Hydrolysis of 4-Nitrophenyl Phosphate by (Amino Acidato)zinc Complexes

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Simple zinc complexes of glycine, histidine, and cysteine are proposed as evolutionary models for hydrolytic enzymes. Catalytic activity of (amino acidato)zinc complexes has been studied towards hydrolysis of 4-nitrophenyl phosphate at 40 °C over the pH range 5.0—10.0 by monitoring release of *p*-nitrophenol at 405 nm. Detailed kinetic investigations on hydrolysis of ester catalyzed by zinc complexes are discussed through formation of (amino acidato)zinc-substrate complex. Mechanistic and evolutionary aspect of this study are discussed in relation to the esterase property of the zinc metalloenzymes.

Most amino acids and peptides possess a number of functional groups which can participate in metal binding processes.[1] The transition metal ions commonly bind to protein through histidine side chains due to σ donor strength of imidazole and moderate pK_a (ca. 7) of imidazolium.[2] Cysteine, also figures prominently in discussion with respect to metal ion binding ability to protein, due to three possible coordination sites, namely, mercapto, amino, and carboxylato groups. In many metalloenzymes, particularly zinc containing hydrolytic enzymes, cysteine and histidine are found as active molecules at coordination sites. Of all known hydrolytic enzymes, very extensively studied enzymes are carboxypeptides-A, carbonic anhydrase, and alkaline phosphatase. Apart from their similar hydrolytic nature, they resemble each other by the fact that they all are zinc containing enzymes and have at least two histidyl residues coordinated to zinc atom. Ligation of the metal ion in active sites of many hydrolases by histidine implies the importance of such units having special chemical feature in the evolutionary selection processes. It can therefore, be conveniently assumed that histidine might have played important role along with metal ions in the evolution of hydrolytic enzymes.

Several successful attempts have been made on the metal ions[3,4] and metal complexes[5—7] catalyzed hydrolytic reactions. A detailed study on divalent metal ion catalyzed hydrolysis of phenolic and aliphatic esters or picolinic acid is reported by Fife and Przystas,[8] where authors have proposed the hydrolysis of substrate to proceed via coordination of pyridine nitrogen to metal ions. Recently, mixed ligand cobalt complexes of the type $[Co(Im)(NH_3)_5]^{2+}$, $[Co(OH)(NH_3)_5]^{2+}$,[9] and $[Co(ImH)(en)_2(H_2O)]^{3+}$, $[Co(ImCH_3)(en)_2(H_2O)]^{2+}$,[10] and *cis*-$[Co(OH)O_3POC_6H_4NO_2(en)_2]$[11] are proposed as possible models for carbonic anhydrase and for alkaline phosphatase respectively. Further, on the basis of higher basicity, $[Co(Im)(NH_3)_5]^{2+}$ was proposed as better nucleophile in hydrolytic reactions.

Although various models have been proposed for hydrolases from time to time, very little attention is given towards evolutionary aspects of these enzymes. The present investigations were carried out to obtain information about probable evolutionary path of zinc containing enzymes. Simple metal complexes which closely resemble metal coordination sites in enzymes have been chosen as catalyst and tested for common hydrolytic reaction. Catalytic activity of zinc amino acid complexes such as bis(glycinato)zinc, bis(cysteinato)zinc, and bis(histidinato)zinc were tested towards hydrolysis of *p*-nitrophenyl phosphate.

Experimental

Materials. Glycine (B. D. H.), L-histidine (Sisco), L-cysteine (Sisco), zinc oxide (B. D. H.), zinc chloride (B. D. H.), *p*-nitrophenyl phosphate (Sisco), were used as supplied and were of Anala R grade. All other chemicals used were of reagent grade.

i) Bis(L- histidianato)zinc(II)Dihydrate, $[Zn(L\text{-}his)_2]\cdot 2H_2O$. Bis(histidinato)zinc complex was synthesised by the method of Ashby et al.[12] zinc oxide (0.01 mol, 0.8 g) was added slowly to an aqueous solution of histidine (0.02 mol, 3.10 g) in 1 : 2 molar ratio and undissolved material was removed by filteration. Ethanol was added to the filtrate slowly until solution became turbid and cooled for 24 h. Needles were formed upon standing and were filtered and dried in vacuo.

ii) Bis(glycinato)zinc(II) Monohydrate, $[Zn(gly)_2]\cdot H_2O$. This complex was prepared as per the procedure reported by Low et al.[13]. To the boiling solution of zinc oxide (0.01 mol, 0.8 g), glycine (0.02 mol, 1.5 g) was added and the solution was boiled for another ten minutes. Undissolved material was filtered off. The complex was precipitated by adding ethanol to the filtrate and recrystallized from ethanol water system.

iii) Sodium Bis(cysteinato)zinc(II) Tetrahydrate, $Na_2[Zn(cys)_2]\cdot 4H_2O$. Sodium bis(cysteinato)zinc(II) tetrahydrate was prepared by the method of Shindo and Brown.[14] To the aqueous solution of cysteine (0.02 mol, 2.42 g) and zinc chloride (0.01 mol, 1.36 g), sodium hydroxide (0.04 mol, 1.6 g) was added. A white precipitate appeared which soon dissolved on stirring. The solution evaporated to dryness and residue was recrystallized from ethanol water system.

