The Reversible Dissociation of the Alkaline Phosphatase of Escherichia coli

I. FORMATION AND REACTIVATION OF SUBUNITS*

Milton J. Schlesinger† and Kathleen Barrett

From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts

(Received for publication, June 9, 1965)

Current investigations of protein structure reveal that an increasing number of enzymes occur in their native state as organized aggregates of polypeptide chains (1, 2). Although it has long been known that some proteins are composed of subunits, the extent and significance of this feature of protein structure has only been recently recognized. For example, it now appears that the mechanism of feedback control, whereby a metabolite unrelated to the substrate or product of an enzymic reaction can affect the activity of that enzyme, consists of an interaction of polypeptide chains that leads to conformational changes at the catalytic site (3–6). For a number of enzymes, this activation or inhibition is observed to alter the molecular weight of the protein and indicates an association or dissociation of polypeptide chains (7–15).

Studies on the interaction of subunits and their role in providing for the tertiary structure of proteins have been greatly facilitated by the recognition that the reversible denaturation of enzymes consists, in many cases, of the dissociation of the protein into subunits (16–27). The alkaline phosphatase of Escherichia coli is an example of such an enzyme. Previously, it had been observed that this protein, which is composed of two identical polypeptide chains (28), could be reversibly denatured by thiol reduction in the presence of urea (29). This treatment led to the dissociation of the dimer. In continuing these studies with proteins antigenically related to alkaline phosphatase that were purified from alkaline phosphatase-negative mutants of E. coli, it was found that acid treatment could reversibly dissociate these proteins (30). Experiments were then carried out to study the effect of acid on the alkaline phosphatase derived from wild-type E. coli. The results of this investigation are presented here, and they provide information on the role of the subunit in the structure and function of this enzyme and on the mechanism of dimer formation from the polypeptide chains. Some of these results have been presented in brief form elsewhere (31, 32).

EXPERIMENTAL PROCEDURE

Methods

Cell Growth and Enzyme Purification—Alkaline phosphatase was purified from lysozyme-prepared protoplasts of E. coli, strain cw 3747, according to methods previously reported (29) except that the cells were grown in 50-liter batches in a fermenter. After protoplast formation and removal of cells by centrifugation, the supernatant solution was dialyzed against 0.01 M Tris-Cl, pH 7.4, and 0.001 M MgCl₂ and subsequently heated at 67° for 30 min in the presence of 0.01 M MgCl₂. Denatured protein was removed by centrifugation, and the supernatant solution was dialyzed against 0.01 M Tris-Cl, pH 7.4, and 0.001 M MgCl₂. The enzyme was adsorbed to a DEAE-cellulose column (1 ml of DEAE-cellulose packed under 10 pounds of pressure for each 50 units of absorbance at 280 μμ) and was eluted with a linear gradient of 0 to 0.4 M NaCl in 0.01 M Tris-Cl, pH 7.4, and 0.001 M MgCl₂. Fractions with high, constant specific enzymic activity were pooled and rechromatographed under conditions identical with those above. The tubes of enzyme with high constant specific activity were pooled, and solutions were dialyzed against 0.001 M NH₄HCO₃ and lyophilized. The enzyme is judged 95% pure according to starch gel electrophoresis and its specific enzyme activity. The lyophilized powder was redissolved in deionized water and kept frozen.

Enzyme Assay—Enzyme was added to p-nitrophenyl phosphate (0.2 mg per ml) in M Tris, pH 8.0, at 37°. After development of color, K₂HPO₄ (0.2 to 0.25 ml of 13% solution per ml of substrate) was added to stop the reaction and the absorbance measured at 410 μμ. One unit is that amount of enzyme that leads to the formation of 1 absorbance unit per min measured in a cuvette of 1-cm path length. The assay conditions were periodically checked by measuring the initial rate of p-nitrophenol production to make sure that no reactivation or inactivation of enzyme was occurring in the assay tubes.

Sedimentation Velocity and Sedimentation Equilibrium Studies—Analyses were performed on a Spinco model E ultracentrifuge, equipped with a phase plate as a schlieren diaphragm. For equilibrium experiments, a double sector cell with 10-mm optical path was used with a 2-mm column. Photographic plates (spectroscopic F III, Kodak) were analyzed with a Gaertner microcomparator. The acidified sample (3.1 mg per ml) was run at 2° for 14 hours at a rotor speed of 11,573 rpm. The untreated enzyme was centrifuged at a concentration of 4 mg per ml at 3° at 9945 rpm for 16 hours before photographs were made. The data were analyzed according to the method of Van Holde and Baldwin (33). The curve of (dc/dr)/r with respect to Δn gave a straight line, indicating that the preparation was homogeneous and that equilibrium had been attained.

