Essential Arginyl Residues in *Escherichia coli* Alkaline Phosphatase†

Frans J. M. Daemen‡ and James F. Riordan*

ABSTRACT: On treatment of *Escherichia coli* alkaline phosphatase with either 2,3-butanedione in borate buffer or phenylglyoxal in bicarbonate buffer both hydrolase and transferase activities are lost concomitantly. Loss of activity is linearly related to the modification of about 15 of the 24 arginine residues present per molecule. No other residues are modified and the protein remains dimeric. Virtually no loss of activity occurs when modification is performed in the presence of pseudosubstrates and inhibitors and two less arginyl residues are modified, *i.e.*, one per active site. Binding of phosphate to the modified enzyme is decreased markedly. The data suggest an essential role for an arginyl residue in the enzymatic mechanism of alkaline phosphatase, probably as a binding site for the negatively charged phosphate group of the substrate.

Alkaline phosphatase, a zinc metalloenzyme from *Escherichia coli*, has a molecular weight of 89,000 (Simpson *et al.*, 1968) and consists of two identical subunits (Rothman and Byrne, 1963; Schlesinger and Barrett, 1965; Simpson *et al.*, 1968). It catalyzes both the hydrolysis of phosphate monoesters and the transfer of phosphoryl groups to suitable alcohol acceptors (Dayan and Wilson, 1964; Reid and Wilson, 1971). Two of its four zinc atoms serve in catalysis and two in maintaining structure (Simpson and Vallee, 1968; Lazdunski *et al.*, 1969). Studies thus far have focussed on genetic and kinetic aspects of the enzyme and the content and function of its metal ion. The amino acid sequence is presently unknown, but recently an electron density map of the enzyme at 7.7-Å resolution has been published (Knox and Wyckoff, 1973).

Phosphate esters and P_i are known to phosphorylate a specific seryl residue of alkaline phosphatase (Engström, 1962; Schwartz and Lipmann, 1961; Pigretti and Milstein, 1965). However, there is little information regarding the possible participation of other amino acid residues in either catalysis or substrate binding. Since all substrates of alkaline phosphatase bear a negatively charged phosphate group, the existence of a corresponding positively charged recognition site(s) on the enzyme would seem likely. The participation of lysyl and histidyl residues in this function has been ruled improbable (Plotch and Lukton, 1965; Tait and Vallee, 1966; Christen *et al.*, 1971) and, hence, zinc and arginyl residues are the remaining possibilities. These need not be mutually exclusive, of course. The present studies, prompted by the development of selective reagents for the chemical modification of arginyl residues under mild conditions in the past few years (Yankeelov *et al.*, 1968; Yankeelov, 1972; Takahashi, 1968; Riordan, 1973), point to a functional role of arginine in alkaline phosphatase. The data demonstrate that two arginyl residues are essential to both the hydrolase and transferase activities of the enzyme. A preliminary report of these findings has appeared (Daemen, 1973).

Materials and Methods

Alkaline phosphatase of *E. coli*, strain C-90, was prepared as previously reported (Simpson *et al.*, 1968). Its specific activity ranged from 40 to 50 μmol per min per mg when measured in 1 M Tris-Cl and from 21 to 25 μmol per min per mg when measured in 1 M NaCl under standard conditions (see below). Other materials (and suppliers) were: 4-nitrophenyl phosphate disodium hexahydrate (Sigma Chemical Co.); 40% aqueous glyoxal, 40% aqueous methylglyoxal, 2,3-butanedione, phenylglyoxal monohydrate, and phenylphosphonic acid (Aldrich Chemical Co.). ^{14}C-labeled phenylglyoxal was prepared from acetophenone-7-^{14}C (ICN Chemical & Radioisotope Division) by oxidation with selenious acid (Fisher Scientific Co.) as described by Riley and Gray (1947). After repeated crystallizations it had a constant specific radioactivity of 84,000 cpm/μmol.

Enzymatic activity was measured with 4-nitrophenyl phosphate as substrate in a Unicam SP-800 spectrophotometer at 25°. The release of 4-nitrophenol was determined at 400 nm, using a molar absorptivity at pH 8.0 of 1.68×10^4. The assay medium contained 1 mM substrate in either 1 M Tris-Cl (pH 8.0), ionic strength 1.0 (prepared by mixing 1 M Tris-Cl with 1 M Tris base, 1 M NaCl to give a pH of 8.0), or 1 M NaCl–0.01 M Veronal (pH 8.0). The enzyme concentration was usually about 0.1 μg/ml ($\sim 10^{-9}$ M). None of the reagents used in this study interfered with the assays at the concentrations employed. The enzymatic activity in 1 M NaCl will be referred to as hydrolase activity and that in 1 M Tris as the sum of hydrolase and transferase activities, Tris being the alcohol acceptor.

Protein concentrations were measured spectrophotometrically using $A_{278}^{1\%} = 7.2$ (Plocke *et al.*, 1962) and a molecular weight of 89,000 (Simpson *et al.*, 1968). A Radiometer Model 22 pH meter with a general-purpose glass electrode was used for determination of pH.

Modification reactions were carried out under conditions given in the figure legends. The modification of specific amino acid residues was determined by amino acid analysis after acid hydrolysis. Aliquots of the reaction mixture were separated from excess reagent by gel filtration over a Bio-Gel P-4 column equilibrated with 0.1 M sodium borate (pH 8.0) at 4°. Protein hydrolyses were carried out in sealed, evacuated ampoules at 110° for 18–20 hr and analyses were performed according to Spackman *et al.* (1958) using a Spinco Model 120C amino acid analyzer.

