Irreversible Inactivation of *Escherichia coli* Alkaline Phosphatase with Active Site Directed Alkylating Reagents

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1. *E. coli* alkaline phosphatase undergoes an irreversible inactivation with the substrate analogues mono- and dichloroacetyl-β-glycerophosphate, and chloroacetyl phosphate. Substrates like glucose-6-phosphate, the reversible inhibitor inorganic phosphate, as well as the irreversible inhibitor diisopropylfluorophosphate protect the enzyme from the attack by the new reagents.

2. The inhibition of the enzyme by these active site reagents was studied as a function of time and pH. In addition, the effect of chloroacetate and other haloacetates on the catalytic activity was studied under similar experimental conditions. These halo-acetates were found to give negligible inactivation.

3. A difference spectrum is presented which shows that the absorption spectrum of the inactivated enzyme differs from the spectrum of the native enzyme in the region of 250 nm.

4. The results obtained in this study, including amino acid analyses on the inactivated enzyme, suggest that the chloroacetyl derivatives of β-glycerophosphate and chloroacetyl phosphate are directed to the active site of *E. coli* alkaline phosphatase and inactivate the enzyme by alkylating the N^1 atom of a histidine residue present in or near the active site.

The alkaline phosphatase of *E. coli* is known to be a zinc metalloenzyme [1] and to be composed of two identical subunits.[2,3] A phosphoryl enzyme in which the phosphate is bound covalently to the hydroxyl group of a particular serine residue in the active site is formed during the action of alkaline phosphatase.[4] We have utilized the fact that the enzyme becomes phosphorylated by either substrate or phosphate [4-6] in studies aimed to give information about the presence of functional groups other than the serine hydroxyl group in the active site. This paper reports the results obtained with monochloroacetyl-β-glycerophosphate, dichloroacetyl-β-glycerophosphate, and chloroacetyl phosphate, *i.e.* substrates containing reactive alkylating groups near the phosphate group.

MATERIALS AND METHODS

Reagents. Tris(hydroxymethyl)aminomethane. Tris Sigma 121 was used. *p*-Nitro-phenylphosphate disodium tetrahydrate from Sigma was utilized. Sodium *β*-glycero-phosphate containing $5\frac{1}{2}$ moles water of hydration ($M = 315.1$) was obtained from British Drug Houses. Analytical grade of all other chemicals was used.

Alkaline phosphatase of E. coli was prepared with a modification of the method of Malamy and Horecker.[7] The complete description of improved growth conditions of the *E. coli* bacteria and the modified purification procedure of alkaline phosphatase will be reported (H. Csopak, manuscript in preparation). Before use, the enzyme was dialysed overnight against distilled water at 4°.

Diisopropylfluorophosphate inactivated alkaline phosphatase was prepared as follows: 1.25×10^{-5} M alkaline phosphatase in 5 ml 0.05 M Tris-HCl buffer, pH 7.5, was incubated with 1.0×10^{-4} M diisopropylfluorophosphate in isopropanol at 25°. For assay 2 μl aliquots of the reaction mixture were withdrawn at appropriate time intervals, the enzymatic activity was measured as described below, and the remaining enzymatic activity was calculated. A control experiment without diisopropylfluorophosphate was performed concomitantly. Under the present experimental conditions the enzyme is completely inhibited by incubation with diisopropylfluorophosphate.

Protein concentrations were determined spectrophotometrically at 278 nm.[7]

Enzyme activity was measured in 1.0 M Tris buffer, pH 8.0, at 27°, with 1.0×10^{-3} M *p*-nitrophenyl phosphate.[7] A unit of enzyme was defined, and the specific activity was expressed as described by Malamy and Horecker.[7] The specific activity of the enzyme preparation used in the experiments had a value of about 2800 units.

Inactivation experiments were carried out at 27°. The enzyme, inhibitors, and buffer in a total volume of 2 ml were incubated in stoppered plastic tubes at 27°. The mixtures were assayed by pipetting samples into assay cells containing substrate and buffer.

