Identification of Functional Groups of Pig Kidney Alkaline Phosphatase by Specific Inhibitors

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Inactivation studies with 17 group-specific inhibitors showed that amino, hystidyl and tyrosyl residues probably are components of the active and/or regulatory sites of pig kidney alkaline phosphatase.

For investigation of the functional groups of the active site(s) of alkaline phosphatase (EC 3.1.3.1) two methods have been commonly used: studies of the influence of pH on the kinetic constants K_m and V (*e. g.* Lazdunski *et al.* [1]) and the application of group-specific inhibitors (*e. g.* Fishman and Ghosh [2]). Previously published studies on the functional groups of alkaline phosphatase are to some extent contradictory. Some authors [2–4] report the presence of the ε-amino group of lysine in the active site, whereas others postulate an imidazole group of histidine [5, 6] or a tyrosine residue [7, 8] taking part in the catalytic process. The results about the presence of SH groups are even more in disagreement. Whereas the alkaline phosphatase from E. coli contains no sulfhydryl group [9, 10], the alkaline phosphatase from intestine is inhibited by thiol-specific reagents [2, 11]. On the other hand, Fosset *et al.* [12] found only a reaction with thiol-specific reagents after the enzyme had been denatured.

Recently Ahlers [13] identified by means of kinetic measurements one functional group with pK = 7.0 and two functional groups with pK = 9.1, involved in substrate binding, and a group with pK = 8.8, which catalyzes substrate conversion. The essential Mg^{2+} ions are bound by a group with a pK value of 10.15. To obtain more information about the amino acid residues belonging to the pK values, in this paper the inhibition of pig kidney alkaline phosphatase by several group-specific inhibitors is examined.

Materials and Methods

Preparation and purification of pig kidney alkaline phosphatase has been described recently [13, 14].

As group-specific inhibitors are seldom absolutely specific, and on the other hand sometimes don't react because of steric hindrance, it is necessary to use a great number of such inhibitors to determine the functional groups of the active and/or regulatory centers of enzymes [15]. Therefore, to get reliable results we used, whenever possible, two or more reagents for each functional group which might participate in substrate binding and con-

Table I. Conditions for preincubation.

No.	Inhibitor	Buffer	Time of preincubation
1	2,4,6-trinitrobenzene-sulfonic acid	0.1 M carbonate, pH = 9.6	15 min
2	1,2-naphtoquinone-4-sulfonic acid	0.1 M carbonate, pH = 8.8	70 min
3	O-methyl-isourea	0.08 M carbonate, pH = 9.6	65 h
4	citraconic anhydride	0.1 M carbonate, pH = 8.8	200 min
5	maleic anhydride	0.02 M diethyl-barbiturate, pH = 7.2	30 min
6	*p*-chloro-mercuri-benzoate (pCMB)	0.08 M carbonate, pH = 9.15	60 min
		0.02 M diethyl-barbiturate, pH = 7.9	60 min
7	N-ethylmaleimide (NEM)	0.02 M diethyl-barbiturate, pH = 7.7	90 min
8	5,5′-dithiobis (2-nitro-benzoic acid)	0.02 M diethyl-barbiturate, pH = 7.7	15 min
9	ethacrynic acid	0.1 M carbonate, pH = 8.8	40 min
10	phenylmethane-sulfonyl-fluoride	0.02 M diethyl-barbiturate, pH = 7.7	30 min
11	N-acetylimidazole	0.02 M diethyl-barbiturate, pH = 7.7	25 min
12	tetranitromethane	0.2 M Tris-Cl, pH = 8.0	60 min
		0.1 M carbonate, pH = 9.2	60 min
13	iodine	0.08 M carbonate, pH = 9.6	10 min
14	N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide	0.02 M diethyl-barbiturate, pH = 7.2	60 min
15	2-hydroxy-5-nitro-benzyl bromide	0.1 M carbonate, pH = 9.6	15 min
16	phenylglyoxal	0.02 M diethyl-barbiturate, pH = 7.7	90 min
17	2,3-butanedione	0.02 M diethyl-barbiturate, pH = 7.2	120 min

