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IMIDAZOLE: AN INHIBITOR OF L-PHENYLALANINE-INSENSITIVE ALKALINE PHOSPHATASES OF TISSUES OTHER THAN INTESTINE AND PLACENTA

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SUMMARY

1. Alkaline phosphatases (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) from brain, kidney, liver, bone, lung and spleen, which are not very sensitive to L-phenylalanine, are strongly inhibited by imidazole, whereas the placental and intestinal enzymes, which are very sensitive to L-phenylalanine, are only slightly affected. This is a new possibility for distinguishing the alkaline phosphatase isoenzymes.

2. The inhibition is apparently of an uncompetitive type, suggesting that the inhibitor interacts with the *ES* complex to form an *EIS* complex.

3. Histidine acts upon all enzymes assayed in this study. Its inhibition is of a mixed type.

4. An interpretation, taking into account the role of the zinc atom(s) present in the active center, is offered.

INTRODUCTION

Since the first findings of Fishman *et al.*[1], it is well known that mammalian alkaline phosphatases are divided into two groups, according to their sensitivity to L-phenylalanine. In a previous paper[2], we discussed the role of metal ions (Mg^{2+}, Zn^{2+}) as major variables in the control of the stereospecific L-phenylalanine effect, and we have shown with the bovine brain enzyme, which is not very sensitive to L-phenylalanine, that several other high molecular weight amino acids (L-tryptophan, L-leucine, L-lysine, L-arginine) also produce a stereospecific effect $\left(\frac{D-L}{D} \times 100\right)$ whereas the very sensitive enzymes are only affected by L-tryptophan, the effect of which is not very different from that of L-phenylalanine and by L-leucine which has a lesser effect.

Fishman and Hsien-Gieh Sie[3] relate similar results with the human bone and liver enzymes and emphasize the important effect of L-homoarginine.

More recently, we found that imidazole is a potent inhibitor of many "not very L-phenylalanine-sensitive" alkaline phosphatases. This is the topic of the present paper.

MATERIALS AND METHODS

Materials

The preparation of brain enzymes from several species has been described previously[4,5]. The placental enzyme was prepared according to the procedure of Ghosh and Fishman[6] as modified by us[2]. The calf intestinal enzyme was a commercial preparation (C. F. Boehringer and Soehne GmbH Mannheim 15436 EPAC; Lot No. 6498213, June 1969). The extracts of rat tissues, rabbit and pigeon intestine and bovine kidney were aqueous layers obtained by butanol extraction: homogenisation of the tissues in water (1 ml/g of tissue), shaking with butanol (1.5 ml/g of tissue, 37 °C for 5 min), centrifugation at 3500 × *g* for 30 min, and siphoning of the aqueous layers.

Imidazole, L-homoarginine, D-phenylalanine, L-phenylalanine, D-histidine, L-histidine were purchased from Fluka.

Enzyme assay

The enzyme reaction was routinely carried out in a total volume of 2.5 ml at 37 °C. The medium contained 25 mM borate at a chosen pH, 5 mM *p*-nitrophenyl phosphate, 1 mM Mg^{2+} and the amount of enzyme required to produce an increase of $A_{400\ nm}$, corresponding to the release of *p*-nitrophenol, of about 0.2 per min. The buffered effector was added at a chosen concentration. The reaction was followed on a Unicam SP 800 spectrophotometer equipped with a SP 20 recorder.

Incubations with metal ions

The purified brain enzymes were incubated at 4 °C for several hours with 5 mM Mg^{2+} in 0.05 M Tris–HCl, 0.1 M NaCl (pH 8.0) and assayed in a medium with or without Mg^{2+} added. Incubations of the bovine brain enzyme were also done with 5 mM Ni^{2+}, Mn^{2+} and Co^{2+}, or with 1.6 mM Zn^{2+}. These metal-modified enzymes were assayed in a medium without Mg^{2+} and were compared to the Mg^{2+} enzyme, which is considered as "native enzyme" and which was also assayed in a medium without Mg^{2+} added. The enzymes were diluted 100-fold in the assay medium, so that the final metal ion concentration was 50 μM for the Mg^{2+}, Ni^{2+}, Mn^{2+} and Co^{2+} enzymes, and 16 μM for the Zn^{2+} enzyme.

RESULTS AND DISCUSSION

Effect of imidazole, phenylalanine and homoarginine on several alkaline phosphatases

From Table I, it can be seen that the enzymes having a moderate sensitivity to L-phenylalanine are strongly inhibited by imidazole. The same enzymes are also affected by L-homoarginine, as was shown by Fishman and Hsien-Gieh Sie[3] for human bone and liver enzymes. Besides, it is clearly established that this inhibition is organ specific and not species specific. From a mixture containing the two kinds of enzymes (bovine brain and human placental enzymes), we are able to determine the quantity

TABLE I

EFFECT OF IMIDAZOLE, PHENYLALANINE AND HOMOARGININE ON SEVERAL ALKALINE PHOSPHATASES AT pH 8.5

Activation is indicated by the sign (−), O = activity without effector, I = activity with 8 mM imidazole. D and L = activities with D and L isomers. The concentration is 20 mM for D- and L-phenylalanine, 10 mM for L-homoarginine. For intestinal or placental enzymes Mg^{2+} was or was not added to the assay medium. For these enzymes as for the others, the effect of imidazole is not influenced by the addition of Mg^{2+}.