Sedimentation velocity experiments were conducted at con-
stant temperature (6-8°) with rotor speeds of 59,780 rpm. Schlieren patterns were photographed usually at 4-min intervals at periods of 30 min and 2 hours after speed had been reached. The observed coefficients were corrected to values corresponding to a solvent with viscosity and density of water at 20° (η\textsubscript{20}, \rho). Kodak metallographic plates were used. The data were analyzed on a digital computer with a least squares program.¹

**Zinc Exchange—**Enzyme (40 μg) was incubated in the presence of 5 μmoles of NaOH, 5 μmoles of sodium malonate, pH 2.3, 16 μmoles of Tris-Cl, pH 7.4, and 0.1 μmole of \(^{65}\text{ZnSO}_4\) (140,000 cpm) in a total volume of 1 ml at 37° for 30 min. Then 0.9 ml was added to a column (0.8 x 10 cm) of Sephadex G-25 and the column was eluted with 0.02 m Tris-Cl buffer, pH 7.4. The flow rate was 0.5 ml per min for the first 12 ml and then was increased to 2 ml per min. In an identical reaction mixture, 40 μg of acid-inactivated enzyme were incubated. After 30 min, at 37°, 30% of the enzymic activity was recovered, and 0.9 ml of this reaction mixture was poured on the Sephadex G-25 column and fractions were collected as indicated above.

**Radioactivity Measurements—**\(^{34}\text{C}\) was determined with a low background, end window gas flow counter. \(^{65}\text{Zn}\) was measured with an NaI crystal well counter.

**Protein Determination—**The concentration of purified enzyme was determined by measuring the absorbance at 280 nm, assuming a value of 0.770 equals 1 mg per ml (28).

**Materials**

**Buffers—**All Tris buffer solutions were adjusted to the proper pH with either HCl or sodium acetate. The final volume is adjusted so that the stated concentration is that of Tris.

**Chemicals—**Sources of chemicals used were as follows: p-nitrophenyl phosphate, Sigma; DEAE-cellulose, guanidine-HCl, EDTA disodium salt, hexadecyltrimethylammonium bromide, 2-mercaptoethanol, and thioglycolic acid (distilled prior to use), Eastman Organic Chemicals; sodium dodecyl sulfate (95%), Matheson Coleman and Bell; Brij 56 (polyoxyethylene (10) cetyl ether), Atlas Chemical Industries, Inc.; 1,10-phenanthroline, The G. Frederick Smith Chemical Company; RNase, Worthington; human serum albumin, Pentex; fluorescein mercuric acetate, gift of Dr. F. Karush; 1\(^{14}\)C-iodoacetic acid, New England Nuclear; 1\(^{14}\)C-Iodoacetamide, Tracerlab; \(^{65}\text{Zn}, \) Nuclear Science and Engineering Corporation.

**RESULTS**

**Reversible Denaturation by Acid—**The alkaline phosphatase of E. coli is an exceptionally stable protein (34); however, incubation of the enzyme at low pH leads to a loss of enzymic activity that can subsequently be recovered. Studies of the mechanism of this inactivation indicate that the denaturation process is complex and consists of at least two different reactions (Fig. 1). At pH values between 5.0 and 3.0 and at 0°, there is an initial rapid but limited loss of activity followed by a much slower denaturation. The initial rate and extent of inactivation under these conditions is relatively unaffected by protein concentration, but increasing ionic strength leads to a greater extent of denaturation. For example, with 25 μg per ml of enzyme at pH 3.15 in glycine buffer, after 60 min at 0°, 60% of the enzymic activity is retained at 0.05 ionic strength, compared to 32% present at 0.2 ionic strength. At 8 μg per ml (ionic strength = 0.05) 52% of the original activity remained. As the pH is lowered (below 3.0), the initial rate of inactivation increases rapidly. Here, the second denaturation process appears to become more rapid (see Fig. 1) and probably accounts for the increase in the initial rate. Higher protein concentrations and high ionic strength solutions were found to lead to increased rate of denaturation at these lower pH values.

The observation that a pH less than 3.0 was required to denature the enzyme rapidly suggested that there might be only a few uncharged carboxyl residues that are essential to proper tertiary structure. This is not the case, however, for a titration curve of the enzyme shows that at pH 3.0, there are still about 40 carboxyl groups remaining to be titrated (Fig. 2). These groups, which are not titrated at pH 3.0 in the native protein, become susceptible to titration at this pH after the enzyme has once been exposed to pH 2.3. This very pronounced hysteresis effect

¹ The computer program was devised by Mr. E. Eikenberry at Massachusetts Institute of Technology.
FIG. 2. Acid titration of alkaline phosphatase. Enzyme, previously dialyzed against 0.1 M KCl, was titrated with 0.1 N HCl in a difunctional recording titrator (International Instruments), at 15°. At indicated points, 10-μl aliquots were removed into 5.0 ml of cold 0.05 M Tris-Cl buffer, pH 7.4, and assayed for activity. The addition of acid was stopped at pH 2.3, and the enzyme was allowed to denature further for 20 min. Alkali (0.082 N KOH) was then added until neutrality was attained. The addition of acid and alkali was at a constant rate of 0.01 ml per min. The original solution contained 8.6 mg of enzyme in 1.0 ml of 0.1 M KCl. O-O, titration with acid; O-O, back titration with base; ▲▲, difference curve.

indicates that a large number of charged groups in the active enzyme are masked. Many of these are carboxyl residues, for the difference curve of Fig. 2 has a maximum at pH 3.2 and comes to zero at pH 5.6.

