Influence of Reagents Reacting with Metal, Thiol and Amino Sites on Catalytic Activity and L-Phenylalanine Inhibition of Rat Intestinal Alkaline Phosphatase

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1. Studies on the inactivation of rat intestinal alkaline phosphatase by several metal-binding agents, namely EDTA, 8-hydroxyquinoline, pyridine-2,6-dicarboxylic acid, $\alpha\alpha'$ -bipyridyl, *o*-phenanthroline and sodium cyanide, indicated the functional role of a metal, probably zinc, in the catalysis. The metal ligands lowered stereospecific uncompetitive inhibition of the enzyme by L-phenylalanine by an extent that paralleled the decline in enzyme activity. 2. The thiol reagents *p*-hydroxymercuribenzoate, iodoacetamide and iodine inactivated rat intestinal phosphatase. The enzyme could be protected from inactivation by either cysteine or substrate. The L-phenylalanine inhibition remained unchanged only in the presence of moderately inactivating concentrations of the thiol reagents. 3. Inactivation of the enzyme by the amino-group-blocking reagent, *O*-methylisourea, provided ample evidence for the participation in the catalysis of the ϵ -amino group of lysine. At the same time, L-phenylalanine inhibition remained unaltered even when the enzyme was strongly inactivated. This ϵ -amino-group-blocked enzyme exhibited no change in migration in starch gel, in contrast with enzyme treated with acetic anhydride, formaldehyde or succinic anhydride. The Michaelis constant of the enzyme was enhanced by such modifications, but the optimum pH remained the same. 4. D-Phenylalanine acted as a competitive or 'co-operative' activator for intestinal alkaline phosphatase after it had been modified by acetylation.

Kinetic evidence indicating that the inhibition of rat intestinal alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) by L-phenylalanine is 'uncompetitive' has been presented previously (Ghosh & Fishman, 1966). The extent of inhibition, the maximal velocities in the presence and absence of the inhibitor and the Michaelis constants, K_m , are dependent on pH. The presence of ionizable groups that influence the enzyme activity was inferred from two discontinuities in the pK_m -pH plot at pH 8.6 and 9.6 and from the symmetrical bell-shaped V_{max} -pH curves.

In the present paper we have attempted to identify the dissociable groups at or near the active site of the enzyme and to evaluate their influence on catalysis and inhibition. The new findings indicate that only the metal site is essential for both catalysis of substrate hydrolysis and inhibition by L-phenylalanine, whereas thiol and amino groups appear to play more of a role in catalysis than in 'uncompetitive' inhibition. Treatment with certain amino-group-blocking reagents does provide

an enzyme species having increased electrophoretic mobility, diminished substrate affinity and the capability of being activated by D-phenylalanine.

A preliminary report of this work has been presented (Ghosh & Fishman, 1965).

MATERIALS AND METHODS

Materials. Materials and their sources are respectively: disodium phenyl phosphate (Sigma Chemical Co., St Louis, Mo., U.S.A.); D- and L-phenylalanine and cysteine (Calbiochem, Los Angeles, Calif., U.S.A.); *O*-methylisourea hydrogen sulphate (Sapon Laboratories, Oceanside, N.Y., U.S.A.); PHMB* and iodoacetamide (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.); iodine and acetamide (Merck and Co. Inc., Rahway, N.J., U.S.A.); EDTA, $\alpha\alpha'$ -bipyridyl and *o*-phenanthroline (Fisher Scientific Co., Pittsburgh, Pa., U.S.A.); pyridine-2,6-dicarboxylic acid (Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A.); 8-hydroxyquinoline sulphate (A. Daigger and Co., Chicago, Ill., U.S.A.); succinic anhydride (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.). All reagents were of the purest grade obtainable.

* Abbreviation: PHMB, *p*-hydroxymercuribenzoate.