Tissue	*Species*	*Imidazole inhibition* $\frac{O-I}{O} \times 100$	L-*Phenylalanine stereospecific effect* $\frac{D-L}{D} \times 100$	L-*Homoarginine effect* $\frac{O-L}{O} \times 100$
Brain	Bovine*	74	38	78
	Rat	70	24	77
	Sheep* $-M_1$	64	30	67
	$-M_3$	74	39	82
	Pig* $-P_1$	70	34	76
	$-P_2$	72	32	76
	Rabbit* $-R_1$	65	31	59
	$-R_3$	60	29	58
	Horse*	77	42	80
Kidney	Bovine	74	40	79
	Rat	72	34	79
Liver	Rat	60	34	71
Bone	Rat	71	30	73
Lung	Rat	69	39	79
Spleen	Rat	57	31	70
Intestine	Bovine*	14	66	4
	Rat	9	77	− 9
	Rabbit	33	67	11
	Pigeon	0	83	−54
Placenta	Human*	10	93	− 8

* Purified enzymes (The observed effect is independent from the state of purification of the enzymes). M_1, M_3, P_1, P_2, R_1, R_3 are fractions isolated by DEAE-cellulose chromatography[5].

of imidazole sensitive enzyme present. This property appears to be of interest in determining the contribution of non-placental or non-intestinal isoenzymes in sera.

Kinetic studies of imidazole inhibition of the bovine brain enzyme

With respect to the nature of the kinetics (Table II and Fig. 1), the data on imidazole action can be compared to those of L-phenylalanine[2,7–11] and L-tryptophan[12]. The inhibition is dependent on the pH (maximum at pH 8.5), greatly dependent on substrate and inhibitor concentrations and is apparently of an uncompetitive type. Indeed, the double reciprocal plots of velocity and substrate concentration in the presence of three different concentrations of imidazole are straight lines parallel to the one obtained without inhibitor.

This phenomenon is non-allosteric in nature because (a) the hyperbolic shape of the curves relating inhibition as a function of inhibitor or substrate concentration; (b) the figure of unity for n, the number of imidazole molecules which combine with one molecule of enzyme; (c) the persistence of inhibition after the tertiary structure of the enzyme has been altered by heat denaturation and treatment with urea.

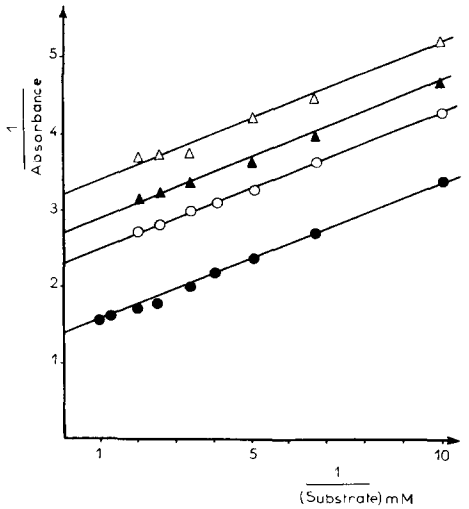

Fig. 1. Lineweaver–Burk plot (pH 8.5) of brain enzyme incubated with Mg^{2+}. ●—●, without imidazole; ○—○, ▲—▲, △--△, with 4, 6, 8 mM imidazole respectively. The substrate concentrations vary from 0.1 to 1 mM.

TABLE II

EFFECT OF IMIDAZOLE AND HISTIDINE ON BOVINE BRAIN AND HUMAN PLACENTAL ALKALINE PHOSPHATASES

Inhibition is given as a function of concentration of the effector. O = activity without effector, I = activity with imidazole, H = activity with D- or L-histidine.

Enzyme Source	*Effector concentration (mM)*	*Imidazole inhibition* $\frac{O-I}{O} \times 100$		*Histidine inhibition* $\frac{O-H}{O} \times 100$		
		pH		*pH*		
		8.5	*10.0*	*8.5*	*10.0*	
Bovine brain	2	42	—	—	15	
	4	58	24	30	44	
	8	74	39	55	80	
	10	—	—	—	85	
	12	—	42	58	89	
	16	—	—	69	92	
		pH		*pH*		
		8.5	*9.6*	*8.5*	*9.6*	*10.0*
Human placenta	4	9	6	—	18	—
	6	—	—	—	28	—
	8	10	6	13	35	43
	10	—	—	—	39	—
	12	—	—	—	—	49

Therefore, as for the action of L-phenylalanine, it is reasonable to suggest that the inhibitor interacts with the enzyme–substrate complex in order to form an inactive or partly active *EIS* complex.