Kinetics of Reactivation—Enzymic activity that is lost as a result of treatment of the alkaline phosphatase at pH 2.0 can be rapidly recovered after neutralizing the solution and incubating the protein at 37°. Routinely, about 70% of the original activity is recoverable and in some cases this value is close to 100%.

The kinetics of reactivation were studied as a function of temperature, protein concentration, and the ionic environment. The rate-limiting reaction is temperature-dependent and obeys bimolecular kinetics (Fig. 3) over an 8-fold range of protein concentration. The Arrhenius plot of reaction velocity versus 1/T shows that these kinetics are valid over the temperature range of 15-37°, and an activation energy of about 12.9 Kcal per mole can be calculated for the reactivation (Fig. 4).

Effect of Ionic Environment on Reactivation—The initial rate of reactivation is sensitive to ionic conditions, particularly with regard to metal ions. In the presence of EDTA, recovery of active enzyme is almost completely inhibited. In addition, the rate of reactivation can be increased up to 7-fold when metals are added to the reaction mixture (Table I). Maximal rates and yields of recovered enzyme, when tested at 100 μg of protein per ml (1.2 × 10^-5 M) in 0.1 M Tris, pH 8.0, are observed with zinc ions at 10^-4 M. The optimum ratio of zinc to protein concentrations depends on several factors (e.g. pH, ionic strength, and the nature of the buffer), and no meaningful stoichiometry has been obtained.

Several other divalent cations can partially substitute for zinc. Cadmium (10^-3 M), manganese (10^-3 M), and cobalt (10^-2 M) are able to reactivate the enzyme to an extent that is 20 to 30% that observed with zinc. Magnesium at 10^-2 M is almost as effective as zinc with respect to yield, but the initial rates are much slower. As indicated below, the influence of metals on reactivation is related to the fact that the native alkaline phosphatase is a zinc-containing enzyme.

The rate and extent of reactivation of acid-denatured alkaline phosphatase is also affected by pH, ionic strength, and the nature of the buffer (Table I). Maximum rates are observed in the pH range of 6.0 to 7.0 at low ionic strengths. The importance of ionic strength to the rate of reactivation is further indicated by the effect of detergents on renaturation. The nonionic detergent, Brij, actually stimulates reactivation (Tables I and IV). In contrast, ionic detergents appear to inactivate the acid-denatured enzyme completely. Phosphate buffers inhibit the rate and extent of reactivation and this effect is not due to inhibition by phosphate of the actual enzyme assay (34, 35). The molar ratio of phosphate to protein required for inhibition is very high, on the order of 10^6, and lower ratios (as used in reactivation)

![Graph](image_url)

FIG. 3. Initial rate of enzyme reactivation as a function of total protein concentration. Enzyme that was inactivated at pH 2.2 at 0° was incubated at the temperature indicated in the presence of 0.1 M Tris-Cl (pH 7.4)-0.00005 M ZnCl_2-0.001 M MgCl_2. Enzymic activities were determined at short intervals during the first 5 min and initial rates were measured. O, rates determined after a 10-hour preincubation at 100 μg per ml of enzyme in 0.1 M Tris-Cl (pH 7.4)-0.001 M EDTA, at 37°. In the reactivation of these samples, the ZnCl_2 concentration was adjusted so that it was twice that of the EDTA in the sample. The dashed curve is the reactivation of enzyme denatured by 6 M guanidine-HCl. In this case, the protein was diluted 100-fold in order to measure reactivation; temperature was 27°.
influence only the rate of reactivation. This inhibition by phosphate is probably not related to the fact that synthesis of the enzyme in wild-type strains of E. coli is repressed when inorganic phosphate is in the medium (36). The phosphate concentrations must be extraordinarily high in order to prevent reassociation, and, in addition, the subunits are not able to bind phosphate in a manner similar to that of the active dimer.2 Possibly, the phosphate inhibition is related to the zinc requirement, and at high concentrations of phosphate insufficient metal is available for reassociation.

Nature of Acid-inactivated Enzyme—In previous studies with mutational altered alkaline phosphatase proteins, it was observed that mild acidification led to the dissociation of the protein into subunits (30). We present here further evidence that the product of the low pH denaturation reaction is a subunit of enzyme. The molecular weight of the protein after treatment at pH 2.3 was determined by sedimentation equilibrium in 0.1 M sodium acetate, pH 4.0, and 10^-3 M EDTA and was found to be 43,000. Untreated enzyme in 0.1 M Tris, pH 7.4, was found to have a molecular weight of 86,000. These values are calculated on the assumption that both enzyme and subunit have a partial specific volume of 0.73, based on the amino acid composition (34). The acidified protein after centrifugation had 8% residual activity and was reactivatable to 62% the original activity.

The sedimentation coefficients of the acidified protein at low pH, at pH 4.0, and at pH 7.4 in the presence of EDTA are presented in Table II, together with s_{20,w} values of other preparations of this protein. The actual patterns of four of the samples are shown in Fig. 5. The subunit of the enzyme can apparently exist in two distinct conformations: one with an s_{20,w} of 2.3 and the other with an s_{20,w} of 3.4. In addition, the alkaline phosphatase subunit has a number of other physical and chemical properties that distinguish it from the intact protein. These are presented in the accompanying paper (37).

