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Studies on the Decarboxylation of Pyruvate. II.[1] Kinetic Studies on the Binding of Thiamine Pyrophosphate to Pyruvate Decarboxylase[2]

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The reconstitution of pyruvate decarboxylase has been studied kinetically in the presence of several concentrations of thiamine pyrophosphate (TPP) and Mn^{2+}. The Km for TPP determined graphically was decreased by increasing Mn^{2+} concentration, while V_{max} was almost unchanged. The values of Kms for Mn^{2+} and V_{max} were affected in mixed manner by the amount of TPP.

The significance of Kms thus determined is discussed and the values are compared to that determined in transketolase.

Yeast cytoplasmic pyruvate decarboxylase (2-oxo acid carboxylase, EC 4.1.1.1) contains 4 molecules of thiamine pyrophosphate (TPP) and the same number of metal ions.[4] The holoenzyme (molecular weight is 175000[4] or over[5]) releases the cofactors at alkaline pH and is dissociated into 2 subunits of one half the original molecular weight. The apoenzyme thus produced is reconstituted to active pyruvate decarboxylase in the presence of a large excess of TPP and metal ions.[6–10]

Schellenberger and his coworkers[11] have shown from their kinetic experiments that there are 2 different binding sites for TPP and metal ions on the enzyme and that the apoenzyme is first changed into an inactive ternary complex (I) followed by the formation of a cyclic enzyme-metal-TPP complex (II) in the reconstitution process as shown below; of several concentrations of TPP, Mn^{2+} and appropriate amount of apoenzyme in citrate buffer of pH 6.0 at 30° for 15 min and the initial velocity was plotted against TPP or Mn^{2+} concentration. Double reciprocal plots of these data fit a straight line in varying the concentrations of TPP or Mn^{2+} as shown in Fig. 1 and 2.

It is apparent from Fig. 1 that Mn^{2+} in the concentration range of 2×10^{-4}—2×10^{-2}M activates apoenzyme in a competitive manner with respect to TPP; the presence of 2×10^{-4}M, 2×10^{-3}M or 2×10^{-2}M of Mn^{2+} reduced the Km value of TPP to 7.14, 1.10 or 0.69 μM, respectively, keeping the maximum reaction rate almost constant. The relations, however, were limited only when TPP concentration was low. As shown in Fig. 2, the *K*m value for Mn^{2+} and the maximum reaction rate are affected in mixed manner by TPP. At high TPP concentrations, the cofactors seemed to irreversibly bind to apoenzyme.[10] The *K*m for Mn^{2+}

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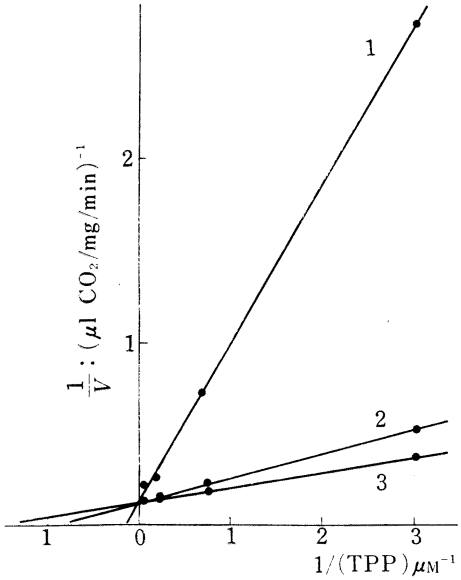

Fig. 1. Reciprocal Velocity plotted against the Reciprocal of the Concentration of TPP at several Constant Concentration of Mn^{2+}