† From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts. *Received February 11, 1974.* This work was supported by Grant-in-Aid GM-15003 from the National Institutes of Health, of the Department of Health, Education, and Welfare.

‡ Present address: Laboratory of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands. Supported in part by a travel grant from The Netherlands Organization for the Advancement of Pure Research (ZWO).

TABLE I: Loss of Hydrolase Activity on Incubation of 15 μM Alkaline Phosphatase with 50 mM Arginine Reagent in 125 mM Buffer (pH 7.5) at 25° for 1 hr.

Reagent	Loss of Activity (%)	
	Borate	Bicarbonate
Phenylglyoxal	12	95
Glyoxal	18	81
Methylglyoxal	29	81
Butanedione	55	47

In the case of modification with [^{14}C]phenylglyoxal, incorporation of radioactivity into the protein was measured in a Packard Tri-Carb scintillation counter using Bray's solution after removal of excess reagent by gel filtration in 0.1 M sodium bicarbonate (pH 8.0) at 4°. Calculations were based on the assumption that two phenylglyoxal molecules condense with one arginyl residue (Takahashi, 1968).

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out according to Weber and Osborn (1969) with the exception that the samples were dissolved in 0.1% sodium dodecyl sulfate, 0.1% β-mercaptoethanol, 0.01 M sodium phosphate buffer (pH 7.0), and preincubated at 37° for several hours. Protein monomers used for the calibration of the gels were: phosphorylase (mol wt 92,000), bovine serum albumin (mol wt 68,000), ovalbumin (mol wt 43,500), and carbonic anhydrase (mol wt 30,000).

Equilibrium dialyses were carried out in duplicate using equal 500-μl volumes of protein and buffer solutions in 1-ml capacity dialysis cells (Chemical Rubber Corp.) as described previously (Simpson and Vallee, 1970). The cells were rotated on a slanted table at room temperature during the 48-hr equilibration. At the conclusion of the experiment, duplicate aliquots of protein, buffer, or standards were removed with a single Carlsberg micropipet of appropriate volume for measurement of total phosphate according to the procedure of Bloch and Schlesinger (1973) except that protein hydrolysis was done at 110°.

Zinc was determined by atomic absorption spectrometry (Fuwa *et al.*, 1964). The usual precautions were taken to avoid metal ion contamination (Thiers, 1957).

TABLE II: Loss of Activity on Incubation of 15 μM Alkaline Phosphatase with Butanedione for 1 hr at 25°: Effect of Reagent, Borate, and H^+ Concentrations.

[Butanedione] (mM)	[Borate] (mM)	pH	Loss of Act. (%)
10	500	7.5	15
50	500	7.5	36
100	500	7.5	44
50	50	7.5	47
50	125	7.5	54
50	250	7.5	49
50	500	7.5	33
100	100	7.5	50
100	250	7.5	60
100	500	7.5	44
50	125	7.0	26
50	125	7.5	50
50	125	8.0	33

TABLE III: Loss of Activity on Incubation of 15 μM Alkaline Phosphatase with Phenylglyoxal in 125 mM Sodium Bicarbonate (pH 7.5) for 1 hr at 25°. Effect of Reagent Concentration.

[Phenylglyoxal], mM	Loss of Act. (%)
0.5	2
5	24
20	55
50	92

Results

Selection of Optimal Conditions for Inactivation. Preliminary experiments demonstrated that a number of α-dicarbonyl reagents, previously thought to be suitable for the modification of arginyl residues in proteins, affected the phosphohydrolase activity of alkaline phosphatase. Results obtained under experimental conditions derived from earlier studies (Riordan, 1973) are summarized in Table I. In 0.125 M sodium borate, using activity loss as a gauge, butanedione is the most effective reagent, while in 0.125 M sodium bicarbonate the α-ketoaldehydes, especially phenylglyoxal, cause the greatest inactivation.

Optimal conditions for studying the inactivation of alkaline phosphatase by butanedione in borate buffer were determined by systematic variation of the concentration of butanedione, the ratio of butanedione to borate, and the pH of the incubation mixture (Table II). At pH 7.5 in 125–250 mM borate, 50–100 mM butanedione results in half-inactivation of the enzyme in about 1 hr, and these conditions were adopted for succeeding experiments. Variation of enzyme concentration between 10 and 50 μM (0.9–4.5 mg/ml) has virtually no effect on the rate of inactivation.

The course of inactivation with phenylglyoxal is shown in Table III. In order to achieve 50% inactivation of the enzyme in 1 hr, a concentration of about 20 mM reagent was required.

Characteristics of the Butanedione Inactivation Reaction. The time course of inactivation of alkaline phosphatase with 60 mM butanedione is depicted in Figure 1. Over a period of 3 hr hydrolase and transferase activities decrease at exactly the same rate to about 25% of the control. This parallel response of both activities has been observed for all conditions employed in this study. On extending the reaction time to 20 hr both activities fall to about 1% of those of the native enzyme, and with higher concentrations of butanedione the enzyme is almost

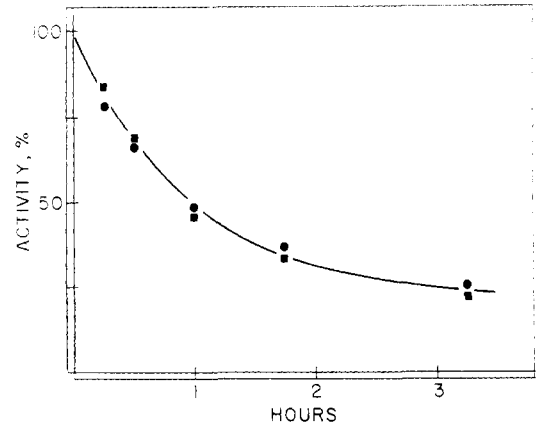

FIGURE 1: Time course of inactivation of alkaline phosphatase by butanedione. Enzyme (20 μM) was incubated at 25° with 60 mM butanedione in 125 mM sodium borate (pH 7.5): hydrolase activity (●); transferase activity (■).