Effect of histidine

The action of histidine is different from that of imidazole. Histidine acts upon all enzymes assayed in this paper, but its action is stronger upon the "not very L-phenylalanine sensitive" enzymes (Table II). The inhibition is of a mixed type, reaching a maximum at alkaline pH (about 10.0). In this respect, its effect can be compared to that of cysteine[13]. D- and L-histidine produce the same inhibition; there is no stereospecific effect. Cox and Griffin[14] have observed the same effect with several other alkaline phosphatases.

Imidazole inhibition of bovine brain enzyme and metal ions

Considering the fact that the action of phenylalanine and other amino acids on the brain enzyme is closely related to the activation by Mg^{2+}, we have previously suggested[2] that the stereospecific effect involves the preliminary fixation of the amino acid on the Mg^{2+} of the enzyme. In the case of imidazole, the action of which is Mg^{2+} independent, the direct complex formation, with probable participation of the constitutive Zn atom(s) of the active center, seems to be the only possibility.

The following experiment is particularly demonstrative. It was done with the brain enzyme, from which Mg^{2+} can be easily eliminated by prolonged dialysis and replaced by other metal ions. It was observed that modified enzymes are obtained which are Mg^{2+} insensitive at the concentration that usually produces maximum activation. The Ni^{2+}, Mn^{2+} or Co^{2+} enzymes are not essentially different from the Mg^{2+} enzyme. They have the same K_m values and react in the same manner with the effectors studied. They are, however, less active. Results obtained with the enzyme modified by Zn^{2+} are more interesting. The K_m value is not modified, but its activity is very low; it is unstable and above all reacts differently with imidazole. The inhibition, which is slightly lower (65%) than for the Mg^{2+} enzyme (74%), is independent from the substrate concentration (non competitive inhibition) (Fig. 2). We have checked that these modifications are independent from the concentration of Zn^{2+} in the assay medium. Indeed, in borate buffer at pH 8.5, Zn^{2+} which was even added to concentrations approaching the solubility limit, does not inhibit the Zn^{2+} or Mg^{2+} enzymes and at the same time does not modify the type of inhibition by imidazole.

Since the modification of the type of inhibition is related to the presence of Zn^{2+} newly bound to the enzyme, it is logical to assign the observed effect to a substrate-independent formation of a complex between this ion and imidazole. It is then easily understood that in the case of "native enzyme" containing Mg^{2+} and constitutive Zn^{2+}, imidazole can act only on the constitutive Zn^{2+}, which is generally considered as participating in the binding of the substrate; hence the possibility of *EIS* complex formation.

In the case of the Zn^{2+}-modified enzyme, if the substrate does not participate in the complex formation, it is probably because the newly bound Zn^{2+} is too distant from the active center. Unlike the constitutive Zn^{2+}, the eventual direct participation of this Zn^{2+} in the Michaelis complex formation or in the mechanism of hydrolysis may then be considered as unlikely. The added Zn^{2+}, occupying here the

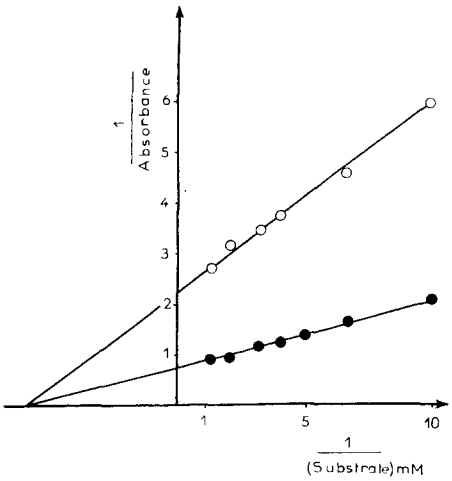

Fig. 2. Lineweaver–Burk plot (pH 8.5) of brain enzyme incubated with Zn^{2+}. ●—●, without imidazole; ○—○, with 8 mM imidazole. The substrate concentrations (*p*-nitrophenyl phosphate) vary from 0.1 to 8 mM. The velocities of hydrolysis were corrected for the decrease of activity of the Zn^{2+} enzyme during the experiment.

position of Mg^{2+} in the hypothesis that we have previously suggested[2] (*i.e.* that Mg^{2+} may have above all a structural role) is strengthened.

Imidazole and placental or intestinal enzymes

The fact that the placental or intestinal enzymes, for which the presence of Zn^{2+} in the active center is well known, are not or are very little inhibited by imidazole, unlike the other alkaline phosphatases described here, suggests several hypotheses. Zn^{2+} may not be equally accessible in the two kinds of enzymes, or it may be more or less strongly bound, so that the binding of imidazole to Zn^{2+} may or may not be possible. Another interpretation which is also satisfactory is suggested from the results obtained for the Zn^{2+} modified brain enzyme. Indeed, it may be possible that the placental or intestinal enzymes contain in addition to catalytic Zn^{2+} another metal ion, perhaps another Zn^{2+}, capable of binding imidazole without producing inhibition, thus preventing it from acting on the catalytic Zn^{2+}. The poor sensitivity to Mg^{2+} of these two enzymes, compared to that of the brain enzyme[2], is in agreement with this view.

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