MECHANISM BASED STUDIES OF THE ACTIVE SITE DIRECTED INHIBITION AND ACTIVATION OF ENZYME TRANSKETOLASE *

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ABSTRACT

Derivatives of phenyl-keto butenoic acids have been reported to be inhibitors of pyruvate decarboxylase, (PDC).

The inhibition of transketolase, a thiamine requiring enzyme such as PDC, by meta nitro phenyl derivative of 2-oxo- 3-butenoic acid (MNPB) is reported here. These studies indicate that the inhibitor binds to the enzyme at the active site. A two step inhibition was observed, first the inhibitor reacts with the enzyme on one site- non cooperative with Mg^{2+}, and TPP, inhibiting the enzyme, second in higher concentration of the inhibitor an abrupt enhancement of the inhibition takes place. In the absence of cofactors the lower concentration of the inhibitor caused an enhancement of activation to 150% of original enzyme activity followed by a drop to a low 50% in 60 minutes. Higher concentration of the inhibitor produced an inhibition with a half life that was pronouncely larger than when cofactors were present ($t_{1/2} \approx 1.9$ min VS. $t_{1/2} \approx 1$ min). We conclude therefore that the enzyme cantains a regulatory and a catalytic site with the regulatory site functional, without the aid of cofactors and that the cofactors are auxiliary for the action of the enzyme.

The lowering effect of the reaction half life by the cofactors is due to the rate enhancement caused by cofactors in the catalytic site of the enzyme.

INTRODUCTION:

Transketolase (EC,2.2.1.1) is found to be active in several different forms, monomeric, dimeric and tetrameric, depending on the variety of organisms. The apo-enzyme requires Mg^{2+} or Ca^{2+} and thiamine pyrophosphate (TPP) for its full activity and the formation of holo-enzyme (1).

The action of transketolase in intermediary metabolism is in the pentose-phosphate pathway, it catalyses two steps of these reactions which are the conversion of xylulose-5-phosphate to seduheptulose and also the conversion of fructose -6-phosphate to tetrose -4-phosphate. In these reactions the transposition of a ketal group from a phosphate sugar to another takes place. Although the mecha-

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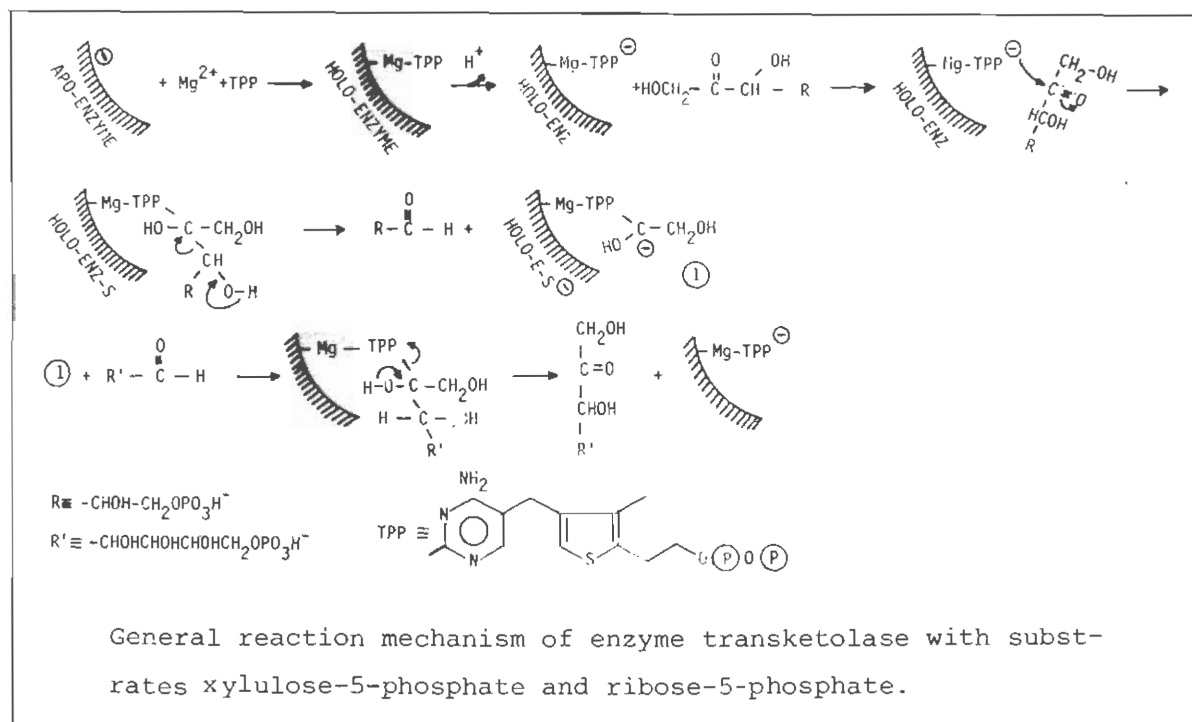
nism of the active site of the enzyme has not been totally known, but the effect of some amino acid residues such as histidine, arginine and cysteine were found to be essential in the binding of the enzyme to its cofactors (2-4).