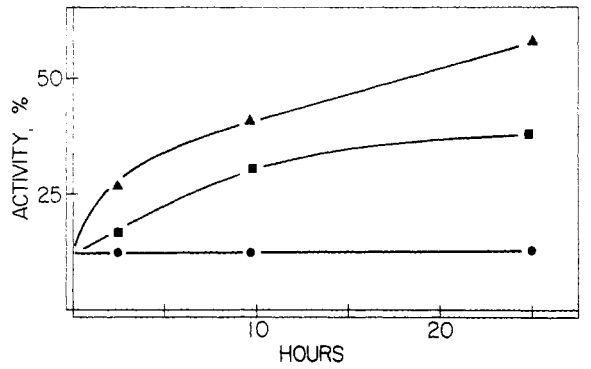

FIGURE 2: Reversibility of alkaline phosphatase inactivation by butanedione. Butanedione-modified enzyme (6-hr incubation under the conditions of Figure 1), retaining 14.5% of its original activity, was gel filtered through a 0.9 × 20 cm Bio-Gel P-4 column in 0.1 M borate (pH 7.5) at 4°. The protein-containing fraction, now 3 μM, retained 12.5% of the original specific activity. This solution was 40× diluted with either 0.1 M borate (●), 0.1 M sodium bicarbonate (■), or 0.1 M Tris-Cl (▲), all at pH 7.5, and incubated at 25° in the dark.

completely inactivated (<0.05%). Initially, the reaction appears to follow pseudo-first-order kinetics with respect to enzyme, but the overall rate of inactivation is not consistent with any simple scheme.

The reversibility of the inactivation reaction was studied both by removing butanedione either *via* dialysis or gel filtration, or by a 40- 100-fold dilution of the reaction mixture. Varying degrees of restoration of activity can be achieved during the early stages of modification depending on the buffer employed, but after prolonged reaction, *i.e.*, 20 hr, essentially no activity can be recovered. For example, an enzyme sample treated with butanedione for 6 hr had about 15% residual activity but on incubation in 0.1 M Tris (pH 8.0) for 25 hr its activity increased to 60% of the unmodified control. Only about half as much restoration of activity occurs on incubation in 0.1 M bicarbonate, and in 0.1 M borate no activity is restored at all (Figure 2).

Chemical Consequences of Inactivation by Butanedione. The residues modified by treatment of alkaline phosphatase with butanedione were identified by amino acid analysis after acid hydrolysis. Loss of activity correlates with a progressive decrease of arginyl residues (Figure 3). When only 1% of the activity remains, 15 of the 24 arginines of alkaline phosphatase

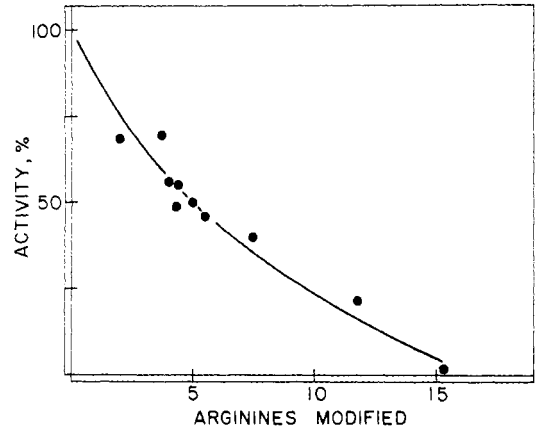

FIGURE 3: Relation between alkaline phosphatase activity and arginine modification upon butanedione treatment. Enzyme was modified under the standard conditions of Figure 1. Aliquots were assayed for enzyme activity. Others were freed of excess reagent by gel filtration through a Bio-Gel P-4 column in 0.1 M borate at 4° and subjected to amino acid analysis after acid hydrolysis. The number of arginines modified is given per mole of enzyme.

TABLE IV: Sodium Dodecyl Sulfate Gel Electrophoresis of Native and Modified Alkaline Phosphatases.

Enzyme	Rel Electrophoretic Mobility	App Mol Wt
Native	0.15	90,000
Butanedione modified	0.16	89,000
Acid dissociated, native	0.38	43,000
Acid dissociated, butanedione modified	0.39	43,000

have been modified. None of the other amino acids is affected. Notably the contents of lysine and histidine are unchanged.

In order to characterize more precisely the cause of the inactivation of the enzyme, various analytical determinations were carried out on an enzyme preparation extensively modified and exhibiting only 1% activity. After removal of excess butanedione, absorption and circular dichroic spectra of this material between 250 and 350 nm are identical with those of the native enzyme. Its zinc content also remains unchanged, 3.2 g-atom/mol *vs.* 3.1 for this particular enzyme preparation.