Role of Zinc Ions—The E. coli alkaline phosphatase is a zinc metalloprotein with 2 atoms of zinc per dimer (38). The stimulation of reactivation of acid-treated material by zinc ions and the demonstration that EDTA prevents reactivation suggested that (a) acidification leads to a release of zinc atoms and (b) the metal may be essential for the dimerization reaction.

The loss of zinc upon acidification of the protein was determined by measuring the extent to which ^{65}Zn could be bound by

<table>
<thead>
<tr>
<th>Condition varied</th>
<th>Initial rate</th>
<th>Extent of untreated enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Presence of Zn^{2+}, ionic strength = 0.025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH = 5.4+</td>
<td>1.34</td>
<td>40</td>
</tr>
<tr>
<td>pH = 5.4−</td>
<td>0.34</td>
<td>27</td>
</tr>
<tr>
<td>pH = 6.3+</td>
<td>7.0</td>
<td>76</td>
</tr>
<tr>
<td>pH = 6.3−</td>
<td>1.0</td>
<td>63</td>
</tr>
<tr>
<td>pH = 7.7+</td>
<td>2.6</td>
<td>67</td>
</tr>
<tr>
<td>pH = 7.7−</td>
<td>0.46</td>
<td>53</td>
</tr>
<tr>
<td>2. Ionic strength, pH = 7.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.016</td>
<td>3.0</td>
<td>70</td>
</tr>
<tr>
<td>0.04</td>
<td>2.2</td>
<td>70</td>
</tr>
<tr>
<td>0.08</td>
<td>1.9</td>
<td>77</td>
</tr>
<tr>
<td>0.16</td>
<td>1.3</td>
<td>80</td>
</tr>
<tr>
<td>3. pH, ionic strength = 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.1</td>
<td>4.8</td>
<td>60</td>
</tr>
<tr>
<td>6.8</td>
<td>2.8</td>
<td>50</td>
</tr>
<tr>
<td>7.0</td>
<td>3.4</td>
<td>50</td>
</tr>
<tr>
<td>7.4</td>
<td>2.3</td>
<td>47</td>
</tr>
<tr>
<td>4. Detergents, pH = 7.4, ionic strength = 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brij 56</td>
<td>1.1</td>
<td>80</td>
</tr>
<tr>
<td>Hexadecyltrimethylammonium bromide</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>5. Phosphate buffer, pH 7.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 M</td>
<td>0.02</td>
<td>29</td>
</tr>
<tr>
<td>0.05 M</td>
<td>0.03</td>
<td>46</td>
</tr>
<tr>
<td>0.025 M</td>
<td>0.07</td>
<td>48</td>
</tr>
</tbody>
</table>

* M. J. Schlesinger, unpublished results.
TABLE II

Sedimentation coefficients of various forms of alkaline phosphatase

Enzyme was titrated to pH 2.0 at 0° with 0.1 N HCl and diluted into the various buffers to give a concentration varying from 1 to 4 mg per ml. In the case of EDTA denaturation, enzyme at 2 mg per ml was dialyzed for 2 days against 4 changes of 0.1 M Tris-Cl, pH 7.4, and 0.001 M EDTA buffer. Enzymic activity was determined after the centrifugation.

<table>
<thead>
<tr>
<th>Treatment; buffer</th>
<th>20S, S</th>
<th>Enzymic activity</th>
<th>Sample</th>
<th>Reactivated</th>
</tr>
</thead>
<tbody>
<tr>
<td>None; 0.1 M Tris-Cl, pH 7.4</td>
<td>6.1</td>
<td>1100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 2.0; 0.01 M HCl, 0.1 M KCl</td>
<td>2.3</td>
<td>5</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td>pH 2.0; 0.1 M sodium acetate, pH 4.2, 0.001 M EDTA</td>
<td>3.4</td>
<td>55</td>
<td>570</td>
<td></td>
</tr>
<tr>
<td>pH 2.0; 0.1 M Tris-Cl, pH 7.4, 0.001 M EDTA</td>
<td>3.7</td>
<td>150</td>
<td>850</td>
<td></td>
</tr>
<tr>
<td>Reactivated; 0.1 M Tris-Cl, pH 7.4</td>
<td>6.1</td>
<td>815</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001 M EDTA; 0.1 M Tris-Cl, pH 7.4, 0.001 M EDTA</td>
<td>5.8</td>
<td>&lt;0.01</td>
<td>1170</td>
<td></td>
</tr>
</tbody>
</table>

the native enzyme and by the enzyme after dissociation and reassociation. After incubation for 30 min in the presence of ^{65}Zn, the various reaction mixtures were applied to a Sephadex G-25 column and the elution profile of ^{65}Zn was determined (Fig. 6). For the native enzyme less than 7% of the added ^{65}Zn is recovered in the active enzyme in contrast to the acidified material where close to 50% of the added isotope is recovered in the region of the reactivated enzyme.