Infrared spectra of zinc amino acid complexes were recorded on Beckmann I. R. Spectrophotometer in KBr pellets. Spectral data are tabulated in Table 1.

Rate Measurements. Kinetics of hydrolysis of *p*-nitrophenyl phosphate [4-NPP] catalyzed by (amino acidato)zinc complexes was studied spectrophotometrically at different

Table 1. Characteristic Infrared Bands of (Amino Acidato)zinc Complexes

I. R. Vibrations (cm^{-1})	$[Zn(gly)_2]\cdot H_2O$	$[Zn(his)_2]\cdot 2H_2O$	$[Na_2[Zn(cys)_2]\cdot 4H_2O$
NH_2 Stretch.	3450	3460	3400
H_2O Stretch.	3270	3290	3360
COO^- Asymm. stretch.	1600	1620	1590
NH_2 Bend	—	1540	1547
COO^- Symm. stretch.	1400	1410	1404
NH_2 Wagging	—	1270	1260
NH_2 Twist.	1100	1110	1100

pH, at various concentrations of substrate and catalyst. The standard solution of 4-NPP was prepared in distilled water only. Reaction was initiated by adding 4-NPP and catalyst to previously thermostated buffer at desired pH at 40 °C. Released *p*-nitrophenol was measured at 405 nm at different time intervals.

Results and Discussion

The catalyzed hydrodysis of 4-NPP was studied over a wide pH range 5.0—10.0 at 40 °C and was found pH dependent. The desired pH of the solution was maintained by using phosphate (KH_2PO_4, 0.01 M + Na_2HPO_4, 0.01 M), borax (boric acid, 0.2 M + borax, 0.05 M), and sodium carbonate (0.1 M)–hydrogen carbonate (0.1 M), buffer (1 M = 1 mol dm^{-3}). The pH rate profiles for studied hydrolysis reactions are shown in Fig. 1. Bis(cysteinato)zinc and bis(histidinato)zinc both showed a remarkable enhancement in rate of hydrolysis with increased pH. It is therefore considered that hydrolysis of 4-NPP proceeds with OH^- catalysis. A pH dependent catalysis of ester indicates the possibility of coordination of OH^- to the available site on tetrahedral bis(cysteinato)zinc complex. In case of irregular tetrahedral bis(histidinato)zinc complex, however, it is considered that one of the distant carboxyl groups is replaced by hydroxyl group. The substrate subsequently binds to another available coordination site on the bis(cysteinato)zinc complex or displaces a second distant carboxyl group on bis(histidinato)zinc complex and thus accelerates the reaction at higher pH. Bis(glycinato)zinc complex did not show catalytic effect towards hydrolysis of 4-NPP. This is probably due to non-availability of coordination sites on trigonal bipyramidal bis(glycinato)zinc complex for substrate and OH^- binding which ultimately could accelerate the hydrolysis reactions and therefore its results are not discussed here.

A plot of initial rate of hydrolysis against concentration of 4-nintrophenyl phosphate (4-NPP) initially, is straight line passing through the origin. Figure 2 shows that hydrolysis rate initially follows first order upto a lower substrate concentration (4-NPP, 2.5×10^{-3} M) after which increase in the rate is not linear with increase in concentration of substrate. The order with respect to catalysts was also found to be one as plot between initial hydrolysis rate versus catalyst concentration is a straight line passing through the origin (Fig. 3). First order dependency of hydrolysis rate on catalyst is further confirmed by plotting log rate versus log [Catalyst] where a straight line with unit slope is obtained [not shown].

