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THE REACTION OF TRIS (HYDROXYMETHYL) AMINOMETHANE WITH CALF INTESTINAL ALKALINE PHOSPHATASE

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Summary

The effect of tris (hydroxymethyl) aminomethane concentrations on the rate of calf intestinal alkaline phosphatase-catalyzed hydrolysis of *p*-nitrophenyl phosphate was studied, in the pH range 8—10, where no transphosphorylation reaction could be detected. Kinetic analysis of the results permitted description of the effect of Tris concentrations T on the rate of enzyme catalyzed hydrolysis (V) by the following equation:

$$V = [E_0]\{k + k_M[T]\} + [E_0]\frac{[T]\{k_{T\ lim}/(1 + K_1/[H]) - k\}}{[T] + A[H]}$$

The rate-accelerating effect of Tris concentrations can be ascribed to two different mechanisms: At moderate Tris concentrations (0.01—0.20 M) the enzyme forms a reversible addition complex with a Tris molecule. This complex has an enhanced catalytic activity. We suggest that the binding of Tris to the enzyme could potentiate a second active site of the enzyme, due to its ionization effect upon an acidic group of the enzyme of pK = 8.9. The modest linear rate accelerating effect of Tris at high concentrations (0.20—0.60 M) could be ascribed to the change of the dielectric constant of the medium, the degree of solvation of the protein, or change in the tertiary structure of the enzyme.

Introduction

The inhibitory effect of phosphate ions on the catalytic efficiency of alkaline phosphatase, and the small numbers of buffers available in the pH

Abbreviations: Nph-*P*, *p*-nitrophenyl phosphate; NpH, nitrophenol.

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region 7—10, led to the extensive use of tris (hydroxymethyl) aminomethane Cl, Tris · Cl buffer, in the kinetic and mechanistic studies of alkaline phosphatase. The presence of three primary alcohol functions in Tris confers, however, nucleophilic properties to the buffer molecule and under certain conditions extensive phosphorylation of the hydroxyl groups can be catalyzed by phosphatases [1,2,3]. The increased rate of decomposition of phosphate esters in the presence of Tris was explained by this transphosphorylation reaction [1,2]. Calf intestinal alkaline phosphatase also catalyzes the transphosphorylation reaction in the pH range 6.9—7.9 from *p*-nitrophenyl phosphate (Nph-*P*), with the formation of appreciable amounts of Tris-phosphate [1,2].

We observed that the increase of Tris concentrations enhances the rate of the enzyme catalyzed hydrolysis of phosphate esters in the pH region 8—10, in spite of the fact that at these pH values no transphosphorylation can be detected. This finding suggests a specific interaction of Tris with the enzyme. The present report is devoted to analysis of this phenomenon.

Experimental procedure

Materials

Materials used were obtained from the following sources: Calf intestinal alkaline phosphatase (EC 3.1.3.1) Lot P34502-Type VII and *p*-nitrophenyl phosphate, (Nph-*P*), were purchased from Sigma Chemical Company. Tris (hydroxymethyl) aminomethane, Tris, was from Fischer Scientific Co. All other chemicals were analytical reagent grade.

Stock solutions

Stock solutions of alkaline phosphatase purchased in suspension (5.0 mg/ml in 3.2 M $(NH_4)_2SO_4$, 0.001 M $MgCl_2$ and 0.001 M $ZnCl_2$ solution) were diluted with conductivity water to a final concentration of 1.0 mg protein/ml, and kept frozen. Before use the enzyme solution was thawed and diluted with conductivity water 10 000 fold. Solutions of Nph-*P* (0.01 M in water) were kept at $-10°C$. Stock solutions of 1.0 M NaCl, 0.1 $MgCl_2$ and 2.0 M Tris were prepared in water. Tris · Cl buffers at the required pH were prepared from 2 M stock solution of Tris by adding the appropriate amounts of HCl (2 M), to reach the desired pH and then diluted to 1.0 M with water or salt solutions.

Enzyme concentrations were calculated on the basis of protein content of the reaction mixture, assuming 100% purity for the enzyme and a molecular weight of 100 000 g/mol.

