Reversible Dissociation of *Escherichia coli* Alkaline Phosphatase at High pH Values

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The inactivation of *E. coli* alkaline phosphatase at high pH values has been investigated. This type of inactivation causes dissociation of the native dimeric enzyme to inactive monomers. This material has sedimentation properties similar to the subunits produced by acid treatment of the native enzyme.

The high pH inactivation is time dependent, and partially reversible.

Reactivation of inactivated enzyme is affected by the presence of zinc ion.

The catalytically active form of alkaline phosphatase from *E. coli* is a dimer consisting of two identical subunits and it is a zinc metalloenzyme. Several procedures for the formation of inactive monomers have been described:

1. According to Levinthal *et al.* thiol reduction in the presence of 8.0 M urea results in the formation of an inactive subunit.
2. The reversible denaturation of the enzyme at low pH produces identical and inactive monomers.
3. Heat treatment of the enzyme causes inactivation and subunits appear to be produced.
4. Incubation of the protein in 6.0 M guanidine-HCl produces inactivation, which appears to be due to the dissociation of the dimer.

However, treatment with metal chelating agents gives an inactive enzyme, which seems to retain the dimeric form.

In the course of metal-binding studies carried out in this laboratory, it has been observed that alkaline phosphatase at elevated pH values is inactivated and to eliminate the possibility of dissociation of the enzyme, the zinc binding could only be studied in a narrow pH region.

The purpose of this communication is to describe briefly several studies carried out, which indicate that the alkaline phosphatase of *E. coli* loses enzymatic activity and dissociates into subunits at high pH. The data presented here indicate that the inactivation at high pH is reversible under certain
circumstances and the extent of reactivation is affected by the addition of zinc ions.

The inactivation at high pH values—which has not been reported so far—is time dependent, and experiments carried out under such conditions must consider the possible effects of dissociation.

**EXPERIMENTAL**

*Protein.* Alkaline phosphatase of *Escherichia coli* was prepared and purified as described in an accompanying paper. Preparation of apophosphatase and method of reconstitution as described earlier. Preparation of apophosphatase and reconstitution as described earlier. Preparation of apophosphatase and method of reconstitution as described earlier.*

*Protein concentration measurements.* The protein concentration was measured spectrophotometrically at 278 nm.

*Enzyme activity measurements.* The enzyme activity was determined in 1.0 M Tris buffer, pH 8.0, at 27°C with 1.0 x 10^-3 M p-nitrophenylphosphate. In the case of metal-free experiments, 1.10-phenanthroline at the final concentration of 1.0 x 10^-4 M was included in the assay mixture to remove traces of contaminating heavy metal ions. A unit of enzyme was defined and the specific activity was expressed as described by Malamy and Horecker.

*Reagents.* p-Nitrophenyl phosphate disodium tetrahydrate from Sigma and Tris(hydroxymethyl)aminomethane, Tris “Sigma 121”, were used. Glycine-buffer substance (Merck analytical grade) was employed. Analytical grade salts were used without further purification as the source of metal ions. 1,10-Phenanthroline (Merck analytical grade) was used without further purification. All solutions were made with deionized water. To the metal-free experiments buffers were extracted with dithizone as described elsewhere. Chelex 100, 200—400 mesh, was a Calbiochem product.

**RESULTS**

*The pH dependence of alkaline phosphatase inactivation.* In this experiment, 2 ml aliquots of a stock enzyme solution containing 0.8 mg of protein per ml, in 0.01 M Tris-HCl buffer, pH 8.0, were adjusted to the various pH values by addition of 2 M NaOH with rapid stirring. The solutions were then incubated at 20°C for 8 min and assayed for phosphatase activity. All activity values are expressed relative to pH 8.0, where it is known that inactivation does not occur. Fig. 1 shows the results of the pH inactivation after 8 min at different values of pH.

*The relative enzyme activity of alkaline phosphatase in 0.1 M KNO₃ solutions at different pH, and after different times.* Aliquots, 2 ml, of a stock solution containing 0.5 mg of protein per ml in 0.1 M KNO₃, were adjusted to the indicated pH values by the addition of 2 M NaOH with concomitant rapid stirring. The solutions were then stored at 20°C for various periods and assayed for phosphatase activity at the indicated times. All activities are expressed relative to pH 8.0. For the results, see Table 1. At the same protein concentration as described above, experiments have been performed at different pH values in 0.1 M Glycine-NaOH buffer. Table 2 shows the results.

*The time course of inactivation of alkaline phosphatase at pH 12.7.* Fig. 2 gives the time course of enzyme inactivation at 20°C, pH 12.7, in 0.01 M Tris-HCl buffer. The volume of the sample was 2 ml, and the enzyme concentration 0.5 mg protein per ml. As a control, enzyme solution at pH 8.0 was used.

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Table 1. The relative enzyme activity of alkaline phosphatase in 0.1 M KNO₃ solutions at different pH values.