A purified specimen of alkaline phosphatase had a specific activity of 20 units/mg. of protein (protein concn. 5.6mg./ml.). The unit of enzyme is defined to be that which hydrolyses 1 μ mole of substrate/ml. in 1min. at 37° in 50mm-Na₂CO₃-NaHCO₃ buffer, pH9.8, containing 18mm-disodium phenyl phosphate. The enzyme was prepared in this laboratory by the method of Portmann and co-workers (Portmann, 1957; Portmann, Ressler & Chardenners, 1960) with rat intestinal mucosa as the source. This is designated the stock undiluted enzyme preparation.

Methods of enzyme assay. Unless otherwise specified each experiment was performed in duplicate or triplicate as follows. In a 10ml. stoppered tube (R), 2.5ml. of 100mm-Na₂CO₃-NaHCO₃ buffer, pH9.2, was mixed with 0.5ml. of 30.0mm-disodium phenyl phosphate and 1.5ml. of water. In another tube (L), 2.5ml. of the same buffer and 0.5ml. of substrate solution were mixed with 0.5ml. of 50.0mm-L-phenylalanine and 1.0ml. of water. In a third tube (D), all the solutions were present in the same proportions as in tube L except that 0.5ml. of 50.0mm-D-phenylalanine was substituted for the equivalent amount of L-phenylalanine. The hydrolysis was initiated by adding 0.5ml. of an appropriate dilution of the untreated or modified enzyme solution to each of the R, L and D tubes, which had been preincubated for 5min. at 37°. Samples (1.55ml.) were withdrawn at zero time and 15min. later and then the reaction was terminated by rapidly mixing in 1.5ml. of 1.5M-formaldehyde. The liberated phenol was assayed by the diazo method as described by Ghosh & Fishman (1966) and was expressed as μ moles of phenol/ml. released in 15min. at 37°. The R-tube values (*R*) were used to determine the changes in the activity of alkaline phosphatase induced by group-specific reagents described below, whereas from the D-tube and L-tube values (*D* and *L* respectively) the percentage inhibition (*I*) by 5mm-L-phenylalanine was calculated, as reported by Ghosh & Fishman (1966), by applying the relation $I = 100(D - L)/D$. It should be pointed out that, unlike L-phenylalanine, D-phenylalanine is not an inhibitor of rat or human intestinal alkaline phosphatase at 5mm concentration.

Conditions for use of group-blocking reagents. (a) PHMB

and iodoacetamide. A 4.0ml. sample of the diluted (1:250) enzyme specimen was treated at 25° for 1hr. with a 1ml. solution of the desired concentration of PHMB at pH7.1. Similar experiments were done at pH6.5 for 16hr. at 25° substituting iodoacetamide for PHMB. The untreated enzyme of the same dilution with and without 5mm-L-phenylalanine served as controls.

(b) Iodine. Samples of the diluted (1:250) enzyme specimen were incubated in an ice bath for 30min. at pH7 with different concentrations of iodine in KI soln. The enzyme specimens treated with iodine were assayed together with an untreated control.

(c) *O*-Methylisourea. *O*-Methylisourea hydrogen sulphate (0.1g.) was treated with 2ml. of water and 0.2g. of Ba(OH)₂·8H₂O. The mixture was shaken thoroughly and allowed to settle for 15min. The heavy white precipitate of BaSO₄ was removed by centrifugation. The clear supernatant, containing *O*-methylisourea, was chilled in an ice bath and was added slowly with shaking to a precooled rat intestinal enzyme specimen (1ml.) in a stoppered tube. The total volume was made up to 3.0ml. and the reaction mixture was maintained at pH10-10.5 at 5°. A control was run simultaneously with water in place of the reagent. Samples of the reaction mixture were withdrawn at different intervals up to 114hr., diluted 1:100, dialysed and assayed for enzyme activity and L-phenylalanine inhibition.

Starch-gel electrophoresis. The acetylated, formaldehyde-acetamide-treated, succinylated and *O*-methylisourea-treated rat intestinal alkaline phosphatase preparations were subjected to starch-gel electrophoresis together with the native untreated specimen. The enzyme preparations treated with the amino-group reagents were not diluted to ensure the appearance of enzyme-positive bands. The enzymogram was developed, after electrophoresis at 4-5° for 18hr. at pH8.6, by incubating with α -naphthyl phosphate for 2hr. and coupling the liberated α -naphthol with azo dye (Fast Blue RR).