Methods

pH measurements were carried out with pH meter model TTT Ic, Radiometer, Copenhagen, using glass electrode type GK2322C and calomel electrode type K4312.

Spectrophotometric measurements were made using a Zeiss model M3 QIII spectrophotometer.

Enzyme assay

The assay mixtures contained 10^{-3} M Nph-*P*, 10^{-2} M $MgCl_2$, 0.2 M NaCl,

the appropriate amounts of Tris · Cl buffer at the required pH. The enzymatic reactions were initiated at 25°C by addition of the enzyme solution. The reaction was followed spectrophotometrically, by reading the absorbance at 400 nm. *p*-Nitrophenol (Nph), one of the products of enzymic hydrolysis absorbs at this wavelength. The molar absorption $\epsilon_{400} = 18 \cdot 10^3$ above pH 8.6. At lower pH values corrections were made for the partial ionization of Nph. In all experiments the substrate concentration was at least 2.5 higher than the K_m of the enzymatic reaction, as determined by Lineweaver-Burk plots at several pH values. In each case the initial velocity was determined by plotting the absorbance vs time, taking into account for calculations only the linear part of the curves.

For the determination of K_m as a function of Tris · Cl concentrations, and of pH, the substrate concentrations varied from 10^{-5} to $2 \cdot 10^{-3}$ M. Inorganic phosphate was determined with the method of Fiske-SubbaRow [4].

Phosphoryl transferase activity of alkaline phosphatase to Tris.

The phosphoryl transferase activity of alkaline phosphatase from NpH-*P* to Tris was studied at pH values 7.2, 7.5, 7.8, 8.0, 8.5, 8.8, 9.0 and 9.5. The reaction mixture contained 0.1 M Nph-*P*, 0.01 M $MgCl_2$ and varying concentrations of Tris · Cl. The reaction was initiated by adding 1.0 ml 0.05 μg/ml enzyme. Reaction mixtures (10 ml) were incubated at 25°C. At given time intervals, aliquots were removed simultaneously to determine the amount of Nph, and inorganic phosphate [4]. The liberation of Nph was followed spectrophotometrically using 0.1 cm light path cuvettes, while the aliquots for P_i determination were added to 1.0 ml of 68% perchloric acid, and the quantity of inorganic phosphate was determined. For the calculation of the rate of phosphoryl transfer reaction, the relationship introduced by Brestkin et al. [5] was used:

$$V_a = V_{ROH} - V_{P_i}$$

Above pH 8.2 the amount of alcohol was found to be equal within experimental error to the amount of P_i. These results were interpreted to show that above pH 8.2 no phosphoryl transfer reaction occurred.

Results

The rate of enzymic hydrolysis of Nph-*P* in saturating concentrations of substrate was measured at various Tris concentrations (0.05 M to 0.6 M), at several pH values between 8.0 and 10.0 in the presence of 0.2 M NaCl. NaCl was added to maintain the ionic strength reasonably constant under the experimental conditions applied. The influence of Tris concentrations on the rate of enzymic hydrolysis is illustrated in Fig. 1 for pH 8.75. At low Tris concentrations, the rate of enzymic hydrolysis rapidly increased with increasing Tris concentrations, displaying the shape of a typical saturation curve. At higher Tris concentrations, the influence of Tris became less pronounced, and appeared to obey a linear relationship. Curves of similar shape were obtained at all other pHs.