The general scheme of transketolase reaction is depicted below.



General reaction mechanism of enzyme transketolase with substrates xylulose-5-phosphate and ribose-5-phosphate.

It is shown that the enzyme is inhibited by sugar phosphates as well as inorganic phosphates (5). Some other compounds such as phenylglyoxal and butanedion were found to inactivate the enzyme(6). Meta nitro derivative of phenyl-keto butenoic acid had been used successfuly with pyruvate decarboxylase, a thiamine requiring enzyme, which catalyses the decarboxylation of pyruvate to acetaldehyde. A dead -end complex was found to be formed between the enzyme and some of these derivatives (7,8).

Based on the above findings, we undertook the study of the possible inhibitory trends of the enzyme transketolase, with MNPB.

Although the active sites of the two enzymes are shown to be different both in amino acid residues involved in catalysis and the polarity (9), but similar trends of inhibition were found in both enzymes as well as differences that could serve as a useful tool for the mechanistic studies.

It has been reported that one of the sites on TK seem to have less affinity for TPP, and the catalytic function may be assigned to only one site (10),The binding of TPP with the regulatory site of TK, had not only shown to have a minor catalytic

effect but is mostly effective in prevention of active site formation after the divalent ion had been added (10).

The active site of the two enzymes have also different polar characteristics, PDC's active site seems to be hydrophobic with some tryptophanyl residues, which are effective in TPP binding to the enzyme (11).

EXPERIMENTAL PROCEDURES:

MATERIAL

Transketolase, ribose-5-phosphate, xylulose-5-phosphate, thiamine pyrophosphate (TPP), triose-phosphate isomerase (TPI), glyceraldehyde dehydrogenase and NADH were purchased from Sigma chemical Co. All the inorganic salts used were analytical grade purchased from May and Baker and Merck Co. the m-nitro phenyl -2 oxo 3-butenoic acid was synthesized according to the procedure by Roushdi (12). Cary 118 spectrophotometer were used through the experiments.

The reactions were started at 25 °C. The inhibition studies were carried out in two different stages, first the direct interaction of the enzyme and MNPB were studied from the time dependent increase in the absorption band at 276 nm both in phosphate and in bicarbonate buffer at pH=7.5. The reaction mixture contained 0.1 M phosphate or bicarbonate buffer, pH=7.5 which was 1×10^{-3} M in inhibitor and 1 mM in both TPP and Mg^{2+} and contained 5 u/mg-18 u/mg of enzyme. The blank contained all the components except the enzyme. From the increase in absorption with time, a first order trend was observed in bicarbonate buffer which was treated according to the classical Guggenheim plot (13).