Michaelis constants and the optimum pH. Michaelis constants of the rat intestinal alkaline phosphatase before and after treatment with amino-group-blocking reagents were determined by the conventional Lineweaver-Burk

Table 1. *Effect of metal-binding agents on enzyme activity and L-phenylalanine inhibition*

The enzyme (1:500) was incubated with metal-binding agents at pH 9.2 and 37° for 15 min. The substrate was then added and the incubation continued for 15 min. Phenol was measured in the experiments with EDTA and sodium cyanide and phosphate in the experiments with 8-hydroxyquinoline and pyridine-2,6-dicarboxylic acid.

Metal-binding agent	Concn. (mm)	Residual activity (%)	Inhibition by 5mm-L-phenylalanine (%)
None	0.0	100.0	68.0
EDTA	1.0	10.9	6.1
8-Hydroxyquinoline	0.01	100.0	67.5
	0.10	42.5	46.5
	1.0	8.0	0.0
Pyridine-2,6-dicarboxylic acid	0.25	40.0	56.0
	0.50	31.0	48.0
	1.00	28.5	28.0
	3.00	0.0	—
Sodium cyanide	2.0	70.0	59.9
	10.0	18.0	31.5
	20.0	0.0	—

method as done by Ghosh & Fishman (1966) with 50mM- Na_2CO_3 - NaHCO_3 buffer, pH 9.2, and phenyl phosphate as substrate. The same buffer in the pH range 8.7–10.6 was used to determine the optimum pH of all these enzyme specimens with 18mM-phenyl phosphate as substrate.

RESULTS

Experiments with metal-binding reagents

Effect of chelating agents on enzyme activity and inhibition by L-phenylalanine. Since other workers (Mathies, 1958; Vallee, 1960; Plocke, Levinthal & Vallee, 1962) have reported that the enzyme (pig kidney and *Escherichia coli*) is a metallo-enzyme containing zinc in the active centre, the changes in the activity of rat intestinal enzyme induced by different metal-binding agents, EDTA, 8-hydroxyquinoline, pyridine-2,6-dicarboxylic acid and sodium cyanide, have been evaluated. Thus both the activities and the percentage inhibitions by 5mM-L-phenylalanine of the enzyme inactivated by chelators and of an untreated control were determined at several concentrations of the ligands. The results are given in Table 1 and are illustrated for EDTA in Fig. 1. The data show that with the increase of the concentrations of the metal-binding agents there is a concomitant decrease in enzyme activity and a decrease in the extent of L-phenylalanine inhibition. The diminution of this inhibition is proportional to the decrease in the enzyme activity, indicating that the metal site is essential not only for catalysis but also for the mechanism of L-phenylalanine inhibition.

Fig. 1. Enzyme activity (○) and L-phenylalanine (5mM) inhibition (△) as a function of EDTA concentration.

Plocke *et al.* (1962) postulated the presence of zinc as a firmly bound component of alkaline phosphatase essential to enzymic activity from data comparing the extent of inhibition of the enzyme by various metal ligands with the respective stability constants of the Zn^{2+} complexes. Similarly, concentrations required to produce 50% inactivation by the metal-binding agents, EDTA, 8-hydroxyquinoline, $\alpha\alpha'$ -bipyridyl, *o*-phenanthroline and sodium cyanide, were determined by plotting V_i/V_c against $\log i$ (Fig. 2), where V_i is the activity of the digest containing metal-binding reagents, V_c is the activity of the untreated control and i is the molar concentration of the inhibitory metal binder. The values obtained from Fig. 2 are listed in Table 2 and are compared with the corresponding stability constants of the Zn^{2+} complexes (Bjerrum, Schwarzenbach & Sillen, 1957–58). The data demonstrate that the order of their effectiveness as a metal binder is similar to the order of the stability constants. It was also observed that the inhibition of the enzyme by EDTA is reversed and that the L-phenylalanine inhibition is restored by the addition of 1.0mM- Zn^{2+} solution to the enzyme specimen dialysed against, or treated with, the chelator (Table 6). In view of these results and of the presence of zinc in pig and *E. coli* alkaline phosphatase, the metal in rat intestinal alkaline phosphatase may be zinc.