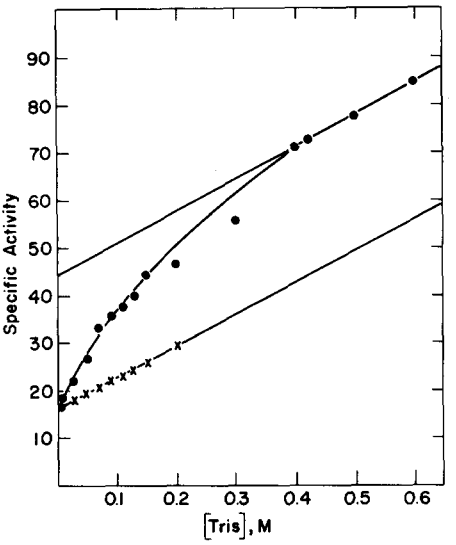

Fig. 1. The dependence of alkaline phosphatase activity on Tris concentrations at pH 8.75. The reaction mixtures contained 0.001 M Nph-*P*, 0.01 M $MgCl_2$, 0.2 M NaCl and various amounts of Tris · Cl at the above pH. The enzyme concentration in the reaction mixtures was 0.05 μg/ml. The kinetic parameters were determined through graphical correction for V by drawing a straight line (lower line) through the extrapolated intersection of the experimental curve with the orginate (kE_0), and parallel to the experimental line at high Tris · Cl concentrations (higher straight line).

Kinetic analysis of the data obtained at each pH value was carried out with the aid of the following assumptions: (1) The enzyme-substrate complexes [ES] present at saturating substrate concentrations are able to bind a Tris molecule [EST]; (2) This ternary complex has a higher rate constant k_T than that of the enzyme substrate complex (k); (3) The small linear dependence of the rate at high Tris concentrations is due to a nonspecific medium effect, which can influence both forms of enzyme–substrate complex (ES and EST).

The reaction described above may be represented by the following Scheme 1:

$$\begin{array}{ccccc} E & + S \overset{K_m}{\rightleftharpoons} & ES & \overset{k}{\longrightarrow} & E + P \\ {\scriptstyle +T}\downarrow\uparrow{\scriptstyle K'_T} & & {\scriptstyle +T}\downarrow\uparrow{\scriptstyle K_T} & & \\ ET & + S \overset{K'_m}{\rightleftharpoons} & EST & \overset{k_T}{\longrightarrow} & ET + P \end{array} \qquad (1)$$

At saturating substrate concentrations, the rate of reaction can be expressed by Eqn 2:

$$V = \frac{dP}{dt} = k[ES] + k_T[EST] + k_M[E_0][T] \qquad (2)$$

where k_M is a rate constant observed at high Tris concentration, and E_0 is the total enzyme concentration. With the auxiliary Eqns 3, 4 and 5:

$$[E_0] = [ES] + [EST] \qquad (3)$$

$$K_T = \frac{[T][ES]}{[EST]} \tag{4}$$

$$[EST] = \frac{[E_0]}{1 + \frac{K_T}{[T]}} \tag{5}$$

Eqn 2 can be transformed to Eqn 6:

$$V = k[E_0] + k_M[T][E_0] + (k_T - k)\frac{[E_0]}{1 + \frac{K_T}{[T]}} \tag{6}$$

Thus the observed rate is the sum of a saturation-type hyperbola and a linear term

$$V_1 = \{(k + k_M[T]\}[E_0] \tag{7}$$

V_1 was obtained graphically by drawing a straight line through the extrapolated intersection of the experimental curve with the orginate (kE_0) and parallel to the experimental curve at high Tris concentrations (lower straight line in Fig. 1). The differences between this line and the experimental points (V_{corr}) were calculated, and represented in Eqn 8.

$$V_{corr} = V - V_1 = \frac{[E_0](k_T - k)}{1 + K_T[T]} \tag{8}$$

or

$$\frac{1}{V_{corr}} = \frac{1}{[E_0](k_T - k)} + \frac{K_T}{[E_0](k_T - k)}\frac{1}{[T]} \tag{9}$$

The Tris dependency of V_{corr} was then analyzed by plotting $1/V_{corr}$ vs $1/[T]$ according to this equation. A representative plot of experiments performed at pH 9.0 is given in Fig. 2. At all pH values where experiments were performed

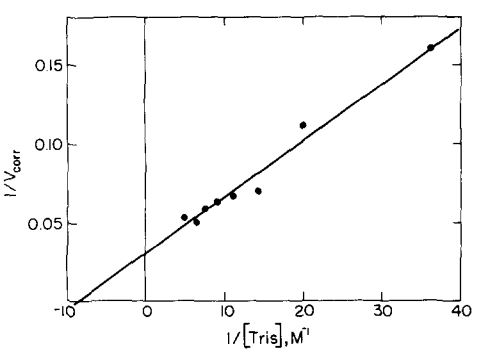

Fig. 2. The Tris dependency of V_{corr}, analyzed by plotting $1/V_{corr}$ vs $1/[T]$ at pH 8.75. From the slope and the intercept of these lines values of K_T and $(k_T - k)$ were calculated. Experimental conditions were identical as described in Fig. 1.

straight lines were obtained showing that the data are consistent with the hypothesis given in Scheme I. From the slope of the line and intercept with the y-axis (see Fig. 2) the values of K_T and $(k_T - k)$ were calculated. The results for pH 9 and other pH values are shown in Table I, together with the values of k, k_T and k_M determined graphically by the methods above.

