COENZYME ANALOGUE INHIBITION IN THE RECONSTITUTION OF YEAST PYRUVATE DECARBOXYLASE AND TRANSKETOLASE*

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SUMMARY

The reconstitutions of the apoenzymes of yeast cytoplasmic pyruvate decarboxylase and transketolase were studied in the presence of thiamine pyrophosphate and its analogues. The reconstitutions of both enzymes were competitively inhibited by 3-methyl, 3-benzyl and "thiazole" pyrophosphates. Stronger inhibition by the latter than the former two in both enzyme experiments, demonstrates secondary importance of the positive charge of coenzyme in the protein binding, though it is essential for its catalytic activity.

INTRODUCTION

The interaction of the apoenzyme of pyruvate decarboxylase (2-oxoacid carboxylase EC 4.1.1.1) with its cofactors, TPP and divalent cation, has been studied by several investigators (1-3). A hydrophobic interaction of the 2' and 4 methyl groups of TPP and the ionic interaction of the pyrophosphate group of the coenzyme with pyruvate decarboxylase seems to be well established. The involvement of positively charged nitrogen in the thiazole moiety of TPP through the charge transfer interaction has also been suggested in model studies (4,5). Further evidence supporting this interaction has been derived from the UV and CD measurements upon the addition of TPP and divalent metal to tryptophan and also to apotransketolase (6-8). The importance of this interaction for binding, however, presents some questions since tetrahydrothiamine pyrophosphate which has no quaternary nitrogen in its molecule had a smaller binding constant than TPP in the kinetic experiments with both pyruvate decarboxylase and transketolase (3,9). In this communication, an attempt has

*Preliminary data were presented at the ICB, Stockholm, July 1973.
Abbreviations: TPP, thiamine pyrophosphate, "Thiazole," 4-methyl-5-(2-hydroxyethyl)-thiazole

been made to elucidate the importance of quaternary nitrogen by use of the inhibitor, "thiazole" pyrophosphate (10) and its analogues in the reconstitution of both enzymes.

MATERIALS AND METHODS

Pyruvate decarboxylase was isolated from "sappors" brewer's yeast as described previously (11). The preparation of apoenzyme was essentially followed by the method of Morey and Juni (2), and the specific activity determined by the coupled assay with alcohol dehydrogenase (12) was 26.4 unit/mg of protein in the presence of excess TPP and Mg^{2+}. For the reconstitution experiment of pyruvate decarboxylase, the apoenzyme was first incubated at 25° for 1 min. in 0.01 M phosphate buffer (pH 6.8) with 25 μmoles of $MgSO_4$, 0.02 μmoles or 0.1 μmoles of TPP with or without coenzyme analogues in a total volume of 1.0 ml. The mixture was added to 250 μmoles of citrate buffer (pH 6.2) containing 0.5 μmoles of NADH and 0.1 mg of alcohol dehydrogenase. The reaction was initiated by the addition of 50 μmoles of pyruvate in a total volume of 3.0 ml. All the assays were carried out at 30° with continuous recording of the absorbancy change at 340 nm for a few minutes before and after the start of the reaction.

The isolation and purification of transketolase from the above mentioned brewer's yeast was followed according to the procedure of Srere *et al.* (13) with some modifications which included repeated purification with DEAE cellulose column and Sephadex G-200 column chromatography upon the complete separation of cofactors from the enzyme in 0.025M glycylglycine buffer (pH 7.5).* The apoenzyme showed the specific activity of 5.8 unit/mg protein in excess TPP and Ca^{2+}.

Reconstitution of transketolase was carried out at 30° using 200 μg of

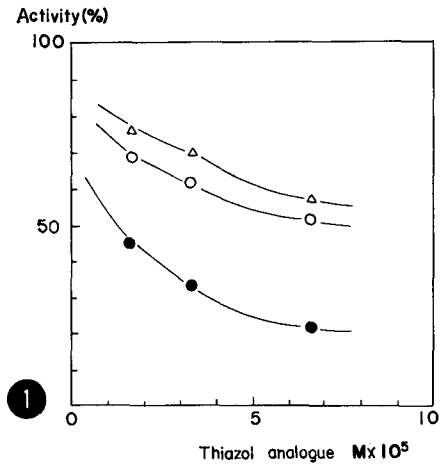
*Apotransketolase was easily separated from the functionally bound enzyme (14), glyceraldehyde phosphate dehydrogenase with Sephadex G-200 and the molecular weight determined by gel filtration was 100,000 ± 6,000, while that determined by SDS polyacrylamide disc electrophoresis showed 50,000 ± 2,500 at pH 7.6, 5% gel. It is thus assumed that apoenzyme is consisted of 2 subunits.

