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A Kinetic and Mechanistic Study of Hydrolysis of Thiamine Pyrophosphate (Cocarboxylase) in Aqueous Buffer and Micellar Media

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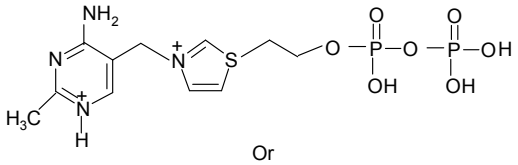
Abstract: Hydrolysis of Thiamine Pyrophosphate (TPP) is too sluggish even at elevated temperatures in aqueous buffer media. However, the hydrolysis reactions of thiamine pyrophosphate (TPP) are dramatically accelerated under micellar conditions even at room temperature by the addition of anionic (SDS) and nonionic (Tx) micelles. Menger – Portnoy's enzymatic model and Piszkiewicz co-operativity model were used to explain the mechanism of hydrolysis under micellar conditions.
Key words: Hydrolysis of Thiamine Pyrophosphate (Cocarboxylase), Kinetic and Mechanistic Study, Aqueous Buffer and Micellar Media.

Introduction:

Hydrolysis is a chemical process in which a certain molecule is split into two parts by the addition of a molecule of water. One fragment of the parent molecule gains a hydrogen ion (H^+) from the additional water molecule. The other group collects the remaining hydroxyl group (OH^-). Hydrolysis is an important process in plants and animals, the most significant example being energy metabolism and storage. All living cells require a continuous supply of energy for two main purposes: for the biosynthesis of small and macromolecules, and for the active transport of ions and molecules across cell membranes. The energy derived from the oxidation of nutrients is used by means of a complex and long sequence of reactions. It is channeled into a special energy-storage molecule, adenosine triphosphate (ATP). In view of this, over the years, the study of hydrolysis of a variety of organic phosphates has become the subject of research interest to chemists and biologists during the past few decades[1-4].

Thiamine (thiamin) or vitamin B_1 is a "thio-vitamin" ("sulfur-containing vitamin"). It is a water-soluble vitamin of the B complex. Its phosphate derivatives are involved in many cellular processes. The best-characterized form of thiamine is thiamine diphosphate (ThDP) or thiamine pyrophosphate (TPP). It is a coenzyme in the catabolism of sugars and amino acids. In yeast, TPP is also required in the first step of alcoholic fermentation. It was also reported that metal ions catalyze the enzymatic and non-enzymatic hydrolysis of large number of phosphate esters [5]. It is understood that metal ions enhance the rate of hydrolysis through complex formation in which neutralization of charge, enhancement of nucleophilicity, polarization of P-O bonds and alignment of reactants facilitate the reaction.

Structures and Schemes of TPP:



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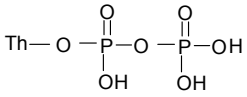


(I) Structure of TPP

During the past few decades there has been an upsurge in exploiting the utility of non-ionic, cationic and anionic surfactants as catalysts in a variety of biologically important reactions owing to their analogous behavior with enzymes. A perusal of literature also shows that surfactants could be effectively employed as catalysts in hydrolysis reactions [6-9]. However, such studies with the phosphate hydrolysis appeared to be scare in literature. In view of this, we have taken up the hydrolysis of kinetic and mechanistic study of thiamine pyrophosphate in aqueous buffer and micellar media. Nevertheless, the kinetics of non-enzymic (spontaneous) hydrolysis and Cu (II) catalyzed hydrolysis of thiamine-pyrophosphate (TPP) has been studied by Khan and Rao [10, 11] in aqueous buffer solution at 56, 64 and 78° C over a pH range of 3.0 to 7.0 at a constant ionic strength1.0 M (KNO_3). The pH rate profiles were analyzed and the overall rate constants resolved into individual specific rate constants relating to various TPP species and Cu (II) bound TPP chelate species in solution. In these studies they have also reported that rate of spontaneous hydrolysis is too slow.