$$\log\frac{A_\infty - A_t}{A_\infty - A_o} = -k/2.303.t$$

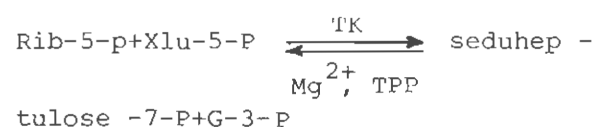
A_∞=infinite absorption (Abs. at the end of the reaction)

A_t=absorption at time t

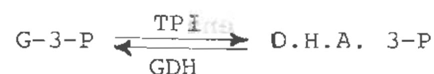
A_o=initial absorption at zero time

$-k/2.303$=the slope of the line at the Δt interval

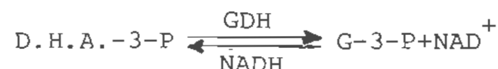
Second, the coupled reaction procedure of de-la Haba (14) was applied using MNPB as the inhibitor. The outline of the reactions are as follows:



$$\text{Rib-5-P+Xlu-5-P} \underset{Mg^{2+},\ TPP}{\overset{TK}{\rightleftharpoons}} \text{seduheptulose-7-P+G-3-P}$$



$$\text{G-3-P} \underset{GDH}{\overset{TPI}{\rightleftharpoons}} \text{D.H.A. 3-P}$$



$$\text{D.H.A.-3-P} \underset{NADH}{\overset{GDH}{\rightleftharpoons}} \text{G-3-P+NAD}^+$$

The activity measurements were carried out using the change in absorbance of NADH at 340 nm converting to NAD^+. The reaction mixture contained 2.5 units of enzyme in 300 µl of 0.1 M bicarbonate buffer. TPP and Mg^{2+} concentrations were kept at 0.1 mM. The enzyme incubation was carried out for 15 minutes at room temperature.

250 μl of this solution was then incubated with 50 μl, of 0.22 M of ribose-5-phosphate for one hour. Aliquots of 50-100 μl of the inhibitor solution was added at this stage and mixed. 25-50 μl of this mixture was then added to 0.5 ml of 0.1 M bicarbonate buffer at pH=7.5 and 0.5 ml of β-NADH solution (0.2 mg/ml in bicarbonate buffer), 100 μl of 20 mM. xylulose-5-phosphate and 10 μl of enzyme mixture (GDH/TPI) (1/5th, diluted with 0.1M bicarbonate buffer) were all mixed and added to the cuvette

The change in absorption was monitored at 340 nm against a blank which contained bicarbonate buffer, TPP and Mg^{2+} in concentrations similar to the test cuvette. All the reagents used were in excess except the enzyme which was kept at a rate controlling concentration. In these reactions the pure enzyme activity was determined at different time intervals. The inhibitor solutions were included in each subsequent runs except that these solutions were added to the reaction mixture after 75 min, of initial incubation time. The final inhibitor concentrations ranged between 5.8-29.5 x 10^{-4} M. The remainning enzyme activity was then assayed at different incubation times. These experiments were repeated without the presence of TPP and Mg^{2+}.

RESULTS AND DISCUSSION

1-DIRECT enzyme-inhibitor binding studies

The direct spectral binding studies of MNPB-TK at pH=7.5 and at 276 nm were carried out in bicarbonate and phosphate buffers.A time dependent increase in absorption was observed which was more detectable in bicarbonate buffer than in phosphate. The blank containing bicarbonate, TPP and Mg^{2+} showed an absorbance which was deducted from the sample absorption at each stage of the reaction. The change in absorption obeyed a first order trend in bicarbonate buffer, thus using the Guggenheim plot the inhibition rate constant was obtained ($k= 8.36x10^{-3}$ s^{-1}).
In phosphate buffer the increase did not follow a first order trend and the Guggenheim plot for the first order reaction produced a well defined curvature that indicated a possible reaction between phosphate and the active site of transketolase (Fig. 2). This effect has been reported before (6,14).