Amino acid analyses. Amino acids were analysed [8] after hydrolysis of the protein for 20 h at 110° in 6 N HCl. Carboxymethyl histidine was determined on samples which had been oxidized with performic acid [9] before hydrolysis.

The pH measurements were carried out with a pH-meter, model pH-meter 25 Radio-meter, with a type CFK electrode. The electrodes were standardized with the aid of standard buffer solutions.

Spectra. All single-wavelength spectrophotometric determinations were done in a Zeiss PMQ II apparatus. Difference spectra were recorded in a Cary 15 recording spectro-photometer. IR spectra were recorded on samples in the solid state (KBr pellets) using a Perkin Elmer 157 apparatus.

Dichloroacetyl-β-glycerophosphate, sodium salt. Sodium *β*-glycerophosphate (3.15 g, 10 mmoles) was dissolved in 10 g of molten chloroacetic acid and chloroacetic acid anhydride (17.1 g, 100 mmoles) was added. The solution was heated for 5 min at 120° and then cooled to room temperature. Dried diethyl ether was added and the precipitate was collected and washed extensively with ether. The product was dried over KOH *in vacuo* and crystallized from methanol and diethyl ether. M.p. 184°. The compound moved as a single spot on thin-layer plates of Kieselgel G with R_F 0.55 with the solvent system 1-butanol:methanol:acetic acid:water (2:1:1:1, volumes). It had a strong and symmetric carbonyl IR absorption band at 5.75 μ. No change in m.p., IR-spectrum, or inhibitor activity was observed by repeating recrystallization. Like many esters of chloroacetic acid the compound was sensitive to moisture (hydrolysis) and fresh material was prepared frequently in order to obtain uniform inhibition results. (Found: C 23.6; H 2.8. Calc. for $C_7H_{10}Cl_2NaO_6P$ (347.0): C 24.2; H 2.9).

Monochloroacetylated β-glycerophosphate, sodium salt. Sodium *β*-glycerophosphate (1.6 g, 5 mmoles) was treated as above but by using only 2.4 g (15 mmoles) of chloroacetic anhydride, *i.e.* with water of hydration in excess. The precipitate obtained (in the same way as described above) had a strong hydroxyl IR absorption at 2.9 μ and about half of the carbonyl absorption at 5.75 μ of that of the diacetylated compound. Thin-layer chromatographic analysis indicated the presence of some unreacted and some dichloro-acetylated *β*-glycerophosphate.

Acyl phosphate of β-glycerophosphate, sodium salt. Sodium *β*-glycerophosphate (3.15 g, 10 mmoles) was dissolved in 40 ml of water and 30 ml of pyridine and the solution was cooled to 0°. Chloroacetic acid anhydride (17.1 g, 100 mmoles) dissolved in 20 ml of

acetonitrile was added dropwise within 5 min and with vigorous stirring. Cold acetone (500 ml) was then added to precipitate the acyl phosphate salt as an oil. The acetone was decanted and the oil dissolved in 20 ml of distilled water and the solution was then immediately freeze-dried. The material was stored *in vacuo* over KOH and P_2O_5 at $-16°$ in a desiccator. It had IR absorption bands corresponding to hydroxyl (3.1 μ) and carbonyl (5.75 μ) groups. Freshly prepared material contained 0.64 moles labile acyl groups/mole (M=270.6) as determined according to Avison.[10] After a few days, considerable decomposition had occurred resulting in lower labile acyl content.

Chloroacetyl phosphate, dilithium salt. Prepared as described for acetyl phosphate.[10] K_2HPO_4 (67 ml 0.25 M solution in water) was allowed to react in 32 g of pyridine with 5.7 g chloroacetic acid anhydride in 10 ml of acetonitrile. Precipitated as the dilithium salt by adding 1.2 g of LiOH in 15 ml of water and then 1 l of absolute ethanol. The precipitate was washed with cold ethanol, dried over P_2O_5 *in vacuo* and stored in the cold in a desiccator. Freshly prepared material contained 0.72 moles labile chloroacetate/mole (M=186.4). The compound showed a similar instability as the above mentioned acyl phosphate.