EXPERIMENTAL DETAILS

All the other chemicals are either Aldrich of E-Merck of AR grade samples. Progress of the hydrolysis of phosphate ester was followed by estimating the amount of phosphoric acid (free phosphate) liberated as a function of time during the course of hydrolysis, according to the method adopted by Khan and Rao [10]. Two ml aliquots of the reaction solution were transferred into a 10 ml volumetric flask containing 5 ml ice cold water and kept for 5-6 hrs at ice cold temperature. Extremely low temperature of ice arrests the progress of hydrolysis completely. The above solution is, then, neutralized by the addition of NH_3 and then 1.0 ml of 0.2% $MgCO_3$ suspension and 1.0 ml of 5% $CaCl_2$ solution. Calcium phosphate precipitates out after sometime. In this procedure $MgCO_3$ acts as entrainer. The precipitate thus obtained was separated by centrifugation and dissolved in 60% $HClO_4$. To this solution 1.0 ml of 5% ammonium molybdate and 0.5 ml of ANS (1-amino-4-naphthol sulfonic acid) reagent were added and the solution was made up to 10 ml with distilled water. The flask was then placed in a constant temperature bath at 25°C for 10 minutes for the development of blue color. The absorbance of the solution was immediately measured at 660 nm. The phosphate content has been computed from standard calibration curve constructed by using standard KH_2PO_4 solutions of various concentrations.

Table-1: Mole fractions of TPP at various pH in aqueous medium

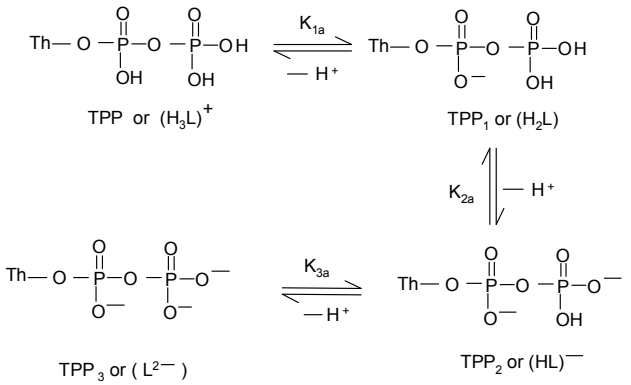
pH	Mo $(H_3L)^+$	M1 (H_2L)	M2 $(HL)^-$	M3 $(L)^{2-}$
3.00	0.030	0.095	0.015	-
4.00	0.003	0.860	0.135	-
5.00	-	0.390	0.602	0.018
6.00	-	0.050	0.740	0.215
7.00	-	0.002	0.250	0.748
8.00	-	-	0.035	0.965
9.00	-	-	0.003	0.985

RESULTS AND DISCUSSION

Salient kinetic features of the study

(i) Hydrolysis of thiamine pyrophosphate (TPP) followed first order kinetics.
(ii) The rate of hydrolysis has been found to be too slow at room temperature (Table-1).
(iii) Rate of hydrolysis has been found to depend on pH.
(iv) The k' Vs pH profile indicated an "S"-type curve without a specific trend.
(v) Data presented in table-3 depicted that in SDS medium even though net rate of hydrolysis has increased the trend is not regular with a variation of [SDS] over a wide concentration range.
(vi) Data presented in table-4 exhibited that in Tx medium, rate of hydrolysis has been dramatically accelerated.

According to the reports of Khan and Rao [10, 11], TPP dissociates in aqueous solution into various dissociated forms as shown in the following equilibria (Scheme 1).



Scheme-1: Dissociation Pattern of TPP

Reactive TPP species and Mechanism of hydrolysis

The foregoing observations indicate that TPP exists in one or more forms at a specific pH as mentioned in literature. The observed rate of hydrolysis, therefore, can be taken up as the algebraic sum of contribution of the above species,

$$k'[TPP]_T = k_0[TPP_0] + k_1[TPP_1] + k_2[TPP_2] + k_3[TPP_3]$$

(or) $$k' = k_0M_0 + k_1M_1 + k_2M_2 + k_3M_3$$

Where k' = observed rate constant; k_o, k_1, k_2 and k_3 are specific rate constants for the species TPP_0, TPP_1, TPP_2 and TPP_3 respectively. M_0, M_1, M_2 and M_3 are the corresponding mole fractions of TPP_0, TPP_1, TPP_2 and TPP_3 respectively.