The limiting values of "k" in phosphate buffer using the initial time interval gave a value of $k=3.99x10^{-3}$ s^{-1}, indicating the interference of phosphate ion with the binding of enzyme and the inhibitor. It was suggested that this binding may be due to the interaction of phosphate ion with pyrophosphate binding locus on the active site (15).

2-Binding studies of TK-MNPB by coupled reactions

Based on the observations obtained from the direct binding., to acertain further, that the binding was directly effective in inhibition, we used coupled reaction technique, In these studies the percent active enzyme, after each period of incubation with MNPB was evaluated.This comparison was made with the active enzyme after similar periods of incubation without the inhibitor (Fig. 2).

After one hour of incubation with ribose -5-P and on the following interval of 2 to 65 minutes the enzyme activity did not change irrespective of the presence or the absence of cofactors. (Figs.2,3) The activity of enzyme when incubated with 100 and 200 mM concentrations of MNPB decreased up to 30 minutes incubation and then increases of 10-20% were observed following that interval (Fig. 2). This may indicate that in the lower inhibitor concentration the inhibitor may detach itself from the enzyme after such periods. In higher MNPB concentration (300-500 mM) the enzyme activity decreased with time and remained unchanged on longer incubation times. In the absence of cofactors and in 100 mM of MNPB the enzyme activity increased to about 150% of its original value in the first minutes of incubation and then decreased to about 50% of its initial activity after 60 minutes. (Figs.3,5). In higher MNPB concentration the inhibition took place obeying the same trend as in the runs with cofactors present but with a half-life that was comparatively larger.

($t_{1/2} \simeq 1.9$ min VS. $t_{1/2} \simeq 1$ min).

The rate of inactivation of Transketolase by varying concentration of MNPB could be estimated from the slope of the plot of log% of the remaining activity in early phase of the reaction (up to 30 minutes) with respect to time (Figs. 4,5)· the logarithm of the rate constant of inac - tivation, versus log(I) for the concentrations of inhibitor ranging from 5.9×10^{-4} - 2.95×10^{-3} M was calculated and drawn (Fig.6). The Plot showed a linear trend for the lowest concentrations of the inhibitor,100-200 mM and produced a 45° angle which may be indicative of one site binding with the inhibitor and is equivalent to n=1 (6).

Higher concentrations of the inhibitor (300-400 mM) produced a secondary linear portion with n=3-4·

In the absence of cofactors, the lowest concentration of the inhibitor (100 mM) caused an enhancement of the rate to about 150% in about 2 minutes followed by a drop to a low 50% after 60 minutes (Fig.5). These observation may signify that the inhibitor, in lower concentrations can induce a po-

sitive effect by its attachment to the regulatory site of the enzyme enhancing the rate of catalysis for the substrate. For such regulation the cofactors may not actively take part. Similar result has been obtained with PDC and Phenyl pyrvate derivatives. Jordan and Coworkers (8) have shown that pyruvate can cause delay of inhibition against phenyl pyruvate derivatives and this effect had been assigned to the allosteric properties of the PDC and the role of pyruvate as a positive effector. It could be concluded that the inhibitor binding is aided by cofactors, causing a faster rate of inactivation and/or the cofactor are taking an active part on the formation of the dimer; the spatial arrangement due to a possible dimerization, a condition prerequisit for the activity, that is caused by cofactors could be destabilized by the inhibitor causing a faster rate of inactivation.

Abreviations:

Transketolase ; TK

Pyruvate decarboxylase ; PDC

Meta nitrophenyl-2-oxo-3-butenoic acid ; MNPB

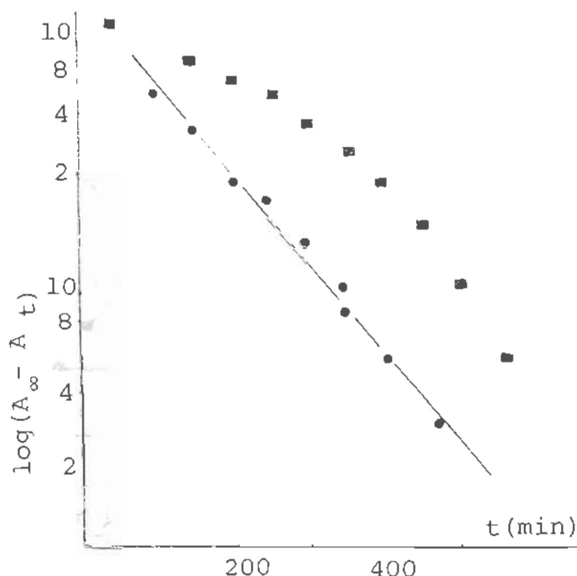
Ribose-5-phosphate ; Rib-5-p: Triose phosphate isomerase=TPI

Thiamine pyrophosphate ; TPP: Glyceraldehyde dehydrogenase=GDH

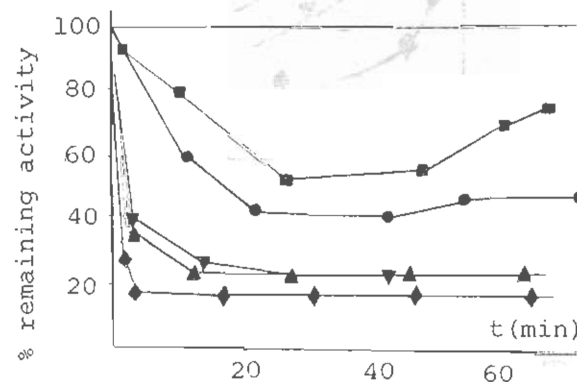
Xylulose-5-phosphate ; Xlu-5-p: Dehydroxyaceton-3-phosphate=D.H.A.3-p

Seduheptulose-7-Phosphate ; seduheptulose-7-p

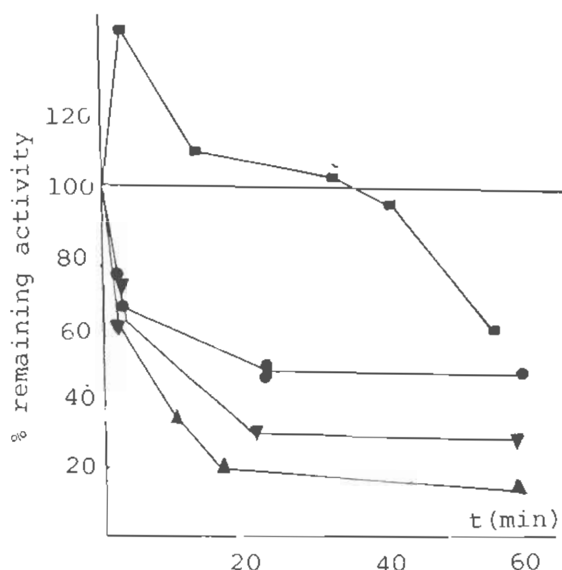
Glyceraldehyde-3-phosphate ; G-3-p



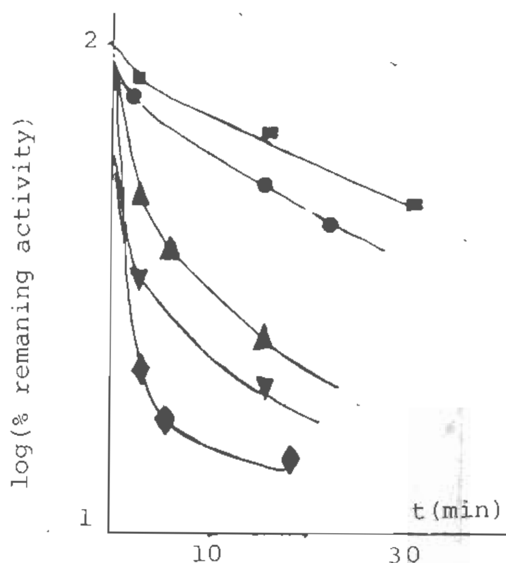
Fig(1) Plot of $\log(A_\infty - A_t)$; A_∞ = product absorbance at the end of the reaction; A_t = absorbance of product at time "t" , vs . time. The slope of the line equals (k/2.303) reaction in phosphate buffer, ■ ; reaction in bicarbonate buffer, ● .