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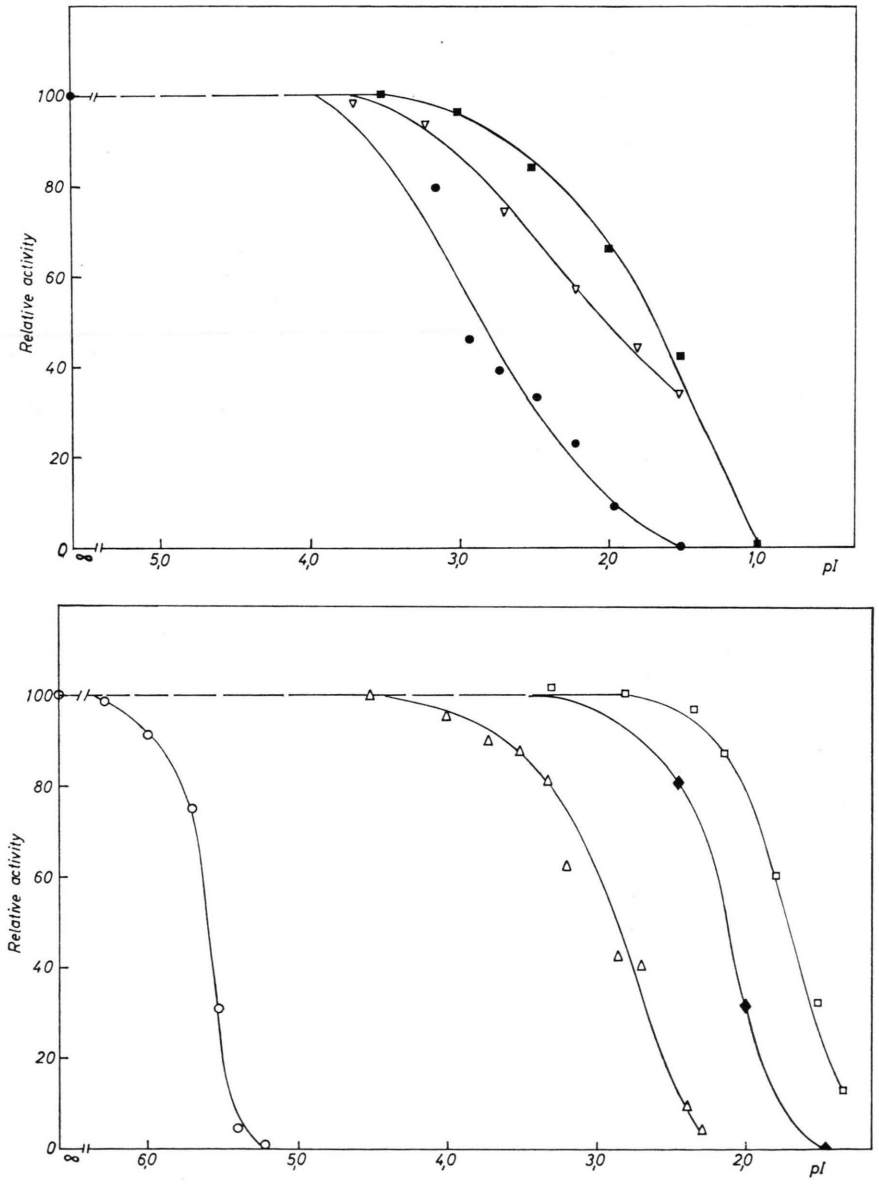
version. We employed the 17 inhibitors given in Table I. Most of them are described in "Methods in Enzymology, **Vol. 25,** Section 8 [1972]".