Effect of PHMB and iodoacetamide

PHMB is considered specific for the thiol group (Fraenkel-Conrat & Mechen, 1949; Van Eys, Ciotti & Kaplan, 1957; Fraenkel-Conrat, 1957, 1959) and, in addition, PHMB produces conformational changes in enzyme protein molecules. The influence of PHMB and iodoacetamide has been examined on our enzyme system. The results (Table 3) show

Fig. 2. Inactivation of rat intestinal alkaline phosphatase as a function of the effective concentration of the metal-binding agents. V_i is the activity of the enzyme treated with the metal binder of concentration i , and V_c that of the untreated control. ■, NaCN; △, *o*-phenanthroline; ○, $\alpha\alpha'$ -bipyridyl; ●, EDTA; ▲, 8-hydroxyquinoline.

Table 2. *Order of stability constants of metal-binding agents compared with their order of effectiveness as inactivators of intestinal alkaline phosphatase*

(a) K_n is the concentration (M) of the metal-binding agent required to produce 50% inactivation. (b) $K_{Zn} = [ZnL_n]/[Zn^{2+}][L]^n$, where L is the ligand and n is the number of molecules of the ligand forming a complex with one Zn^{2+} ion. Values are taken from Bjerrum *et al.* (1957-58).

Metal-binding agent	Inactivation constants		Stability constants	
	$-\log K_i$	Ratio to NaCN value	$\log K_{Zn}$	Ratio to NaCN value
Sodium cyanide	2.80	1.00	12.6	1.00
$\alpha\alpha'$ -Bipyridyl	3.15	1.12	13.5	1.07
1,10-Phenanthroline	3.26	1.16	17.0	1.34
8-Hydroxyquinoline	4.00	1.43	20.8	1.65

Table 3. *Effect of thiol-specific reagents on enzyme activity and L-phenylalanine inhibition*

Reagent	Concn. of thiol reagent	Residual enzyme activity (%)	Inhibition with 5mm-L-phenylalanine (%)
PHMB (mm)	0.00	100.0	67.0
	0.25	93.3	67.0
	0.50	90.8	67.0
	0.75	85.0	67.0
	1.00	75.0	65.0
	2.00	64.1	62.4
	10.0	38.0	48.8
	30.0	22.0	30.0
	50.0	7.30	19.0
	75.0	0.50	0.00
	150.0	0.00	—
Iodoacetamide (mm)	0.00	100.0	72.6
	0.60	100.0	72.6
	1.00	67.0	65.0
	5.00	19.50	71.0
	45.0	5.00	53.0
	60.0	0.00	—
	100.0	0.00	—
Iodine (μ M)	0.00	100.0	69.0
	1.00	85.0	70.7
	3.00	79.0	69.0
	5.00	76.0	68.2
	40.00	60.0	67.0
	77.50	10.0	45.0
	100.00	0.0	—

an enhancement of the loss in the enzyme activity as the concentrations of PHMB are increased. Inhibition by L-phenylalanine remained constant only up to 0.75mm-PHMB, above which it diminished. Iodoacetamide (5mm) eliminated 80% of the original activity but did not alter L-phenylalanine inhibition. Similar results were obtained with well-dialysed enzyme preparations that had first been treated with the thiol reagents for the required length of time. Under the present experimental conditions 30mm-PHMB produced a 78% loss in activity and 60mm-iodoacetamide completely inactivated the enzyme. If the enzyme was pretreated with phenyl phosphate at pH 7.1 before the reaction with iodoacetamide, the substrate

protected the enzyme from the iodoacetamide inhibition (Fig. 3). These findings suggest that the groups, presumably thiol groups, capable of reacting with iodoacetamide are ones that play a part in the catalysis. The inability of substrate to protect against PHMB inactivation may be related to the fact that this mercurial, in addition to reacting with thiol groups, may cause damage to the steric configuration of the active site, as discussed by Bray & Watts (1966) and Brumby, Miller & Massey (1965).