The specific activities (counts per min per unit) were 13 for the native enzyme and 107 for the acid-treated protein. All the enzymic activity (90 to 100% of that added) was recovered in the 3- to 6-ml fractions of the column. When a sample of enzyme was incubated at pH 4.4 (Tris-acetate buffer) at 0° for 30 min in the presence of ^{42}PZn and subsequently incubated for 30 min at pH 7.4 at 37°, only about 1% of zinc was eluted from the column in the enzyme region. This enzyme had 85% of the native activity after pH 4.4 incubation. The lack of zinc exchange at this intermediate pH value is discussed below.

To test whether zinc is essential for association of subunits or activates a preformed dimer, high concentrations of acidified enzyme were incubated in the presence of EDTA at 37° at neutral pH. Aliquots were then diluted at different protein concentrations into a buffer containing zinc, and the rate of reactivation was determined. The data for two different concentrations are plotted in Fig. 3 (circles on the 21° reactivation curve). They fall on the line plotted for bimolecular kinetics and indicate that subunits are unable to dimerize in the presence of EDTA. The inability of the subunits to associate in the presence of EDTA is further suggested by the subunit value of acid-treated enzyme tested at pH 7.4, 0.001 M EDTA (Table II). Thus, the zinc atoms are essential for the dimerization process.

Sulfhydryl Groups—According to amino acid analysis of the alkaline phosphatase (28), there are about 7 cysteine residues per dimer. In order to test whether these are present as free —SH or as —S—S— bridges, two sets of experiments were carried out. In Table III, we note that there are 4 disulfide residues detectable by a method which measures the quenching of fluorescence of fluorescein mercuric acetate (39). There are no free —SH residues detectable in either the native or guanidine-denatured enzyme by this method. Consistent with these data are the results of alkylation experiments in which ^{14}C-iodoacetic acid or ^{14}C-iodoacetamide were incubated with various forms of the alkaline phosphatase. Only when the enzyme had been

*Fig. 5. Sedimentation pattern of the different forms of alkaline phosphatase. Top to bottom: native enzyme in 0.01 M Tris-Cl (pH 7.4); 0.001 M MgCl₂; acid-denatured enzyme in 0.01 M HCl-0.1 M KCl; acid-denatured enzyme in 0.05 M sodium acetate, pH 4.0; guanidine-denatured enzyme in 0.01 M Tris-Cl (pH 7.4)-0.001 M EDTA-1.3 M guanidine-HCl. All preparations (3.8 mg of protein per ml) were centrifuged at 59,950 rpm at 7°. Photographs were taken approximately 35 min after reaching speed. Enzymic activities were, respectively, 830, 23, 23, <1 units per mg, measured after centrifugation.*
subjected to thiol reduction was there significant alkylation (Table IV). Enzyme that had been denatured by acid, guanidine, EDTA, or o-phenanthroline had less than 0.5 —SH residue per dimer. In contrast, reduced material can incorporate 9 to 10 alkyl groups. These results are also consistent with data which showed that sulfhydryl-binding agents had no effect on the reactivation of alkaline phosphatase subunits (32) or on formation of active enzyme from mutationally altered subunits

Addition of mercaptoethanol to the guanidine solution gave an amount of quenching very close to that measured in 0.05 M Tris-Cl alkaline phosphatase buffers at pH 7.4.

Human serum albumin was noted, and measured at 490 nm excitation and 530 nm emission. RNase was used as a standard for the N NaOH determinations and mercaptoethanol was the standard at pH 7.4 (0.05 M Tris-Cl) constant. The percentage of quenching was compared to the

minimations were made after the rate of quenching had reached of 0.1 to 0.6 mole of —S—S— or —SH per mole of reagent. Deter-

Details of the procedure are presented in "Methods." Untreated incubated at pH 7.4, O-O.

Fluorescein mercuric acetate was used at 10·6 M in the solution noted, and measured at 490 nm excitation and 530 nm emission. RNase was used as a standard for the N NaOH determinations and mercaptoethanol was the standard at pH 7.4 (0.05 M Tris-Cl) and in the guanidine solutions. Quenching was linear for ratios of 0.1 to 0.6 mole of —S—S— or —SH per mole of reagent. Determinations were made after the rate of quenching had reached reached zero (15 min) or, in the case of guanidine, had become constant. The percentage of quenching was compared to the standard curve in order to obtain the —S—S— or —SH residues. Addition of mercaptoethanol to the guanidine solution gave an amount of quenching very close to that measured in 0.05 M Tris-Cl buffers at pH 7.4.

**TABLE III**

**Sulfhydryl determination with fluorescein mercuric acetate**

Fluorescein mercuric acetate was used at 10·6 M in the solution noted, and measured at 490 nm excitation and 530 nm emission. RNase was used as a standard for the N NaOH determinations and mercaptoethanol was the standard at pH 7.4 (0.05 M Tris-Cl) and in the guanidine solutions. Quenching was linear for ratios of 0.1 to 0.6 mole of —S—S— or —SH per mole of reagent. Determinations were made after the rate of quenching had reached reached zero (15 min) or, in the case of guanidine, had become constant. The percentage of quenching was compared to the standard curve in order to obtain the —S—S— or —SH residues. Addition of mercaptoethanol to the guanidine solution gave an amount of quenching very close to that measured in 0.05 M Tris-Cl buffers at pH 7.4.