E. coli alkaline phosphatase exhibits unusual stability toward sodium dodecyl sulfate that provides a means to study the quaternary structure of the modified enzyme. In 1% sodium dodecyl sulfate the native enzyme retains complete activity for at least 1 week at 20°. Indeed, on polyacrylamide gel electrophoresis in sodium dodecyl sulfate, both native and modified alkaline phosphatases migrate with apparent molecular weights of about 90,000 (Table III). Acid denaturation is known to dissociate the native enzyme into monomers of app mol wt ~44,000 (Schlesinger and Barrett, 1965), and these do not reassociate on neutralization in the presence of ionic detergents. This behavior is characteristic of both native and butanedione-inactivated alkaline phosphatases (Table IV).

Protection against Butanedione Inactivation: Differential Labeling. Modification of alkaline phosphatase with butanedione in the presence of competitive inhibitors of the enzyme, either 15 mM P_i or phenylphosphonate, almost completely prevents inactivation (Figure 4). The modified enzyme is fully active after removal of inhibitor and excess reagent by gel filtration through a Bio-Gel P-4 column equilibrated with 0.1 M borate. Exposure of this product to butanedione, now in the absence of inhibitor, decreases its activity just as with the native enzyme.

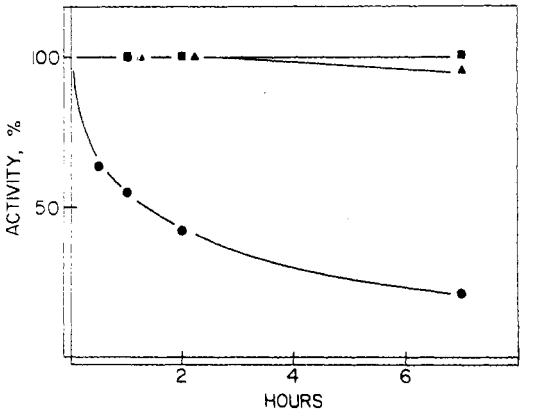

FIGURE 4: Effect of competitive inhibitors of alkaline phosphatase on enzyme inactivation by butanedione. Enzyme (20 μM) was incubated with 50 mM butanedione in 125 mM borate (pH 7.5) at 25°. Either 15 mM sodium phosphate (■) or 15 mM sodium phenylphosphonate (▲) or no inhibitor at all (●) was present.

TABLE V: Differential Labeling of Alkaline Phosphatase by Butanedione in the Absence and Presence of Phosphate; Relation to Enzyme Activity.[a]

Reaction Time (hr)	Enzyme Act. (%)		Arginines Protected by P_i (Mol/Mol of Enzyme)
	$-P_i$	$+P_i$	
2	44	100	0.6
7	22	100	1.7
18	1	89	2.6

[a] Enzyme (1.5 mM) was treated with 50 mM butanedione in 125 mM sodium borate (pH 7.5) in the absence and presence of 15 mM phosphate. Aliquots were assayed for activity and others were freed of excess reagents and subjected to amino acid analysis. Enzyme activity refers to both hydrolase and transferase activity which are affected equally.

Such experiments indicate the possibility that differential labeling in the presence and absence of phosphate would distinguish enzymatically essential arginyl residues from all others. Two samples of alkaline phosphatase were therefore modified with butanedione, one in the presence and the other in the absence of 15 mM phosphate, but under otherwise identical conditions. The time course of arginine modification of the two samples is depicted in Figure 5. Control experiments demonstrated that the presence of this concentration of phosphate does not influence the rate of modification of free arginine. The presence of phosphate protects against loss of activity and extrapolation of data obtained from intermediate degrees to complete inactivation indicates that 2–2.5 arginyl residues/molecule are essential for activity (Table V).

Inactivation by Phenylglyoxal. The role of arginyl residues in alkaline phosphatase was also examined with phenylglyoxal both apart from and in conjunction with butanedione. In 125 mM bicarbonate buffer at pH 7.5, 20 mM phenylglyoxal rapidly and concomitantly inactivates both the hydrolase and the transferase activities of alkaline phosphatase (Figure 6). After 3 hr activity decreases to about 25% of the control and after 12 hr it is almost completely abolished. Pseudo-first-order kinetics pertain over a greater portion of the reaction than is the case with butanedione. In the early stages of modification phenylglyoxal inactivation is readily reversible, especially in Tris

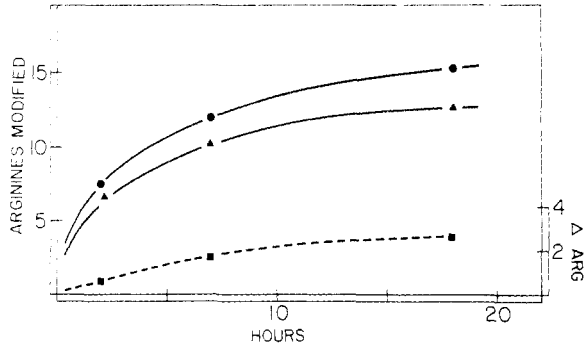

FIGURE 5: Effect of the presence of phosphate on the arginine modification of alkaline phosphatase by butanedione. Enzyme (20 μM) was incubated with 65 mM butanedione in 125 mM borate (pH 7.5) at 25° in the presence (▲) or absence (●) of 15 mM phosphate. Aliquots were removed at various times, strictly in parallel, freed of excess reagent by gel filtration in 0.1 M borate at 4° and subjected to amino acid analysis after acid hydrolysis. The solid lines indicate the moles of arginine modified per mole of enzyme; the dashed line shows the difference between modification in the presence and absence of phosphate.