Reaction with iodine. The results shown in Table 3 also indicated that the enzyme was totally inactivated by 100 μ M-iodine solution, whereas L-phenylalanine inhibition of iodine-inactivated enzyme

Fig. 3. Protection by substrate, phenyl phosphate, of the enzyme inhibition (a) by iodoacetamide and (b) by iodine. Curve 1 in (a) was obtained by pretreatment of the enzyme with 7mM-phenyl phosphate at pH 7.1 and then treatment with iodoacetamide as described in the text. Curve 2 in (a) represents the inhibition of the enzyme without pretreatment with substrate. The corresponding curves for iodine inhibition of the enzyme pretreated with 26mM-phenyl phosphate and that without substrate treatment are curves 1 and 2 respectively in (b).

Fig. 4. Reversal by cysteine of the enzyme inhibition by PHMB, iodoacetamide and iodine. Curves 1, 2 and 3 are the activity curves for enzymes treated with iodoacetamide, PHMB and iodine respectively without treatment with cysteine. Curves 1+, 2+ and 3+ are corresponding curves for enzymes treated first with these reagents and then subsequently with 2mM-, 4mM- and 20mM-cysteine respectively.

was not changed appreciably up to 40 μM-iodine concentration. The iodine and iodoacetamide inhibition may be prevented by pretreatment of the enzyme with the substrate (Fig. 3).

Fig. 5. Effect of cysteine concentration on the enzyme activity (O) and L-phenylalanine (5mM) inhibition (Δ).

Iodination of enzyme proteins may either oxidize thiol groups to disulphide bridges or displace the hydrogen of thiol groups or of the benzene nucleus of tyrosine (Fraenkel-Conrat, 1957). On the basis of the inhibition of alkaline phosphatase by iodine in potassium iodide, Sizer (1942) concluded that the enzyme needs the tyrosine hydroxyl groups for catalysis. However, since rat intestinal alkaline phosphatase is inhibited by well-known thiol-specific reagents and since this iodine inhibition can be reversed by cysteine (Fig. 4), there is no need to invoke the tyrosinyl hydroxyl groups into the explanation of the effects of iodination. These findings are consistent with similar results reported in ox intestinal alkaline phosphatase (Lazdunski & Ouellet, 1962) and other esterases (Oosterbaan & Cohen, 1964) that are inhibited by thiol reagents.

Effect of cysteine. The influence of cysteine in a range of concentrations (0.1–2mM) on the untreated enzyme were determined and the results (Fig. 5) indicate that cysteine activates the enzyme up to a concentration of 0.5mM, above which considerable inhibition is observed, the activity being totally destroyed above 2mM. Also enzyme that has been inactivated by PHMB, iodoacetamide or iodine regained and surpassed its original activity when cysteine was added before assaying the inhibited material (Fig. 4). These findings are in agreement with those observed with

Table 4. *Effect of amino-group reagents on enzyme activity and L-phenylalanine inhibition*

Acetylation was done with acetic anhydride in half-saturated sodium acetate, pH 8.0, at 0° and the deamination by HNO_2 was done at 1–2°. The succinylation with succinic anhydride was performed at pH 8.0 at 5–6°. The reaction with formaldehyde was carried out at pH 6.0 at 1–2°.

Reagent	Time of reaction (hr.)	Concn. of reagent (mM)	Residual enzyme activity (%)	Inhibition by 5mM-L-phenylalanine (%)
Acetic anhydride	0.00	91.0	100	70
	0.16	91.0	6	74
Nitrous acid	30.0	0.0	100	70
	30.0	200.0	53	73
Succinic anhydride	0.0	500.0	100	66
	48.0	500.0	18	59
Formaldehyde	0.5	0.0	100	70
	0.5	1500.0	42	69
	0.5	4500.0	13	64
Formaldehyde + acetamide	0.0	2500.0	100	74
	24.0	2500.0	18	74
O-Methylisourea	0.0	175.0	100	66
	12.0	175.0	75	64
	40.0	175.0	65	62
	58.0	175.0	38	61
	90.0	175.0	35	61
	114.0	175.0	32	61
	154.0	175.0	32	61