RESULTS

pH dependence of inactivation by dichloroacetyl-β-glycerophosphate. The reaction between alkaline phosphatase and the inhibitor was carried out at various pH values and the results are shown in Fig. 1. The rate of inactivation of

Fig. 1. Rate of inactivation of alkaline phosphatase by dichloroacetyl-β-glycerophosphate at different pH values. The reaction mixture in a volume of 2.0 ml contained the following: 0.01 M buffer, 6.25×10^{-6} M enzyme, and 3.0×10^{-4} M dichloroacetyl-β-glycerophosphate. Buffers used were as follows: sodium acetate buffer for pH 3.0—6.0, Tris-HCl buffer above pH 6.0. The reaction was carried out at 25° for 48 h. The pH of the alkylation mixture remained within ± 0.08 pH unit during the course of reaction. For assay, 5 μl aliquots of the reaction mixture were withdrawn from time to time, and the enzymatic activity was measured as described in the text. Each value represents a double activity measurement, *i.e.* the difference between the reagent treated aliquot and the control aliquot.
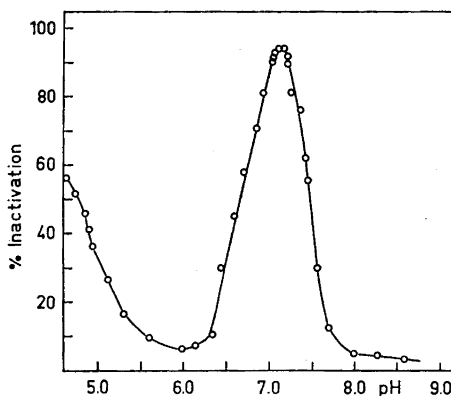
alkaline phosphatase by dichloroacetyl-β-glycerophosphate increases markedly below pH 5.8, is almost constant from pH 5.8 to 6.1, and then exhibits a sharp inhibition peak around pH 7.0.

Enzyme activity in the presence of mono- and dichloroacetyl-β-glycerophosphate. The rates of inactivation by these two reagents are illustrated by Table 1. The monochloroacetylated material, prepared in the presence of sufficient water to hydrolyse any acyl phosphate formed in the preparation procedure, inactivates the enzyme somewhat slower than does the diacetylated compound.

Alkaline phosphatase inactivation at pH 5.60. In order to get further details of the nature of the inactivation, experiments were carried out with dichloroacetyl-β-glycerophosphate and different concentrations of monochloroacetyl-β-
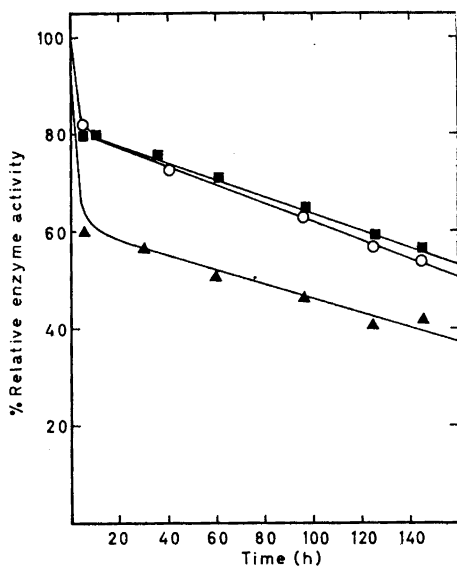
Table 1. Inactivation of alkaline phosphatase by mono- and dichloroacetyl-β-glycerophosphate at pH 6.33. The reaction mixture in a volume of 2.0 ml contained the following: 0.01 M Tris-HCl buffer, 5.0×10^{-6} M of enzyme, 2.5×10^{-4} M of the reagents. For assay, 5 μl aliquots of the reaction mixture were withdrawn from time to time, and the enzymatic activity was measured as described under Methods. Each value in the table represents a double activity measurement, *i.e.* the difference between the reagent treated aliquot and the control aliquot.