<table>
<thead>
<tr>
<th>Material and concentration</th>
<th>Solution</th>
<th>—SH residues/mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>N NaOH</td>
<td>3.8</td>
</tr>
<tr>
<td>0.7 · 10⁻³ M</td>
<td>N NaOH</td>
<td>4.2</td>
</tr>
<tr>
<td>1.0 · 10⁻³ M</td>
<td>N NaOH</td>
<td>4.2</td>
</tr>
<tr>
<td>1.5 · 10⁻³ M</td>
<td>pH 7.4, 0.5 M</td>
<td>0.1</td>
</tr>
<tr>
<td>3 · 10⁻⁷ M</td>
<td>pH 7.4, 0.6 M</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>3 · 10⁻⁷ M</td>
<td>pH 7.4, 0.6 M</td>
<td>guanidine-HCl</td>
</tr>
<tr>
<td>RNase</td>
<td>pH 7.4, 0.05 M</td>
<td>0.1</td>
</tr>
<tr>
<td>3 · 10⁻⁷ M</td>
<td>pH 7.4, 0.6 M</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>pH 7.4, 0.05 M</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* The activity of the enzyme was 1% native at the time the —SH determination was made.

**FIG. 6** Separation of ⁶⁵Zn-labeled enzyme by Sephadex G-25. Details of the procedure are presented in "Methods." Untreated incubated at pH 7.4, O-O.

**TABLE IV**

Sulfhydryl group determination of alkaline phosphatase by ⁴⁰C-alkylation

In Treatment 1, ¹⁴C-iodoacetate (2 µC per pmole) was allowed to react with 2.5 mg of enzyme at a reagent to protein ratio of 40:1. In the case in which thioglycolate was used, 1.5 mg of enzyme were used and an iodoacetate to total thiol ratio of 1:5:1 was used. After a 20-min incubation at 23°C in 0.1 M Tris-Cl, pH 8.0, mercaptoethanol was added in excess, and the reaction mixtures were dialyzed against two changes of 0.002 M NH₄HCO₃. Aliquots were precipitated with 5% trichloroacetic acid and counted. In Treatment 2, ¹⁴C-iodoacetamide (1.53 µC per pmole) was allowed to react with enzyme at a molar ratio of reagent to protein of 400:1 except in the presence of thioglycolate when a molar ratio of 1:1 of reagent to thiol was used. Reduction was allowed to proceed for 4 hours, and reaction with reagent occurred at 23°C for 30 min. A 0.1-mg portion of m mercaptoethanol was added, and the material was dialyzed against two changes of 0.005 M NH₄HCO₃. In all reaction mixtures, 1 mg of protein was used.

<table>
<thead>
<tr>
<th>Preincubation treatment</th>
<th>cpm/mg protein</th>
<th>Enzymic activity prior to alkylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thioglycolate + 8 M urea</td>
<td>120,000</td>
<td>520</td>
</tr>
<tr>
<td>Thioglycolate + 8 M urea</td>
<td>160,000</td>
<td>7</td>
</tr>
<tr>
<td>Thioglycolate + 6 M guanidine-HCl</td>
<td>112,000</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Thioglycolate + 6 M guanidine-HCl</td>
<td>190</td>
<td></td>
</tr>
<tr>
<td>Thioglycolate + 6 M guanidine-HCl</td>
<td>2,380</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Thioglycolate + 6 M guanidine-HCl</td>
<td>5,400</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Thioglycolate + 6 M guanidine-HCl</td>
<td>5,450</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Thioglycolate + 6 M guanidine-HCl</td>
<td>2,200</td>
<td>1,650</td>
</tr>
</tbody>
</table>

These observations together with the evidence that acidification or exposure to 0 M guanidine can dissociate the enzyme indicates that the disulfide bridges of the alkaline phosphatase are intrachain.

**Nature of Reactivated Enzyme**—The renatured protein is very similar to the native enzyme. No differences are observed between the native and reactivated enzyme when analyzed by starch gel electrophoresis, ultracentrifugation (see Table III for the reactivated enzyme when analyzed by starch gel electrophoresis, ultracentrifugation (see Table III for the nature of Reactivated Enzyme). The resistance to proteolysis and the heat stability of the native and renatured preparations are comparable when examined after 10 min at 85°C. The Kₘ for p-nitrophenyl phosphate (1.8 · 10⁻⁴ M) and Kᵢ for phosphate (a competitive inhibitor, Kᵢ = 5.6 · 10⁻⁵ M) are also identical. In only one test has the renatured enzyme proved to be distinguishable. In the assay solution (M Tris, pH 8.0) the rate of formation of nitrophenol becomes nonlinear after 5 min at 37°C unless the renatured enzyme is pretreated with magnesium or is assayed in the presence of magnesium. The reason for this difference is not completely understood; however, metals such as magnesium and zinc are known to protect enzyme against heat denaturation (34).