A tentative mechanism may be considered for zinc

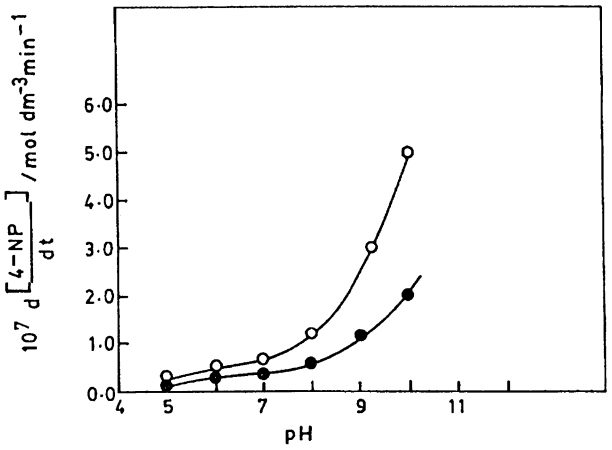


Fig. 1. Hydrolysis of 4-nitrophenyl phosphate rate as a function of pH: $[4\text{-NPP}]_0 = 2\times10^{-3}$ M; [Catalyst] = 1.0×10^{-3} M; Temp 40±1°C. o = $[Zn(his)_2]$, • = $Na_2[Zn(cys)_2]$.

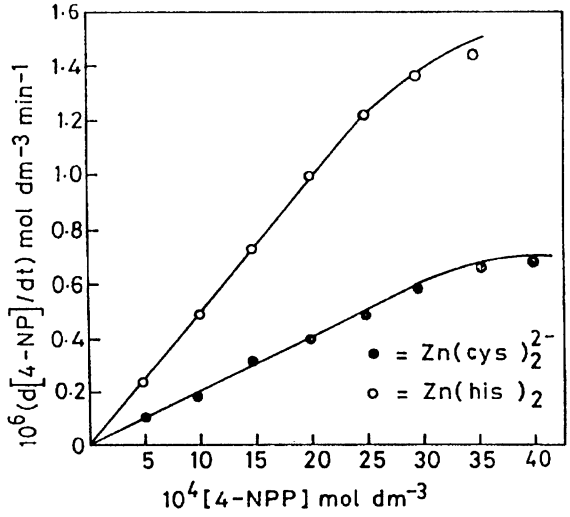
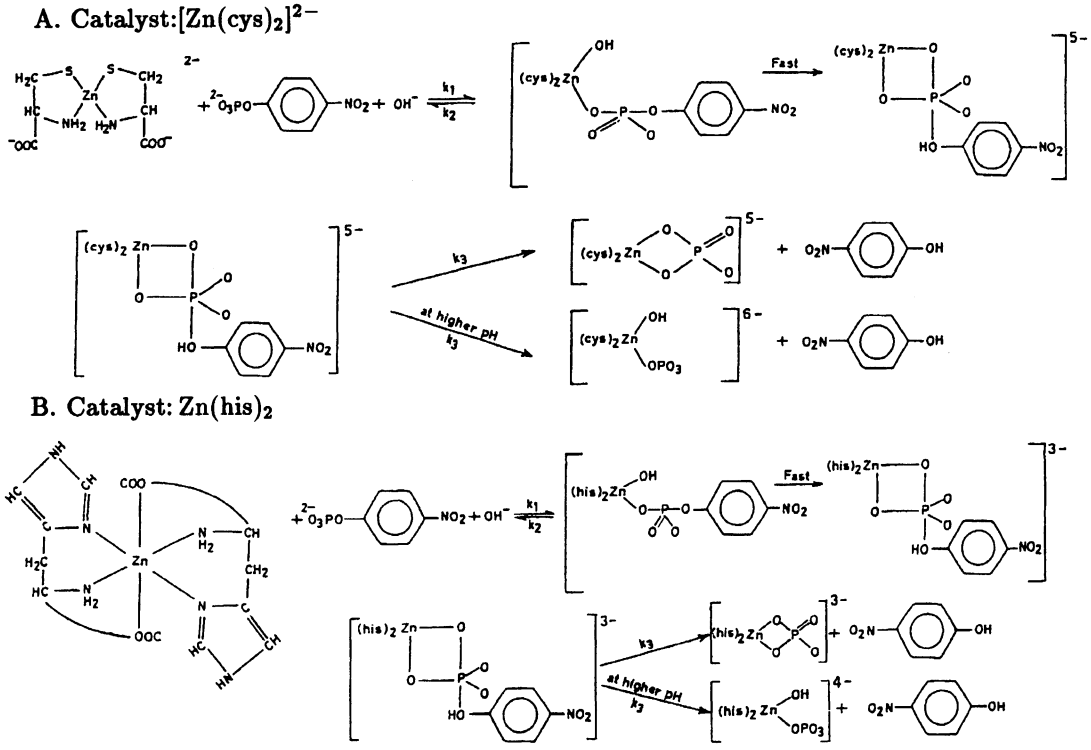


Fig. 2. Hydrolysis of 4-nitrophenyl phosphate rate as a function of $[4\text{-NPP}]_0$ at pH=10.0; [Catalyst] = 2×10^{-4} M; Temp 40±1°C.