Mole fractions of various species and corresponding specific rate constants have been calculated according to the method discussed in earlier section and the data are compiled in table-1. Data presented in table-1 indicate that cationic species of TPP (H_3L^+ or M_0) is present in very small anionic (3%) in pH range 3.0 to 4.0. It appears that H_3L^+ becomes appreciable only when high acid concentration is used. Therefore, H_2L appeared to the main species in the experimental pH-range (3.0 to 7.0); HL^- appeared to be important in in the experimental pH-range (3.0 to 9.0); while L^{2-} predominated in the experimental pH-range (5.0 to 9.0). It was therefore reasonable to consider the observed rate constant as the sum of contributions of M_1, M_2 and M_3 in the experimental pH-range (3.0 to 9.0). Distribution of species also indicate that H_2L (M_1) is highly significant in the pH range 3.0 to 4.0 and appeared to be very high at pH 3.0, while HL^- (M_2) would be high at pH 6.0 and L^{2-} is highly predominant in alkaline pH range (above 7.0).

$$k' = k_1M_1 + k_2M_2 + k_3M_3$$

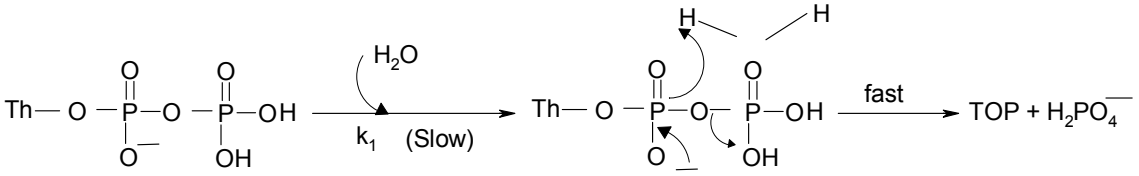
Accordingly specific rate constants k_1, k_2 and k_3 were determined and corresponding activation parameters were also presented in **table-2.** From the data it appeared that rate of hydrolysis of L^{2-} (highly deprotonated) form is the most active species for hydrolysis.

Activation parameters are highly significant in the interpretation of mechanisms of nucleophilic reactions and in particular S_N1 and bimolecular S_N2 mechanisms of ester hydrolysis reactions. The observed small positive entropy of activation coupled with the conclusions of Schalaeger etal may lead to propose SN_1 mechanism for the hydrolysis of TPP. On the basis of observed small entropies of activation, the mechanism of hydrolysis of TPP_1 and TPP_2 may probably be explained due to a cyclic transition state and internal proton transfer. Reaction sequences have been, however, depicted in Schemes 2 and 3.

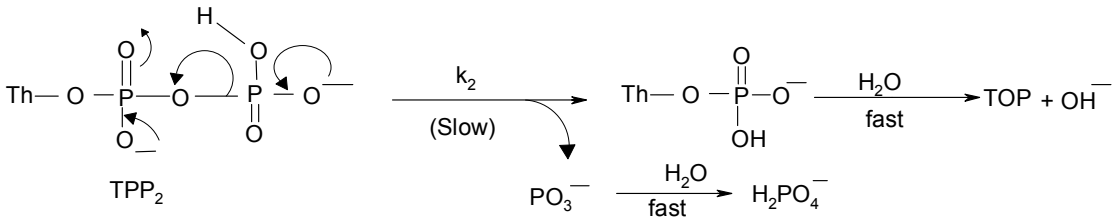
Table-2: Specific rate constants and activation parameters

Specific rate constants (s^{-1}) (323K)	$\Delta H^{\#}$ $kJmol^{-1}$	$\Delta G^{\#}$ $kJmol^{-1}$	$\Delta S^{\#}$ $JK^{-1}mol^{-1}$
$10^7k_1 = 1.90$	80.8	80.4	0.840
$10^7k_2 = 0.750$	90.9	82.9	23.9
$10^7k_3 = 0.850$	88.3	76.2	36.8

Scheme - 2: Hydrolysis of TPP_1



Scheme - 3: Hydrolysis of TPP_2



Mechanism of hydrolysis of the fully deprotonated form of TPP (TPP_3) could be explained on the lines of hydrolysis of acetyl phosphate dianion and salicylic phosphate dianion. Accordingly the reaction may proceed through the formation of transient meta phosphate in the rate limiting step, as shown in Scheme - 4. It was also, further noted that the cleavage of P-O bond in the two processes involve same energy as evidenced from the more or less same enthalpies of activation. However, the relatively large observed entropy of activation over other steps may probably exhibit its importance apart from enthalpy of activation. The lowest entropy of activation ($\Delta S_1^{\#}$) corresponding to (TPP_1) may probably indicate a greater solvation in the transition state resulting from the rearrangement of two protons.