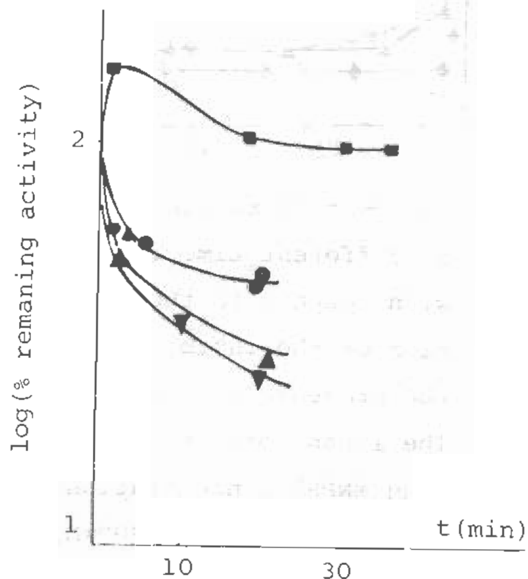
Fig(2) Per cent TK remaining activity at different time intervals with respect to the concentration of the inhibitor (MNPB) in the presence of TPP and Mg^{2+}. The lines refer to the following (MNPB) concentrations; 100 mM, ■ ; 200mM, ● ; 300mM, ▼ ; 400mM, ▲ ; 500mM, ♦ ;



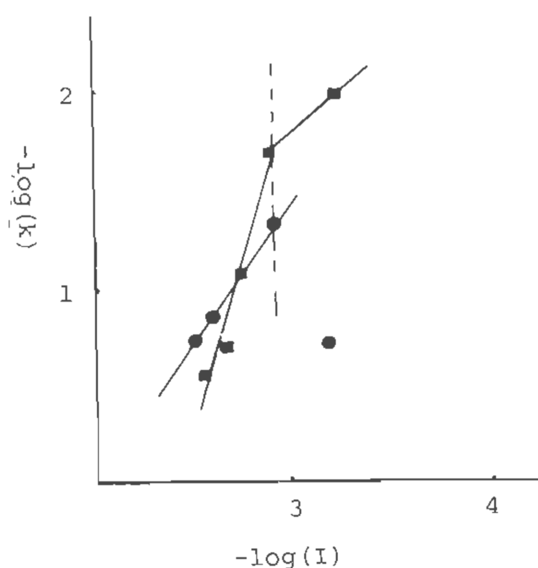
Fig(3) Per cent TK remaining activity vs.time for different inhibitor(MNPB),concentrations.TPP and Mg^{2+} were not included in the reaction mixture.Using the original stock concentrations, the lines refer to the following MNPB concentrations; 100 mM, ■ : 200mM, ● ; 300mM, ▼ ; 400mM, ▲ ;



Fig(4) Logarithm of per cent remaining activity vs.time for different concentrations of MNPB, in the presence of TPP and Mg^{2+}.The slope of the lines in the early periods of the reaction is equal to the inactivation rate constant,Inhibitor concentrations based on stock solutions used were; 100mM,■; 200mM,● ; 300mM, ▲ ; 400mM , ▼; 500mM, ◆ ;



Fig(5) Logarithm of per cent remaining activity vs. time for different concentrations of MNPB in the absence of cofactors . The slope of the line in the early periods of the reaction is equal to the inactivation rate constant for inhibitor concentrations; 100mM,■ ;200 mM,● ; 300mM, ▲ ; 400mM, ▼ ;



Fig(6) The change of logarithm of inactivation rate vs. the logarithm of the inhibitor concentrations; In the presence of cofactors, ■ ; In the absence of cofactors, ● ;

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