Reagents	Relative enzyme activity after several hours (%)									
	1	7	20	35	50	63	75	100	120	150
Monochloroacetyl-β-glycerophosphate	99	98	96	93	86	83	78	69	62	48
Dichloroacetyl-β-glycerophosphate	98	97	94	87	81	71	64	43	36	14

Table 2. The effect of different reagents on the alkaline phosphatase activity. The reaction mixture in a volume of 2 ml contained the following: 0.01 M buffer, 1.0×10^{-5} M enzyme and reagents at the concentrations indicated below. Buffers used were as follows: Sodium acetate buffer for pH 3.0—6.0, and Tris-HCl buffer above pH 6.0. For assay, 2 μl aliquots of the reaction mixture were withdrawn from time to time, and the enzymatic activity was measured as described under Methods. The values for a single pH run represent a triplicate activity determination for both the reagent treated enzyme aliquot and the control aliquot.

Reagents added, moles/mole enzyme		pH	Relative enzyme activity % after several days						
			1	2	3	10	15	20	28
Chloroacetylphosphate	250	7.23	80	50	11	0	—	—	—
Dichloroacetyl-β-	50	7.23	89	65	2	—	—	—	—
Monochloroacetyl-β-glycerophosphate	50	7.24	92	80	62	0	—	—	—
Chloroacetate [a]	100	7.23	100	99	98	100	98	98	98
Chloroacetate	200	7.23	100	100	97	99	99	97	98
Chloroacetate	100	6.90	100	100	100	98	100	99	99
Chloroacetate	100	6.30	100	99	99	98	97	97	97
Chloroacetate	100	5.90	100	100	98	98	99	99	99
Chloroacetate+50 equiv. β-glycerophosphate	100	7.24	98	98	96	97	98	98	98
Chloroacetate+50 equiv. β-glycerophosphate	50	6.90	100	98	96	99	97	97	97
Acylphosphate of β-glycerophosphate [b]	100	6.80	100	100	100	86	60	28	5

[a] Reactions with iodoacetate (5000 moles/mole enzyme) at pH 7.0 resulted in 33 % loss of activity after 5 days. Bromoacetate (5000 moles/mole enzyme) at pH 7.0 gave 43% inhibition after 3 days.

[b] During 5 days incubation this diester derivative did not affect the enzymatic activity. Phosphodiester bonds are hydrolysed very slowly or not at all by *E. coli* alkaline phosphatase.[11]

glycerophosphate at low pH. Fig. 2 clearly demonstrates that despite the various concentrations of the reagent the shape of the curves are similar. Note, however, that the level of inactivation at a given phosphate concentration $(2.5 \times 10^{-4}$ M reagent) is the same whether the chloroacetyl concentration is equal to (curve B) or double that of the phosphate concentration (curve A). Higher phosphate concentration $(5 \times 10^{-4}$ M) gives a higher level of inactivation (curve C).