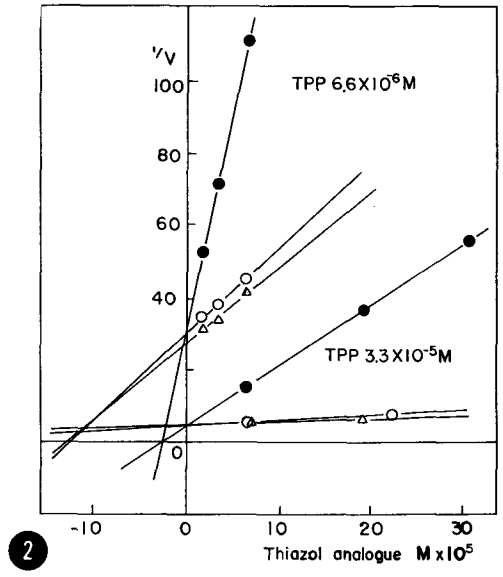
apotransketolase, 0.51 mμmoles of TPP with or without coenzyme analogues, 9 μmoles of $CaCl_2$, 0.3 μmoles of NADH, 1.5 mg α-glycerophosphate dehydrogenase, triose phosphate isomerase prepared by the procedure of Srere, et al. (13), 7.5 μmoles of pentose-5-phosphate equilibrium mixture and 100 μmoles of glycylglycine buffer (pH 7.6) in the total volume of 3.0 ml. The absorbancy decrease of NADH was measured in the same manner as described for pyruvate decarboxylase. The colorimetric method (15) was also employed to determine the sedoheptulose formed in the transketolase mediated reaction. Protein determination was made by the method of Lowry, et al. (16).

"Thiazole" pyrophosphate was prepared by cleaving TPP with sodium sulfite (17) and was purified by Amberlite CG-50 (20 x 540 mm). Methyl "thiazole" and benzyl "thiazole" were prepared as described by Clarke (18) and Maier, et al. (19) respectively. Their phosphorylation was carried out at 100° for 15 minutes with phosphoric acid preheated at 100° in an analogous manner to thiamine phosphorylation (2) and the diphosphate esters were separated from mono- and polyphosphate esters by Amberlite CG-50. The pyrophosphate esters of "thiazole," methyl "thiazole" and benzyl "thiazole" showed Rf values of 0.24, 0.15 and 0.60 respectively in the thin layer chromatography with the solvent system of n-propanol: 1M-ammonium formate: water = 65:15:20. Their molar absorbancies were 4.6×10^3 at 250 nm, 4.3×10^3 at 258 nm and 5.8×10^3 at 258 nm, respectively.

RESULTS

As shown in Fig. 1, the reconstitution of pyruvate decarboxylase with 6.6×10^{-6}M of TPP was strongly inhibited by the addition of "thiazole" pyrophosphate. The amount of TPP used in this experiment was the amount required to saturate about 80% of the apoenzyme. It was also inhibited by methyl and benzyl "thiazole" pyrophosphate though the extent of the inhibition was less than that by "thiazole" pyrophosphate. "Thiazole" esters of non-phosphorylated or monophosphorylated forms were inactive. Fig. 2 shows the Dixon plots for the competitive nature of the inhibition of pyruvate decar-





—●— "Thizaole" pyrophosphate

—○— Methyl "thiazole" pyrophosphate

—△— Benzyl "thiazole" pyrophosphate

Fig. 1 - Effects of "thiazole" analogues on the reconstitution of pyruvate decarboxylase. —●— "thiazole" pyrophosphate; — O — methyl "thiazole" pyrophosphate; — △ — benzyl "thiazole" pyrophosphate. 0.02 μmoles of TPP and 25 μmoles of $MgSO_4$ were used for the reconstitution of pyruvate decarboxylase.