Scheme - 4: Hydrolysis of TPP_3

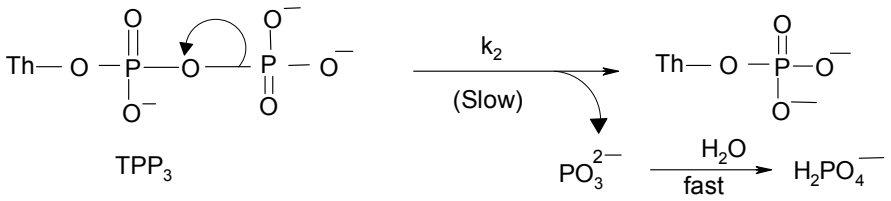


Table-3: Effect of pH on [TPP] in presence of [SDS] micelle

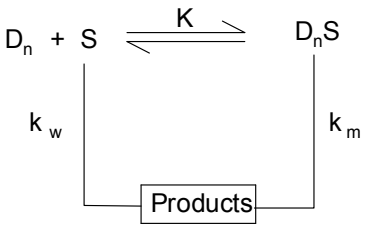
	$10^6 k_\phi$ (sec^{-1}) at pH					
10^3[SDS]	4.80		3.00		3.80	
mol dm^{-3}	303K	313K	303K	313K	303K	313K
2	7.87	17.0	15.0	31.0	17.3	32.1
4	7.27	17.5	14.5	30.3	17.3	31.6
8	9.06	18.3	12.3	27.1	16.1	30.1
10	9.10	18.6	11.6	25.6	15.6	29.6
12	9.10	18.8	11.2	24.6	15.4	29.0
14	9.06	18.9	10.9	23.6	15.2	28.3
16	8.95	19.0	10.8	22.5	15.1	27.9
18	8.82	19.1	10.7	22.3	14.9	27.7
22	8.82	19.1	10.6	22.1	14.5	27.6

Mechanism of hydrolysis of thiamine pyrophosphate in the presence of anionic micelles

(i) In order to gain further insight into the mechanism of hydrolysis of TPP, the reaction has been studied in the presence of anionic (sodium lauryl sulphate - SLS).

(ii) Variation of [SLS] on rate of hydrolysis has been extensively studied under different pH conditions using phthalate buffers. It was noted that below pH 4.00, rate of hydrolysis was substantially inhibited with an increase in [SLS], while above pH 4.00 rate of hydrolysis was significantly catalyzed. As typical cases the rate data are presented in table-2.

Surfactants dissolve completely in water at very low concentrations, but above a certain level, the critical micelle concentration (CMC), the molecules form globular aggregates, called micelles. Micellar catalysis of' reactions in aqueous solution is generally explained in terms of a distribution of reactants between water and the micelles, with reactions occurring in both environments. Data presented in table-1 show that H_2L, HL^- and L^{2-} (dianionic species) are significant and in the range of pH 3.00 to 9.00. The observed inhibition of SLS could be probably explained as the unfavorable electrostatic repulsions between negatively charged TPP species and negatively charged surface of SLS. The mechanism in the presence of SDS in this range could be reasonably explained by the cooperatively model suggested by Piskiewicz [12].



Rate law for this scheme could be given as,

$$\log [(k_\phi - k_w) / (km - k_\phi)] = n \log[D] + \log K$$

According to the above equation plots of $\log [(k_\phi - k_w)/ (km - k_\phi)]$ as a function of log [D] have been found to be linear. Minimum values of observed rate constants k' in the presence of micelles were taken as k_m values. Binding constants (K) were evaluated at different temperatures and corresponding thermodynamic parameters were also presented in table-5. Observed catalysis for TPP hydrolysis above pH 4.00 could be better explained due to favorable hydrophobic interactions although the electrostatic repulsions between dianionic species of TPP and negatively charged surface of micelle are expected to prevail. Considerably greater rate enhancement at higher pH and retardation in lower pH could also be reasonably attributed to saturation of the anionic micelles by hydronium ions at higher acidities there by rendering them catalytically ineffective at higher acidities [6]. Mechanism in the SDS in this range could be explained due to cooperative model. Micelle substrate binding constants were evaluated by using the following equation as cited by Menger and Portnoy [13].

$$\frac{k_\phi - k_w}{k_m - k_\phi} = \frac{k_w + k_m K C_D}{1 + K C_D}$$

$$\log\left\{\frac{k_\phi - k_w}{k_m - k_\phi}\right\} = n \log[D] + \log K$$

Plots of the data according to the above equation for the inhibition of TPP hydrolysis have been found to be linear. Minimum values of observed rate constants k' in the presence of micelles were taken as k_m values. Binding constants K were evaluated at different temperatures and corresponding thermodynamic parameters were also presented in table-4.