The reagents **1** to **5** are specific for the amino group, and imidazole residues may also react. The measurements can be carried out under mild conditions, so that enzyme denaturation does not readily occur. Maleic anhydride may also react with sulfhydryl or hydroxyl groups. Reagents **6** to **9** are more or less specific for sulfhydryl groups. NEM and the large molecule 5,5′-dithiobis(2-nitrobenzoic acid) react only with the most accessible SH groups. pCMB may lead to enzyme denaturation. Ethacrynic acid also reacts, to a lesser extent, with the amino group of lysine and the imidazole group of histidine [16]. Phenylmethane sulfonyl fluoride reacts with the alcoholic OH group of serine. The reagents **11** to **13** react with tyrosyl and with sulfhydryl residues. Iodine may react with histidine and tryptophane. The reagent **14** blocks the carboxyl group of a protein to give an O-acylisourea, No. **15** reacts with tryptophane, and Nos **16** and **17** with the guanido group of arginine.

To measure the influence of group-specific inhibitors, the test medium contained 0.05 M carbonate/hydrogencarbonate buffer, pH 9.6, 5×10^{-3} M $MgCl_2$, enzyme, and 10^{-2} M β-glycerophosphate. The enzyme was preincubated under the conditions



Figs 1 and 2.
Plots of *v vs* pI for various group-specific inhibitors. Incubation time and conditions are given in Methods. Incubation temperature was 22 °C.
● = Maleic anhydride;
▽ = 1,2-naphthoquinone-4-sulfonic acid;
■ = 2,4,6-Trinitrobenzene-sulfonic acid;
□ = O-methyl-isourea;
◆ = Tetranitromethane;
△ = N-acetylimidazole;
○ = iodine.

given in Table I and with concentration of inhibitors given in the legends to Figs 1 and 2.

At the end of the preincubation, the enzyme was transferred to the test medium and the reaction was immediately initiated by adding β-glycerophosphate as described recently [13, 14].

Results and Discussion

In Figs 1 and 2 the inhibition of pig kidney alkaline phosphatase is shown as a function of the concentration of several group-specific inhibitors. One can see that all examined amino-group-specific reagents inhibit the alkaline phosphatase under reasonable moderate conditions. Using sulfhydryl-specific reagents (**6 – 9**) we did not detect an inhibition up to a concentration of 1 – 2 mM. Thus no accessible SH groups play a part in the enzymatic action of the alkaline phosphatase from pig kidney.

From Figs 1 and 2 it can furthermore be seen that the reagents **11 – 13** (Table I) inhibit the alkaline phosphatase. In the absence of sulfhydryl groups this inhibition is evidence for the presence of tyrosyl residues in the active and/or regulatory sites.

2-Hydroxy-5-nitrobenzyl bromide (tryptophane specific), phenylglyoxal and 2,3-butanedione (specific for the guanido group of arginine residues) do not inhibit the alkaline phosphatase from pig kidney.

Phenylmethane sulfonyl fluoride also failed to inhibit. Since there is much evidence that a serine residue is phosphorylated in the course of substrate metabolism [17, 18], one must conclude that this serine residue is probably inaccessible to the inhibitor.

Carbodiimide reacts with a carboxyl group of a protein to give an O-acylisourea, which can be attacked by a nucleophile. In the case of water, the carboxyl group will be regenerated. If the reaction has to be carried out in an aqueous environment, one can add a nucleophile to slow the regeneration of the carboxyl group. As nucleophilic reagents also interfere with substrate hydrolysis by alkaline phosphatase, it was not possible to add nucleophiles. Thus the results that the alkaline phosphatase is not inhibited by a carbodiimide is only a hint that there are no carboxyl groups necessary for enzyme activity, but not complete proof of this assumption.

A further indication for the assumption that no carboxyl groups are within the active or regulatory sites of pig kidney alkaline phosphatase is the fact that ATPase is inhibited under similar conditions [19].

Recently from the results of [S], [Mg^{2+}] and pH variations the pK values of functional groups have been deduced, and a model for substrate binding and conversion at the active site of pig kidney alkaline phosphatase was discussed [13]. The experiments with group-specific inhibitors support these interpretations. As our results suggest the presence of tyrosyl, amino and/or imidazole groups, we can make the following assignments: the group with pK = 7.0 might be an imidazole group of histidine or an α-amino group. The groups with pK = 9.1 and the group with pK = 8.8 could be ε-amino groups of lysine. The group with pK 10.15, binding Mg^{2+} ions, is probably the phenolic OH group of tyrosine.

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