other thiol phosphatase enzymes such as fructose 1,6-diphosphatase (Walsh & Walsh, 1948) and adenosine triphosphatase (Barron & Singer, 1945). Moreover, Fig. 5 also indicates that the extent of L-phenylalanine inhibition remains unaltered in the concentration range of cysteine within which it acts as an activator. When the concentration of cysteine was higher than 1mM the enzyme lost activity. Under these conditions the extent of L-phenylalanine inhibition diminished gradually as the concentration of cysteine was increased. The loss in sensitivity of intestinal enzyme to L-phenylalanine at high cysteine concentrations may be due to the blocking of the metal site by cysteine, as Agus, Cox & Griffin (1966) have reported for alkaline phosphatase.

Experiments with amino-group-modifying reagents

In general, enzyme activity is greatly diminished as a function of increasing concentration of the amino-group-modifying reagents, acetic anhydride, nitrous acid, succinic anhydride (Buttkas, Clark & Feeney, 1965), formaldehyde and formaldehyde-acetamide, or of time of exposure to the reagent without a concomitant reduction in inhibition by L-phenylalanine. The results are given in Table 4.

Effect of O-methylisourea. To obtain unequivocal evidence for the presence of the ϵ -amino group of lysine in the active centre, the enzyme was treated

with O-methylisourea, which is a specific reagent for this group and transforms it into a guanidino derivative (Fraenkel-Conrat, 1959) as follows:

The results (Table 4) show that the enzyme loses nearly 70% of its activity whereas the extent of inhibition of the treated enzyme by 5mM-L-phenylalanine does not change substantially. It is also noteworthy that inactivation of the enzyme by O-methylisourea (Table 4) remains constant at 68% from 114 to 154 hr. or more of reaction although an excess of O-methylisourea was used. Assuming that all lysyl ϵ -amino groups have reacted, the survival of so much activity argues against the indispensability of such groups for the catalytic event, but the ϵ -amino groups could have an important role in binding the substrate.

The starch-gel electrophoresis of the enzyme treated with acetic anhydride or succinic anhydride showed that these modified enzymes had greater mobility towards the anode than the untreated sample, possibly owing to the replacement of the positively charged $\epsilon\text{-NH}_3^+$ group by negatively charged carboxylate groups. However, the O-methylisourea-treated enzyme did not exhibit an

Table 5. *Effect of chemical modification of amino groups on the Michaelis constants of the rat intestinal alkaline phosphatase*

Nature of the enzyme specimen	Relative activity (%)	Optimum pH	Michaelis constant (K_m) (mM)
Native	100	9.75	0.95
Acetylated	10	9.65	1.50
Formaldehyde-acetamide-treated	10	9.75	2.70
Succinylated	18	9.70	4.00
O-Methylisourea-treated	32	9.70	10.00

Table 6. *Stereospecific inhibition by L-phenylalanine and activation by D-phenylalanine of rat intestinal alkaline phosphatase*

The times of reaction of EDTA, EDTA + Zn²⁺, PHMB, acetic anhydride and O-methylisourea were 0.25, 0.25, 1.0, 0.08 and 154 hr. respectively. U and T refer to untreated and treated enzyme specimens respectively.

Reagent or condition	Concn. (mM)	10 ³ × Enzyme activity (μmoles of phenol/ml./15 min.)			Inhibition by L-phenylalanine (%) [100(D-L)/D]	Activation by D-phenylalanine (%) [100(D-R)/R]
		R	L	D		
EDTA	U	0	49.0	16.5	68	2
	T	1	5.3	5.1	4	0
EDTA + Zn ²⁺	U	0	49.0	16.5	68	2
	T	1	29.3	12.8	56	0
PHMB	U	0	146.8	42.6	71	0
	T	30	31.9	31.9	33	50
Acetic anhydride	U	0	68.1	20.2	70	0
	T	91	7.0	3.7	77	131
O-Methylisourea	U	0	192.5	63.8	67	0
	T	175	61.0	31.9	62	40

appreciable increase in mobility, possibly because the guanidino side chain introduced in the protein did not alter the number of negative charges. The results (Table 5) show no appreciable change in the optimum pH of the treated enzyme. But an increase of the Michaelis constant is interpreted as a decrease in the affinity of the enzyme for the substrate resulting from the blocking of the amino groups in the enzyme protein.