**Reversible Denaturation by Other Methods**—In the course of these investigations we observed that the alkaline phosphatase could be reversibly denatured by heat and by 6 M guanidine-HCl. Both of these treatments appear to inactivate the enzyme by...
Reversible Dissociation of Alkaline Phosphatase of E. coli. I

Vol. 240, No. 11

Reversible dissociation of the dimer, although we cannot yet rule out other forms of inactivation. Reversible denaturation by heat is accomplished by brief treatment at high temperatures (91–93°) in the absence of magnesium. Under such conditions, the enzyme is extensively inactivated (25% activity remains) and with cooling it is renatured to 60% of the original activity. Trypsin inhibits the reactivation. The guanidine-inactivated product is denatured and, for guanidine at 0.1 M, inhibits re-formation of acid-dissociated enzyme as well as the guanidine-denatured product. The reactivation of guanidine-inactivated enzyme must be dialyzed, or greatly diluted, for guanidine at 0.1 M inhibits re-formation of acid-dissociated enzyme as well as the guanidine-denatured product. The reactivation of guanidine-inactivated enzyme obeys bimolecular kinetics (Fig. 3, dashed line), and the sedimentation pattern is identical with acid-treated enzyme (Fig. 5). The alkaline phosphatase is not denatured by 6 M urea at 0°, but this concentration of urea inhibits enzymic activity when present in the assay solution.

Previously, it has been reported that EDTA can reversibly inactivate the alkaline phosphatase (34, 38). The denatured product in this case, however, appears to be still a dimer molecule, for the $s_{20,w}$ value remains almost the same as that of the native enzyme (Table II). Furthermore, the denatured material is instantaneously reactivated at concentrations as low as 0.03 µg per ml when excess zinc is added to the enzyme.

**DISCUSSION**

The E. coli alkaline phosphatase can be reversibly denatured by at least three distinct mechanisms, each leading to a different form of inactive protein. (a) The experiment of Levinthal et al. (29) indicated that the inactive product formed by thiol reduction in the presence of 8 M urea is a subunit containing free sulfhydryl residues. Reactivation of this material to active enzyme required the presence of a thiol-containing buffer, and one of the reactions was the reoxidation of the sulfhydryl group. This part of the renaturation may be analogous to the reactivation of reduced ribonuclease (40, 41), with the formation of intrachain disulfide bonds. (b) The reversible denaturation of the enzyme that is brought about by simply lowering the pH to values less than 3.0 leads to subunits that are neither inactivated by sulfhydryl-binding agents nor alkylated by iodoacetamide or iodoacetate. Thus, the polypeptide chain with intact --S--S-- bridges represents another form of the reversibly denatured enzyme. This type of subunit also appears to be produced by heating briefly to 90° or by incubating the protein in 6 M guanidine hydrochloride. (c) Previously, Garen and Levinthal (34) observed that metal chelators, such as EDTA, reversibly inhibited the enzyme. Plocke et al. (38) studied this type of inactivation in considerable detail and showed that the alkaline phosphatase contained 2 zinc atoms that are essential to activity. Removal or blockage of the zinc led to inactivation; but in this case, the protein was rapidly reactivated on restoration of its metal content. Malamy and Horecker have found a similar effect with cysteine (42). Our finding that enzyme inactivated by EDTA or o-phenanthroline is a dimer indicates that the zinc atoms are not essential for maintaining the dimer form. In this respect, the alkaline phosphatase is distinct from the Bacillus subtilis amylase (43) and yeast alcohol dehydrogenase (44), both of which dissociate in the presence of o-phenanthroline. It should be pointed out that in our studies with this reagent, no measurements were made to determine that zinc was actually removed, although we followed the procedure of Plocke et al. (38), who measured the loss of zinc. Possibly, other chelators inactivate the enzyme by binding to and blocking the metal on the protein. The various forms of the alkaline phosphatase which have been examined here and the possible relation between them are noted in Fig. 7.

The existence of multiple forms of reversibly denatured alkaline phosphatase may provide some basis for explaining the complex character of the acid denaturation reaction. A number of experiments were carried out in order to determine whether the denatured protein obtained at pH 4.0 represented subunits in equilibrium with the dimer. This explanation seems unlikely since it was found that pepsin does not affect the extent of inactivation at pH 4.0, but yet is able to inactivate subunits (prepared by pH 2 treatment) that are incubated at pH 4.0 in the presence of this proteolytic enzyme. Further, such an equilibrium should be affected by the concentration of enzyme since the reassociation is a bimolecular reaction. It was noted, however, that there was little or no effect of protein concentration on the extent of inactivation at pH 4.0. Ultracentrifuge analysis of the inactivated enzyme at pH 4.0 shows essentially a single peak with sedimentation similar to normal enzyme. However, according to Gilbert (45) a monomer-dimer system in rapid equilibrium would produce only a single moving boundary during a sedimentation run.