Scheme 1. Hydrolysis of *p*-nitrophenyl phosphate.

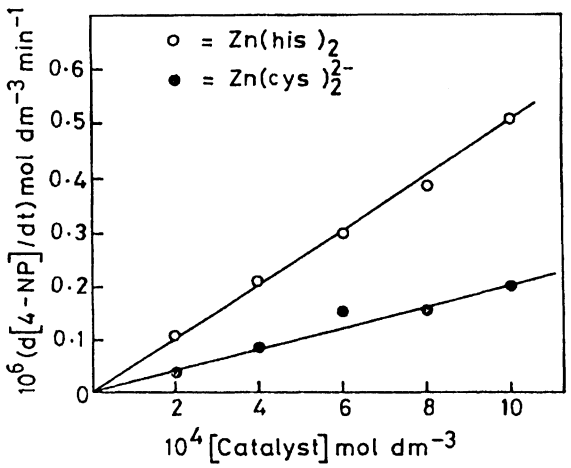


Fig. 3. Hydrolysis of 4-nitrophenyl phosphate rate as a function of [Catalyst]; $[4\text{-NPP}]_0 = 2\times10^{-3}$ M; pH= 10.0; Temp 40±1°C.

amino acid complexes catalyzed hydrolysis of 4-NPP through Scheme 1. A partial support to the proposed mechanism was obtained on the basis of infrared spectra of product. Products were separated with ethanol as solid phosphate derivatives of (amino acidato)-zinc complexes. Infrared spectral data are tabulated in Table 2 and compared with spectral data of original (amino acidato)zinc complexes. Shifts in frequencies of phosphate observed may be due to their coordination to metal ions in bis(histidinato)zinc or bis(cysteinato)-zinc complexes. It appears that reaction mechanism is similar to the typical enzyme catalysed reaction viz.,

$$\text{Enzyme} + \text{Substrate} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{enzyme-substrate complex} \quad (1)$$

$$\text{Enzyme-substrate complex} \xrightarrow{k_3} \text{Product} \quad (2)$$

Rate law for the above reaction can be expressed as follows:

$$V = \frac{k_3[\text{Enzyme}][\text{Substrate}]}{K_M + [\text{Substrate}]}$$

where, V = reaction rate; $K_M = \frac{k_2+k_3}{k_1}$. A tentative mechanism for the hydrolysis of 4-NPP represented in Scheme 1 may be considered as below:

$$C + S + OH^- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (C_1 \xrightarrow{\text{fast}} C_1') \quad (3)$$

$$C_1' \xrightarrow{k_3} \text{Product}, \quad (4)$$

where C=zinc complex of histidine or cysteine, S=4-NPP, C_1 and C_1'=unstable substrate catalyst complex. For the (amino acidato)zinc complexes catalyzed hydrolysis of 4-NPP, following rate law is obtained assuming steady state approach to catalyst-substrate complex C_1'.

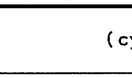
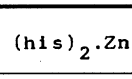
$$V = \frac{k_3[C_T][S][OH^-]}{K_M + [S][OH^-]} \quad (5)$$

Rearranging equation (5) we get:

$$\frac{1}{V} = \frac{K_M}{k_3[C_T][S][OH^-]} + \frac{1}{k_3[C_T]} \quad (6)$$

Linearity of the plot of V^{-1} versus $[S]^{-1}$ with a positive intercept (Fig. 4) suggests that the reaction follows Michaelis–Menten type kinetics and thus it would

Table 2. Characteristic Infrared Spectra of Products of Esters Hydrolysis

I. R. Vibrations (cm^{-1})	$(cys)_2.Zn$ 	$(his)_2.Zn$ 
NH_2 Stretch.	3420_S	3450_S
H_2O Stretch.	3320_S	3310_S
COO^- Asymm. stretch.	1600_M	1620_M
NH_2 Bend.	1535_M	1545_M
COO^- Asymm. stretch.	1400_W	1415_W
$\nu_{P=0}$	1290_{VW}	1270_{VW}
P–O–P	990_{VW}	985_{VW}

S=Strong, M=Medium, W=Weak, VW=Very Weak.