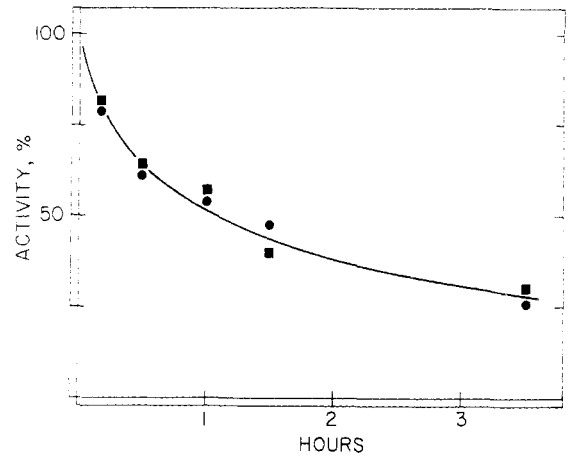

FIGURE 6: Time course of inactivation of alkaline phosphatase by phenylglyoxal. Enzyme (20 μM) was incubated at 25° with 20 mM phenylglyoxal in 125 mM sodium bicarbonate (pH 7.5): hydrolase activity (●); transferase activity (■).

buffer, but if inactivation proceeds to >90%, then the reaction is essentially irreversible. Only arginyl residues are modified during the first 3-hr reaction, but with longer times lysyl, and some histidyl residues, also undergo modification as well. The quantitative relation between inactivation and number of arginines modified is very similar to that found for inactivation by butanedione (Figure 3). For example, with 10% of the activity remaining, 12 of the 24 arginines of the enzyme are modified. Phosphate, and to a lesser extent phenylphosphonate, inhibit the phenylglyoxal inactivation (Figure 7), but not as effectively as they do with butanedione (Figure 4). The availability of [^{14}C]phenylglyoxal allowed a differential labeling experiment in which modification of arginine with butanedione could be followed both by amino acid analysis and by incorporation of radioactivity. Two samples of the enzyme were modified with [^{14}C]phenylglyoxal in parallel, one in the presence and the other in the absence of 15 mM phosphate. Aliquots were examined after 1.5- and 2.5-hr reaction and the resultant enzyme activities and arginine modifications are listed in Table VI. Phosphate prevents inactivation of the enzyme and simultaneously protects arginyl residues from modification. Extrapolation of these data to give the difference between a completely inactivated enzyme and a completely protected one reveals 2–2.5 essential arginyl residues/mol of phosphatase.[1] These data are equivalent to those obtained in the analogous experiment using butanedione (Table V).

In order to demonstrate that butanedione modification affects the binding of P_i to alkaline phosphatase samples of native and modified enzyme were dialyzed against Tris or Tris-phosphate buffers for 48 hr (Table VII). At equilibrium, both enzymes are found to contain less than stoichiometric amounts of tightly bound phosphate even though they have not been exposed to phosphate buffer. This has also been observed recently by Bloch and Schlesinger (1973). With 2×10^{-5} M phosphate present in the dialysis medium, approximately 1 additional mol of phosphate binds per mol of native enzyme but only 0.2 mol binds to the modified enzyme. A further 5-fold increase in phosphate concentration brings about binding of an additional

[1] Preparations of alkaline phosphatase, modified in the presence of phosphate with butanedione or unlabeled phenylglyoxal and hence enzymatically active, could not subsequently be differentially labeled with ^{14}C-phenylglyoxal. Although such experiments indicated that approximately 2 arginines are protected by the presence of phosphate, the total radioactivity incorporated and, hence, the background, was much higher than expected, suggesting that exchange with previously modified arginines might have occurred.

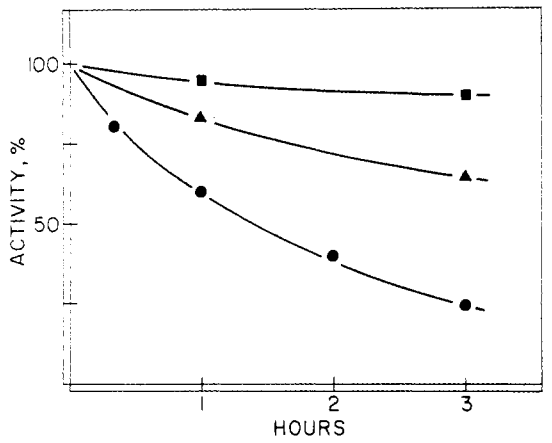

FIGURE 7: Effect of competitive inhibitors of alkaline phosphatase on enzyme inactivation by phenylglyoxal. Enzyme (20 μM) was incubated with 20 mM phenylglyoxal in 125 mM sodium bicarbonate (pH 7.5) at 25°. Either 15 mM sodium phosphate (■) or 15 mM sodium phenylphosphonate (▲) or no inhibitor at all (●) was present.

0.2 mol of phosphate/mol of enzyme in each case. Clearly, phosphate binding is markedly reduced as a consequence of butanedione modification.

Discussion

The recognition of the involvement of particular amino acid side chains in the mechanism of action of enzymes has been facilitated by the availability of specific modification agents. Various dicarbonyl reagents have recently been shown to be suitable for the identification of functional arginyl residues (Yankeelov *et al.*, 1968; Yankeelov, 1972; Takahashi, 1968; Riordan, 1973). Although the selectivity of none of these has proven ideal, nevertheless, arginine modification, when quantitated by amino acid analysis, is reliable. The potential lability of the product of the arginine–dicarbonyl reaction still constitutes a problem if the exact location of the modified arginine in the primary sequence of a protein is to be determined. But this is not a major problem for the present study since the amino acid sequence of alkaline phosphatase is as yet unknown.