Activation of the modified enzyme by D-phenylalanine. Some representative data depicting enzyme activity, inhibition by L-phenylalanine and activation by D-phenylalanine before and after chemical treatment are given in Table 6. In several instances (e.g. amino-group-blocking reagents), treatment makes the enzyme now capable of being markedly activated by D-phenylalanine, a phenomenon not observed with the untreated enzyme. The extent of activation increased the more the enzyme was inactivated by acetylation. Papain hydrolysis and chelators did not produce activatable enzymes.

The extent of activation was a function of substrate concentration and the activation was competitive (Fig. 6) or 'co-operative' (Ogston,

1955). The results, moreover, are consistent with Frieden's (1964) non-limiting case 3D. It may be assumed that both enzyme-substrate complex and enzyme-modifier-substrate complex decompose to give products. Unlike the stable enzyme-inhibitor-substrate complex with L-phenylalanine, the complex that is formed with D-phenylalanine can be expected to be more unstable. Thus D-phenylalanine does appear to restore the catalytic properties of the altered molecule associated with the substrate-binding function of thiol and amino groups, but not metal site. Consequently, it may be inferred indirectly that alkaline phosphatase, which undergoes activation by D-phenylalanine, represents modified enzyme and not surviving native enzyme molecules.

DISCUSSION

On the basis of the present experimental evidence, metal, thiol and amino groups can be implicated in the mechanism of catalysis of hydrolysis of monophosphoric acid esters by rat intestinal alkaline phosphatase. The present findings and interpretations are consistent with our earlier observations (Ghosh & Fishman, 1966) in which

Fig. 6. Activation of the acetylated rat intestinal alkaline phosphatase by D-phenylalanine (5mM) at various substrate concentrations. The main Figure shows Lineweaver-Burk plots for experiments carried out in the absence (○) and presence (Δ) of 5mM-D-phenylalanine. The inset indicates the percentage activation by D-phenylalanine at different substrate concentrations.

the Dixon plot revealed two discontinuities at pH 8.6 and 9.6 and suggested that L-phenylalanine does combine with the enzyme-substrate complex to form an enzyme-inhibitor-substrate complex. This latter complex breaks down more slowly than does the enzyme-substrate complex in a manner which is considered in more detail elsewhere (Fishman & Ghosh, 1967).

Other facts are compatible with the above interpretation but do not necessarily prove it. Thus the inability of α -phenyl- β -alanine and N-formyl-phenylalanine to inhibit rat intestinal alkaline phosphatase (Fishman, Green & Inglis, 1962) may be because the former compound does not have amino and carboxyl groups in the α -position. In the latter reagent the free amino group is blocked by the formyl group. Also, glycylphenylalanine and glycyltyrosine are not inhibitors, possibly because the α -amino groups are blocked. The position of the benzyl groups in phenylalanine may be a determining factor, as α -phenyl- β -alanine does not inhibit rat intestinal alkaline phosphatase.

With regard to the possibility of other essential groups, Morton (1957), in his interpretation of the Dixon (pK_m -pH) plot obtained with calf intestinal alkaline phosphatase, pointed out that the phenolic hydroxyl groups of tyrosine might be in the active site. Morton (1957), however, gave equal weight to the alternative possibility that the essential groups could well be the ϵ -NH₃⁺ group of lysine since the pK values for the tyrosine hydroxyl and the ϵ -amino group of lysine are very close to each other.

The present findings are related to the studies

by Gould (1944), Anagnostopoulos (1950) and Lazdunski & Ouellet (1962) on the effects of thiol and amino group reagents on alkaline phosphatase.

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