Fig. 2. Rate of inactivation of alkaline phosphatase by mono- and dichloroacetyl-β-glycerophosphate at pH 5.60. The reaction mixture in a volume of 2.0 ml contained the following: 0.01 M sodium acetate buffer, pH 5.60, 5.0×10^{-6} M enzyme, and mono- or dichloroacetyl-β-glycerophosphate. For assay, 5 μl aliquots of the reaction mixture were withdrawn at appropriate time intervals and the enzymatic activity was measured as described in the text. Each value represents a double activity measurement, *i.e.* the difference between the reagent treated aliquot and the control aliquot. Curve A, ■, 2.5×10^{-4} M dichloroacetyl-β-glycerophosphate; curve B, ○, 2.5×10^{-4} M monochloroacetyl-β-glycerophosphate; curve C, ▲, 5.0×10^{-4} M monochloroacetyl-β-glycerophosphate.
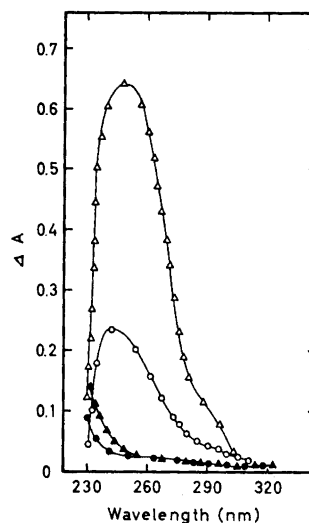
Fig. 3. Difference spectra of the dichloroacetyl-β-glycerophosphate inhibited alkaline phosphatase. Native enzyme and the enzyme inactivated with dichloroacetyl-β-glycerophosphate were utilized. The sample in a volume of 1 ml contained the following: 0.005 M buffer (sodium acetate buffer for pH 3.0−6.0, Tris-HCl buffer above pH 6.0), 5.0×10^{-6} M enzyme, native or inhibited. The inactivation reaction was carried out as described before. ●, dichloroacetyl-β-glycerophosphate *vs.* buffer; ▲, dichloroacetyl-β-glycerophosphate inhibited enzyme at pH 5.60 *vs.* native enzyme at pH 5.60; ○, dichloroacetyl-β-glycerophosphate inhibited enzyme at pH 6.30 *vs.* native enzyme at pH 6.30; △, dichloroacetyl-β-glycerophosphate inhibited enzyme at pH 6.50 *vs.* native enzyme at pH 6.50.

Effect of haloacetates, chloroacetate + sodium β-glycerophosphate and a diester on enzyme activity. It has been shown in this paper that mono- and dichloro-acetyl-β-glycerophosphate are inhibitors of alkaline phosphatase activity. In order to establish whether free chloroacetate of chloroacetate + free β-glycerophosphate have similar effect, we have treated enzyme samples with these reagents. The results obtained (Table 2) show that no loss of catalytic activity was obtained with haloacetates or free chloroacetate and free glycero-phosphate under the conditions where mono- and dichloroacetyl-β-glycero-phosphate inhibit the activity. The diester, $(HOCH_2)_2CH \cdot O \cdot PO(ONa)OCOCH_2Cl$ (the acyl phosphate of β-glycerophosphate) was also ineffective as inhibitor.

Absence of dichloroacetyl-β-glycerophosphate inactivation in the presence of an active site protecting inhibitor or a substrate. Inorganic phosphate is a strong reversible inhibitor for alkaline phosphatase and binds to the reactive serine residue in the active center.[4-6] Fig. 4 shows that 6.0×10^{-4} M inorganic phosphate completely prevents irreversible inactivation of alkaline phosphatase by dichloroacetyl-β-glycerophosphate at reaction times up to 3 days. A similar

Table 3. Amino acid analyses of native and inhibited *E. coli* alkaline phosphatase.

Amino acid	Amino acid residues per molecule of protein						
	Native alkaline phosphatase			Dichloroacetyl-β-glycerophosphate treated alkaline phosphatase (A.P.)			
	Rothman et al.[3]	Simpson et al.[22]	Our results	A.P.	P_i-A.P.[a]	DFP−A.P.[b]	G−6−P A.P.[c]
Lysine	50	50.3	49.1	49.9	50.6	49.8	50.4
Histidine	17	16.4	16.3	15.1	16.0	16.2	16.1
Arginine	23	23.1	23.4	23.2	23.6	23.0	23.5
Aspartic acid	84	92.5	92.6	94.5	92.2	93.0	95.0
Threonine	70	42.0	69.1	70.6	69.2	70.6	69.5
Serine	40	76.0	40.3	41.6	41.6	41.5	41.3
Glutamic acid	79	88.4	86.8	88.2	86.4	86.6	87.6
Proline	36	39.4	38.0	39.6	38.1	38.9	38.7
Glycine	77	87.0	85.4	86.8	85.9	84.6	86.0
Alanine	110	124.4	116.0	117.9	115.0	116.5	116.8
Half-Cystine	7	8.0	7.8	7.8	8.0	7.9	7.8
Valine	43	43.0	42.2	43.2	42.5	42.7	42.9
Methionine	12	14.4	14.8	14.7	14.6	14.7	14.8
Isoleucine	26	27.7	26.0	25.0	25.4	25.3	25.1
Leucine	66	75.4	70.0	72.2	74.0	73.0	72.5
Tyrosine	19	20.3	20.1	20.0	20.3	20.1	20.0
Phenylalanine	15	16.0	16.0	16.1	15.8	16.0	16.2
Tryptophan	7	7.9	8.0	7.9	7.6	7.9	7.8

