketoglutarate–glyoxylate carboligase assay. α-Ketoglutarate dehydrogenase complex protein concentration was 112 μg/flask. The remaining assay parameters were as that described in Figure 1 at pH 6.5.

with thiamine treatment. Glyoxylate is presumably derived from glycine (Laing, 1963), but cannot be detected in normal blood (Buckle, 1963). Both complexes are thiamine-containing enzymes capable of reacting with glyoxylate, and both reactions represent potential pathways for the normal metabolism of glyoxylate. The ease with which thiamine pyrophosphate is removed from the pyruvate as compared with the α-ketoglutarate complex and the concomitant defect in glyoxylate metabolism during thiamine deficiency suggest a major role for the pyruvate reaction. However, in mammalian tissues, the reaction of glyoxylate with pyruvate is much slower than its reaction with α-ketoglutarate (unpublished), and it is not yet clear which reaction is more important in preventing a hyperoxaluria (Koch *et al.*, 1967).

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Schiff Bases of Pyridoxal Phosphate with Active Center Lysines of Ribonuclease A†

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ABSTRACT: RNase A and pyridoxal phosphate react rapidly in aqueous solution, forming a Schiff base complex. Ultraviolet absorption spectra indicate that the formation of this aldimine species is extensive, even under conditions which render the model compound *N*-α-acetyllysine completely unreactive toward pyridoxal phosphate. The protein-bound cofactor is optically active, provided the tertiary structure is intact. The molar ellipticity at 408 nm as a function of pH is a bell-shaped curve and is in close agreement with a theoretical curve generated using the simple model $EPH_2 \leftrightarrows EPH \leftrightarrows EP$ where only EPH is optically active. A stable chemical modification is obtained by reduction of the complex with sodium borohydride. Modification with increasing amounts of coenzyme lowers the specific activity toward cytidine 2′,3′-cyclic phosphate to 20% at a fivefold ratio of coenzyme to enzyme, but the residual activity cannot be reduced further even with a 35-fold molar excess of pyridoxal phosphate. Amino acid analysis of the phosphorylpyridoxyl peptides obtained from tryptic–chymotryptic digests indicates that Lys-7 and Lys-41 are modified in a ratio of 2:3, when an equimolar complex is reduced with $NaBH_4$. This product distribution is constant as a function of temperature, pH, and borohydride concentration. Phosphate ions protect against modification at both sites by inhibiting the formation of the aldimine complex. The preferential binding of pyridoxal phosphate at the active site of RNase A is interpreted in terms of 3-dimensional structure of the enzyme.

The active center of an enzyme not only contains amino acid residues which function in catalysis but also those which form specific binding sites for substrates or cofactors. The specificity of both processes is highly dependent on local tertiary structure. Anomalous pK_a values have often been found for such residues, and their reactivity has been observed

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