Using now standard methods of arginine modification, we have been able to study the function of this residue in *E. coli* alkaline phosphatase. Four arginine reagents, butanedione and three different glyoxals, all rapidly inactivate the enzyme (Table I). Both butanedione (Figure 1) and phenylglyoxal (Figure 6) can completely inactivate the hydrolase and the transferase activities of the enzyme in parallel. All experiments performed support the conclusion that two arginyl residues are essential to the enzymatic activity of alkaline phosphatase. Thus, the butanedione inactivation is reversible in accord with the chemical details of arginine modification by this reagent. Further, analysis of the inactivated enzyme shows that only arginine modification can account for the loss of function. Moreover, substrate analogs protect from inactivation by preventing two arginines per molecule of enzyme from being modified, as would be expected for the modification of functionally essential residues.

The reversibility of the butanedione inactivation in various buffers is also in accord with present views on the reaction between arginine and butanedione. It has been proposed (Riordan, 1973) that a dihydroxyimidazolidine derivative, the primary condensation product of this reaction, readily dissociates into its original components. This product can be stabilized by borate which forms a complex with its vicinal hydroxy groups. Indeed, activity can be restored, albeit partially, to butanedione-modified phosphatase on incubation in bicarbonate buffer. Tris buffer stimulates reactivation (Figure 2) probably by reacting either with borate or butanedione and thereby displacing the equilibrium toward free arginine. Conversely, borate buffer completely prevents reactivation by stabilizing the complex. Analysis of the modified enzyme provides direct evidence for the involvement of arginyl residues in function. The degree of inactivation brought about by both butanedione and phenylglyoxal is directly proportional to the number of arginyl residues modified per mole of enzyme (Figure 3). None of the amino acids other than arginine were found to be modified in

TABLE VI: Differential Labeling of *E. coli* Alkaline Phosphatase with [^{14}C]Phenylglyoxal in the Absence and Presence of Phosphate; Relation to Enzyme Activity.[a]

Reaction Time (hr)	Act. (%)		Arginines Protected by P_i (Mol/Mol of Enzyme)	
	$-P_i$	$+P_i$	From Radioactivity Measurements	From Amino Acid Anal.
1.5	36	84	1.0	0.8
2.5	20	79	1.5	1.5

[a] Enzyme (1.1 M) was treated with 40 mM [^{14}C]phenylglyoxal in 0.125 M sodium bicarbonate (pH 7.5) in the absence and presence of 15 mM phosphate. Some aliquots were assayed for both hydrolase and transferase activity. Others were freed of excess reagent and subjected either to amino acid analysis or to radioactivity measurement. Calculation of arginine modification from radioactivity incorporation is based on two phenylglyoxals reacting with one arginyl residue (Takahashi, 1968).

TABLE VII: Phosphate Binding to Native and Butanedione Modified Alkaline Phosphatases.[a]

Dialysis Buffer	Mol of P_i Bound/Mol of Enzyme	
	Native	Butanedione Modified
0.01 M Tris–0.1 M NaCl (pH 7.64)	0.57	0.22
0.01 M Tris–0.1 M NaCl (pH 7.64) + 2×10^{-5} M P_i	1.64	0.41
0.01 M Tris–0.1 M NaCl (pH 7.64) + 1×10^{-4} M P_i	1.80	0.66

[a] Equal 500-μl aliquots of protein (3.5×10^{-5} M) and buffer were dialyzed for 48 hr at room temperature. Phosphate analyses were performed essentially according to the procedure of Block and Schlesinger (1973). Appropriate aliquots were evaporated to dryness, taken up in 6 N HCl, hydrolyzed *in vacuo* at 110° for 20 hr and again evaporated to dryness. Each sample was taken up in 100 μl of H_2O, 900 μl of freshly prepared molybdate solution was added, and absorbance was measured at 820 nm after incubation at 45° for 20 min. The results are the average of two different samples analyzed in duplicate. The activity of the modified enzyme, prepared by reaction with 60 mM butanedione for 28 hr at 20° under standard conditions, was less than 1% that of the native enzyme.

the butanedione-inactivated enzyme, and its zinc content was equal to that of the native enzyme. Although tryptophan cannot be determined adequately by the amino acid analysis procedures employed here, this residue is known not to react with butanedione (Yang and Schwert, 1972) consistent with the unaltered absorption spectrum observed for the modified enzyme. Further, the modified enzyme retains its dimeric structure (Table III).

Substrates or competitive inhibitors, such as phosphate or phenylphosphonate, in relatively low concentrations prevent or retard the inactivation by both butanedione (Figure 4) and phenylglyoxal (Figure 7), and the number of arginyl residues modified differs in the presence and absence of inhibitor. After removal of inhibitor, treatment with either reagent leads to inactivation. Hence, the inhibitors protect against inactivation by preventing arginyl modification.

These circumstances permitted differential labeling, *i.e.*, exclusion of arginines not essential to enzyme activity by modification in the presence of substrates or inhibitors. In the presence of phosphate about 13 arginyl residues/molecule are modified by butanedione without affecting activity, while in its absence about 15 arginines are modified with complete loss of activity. Thus, loss of activity occurs concomitantly with the modification of approximately two arginyl residues per dimeric molecule. Similar results with phenylglyoxal (Table VI) were confirmed using radioactive reagent.[2] Hence, the involvement of two arginyl residues in the catalytic function of alkaline phosphatase could be demonstrated with two different reagents and by two independent means of analysis.