[a] Alkaline phosphatase in the presence of inorganic phosphate (P_i); (*cf.* legend to Fig. 4).

[b] Alkaline phosphatase inactivated by diisopropylfluorophosphate (DFP); see also under Materials and Methods and legend to Fig. 4.

[c] Alkaline phosphatase in the presence of glucose-6-phosphate (G-6-P); experimental conditions as described in the legend to Fig. 4 except that 6.0×10^{-4} M glucose-6-phosphate was subsituted for KH_2PO_4.

protection against the reaction with dichloroacetyl-β-glycerophosphate is shown by the enzyme after treatment with the irreversible inhibitor diisopropyl-fluorophosphate (Table 3). The substrate, glucose-6-phosphate also prevents the enzyme from reacting with dichloroacetyl-β-glycerophosphate (Table 3).

Difference spectra of the mono- and dichloroacetyl-β-glycerophosphate inhibited alkaline phosphatase. Spectra of the thoroughly dialysed mono- and dichloro-acetyl-β-glycerophosphate inhibited alkaline phosphatase were recorded at different pH values. The reference and sample solutions were identical in protein and buffer concentrations, and also in pH values. As shown in Fig. 3,
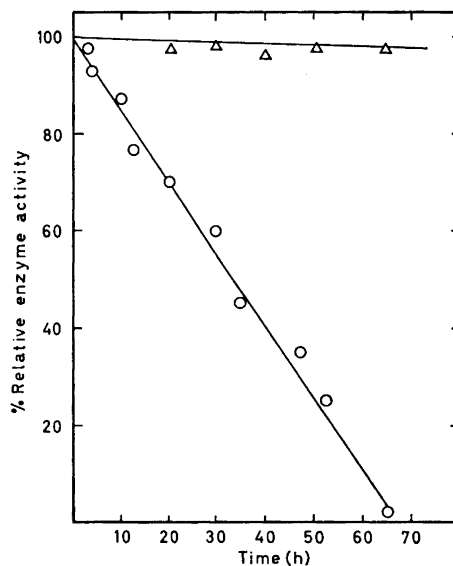
Fig. 4. Inorganic phosphate protection of alkaline phosphatase from reaction with dichloroacetyl-β-glycerophosphate. The reaction mixture in a volume of 2.0 ml contained the following: 0.01 M Tris-HCl buffer, pH 7.0, 1.6×10^{-5} M enzyme, and 6.0×10^{-4} M dichloroacetyl-β-glycerophosphate. For assay 2 μl aliquots of the reaction mixture were withdrawn at appropriate time intervals and the enzymatic activity was measured as described in the text. Each value represents a double activity measurement, *i.e.* the difference between the reagent treated aliquot and the control aliquot, O, no phosphate; $\triangle$, in the presence of 6.0×10^{-4} M KH$_2$PO$_4$.

at pH 6.5 there is a peak in the region of 250 nm and the increase in absorbancy in this region is in proportion to the amount of enzymatic activity lost. No difference spectrum was observed from 230 nm to 300 nm at pH 5.6 despite the fact that the remaining activity was 80 %. However, as is illustrated by Fig. 3, at the same extent of inhibition, at pH 6.30, a difference peak at around 240 nm has appeared.