Table-4: Effect of pH on [TPP] in presence of [Triton-X]

	$10^6 k_\phi$ (sec^{-1}) at pH							
[Triton-X]	2.20		3.00		3.80		4.80	
% (v/v)	303K	313K	303K	313K	303K	313K	303K	313K
0.15	1.15	2.66	6.38	14.2	7.78	15.5	8.58	17.7
0.25	1.05	2.24	5.24	13.9	7.24	14.7	8.22	15.2
0.45	0.95	1.98	5.13	13.3	6.46	14.1	6.06	13.6
0.55	0.75	1.52	1.84	2.65	3.88	9.45	3.48	7.30
0.65	0.62	1.36	0.80	1.75	2.16	5.95	2.00	4.95
0.75	0.54	1.24	0.40	1.30	1.60	5.40	1.56	4.60
0.85	0.48	1.16	0.36	1.20	1.40	5.20	1.40	4.40
0.95	0.44	1.12	0.04	1.15	1.28	5.10	1.28	4.25

Table-5:Binding constants (K_S) and thermodynamic parameters

pH	K_S (303K)	ΔH $kJmol^{-1}$	ΔG $kJmol^{-1}$	ΔS $JK^{-1}mol^{-1}$
		(A) SDS system		
3.00	22.9	1.25	- 7.86	30.1
3.80	7.63	- 2.87	- 5.10	7.35
		(B) Triton-X system		
2.20	4.35	- 6.63	- 3.69	- 9.70
3.00	134	- 38.5	- 12.3	- 86.5
3.80	7.56	- 15.9	- 5.08	- 35.7
4.80	8.57	- 10.7	- 5.40	- 17.5

Table-6: Rate constants and activation parameters

pH	$10^6 k_m$ (303K)	ΔH $kJmol^{-1}$	ΔG $kJmol^{-1}$	ΔS $JK^{-1}mol^{-1}$
		(A) SDS system		
4.80	7.27	73.4	104	101

Observed catalysis for TPP hydrolysis above pH 4.00 could be better explained as due to favorable hydrophobic interactions although the electrostatic repulsions between dianionic species of TPP and negatively charged surface of micelle are expected to prevail. Considerably greater rate enhancement at higher pH and retardation in lower pH could also be reasonably attributed to saturation of the anionic micelles by hydronium ions at higher acidities there by rendering them catalytically ineffective at higher acidities [7]. Mechanism of the reaction in this range could be explained due to cooperative model. Micelle substrate binding constants were evaluated by using the following equation.Values of k_m and K are presented in tables 5 & 6.

Mechanism in the presence of Triton X-100

It has been recorded that rate of hydrolysis decreased with an increase in [Tx] over a wide range of pH. Although the surface of Tx-100 micelle is neutral, the oxygen of polyoxy ethylene group could create a negative surface which could develop an electrostatic repulsion with the mono ionic TPP species (which is the main species in the pH range below 4.00) causing observed rate inhibition. The observed trends could be conveniently explained by the cooperativity model [12].

In the case of Triton-X 100, the rate of hydrolysis was inhibited in all the media (at all pH) as could be seen from table-3. Sepulveda and Mackitchoie earlier stated that phosphate ester is buried in the interior of the nonionic micelle when water is present (in the nonionic micelles), there by rendering the access of the nucleophile to the reaction center more difficult[6]. The observed trends and mechanism of hydrolysis is explained by the cooperativity model[13] in a usual manner. Binding constants and rate constants have been evaluated and corresponding thermodynamic parameters presented in tables 4 & 5.

CONCLUSIONS

1. Hydrolysis reactions of thiamine pyrophosphate (TPP) are dramatically affected by the addition of anionic (SDS) and nonionic (Tx) micelles. However, precipitate formation is noticed when cationic micelles are added to the solutions of TPP.
2. Rates of phosphate (TPP) hydrolysis have been found to be pH dependent in micellar media also.
3. Rate of hydrolysis has been found to be sensitive to both hydrophobic and electrostatic interactions.

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