1: Mn^{2+} 2×10^{-4}M, 2: Mn^{2+} 2×10^{-3}M, 3: Mn^{2+} 2×10^{-2}M

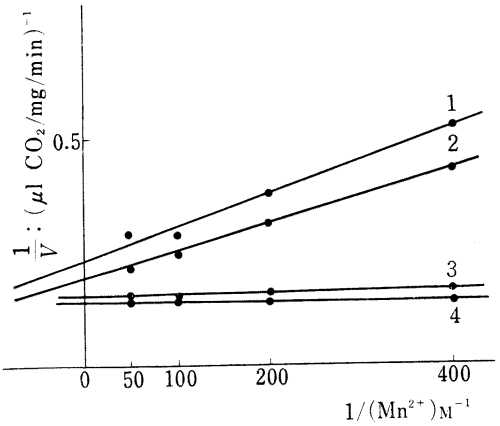

Fig. 2. Reciprocal Velocity plotted against the Reciprocal of the Concentration of Mn^{2+} at several Constant Concentration of TPP

1: TPP 3.3×10^{-7}M, 2: TPP 7.0×10^{-7}M, 3: TPP, 1.65×10^{-4}M, 4: TPP 1.65×10^{-3}M

in high TPP concentration could not be determined, whereas in the presence of 3.3×10^{-7}M or 7×10^{-7}M of TPP, the *K*m was 2.98 mM or 2.63 mM, respectively. The *K*m's for TPP and Mn^{2+} in pyruvate decarboxylase thus obtained should receive careful considerations.

$$E + M \rightleftarrows E.M \quad (1) \qquad EM + TPP \rightleftarrows E.M.TPP \quad (3)$$

$$E + TPP \rightleftarrows E.TPP \quad (2) \qquad E.TPP + M \rightleftarrows E.M.TPP \quad (4)$$

If we assume the following independent reactions in reconstitution process and that the combination of enzyme with TPP and with metal are not independent, that is, the affinities of E and EM for TPP are different, the equation for the

$$\text{enzyme} + \text{TPP} + \text{metal} \longrightarrow \underset{\text{I}}{\text{enzyme}\begin{matrix}\nearrow\text{TPP}\\ \searrow\text{metal}\end{matrix}} \longrightarrow \underset{\text{II}}{\text{enzyme}\begin{matrix}\nearrow\text{TPP}\\ |\\ \searrow\text{metal}\end{matrix}}$$

In the previous paper,[1] we have investigated the effects of order of addition of TPP, Mn^{2+} (the most effective ion among 9 divalent metal ions tested[1]) and pyruvate on the activity of the resolved yeast pyruvate decarboxylase. The addition of Mn^{2+} (10 mM) to the preincubated mixture of TPP (3.3×10^{-7}M), pyruvate (6.6×10^{-2}M) and apoenzyme in citrate buffer of pH 6.0 for 15 min at 30° gave instant CO_2 formation, while the addition of the same amount of TPP to the preincubated mixture of Mn^{2+}, pyruvate and apoenzyme in the citrate buffer resulted in the slowest rate of CO_2 formation among 4 experiments of different order of addition. As the reconstitution seemed to be greatly affected by the presence of TPP and Mn^{2+}, the initial reaction rates of the decarboxylation of pyruvate were reexamined for each cofactors.

Pyruvate decarboxylase was isolated from "Sapporo" brewer's yeast[1] and was resolved according to the procedure of Morey and Juni.[10] The specific enzyme activity was 29.3 units per mg of protein and showed no activity in the absence of cofactors or in the presence of metal

only. Activity was determined after the addition of pyruvate (6.6×10^{-2}M) to the preincubated mixture. The velocity is then[12]

$$v=\frac{ke}{\left(1+\frac{K'_{TPP}}{TPP}\right)\left(1+\frac{K_M}{M}\cdot\frac{(1+TPP/K_{TPP})}{(1+TPP/K'_{TPP})}\right)} \quad (5)$$

where k denotes the velocity constant for the dissociation of the E.M. TPP complex and e is the total concentration of apoenzyme. The dissociation constants of the enzyme or enzyme complex with respect to Mn^{2+} and TPP in the equations (1), (2), and (3), are expressed as K_M, K_{TPP} and K'_{TPP} respectively. If TPP concentration is low and enzyme complex is scarcely formed without the presence of Mn^{2+}, K_{TPP} is near infinite and then the equation (5) reduces to

$$v=\frac{ke}{\left(1+\frac{K'_{TPP}}{TPP}\right)\left(1+\frac{K_M}{M(1+TPP/K'_{TPP})}\right)} \quad (6)$$