Amino acid analyses. Since the loss of catalytic activity by the treatment with chloroacetylated β-glycerophosphate is irreversible (no enzyme activity could be recovered after extensive dialysis), the possibility exists that the chloroacetyl group is transferred to the enzyme to form a covalently bound alkylation substituent. Evidence that this is the case was obtained by amino acid analyses of hydrolysates of inactivated enzyme samples. Typical analyses are given in Table 3. Included for comparison are analyses of untreated enzyme as well as enzyme samples treated with dichloroacetyl-β-glycerophosphate in the presence of inorganic phosphate, diisopropylfluorophosphate, and glucose-6-

phosphate. The results show that modification of histidine occurs, and apparently only of this amino acid. The yield of N^1-carboxymethyl histidine was 0.72 residues per molecule of enzyme.

DISCUSSION

The investigation of the active sites of a number of enzymes using bifunctional reagents has met with considerable success.[11-14] In the case of *E. coli* alkaline phosphatase, no amino acid residues of importance for enzymatic activity other than the reactive serine have been demonstrated. In order to locate additional reactive amino acid side chains in the active site and to learn more about the structural features of this site, a substrate analogue, capable of carrying reactive groups into the active center region, was sought. Chloroacetylated derivatives of the well-known phosphatase substrate β-glycerophosphate as well as chloroacetyl phosphate were chosen and were found to inactivate the enzyme in a specific manner.

The hypothesis underlying this choice of reagents is depicted in Fig. 5. The phosphate group of the reagent is supposed to bind to the reactive serine residue in the active site and then be removed from the glycerol part of the reagent forming the usual phosphoryl enzyme. During the time interval necessary for binding, cleavage of the phosphate-glycerol bond, and removal of the glycerol part of the substrate, the potential alkylating groups of the latter might become favourably located to be attacked by nucleophilic amino acid side chains present in the active site. Such an attack would result in
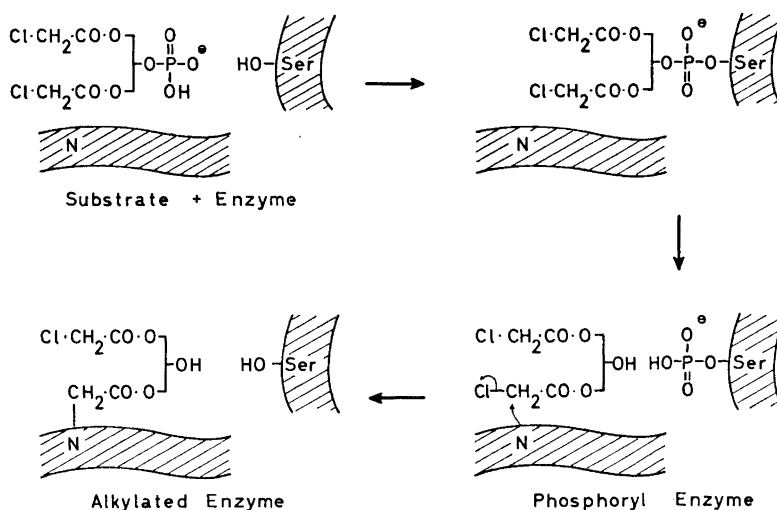

Fig 5. Hypothetical course of the reaction between the active site of *E. coli* alkaline phosphatase and dichloroacetyl-β-glycerophosphate, suggesting the presence of a nucleophile (N) in the active site besides the reactive serine hydroxyl group.

alkylation of the side chain forming an irreversibly modified (in the case of nucleophiles like imidazole-N) or possibly reversibly modified (nucleophiles like carboxylates, *i.e.* forming a hydrolysable ester bond to the reagent) enzyme. This modification in the active site should probably destroy or at least alter the enzymatic activity, as is known to be the case with enzymes such as chymotrypsin, trypsin, and carbonic anhydrase.[11-14]