The pH dependency of K_T and k_T was analyzed by plotting the logarithm of the constants vs pH. In the case of K_T the data yielded a straight line with a slope of −1 (Fig. 3), indicating that the binding of Tris to the enzyme is inversely proportional to the hydrogen ion concentration. The plot of k_T vs pH gave a typical sigmoidal curve (Fig. 4). From the graphical analysis of the pH dependency of k_T given in Fig. 4 one obtains the relations:

$$k_T = \frac{810}{1 + (2 \cdot 10^{-9})[H^+]} \text{ s}^{-1} \tag{10}$$

i.e. $k_{T\,\text{lim}} = 810\ \text{s}^{-1}$ and $pK = 8.7$

A rather large scattering was observed for the values of k and k_M. This may be due to the fact that their values were determined by graphical extrapolation. Within this large uncertainty, the rate constant k is probably pH independent, whereas k_M shows a slight decrease at lower pH values. The latter findings may imply that k_M is dependent on the concentration of unprotonated Tris molecules in the reaction mixture.

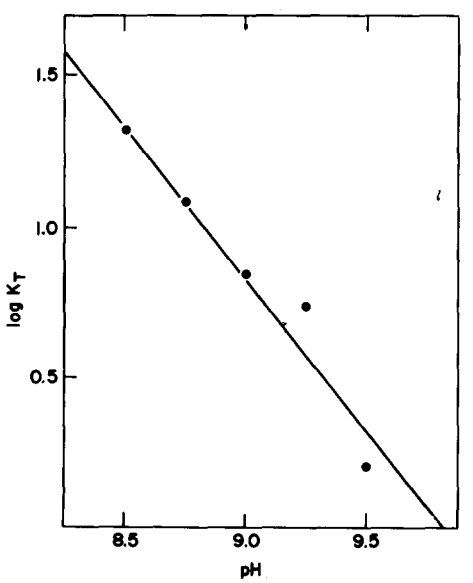

Fig. 3. The pH dependency of K_T analyzed by plotting the constants vs pH. The slope of the line is equal −1.

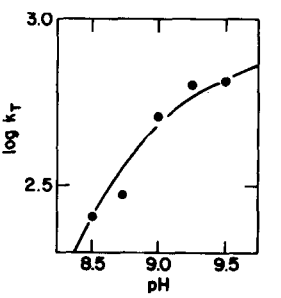

Fig. 4. The pH dependency of k_T was analyzed graphically, by plotting the constants vs pH. The plot yields a typical sigmoidal curve. The solid line is the theoretical curve, according to Eqn 10.

TABLE I

The dependence of $k_T - k$, k_T, k_M and K_T on pH of calf intestinal alkaline phosphatase in 0.2 M NaCl, 0.01 M $MgCl_2$, Nph-*P* 0.001 M as substrate.

pH	$k_T - k$ (s^{-1})	k_T (s^{-1})	k_M ($M^{-1}\ s^{-1}$)	$K_T \times 10^2$ M (M)
8.50	173	253	367	21
8.75	100	300	440	12
9.00	273	513	747	7.0
9.25	100	647	713	5.5
9.50	113	653	693	1.6

Discussion

On the basis of the results obtained from the calf intestinal alkaline phosphatase catalyzed hydrolysis of Nph-*P* at the saturating substrate concentration and at alkaline pH values, the rate-enhancing effect of Tris on this enzyme has to be ascribed to at least two different mechanisms. The pH-dependent rate of hydrolysis is correctly described by Eqn 11:

$$V = [E_0]\{k + k_M[T]\} + E_0 \frac{[T]\{k_{T\ lim}/(1 + (K/[H^+])) - k)\}}{[T] + A[H^+]} \qquad (11)$$

where A is a constant. Eqn 11 is valid only at pH values where no transphosphorylation is observed.