Equation (6) is of the well known form $v=\frac{V_a}{(1+k_{AP}/M)}$ where $V_a=\frac{ke}{1+K'_{TPP}/TPP}$ and K_{AP} (apparent dissociation constant of EM)$=\frac{K_M}{1+TPP/K'_{TPP}}$. As $K_{AP}=K_M$ when TPP$\ll K'_{TPP}$, Km value for Mn^{2+} determined in Fig. 2 equals to the true dissociation constant only at low TPP concentration. Apparent TPP constant (K_{AP}') is also deduced from equation 5 and when K_{TPP} is near infinite, $K_{AP}'=K'_{TPP}(1+K_{TPP}/M)$. Thus the graphically determined constant for TPP (K_{AP}') must be true K'_{TPP} only in the presence of high concentration of Mn^{2+}.

Kochetov and Philippov[13] determined Km for TPP(K_{AP}') in transketolase (D-sedoheptulose-7-phosphate: D-glyceraldehyde-3-phosphate glycolaldehyde transferase E,C.2.2.1.1.), an another TPP dependent enzyme. They observed that the increase in Ca^{2+} concentration caused both a decrease in K_{AP}, and an increase in the maximum reaction rate. The value of 4 μM in the presence of 5×10^{-3}M Ca^{2+} in transketolase is comparable to that of 1.1 μM in the presence of 2×10^{-3}M Mn^{2+} in pyruvate decarboxylase. The situation for determining Km value, however, seems to be complicated in pyruvate decarboxylase since TPP and metal both paricipate in subunits association step as well as the catalytic process.[5] Kinetic studies along this line is under progress.

Experimental

Materials——Pyruvate decarboxylase was extracted from "Sapporo" brewer's yeast as described in the previous paper.[1] Purification of the enzyme and resolution of the cofactors were carried out according to the procedure of Ullrich, *et al.*[4] and Morey, *et al.*[10] respectively. The specific enzyme activity was 29.3 units/mg protein in the presence of excess cofactors. Sodium pyruvate was prepared from freshly redistilled pyruvic acid.[14] NADH and alcohol dehydrogenase(yeast) were purchased from Oriental Yeast Ind. TPP were obtained from Wako Pure Chemical and the solution was neutralized before use.

Assay Systems——Warburg vessels contained 8.3×10^{-2}M citrate buffer pH 6.0, 2×10^{-4}M—2×10^{-2}M Mn^{2+}, 3.3×10^{-7}M—1.65×10^{-3}M TPP, and an appropriate amount of apoenzyme were incubated at 30° for 15 min and the reaction was started by addition of 6.6×10^{-2}M sodium pyruvate from the side arm in a total volume of 3.0 ml. The activity of the enzyme being assayed was determined as the amount of CO_2 evolved during the first 10 min of the reaction. Specific activity of pyruvate decarboxylase was determined by measuring the decrease of NADH absorption at 340 nm for 5 min[4] at 24° in the reaction mixture containing 2 ml of 1.1M K_2HPO_4, 1 ml of the test solution, 0.05 ml of NADH (8×10^{-3}M), and 0.05 ml of alcohol dehydrogenase (10 mg/ml). The enzyme was first incubated for 2 min at 4° in 0.09M phosphate

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buffer pH 6.8 containing 0.02M $MgSO_4$ and 1.5×10^{-3}M TPP. Mn^{2+} was determined by HITACHI 208 atomic absorption spectrophotometer.

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