The results obtained in the present studies suggest that a reaction of the type shown in the above reaction sequence (Fig. 5) actually may occur. While free haloacetates, a mixture of chloroacetate and β-glycerophosphate or a chloroacetylated phosphate diester caused no loss in enzymatic activity within several days, dichloroacetyl-β-glycerophosphate under the same conditions gave 100 % irreversible inhibition. The pH-dependence of the inactivation supports the conclusion that both phosphoryl enzyme formation (maximum at around pH 4.0) and alkylation (pH about 7.0) occur, *i.e.* the reagent acts as a normal substrate besides its function as alkylating reagent. It should be noted, that the low pH part of Fig. 1 is in remarkably good agreement with the phosphate incorporation in the pH range 4 — 6 found by Schwartz.[15]

The pH optimum of the irreversible inactivation indicates that a histidine residue might be involved. Another fact favouring this hypothesis is the spectral change observed as a result of the inactivation around this pH. Acetyl imidazole has a broad absorption band around 244 nm [16] and an increase in absorption at 250 nm has been reported to accompany the photooxidation of histidine residues in lactic dehydrogenase [17] and in 6-phosphogluconate dehydrogenase.[18] It is to note, that no such spectral change occurs at pH around 5.6, where the inhibition probably is the result of phosphoryl enzyme formation. Spectral changes may of course be caused by unspecific conformational changes in the enzyme, but such conformational changes seem more probable at lower pH, where the enzyme is known to be more sensitive to dissociation of the dimer to monomers. The absence of such dissociation around pH 7 was furthermore demonstrated by molecular weight determination in gelfiltration experiments.[19]

Final proof for the modification of histidine was obtained by amino acid analyses of the dichloroacetyl-β-glycerophosphate-inactivated enzyme showing the formation of 1-carboxymethyl histidine, *i.e.* the product expected from the reaction sequence of Fig. 5, if the nucleophile N is the N^1 atom of the imidazole ring of a histidine residue. The yield of this modified histidine was approximately one residue per enzyme molecule, although considerably more than the equimolar concentration of the inactivator had to be used to obtain reasonable inactivation rates. A corresponding loss of one residue of histidine was observed. The necessarily high concentration of inactivator may partly be the result of its instability, and it made accurate determination of K_I values difficult. In spite of these high concentrations, no signs of unspecific modification of, *e.g.*, lysine or methionine residues could be detected (the enzyme does not contain free cysteine [20]). It therefore seems rather probable that the reagent is specific for histidine in the enzyme, and that this histidine is located at or near the active site of the enzyme. The evidences implicating that the inactivation takes place in the active site region are the following:

a) Only haloacetates of monoalkyl phosphate type, *i.e.* substrate analogues, were found to be effective inactivators. Chloroacetate or other simple

haloacetates do not cause a similar inactivation. When the enzyme was incubated with the P-chloroacetyl derivative of β-glycerophosphate, *i.e.* a phosphodiester, the activity of the enzyme was not altered. It is known that diesters are not substrates for alkaline phosphatase of *E. coli*.[21]

b) Specific reversible inhibitors (inorganic phosphate) and irreversible inhibitors (DFP) as well as the substrate, glucose-6-phosphate, protect the enzyme from the attack by dichloroacetyl-β-glycerophosphate, as shown by the absence of irreversible inhibition when the incubation was done in the presence of inorganic phosphate or glucose-6-phosphate and the absence of any significant changes in the amino acid composition of the enzyme when incubation with dichloroacetyl-β-glycerophosphate was done on DFP-inactivated enzyme. It is well known that the three protectors combine with the reactive serine residue in the active site of the enzyme.

The results of this study therefore indicate that *E. coli* alkaline phosphatase, like several other hydrolytic enzymes such as chymotrypsin and trypsin, has at least one reactive histidine residue in its active site, as well as a reactive serine residue. A study of the amino acid sequence around such a reactive histidine residue, employing radioactive chloroacetic anhydride in the preparation of the inhibitor, is contemplated.

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