It is possible that the two-stage inactivation could be due to

---

**Fig. 7. Various forms of the E. coli alkaline phosphatase**
inhomogeneity of the E. coli alkaline phosphatase. Purified preparations of this enzyme are found to be isoenzymic, containing at least three electrophoretically distinct forms (46, 47). One of these might be more easily denaturable than the others at the intermediate pH ranges.

Another explanation is that, initially, there is a loss of zinc from the protein. At a pH between 3 and 5 there would be an equilibrium established between zinc-free, enzymically inactive dimer and active enzyme. As the pH is lowered the zinc-free dimer dissociates into single chains. There are several observations, however, that do not support this view: EDTA at pH 4.0 had only a slight effect on enzyme inactivation and the presence of zinc did not show a significant protection. In addition, almost no exchange of $^{65}$Zn took place when enzyme was incubated at pH 4.4. Pigretti and Milstein (48) have reported a similar two-step complex pattern for acid inactivation of the E. coli alkaline phosphatase but, in their studies, zinc and phosphate did protect against denaturation at pH 4.0. Yet, their data also were not consistent with a simple equilibrium between active and inactive forms of the enzyme.

The kinetics of dimerization measured by return of enzymic activity show that the rate-limiting reaction is second order. Similar kinetics are reported for the association of subunits of swine muscle fumarase that had been dissociated into four subunits by urea (19). However, in the association of subunits of rabbit muscle aldolase to form the enzymically active trimer, half-cystine residues of alkaline phosphatase exist as disulfide bridges that are intrachain.

The foregoing scheme is consistent with several experimental observations, besides those reported here. First, Neu and Heppel (49) have recently shown that the E. coli alkaline phosphatase is released from the cell on treatment with EDTA and sucrose alone, and previously Malamy and Horecker (50) reported that the alkaline phosphatase was quantitatively released by lysozyme treatment of E. coli. These observations suggest that the enzyme lies exterior to the cell membrane and is perhaps associated noncovalently with cell wall structures. Second, no significant amount of active or "activatable" alkaline phosphatase has been detected on ribosomes or polyribosomes of E. coli. Third, the rate of dimerization of subunits is greatly affected by ionic strength and proceeds most rapidly in a low ionic strength buffer. The stimulation of reactivation by a nonionic detergent also supports the notion that a membrane-wall environment is more suitable for the association of subunits than is the cell cytoplasm. Finally, the experiments on intracistronic complementation demonstrate that subunits of this enzyme must be free to interact at some stage in the synthesis of the dimer.

From the complementation studies, it was observed that certain polypeptide chains of this enzyme, that differed in their amino acid composition (as a result of mutation), could interact to produce a partially active "hybrid" protein. Hybrid formation in vitro required that at least one of the participating dimers be first dissociated into subunits (30, 32). It seems unlikely that these different subunits, encoded by separate messenger RNAs, could interact while attached to ribosomes. An alternative model, which cannot be excluded on evidence so far obtained, is that dimerization occurs on the polysome and the intact enzyme diffuses rapidly into the region between cell wall and membrane. In such a case, however, complementation can only be explained by one of the mutationally altered dimers interacting at the ribosome with a differently mutated chain.

The reactivation of the acid-prepared monomers in vitro to active enzyme proceeds at a very rapid rate in buffers of low ionic strength or in the presence of a nonionic detergent. Together with the low activation energy measured, these observations suggest that dimerization in the cell does not require the presence of special enzyme-mediated reactions. It would, in fact, seem that the sequence of the amino acids in the polypeptide chain is sufficient to assure dimer formation. Only a requirement for a zinc atom is necessary in order to form active enzyme. Our studies with subunit prepared in vitro and the mechanism of dimerization suggest that this zinc requirement affords a possible mechanism for localizing and activating the final stages of synthesis of E. coli alkaline phosphatase.

**SUMMARY**

Treatment of purified preparations of the Escherichia coli alkaline phosphatase with dilute acid reversibly inactivates the enzyme. Kinetic studies indicate that the mechanism of inactivation is complex, but that, below pH 3.0, the enzyme dissociates into subunits. These enzymically inactive subunits have a reduced molecular weight half of that of the native enzyme and an $s_{20, w}$ of 2.3 at pH 2.0 (active protein, $s_{20, w} = 6.1$). Reassociation of acid-prepared subunits to active enzyme is a temperature-dependent, bimolecular reaction that is most rapid in low ionic strength buffers containing zinc ions. EDTA inhibits dimerization.

The acid titration curve of active enzyme shows a strong hysteresis effect suggesting that a large number of carboxyl residues are "masked" in the native protein. Acidification also releases the zinc ions that are essential for enzymic activity. The half-cystine residues of alkaline phosphatase exist as disulfide bridges that are intrachain.

High temperature and guanidine-HCl also dissociate the enzyme into subunits with properties similar to those formed by acid.

**Acknowledgments**—We wish to express our appreciation to Professor C. Levinthal and Dr. A. Torriani for their advice and help in the various phases of this investigation. Excellent technical assistance was provided by Mrs. M. Misch. The authors wish to thank Mr. E. Eikenberry and Dr. R. Noble for their assistance in the ultracentrifuge work.

**REFERENCES**


---

*2* A. Torriani, unpublished results.