At moderate Tris concentrations (0.11—0.20 M) the enzyme forms a reversible addition complex. This complex has a greater catalytic activity toward the substrate than the enzyme in its original form. Since the ionization of Tris is virtually complete in the pH region studied (8.5 to 10), the observed pH dependency of K_T therefore requires formally the participation of a hydroxide ion in the complex formation. In terms of prototopic equilibria the above statement allows one to postulate an ionization equilibrium for the enzyme in the highly alkaline pH region ($pK_E \gg 9.5$), and the binding of Tris to the unprotonated form of the enzyme only, according to Schemes 12 and 13:

$$E_iH \rightleftharpoons^{K_E} E_i + H \qquad (12)$$

$$E_i + T \rightleftharpoons^{K_L} E_iT \qquad (13)$$

where

$$K_E = \frac{[E_i][H]}{[E_iH]} \quad \text{and} \quad K_L = \frac{[E_i][T]}{[E_iT]}$$

If this prototropic equilibrium does indeed occur, then the experimentally determined K_T has to be defined as in Eqn 14:

$$K_T = \frac{\{[E_iH] + [E_i]\}[T]}{[E_iT]} \tag{14}$$

Eqn 14 can be transformed to Eqn 15:

$$K_T = K_L \left\{1 + \frac{[H]}{K_E}\right\} \tag{15}$$

or if $H \gg K_E$, then Eqn 15 can be written as Eqn 16:

$$K_T = \frac{K_L}{K_E}[H] \tag{16}$$

Eqn 16 is consistent with the observed pH dependency of K_T. Thus, the true, thermodynamic dissociation constant of enzyme-Tris complex shows a relatively strong binding ($K_L \gg 10^{-2}$), requiring the deprotonated form of an ionizable group of the enzyme. Assuming normal ionization behavior of the amino acid residues of the enzyme, in the pH region of the experiments, the only unprotonated groups are either the phenolic group of a tyrosine residue or the ϵ-amino group of a lysine residue. The binding of Tris to the enzyme molecule, therefore, probably occurs through hydrogen bond formation between the basic form of either phenol or ϵ-amino group of the enzyme and the hydroxyl proton of Tris.

We can, at this time, only speculate on the reason that Tris greatly enhances the catalytic efficiency of the calf intestinal alkaline phosphatase. Our observations may fit the reported explanation of Chlebowski and Coleman [6] on the mechanism of *E. coli* alkaline phosphatase. According to these authors, at pH $\gg$ 8.5, a second active site of *E. coli* alkaline phosphatase is activated by high substrate concentration. It was also reported by Fernley and Walker [7] that the enzymatic activity of calf intestinal alkaline phosphatase increased by a factor of six upon the deprotonation of the complex between the enzyme and 4-methyl umbelliferyl phosphate with a pK of 8.9. In the light of these reports, it is reasonable to assume that Tris also can potentiate a second active site, with the ionization of an acidic group of pK 8.7, consequently increasing the observed rate of enzymatic hydrolysis of the substrate.

Finally, the modest rate acceleration (k_M) at high Tris concentrations (0.2—0.6 M) could perhaps be attributed to the high concentrations of buffer (7% of the solvent water is replaced by Tris) inducing severe changes in the medium. Such large concentration changes of Tris must be accompanied by considerable gradual alteration of the dielectric constant of the medium, the degree of solvation of the protein, and may also effect the tertiary structure of the enzyme. Either one of these changes may easily account for the observed Tris-concentration dependency of enzymic activity in the region of 0.2—0.6 M Tris.

This ability of Tris to influence to such an extent the intestinal alkaline phosphatase activity raises some speculations concerning the possible physiological significance of the existence of an activator-site on the enzyme. The abundance of alkaline phosphatase in the intestine is well documented. A

naturally occurring activator might have a role in control of the activity of this enzyme in the intestine.

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