The precise number of arginines protected from modification by phosphate found in this study is subject to some experimental uncertainty since it represents the difference between two relatively large numbers. Triplicate experiments using butanedione gave an average difference of 2.3 ± 0.4, while using phenylglyoxal it was 2.8 ± 0.3. The butanedione-modification data are somewhat more certain than those using phenylglyoxal (see below), and hence the modification of 2 rather than 3 residues would seem to be the result more likely to be correct.

Although butanedione and phenylglyoxal gave virtually identical functional results, phenylglyoxal seemed somewhat more effective. The rates of inactivation with 20 mM phenylglyoxal were about the same as those with 60 mM butanedione (Figures 1 and 6). This could likely be due to, in part, an influence of the different buffers employed, however (Riordan, 1973). On the other hand, phenylglyoxal seems to be less specific for arginine than is butanedione, especially when reaction times are long. Moreover, phosphate gives less protection from inactivation by phenylglyoxal (Figures 4 and 7). Both of these factors render quantitation of the phenylglyoxal modification complex. It should also be noted that the product of phenylglyoxal modification contains two molecules of phenylglyoxal per arginyl residue (Takahashi, 1968) and its bulkiness might be more likely to disrupt the three-dimensional structure of the enzyme. Finally, in the butanedione modification, variation of the borate concentration allows control of the reversibility of the inactivation, a feature not present with phenylglyoxal.

At present the location of the two essential arginine residues in the primary structure cannot be ascertained since the amino acid sequence of the enzyme is unknown. However, it would be important to determine whether or not these two arginines are located on the same or on different subunits of alkaline phosphatase. Since a considerable number of arginyl residues are modified without affecting activity and since difference measurements are unavoidably imprecise, meaningful kinetic analysis of inactivation rates as a function of arginine modifications were precluded, much as they might have elucidated this problem. It is interesting to note that studies of phosphate binding to alkaline phosphatase at equilibrium are consistent with the presence of two binding sites for phosphate, *i.e.*, one for each subunit (Simpson and Vallee, 1970). Under the conditions used in the phosphate protection experiments, 15 mM phosphate should saturate both these sites, as is indeed observed. It would therefore seem quite likely that the essential arginines protected from modification by phosphate are part of two different phosphate binding sites. There are indications that the essential zinc atom of the enzyme is also involved in phosphate binding (Gottesman *et al.*, 1969). A positively charged substrate recognition site in which both an arginyl residue and the zinc atom might play a role in recognizing the double negative charge of the substrate could account for both sets of data.

Binding of phosphate by the modified enzyme is markedly reduced relative to the native enzyme (Table VII) consistent with the idea that arginyl residues participate in the formation of the enzyme–phosphate complex. It is not surprising that the stoichiometry of phosphate binding to either enzyme is less than 2 mol/mol. Previous studies have indicated that there are two different binding constants for phosphate binding to native alkaline phosphatase (Simpson and Vallee, 1970). The maximum concentration of phosphate employed in these studies was 10^{-4} M, which is probably less than the K for the second mole of phosphate. The manner in which the arginyl residue and the functional zinc ion participate in the catalytic mechanism of alkaline phosphatase remains unresolved. Recent studies on the interaction of methylguanidine and phosphate (Cotton *et al.*, 1973) show that the structure of the guanido group is remarkably well suited to interact strongly with phosphate through hydrogen bonds, and also suggest the possibility that this interaction might accelerate the hydrolysis reaction. Neutralization of negative charges on the phosphate is thought to partially polarize it by drawing negative charge from the phosphorus atom, thus facilitating nucleophilic attack. It is tempting to speculate that the attacking nucleophile might be the hydroxyl group of the specific seryl residue thought to form a phosphoryl-enzyme intermediate during catalysis (Engström, 1962).

The completely parallel behavior of hydrolase and transferase activities upon arginine modification further supports a role for arginine in substrate binding. Binding of substrate is the most obvious common step in the mechanism of both hydrolase and transferase reactions. Almost all other chemical modifications of alkaline phosphatase performed thus far (Plotch and Lukton, 1965; Tait and Vallee, 1966; Christen *et al.*, 1970) alter hydrolase and transferase activities differently. In those instances loss of activity might result from nonspecific changes such as *e.g.*, effect on the conformation of the active center. This hypothesis is consistent with the experiments of Attias *et al.* (1969) who isolated an active alkaline phosphatase-like protein from an arginine auxotroph of *E. coli* K-12, grown with the arginine analog canavanine [2-amino-4-(guanidinooxy)-butyric acid] in the absence of arginine. The enzymatic properties of this protein in which 60–85% of the original arginine was replaced by canavanine were rather similar to those of the native enzyme. However, while the specific transferase activities of the arginine and canavanine enzymes were almost equal, the specific hydrolase activity of the canavanine-containing enzyme was lower by more than 60%. Hence, modifications of arginines of alkaline phosphatase which do not affect its guanido

[2] ^{14}C-labeled butanedione is available commercially (Mallinckrodt) but only as a solution in glacial acetic acid. Difficulties in handling this material precluded its use in the present studies.

group result in specific functional effects more akin to those observed with most other chemical modifications. On the other hand, selective modification of the guanido groups of but two specific arginyl residues apparently affects both activities of the enzyme in an identical and unambigious fashion.