Table 3. Kinetic Constants for Hydrolysis Reaction by (Amino Acidato)-zinc Complexes

Catalyst	$V_{max.}$ ($M\ min^{-1}$)	$^*K_M$ (M)	Turnover number (min^{-1})
For hydrolysis of 4-NPP			
$[Zn(cys)_2]^{2-}$	3.3×10^{-6}	0.030	1.6×10^{-2}
$[Zn(his)_2]$	1.0×10^{-5}	0.036	5.0×10^{-2}

$^*K_M=K_M/[OH^-]$.

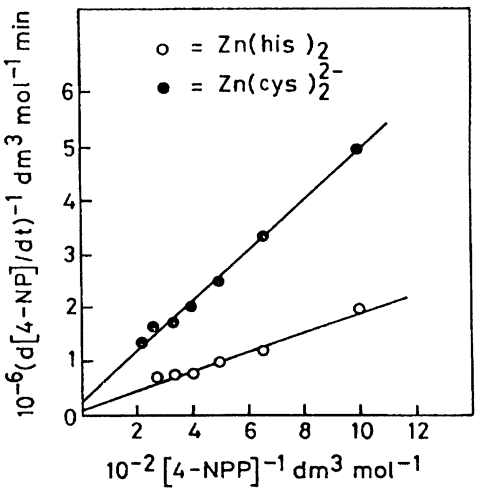


Fig. 4. Lineweaver Burk-plot.

be expected for such type of catalyzed reaction to proceed via formation of substrate-catalyst complex which subsequently breaks down to give products. The proposed mechanism was found to be consistent with rate equation and verification was done by plotting V^{-1} versus $[C_T]^{-1}$, a straight line passing through the origin as warrant by Eq. 6 is obtained. V_{max}, K_M, and turnover number for the catalysts are tabulated in Table 3. Evaluation of the above terms have been done by usual method and reported $^*K_M$ values are equals to $K_M/[OH^-]$.

Catalyzed hydrolysis reactions of esters initially by imidazole,[15] later on by transition metal ions[16—22] and by transition metal complexes[23,24] have been the major focus of recent kinetic and thermodynamic investigations. Recently Ito et al.[25] reported hydrolysis of esters by polypeptide containing histidine. Extensive studies on metal ion catalysis were carried out by Fife and Przystas[8,22] using various esters and amides as substrate for hydrolytic reaction over much wider pH range 3.0—11.0. A pronounced catalytic effect was observed when higher metal ion concentration was employed (100 fold excess over ester). Although it is well-established that metal ions, as such show remarkable catalytic activity towards hydrolysis of esters. However, in our studies we found that lower concentrations of zinc ion (1.0×10^{-3} M) alone did not show notable catalytic activity towards hydrolysis of 4-NPP. Cysteine, histidine, or buffer alone was also not effective towards hydrolysis of 4-NPP (Fig. 5).

The Mode of coordination of metal ions to amino acid residues is important in enzymic catalysis. Of the twenty amino acids commonly occuring in proteins, only few participate actively in enzyme mechanism, other amino acid residues are effective in determining the tertiary, quarternary structure of proteins and binding to

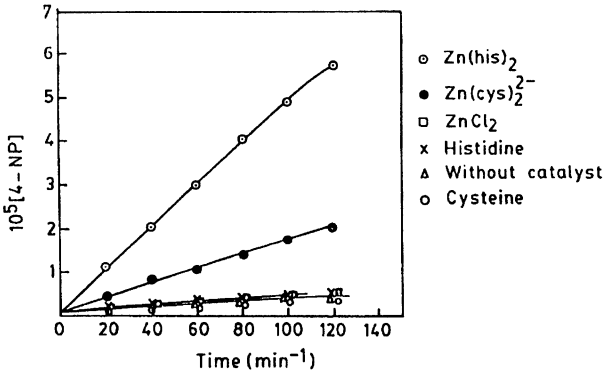


Fig. 5. Hydrolysis of 4-NPP. [4-NPP]=2×10^{-3} M; [Catalyst]=1×10^{-3} M; pH=10.0; Temp 40±1°C.

substrate. Such complexity of biological systems renders the need of detailed study of their mechanism. An increasing popular method of elucidation of structure and mechanism is that of using simple models wherein aspects of structure of properties of these biological systems are imitated by simple chemical system or compound. In the present paper similar procedure was adopted here. We have selected simple amino acids, histidine and cysteine which commonly occur at the active sites of zinc hydrolases and the (amino acidato)zinc complexes as models for zinc hydrolases. The (amino acidato)zinc complexes showed remarkable increase in catalytic activity towards hydrolysis of 4-NPP when compared to metal ions alone. Many more hydrolytic reactions remain to be tested by the (amino acidato)-zinc complexes (Future communication). It is expected that if biologically more complicated ligands coordinate to metal ions, the catalytic activity will increase correspondingly.

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