Fig. 2 - Inhibition of reconstitution of pyruvate decarboxylase by "thiazole" analogues. 0.1 mg of apopyruvate decarboxylase was incubated for 1 min. in 0.01M phosphate buffer (pH 6.8) at 25° with 8.3 x 10^{-3}M $MgSO_4$, 6.6 x 10^{-6}M TPP (upper 3 lines) or 3.3 x 10^{-5}M TPP with or without coenzyme analogues. —●— "thiazole" pyrophosphate; —O— methyl "thiazole" pyrophosphate; — △ — benzyl "thiazole" pyrophosphate.

boxylase by "thiazole" analogues. The upper 3 lines represent the inhibition at 6.6 x 10^{-6} MTPP, while the lower 3 lines show the inhibition at 3.3 x 10^{-5}M TPP. TPP concentrations of 6.6 x 10^{-6}M and 3.3 x 10^{-5}M activate the apoenzyme approximately 80% and 100% respectively. The K_is obtained from this graph were 3.1, 12.5 and 14.0 x 10^{-5}M for "thiazole," methyl "thiazole" and benzyl "thiazole" pyrophosphates respectively. The inhibition of the reconstitution of transketolase with TPP by these 3 coenzyme analogues is shown in Fig. 3.

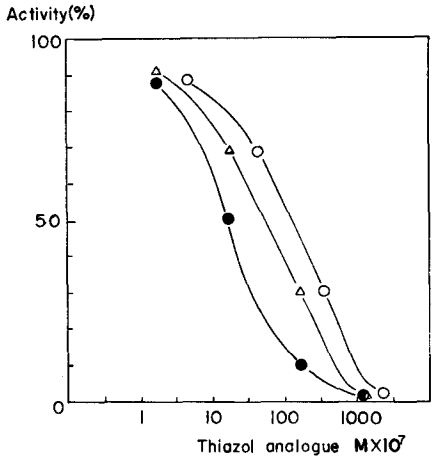


Fig. 3 - Effects of "thiazole" analogues on the reconstitution of transketolase. —●— "thiazole" pyrophosphate; —O— methyl "thiazole" pyrophosphate; —△— benzyl "thiazole" pyrophosphate. 0.51 mμmoles of TPP and 9 μmoles of $CaCl_2$ were used for the reconstitution of transketolase.

The TPP concentration of 1.7×10^{-7}M used in these experiments is approximately the K_m of TPP for transketolase. Competitive inhibition by the 3 coenzyme analogues was demonstrated again in the reconstitution of transketolase. In this case, however, inhibition by the benzyl analogue was a little stronger than that by the methyl analogue, which was opposite of the case with pyruvate decarboxylase.

DISCUSSION

2-Methyl and 2-benzyl "thiazoles" are known to have nonenzymatic catalytic activities of 2% and 38% of that of thiamine respectively at pH 8.7 - 9.0 (21); however, their phosphorylated esters were inactive with apopyruvate decarboxylase or apotransketolase. This may be partly due to their higher K_i values compared to the K_m for TPP because of the lack of pyrimidine structure. The importance of the pyrimidine moiety in TPP has been proposed in its NMR studies (22, 23) as well as in the coenzyme binding studies with apopyruvate decarboxylase (1,3) and apotransketolase (24), the 2'-methyl causing hydrophobic interaction and the 1' N possibly causing hydrogen bonding involving a water molecule in the first coordination sphere. Thus it is not sur-

prising that the K_i of "thiazole" pyrophosphate, 31 μm, is an order of magnitude greater than the K_m for TPP (25). It was unexpected, however, that "thiazole" pyrophosphate would show stronger inhibition than the other two derivatives. It was also surprising that the effect of benzyl "thiazole" pyrophosphate on the reconstitution of pyruvate decarboxylase was not greater than that of methyl analogue in spite of the inductive effect of the aromatic ring of the former. Wittorf and Gubler (3) reported that tetrahydrothiamine which has no quaternary nitrogen in its molecule was the most potent inhibitor among the compounds tested in their reconstitution studies of pyruvate decarboxylase. Heinrich, et al. (7) also reported a high affinity of this compound in their studies with transketolase. These results with the data presented in this paper seem to suggest that quaternary nitrogen of thiazole is playing a less vital role than predicted in the nonenzymic model studies (4,5), and it may be assumed that there would even be an ionic repulsion with charged group(s) on the apoenzyme in the vicinity of the binding site. Therefore, the presence of "pyrimidine" might be important not only for lowering the pKa of C-2 but also for tightening the binding of the coenzyme by overcoming the probable ionic repulsion.

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