References

Attias, J., Schlesinger, M. J., and Schlesinger, S. (1969), *J. Biol. Chem. 244,* 3810.

Bloch, W., and Schlesinger, M. J. (1973), *J. Biol. Chem. 248,* 5794.

Christen, P., Vallee, B. L., and Simpson, R. T. (1971), *Biochemistry 10,* 1377.

Cotton, F. A., Day, V. W., Hazen, E. E. and Larsen, S. (1973), *J. Amer. Chem. Soc. 95,* 4834.

Daemen, F. J. M. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32,* 473 Abstr.

Dayan, J., and Wilson, I. B. (1964), *Biochim. Biophys. Acta 31,* 620.

Engström, L. (1962), *Biochim. Biophys. Acta 56,* 606.

Fuwa, K., Pulido, P., McKay, R., and Vallee, B. L. (1964), *Anal. Chem. 36,* 2407.

Gottesman, M., Simpson, R. T., and Vallee, B. L. (1969), *Biochemistry 8,* 3776.

Knox, J. R., and Wyckoff, H. W. (1973), *J. Mol. Biol. 74,* 533.

Lazdunski, C., Petitclerc, C. and Lazdunski, M. (1969), *Eur. J. Biochem. 8,* 510.

Pigretti, M. M., and Milstein, C. (1965), *Biochem. J. 94,* 106.

Plocke, D. J., Levinthal, C., and Vallee, B. L. (1962), *Biochemistry 1,* 373.

Plotch, S., and Lukton, A. (1965), *Biochim. Biophys. Acta 99,* 181.

Reid, T. W., and Wilson, I. b. (1971), *Enzymes, 4, 3rd Ed.,* 373.

Riley, H. A., and Gray, A. R. (1947), *in* Organic Syntheses, Collect. Vol. II, Blatt, A. H., Ed., New York, N. Y., Wiley, p 509.

Riordan, J. F. (1973), *Biochemistry 12,* 3915.

Rothman, F., and Byrne, R. (1963), *J. Mol. Biol. 6,* 330.

Schlesinger, M. J., and Barrett, K. (1965), *J. Biol. Chem. 240,* 4284.

Schwartz, J. H., and Lipmann, F. (1961), *Proc. Nat. Acad. Sci. U. S. 47,* 1996.

Simpson, R. T., and Vallee, B. L. (1968), *Biochemistry 7,* 4343.

Simpson, R. T., and Vallee, B. L. (1970), *Biochemistry 9,* 953.

Simpson, R. T., Vallee, B. L., and Tait, G. H. (1968), *Biochemistry 7,* 4336.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.

Tait, G. H., and Vallee, B. L. (1966), *Proc. Nat. Acad. Sci. U. S. 56,* 1247.

Takahashi, K. (1968), *J. Biol. Chem. 243,* 6171.

Thiers, R. E. (1957), *Methods Biochem. Anal. 5,* 273.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.

Yang, P. C., and Schwert, G. W. (1972), *Biochemistry 11,* 2218.

Yankeelov, J. A. (1972), *Methods Enzymol. 25,* 566.

Yankeelov, J. A., Jr., Mitchell, C. D., and Crawford, T. H. (1968), *J. Amer. Chem. Soc. 90,* 1664.

An Active Proteolytic Fragment of *Gonyaulax* Luciferase†

Neil Krieger,‡ David Njus,§ and J. W. Hastings*

ABSTRACT: Soluble extracts of the bioluminescent dinoflagellate *Gonyaulax polyedra* contain luciferase activity in several molecular weight forms (400,000, 130,000, and 35,000). The active monomeric chain *in vivo* has a molecular weight of about 130,000, as shown by extraction followed by gel filtration, both in 6 M guanidine·HCl. The 400,000 molecular weight form includes at least one chain of the 130,000 molecular weight species, while the 35,000 molecular weight species is a lytic fragment thereof. When extractions are made in buffer at pH 6, all the luciferase is obtained in the lowest molecular weight form due to an endogenous lytic enzyme most active at acid pH's. Digestion of the 400,000 molecular weight species with a low concentration of subtilisin also produces the 35,000 molecular weight form. In both cases the low molecular weight form is heterogeneous, including active species which can be distinguished both by ion-exchange chromatography and by differences in their pH–activity profiles.

Studies concerning the molecular mechanism of circadian rhythms have been hampered by a lack of molecular correlates. In the marine dinoflagellate, *Gonyaulax polyedra,* there is a rhythm of extractable luciferase activity which correlates with a well-defined circadian rhythm of bioluminescence (Hastings and Sweeney, 1957; Hastings and Bode, 1962). Recent experiments concerned with the molecular basis for this activity rhythm were equivocal, partly because the enzymatic activity occurs in several molecular forms whose interrelationships were not well understood (McMurry, 1971; McMurry and Hastings, 1972). The present study clarifies this matter.

The *in vitro* light-producing system from *Gonyaulax* was first characterized by Hastings and Sweeney (1957) in the supernatant from a 36,000 *g* cell extract. The activity was shown to require a dialyzable heat-stable substrate (*Gonyaulax* luciferin), a heat-labile protein (*Gonyaulax* luciferase), and oxy-

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‡ N. I. H. Predoctoral Trainee, Department of Biochemistry and Molecular Biology. Present address: Department of Pharmacology, Yale University School of Medicine, New Haven, Conn.

§ N. I. H. Predoctoral Trainee in Biophysics.