<table>
<thead>
<tr>
<th>pH</th>
<th>Relative enzyme activitya (%) at times</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 day</td>
</tr>
<tr>
<td>7.0</td>
<td>100</td>
</tr>
<tr>
<td>7.3</td>
<td>100</td>
</tr>
<tr>
<td>8.0</td>
<td>100</td>
</tr>
<tr>
<td>9.0</td>
<td>100</td>
</tr>
<tr>
<td>9.3b</td>
<td>100</td>
</tr>
</tbody>
</table>

a The specific activity of the native alkaline phosphatase was 2200 units. (For definition of unit, see Ref. 7.)
b Experiments conducted under the same experimental conditions at pH 13.0 and 13.8 show that there is no measurable activity after 4 min and 0.5 min, respectively.

Table 2. The relative enzyme activity of alkaline phosphatase in 0.1 M glycine-NaOH buffer of different pH values.

<table>
<thead>
<tr>
<th>pH</th>
<th>Relative enzyme activitya (%) at times</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 day</td>
</tr>
<tr>
<td>9.6</td>
<td>100</td>
</tr>
<tr>
<td>10.0</td>
<td>95</td>
</tr>
<tr>
<td>10.6</td>
<td>85</td>
</tr>
</tbody>
</table>

a The specific activity of the native alkaline phosphatase was 2200 units (a unit defined as in Ref. 7).

Fig. 1. The pH dependence of alkaline phosphatase inactivation. The specific activity of the native alkaline phosphatase used was 2200 units. For experimental details, see text. ○, inactivation of enzyme, measured 8 min after pH adjustment; ●, reactivation of enzyme, measured 180 min after readjustment of pH to 8.0, in the presence of 2.5 × 10⁻³ M ZnCl₂; ×, reactivation of enzyme measured 180 min after readjustment of pH to 8.0, no ZnCl₂ added.

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Fig. 2. The time course of inactivation of alkaline phosphatase at pH 12.7, in 0.01 M Tris-HCl buffer. The specific activity of the native alkaline phosphatase was 2200 units. For experimental details, see text.
The inactivation of apoenzyme at high pH, and the effect of the zinc on reactivation. The experiments described above were carried out with native alkaline phosphatase, which contains zinc. To obtain information on the possible role of zinc ions in the reactivation of the high pH inactivated enzyme, experiments were carried out on apophosphatase. The pH of 2 ml of apoenzyme solution (0.2 mg protein per ml) in 0.1 M KNO₃ in the presence of $3.0 \times 10^{-6}$ M 1,10-phenanthroline was adjusted with 2 M NaOH to pH 12.7. The solution was incubated at 20°C for 50 min and the activity measured as described, with the exception that $1.0 \times 10^{-4}$ M 1,10-phenanthroline was included in the substrate solution to reduce the effect of metal ion contamination. The sample did not show any enzyme activity. Ultracentrifugation carried out with 5.0 mg of protein per ml apophosphatase solution, in 0.1 M KNO₃, and in the presence of $5.0 \times 10^{-5}$ 1,10-phenanthroline shows that the apoenzyme is a subunit at pH 12.7. The pH of the apoenzyme solution was then adjusted with 2 M HCl to pH 8.0. No activity could be detected, and ultracentrifugation carried out at pH 8.0 the same way as described for pH 12.7 did not show the presence of any dimer. Then zinc, in excess of the 1,10-phenanthroline present, was added to the sample and the enzymatic activity was measured after different time intervals. The results of this experiment showed that about 6% of the original enzyme activity was regained 70 min after the addition of zinc to the alkaline-treated apophosphatase, and that the reactivation is time dependent (see Fig. 3). However, addition of zinc to the untreated apoenzyme under the same experimental conditions gives 100% of the original enzymic activity and the reactivation is instantaneous.

![Graph](image)

**Fig. 3.** Reactivation of alkali inactivated apophosphatase with zinc. The specific activity of the native alkaline phosphatase was 2200 units. For experimental details, see text.

**DISCUSSION**

Previously, Schlesinger et al.⁴ have reported that incubation of the *E. coli* alkaline phosphatase at low pH leads to a loss of enzymic activity. These authors also found that the inactive protein is a subunit of the enzyme.

In the present study an experiment has been described which demonstrates the inactivation of alkaline phosphatase at high pH. The inactivation process has been investigated only briefly; however, the data obtained indicate that the inactivation is time dependent (see Tables 1, 2 and Fig. 2). Table 1 provides information on the stability of the enzyme. While after 50 days the protein showed unchanged activity at pH 8.0, already a slight pH change altered the

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enzyme activity. This information is very important in connection with the storage of the enzyme.

When solutions of the high pH inactivated enzyme were subjected to ultracentrifugation, the results shown in Table 3 were obtained. The value of the sedimentation coefficient for the high pH inactivated enzyme is similar to the one reported by Schlesinger for low pH inactivated alkaline phosphatase. This indicates that at high pH the protein probably gives the same type of monomer as at low pH values. As shown in Table 3, in one case a lower value of the sedimentation coefficient was obtained. It is not fully understood if this represents an additional form of subunit formation or originates from some other changes in the protein.

Table 3. Sedimentation coefficients of alkaline phosphatase. Enzyme concentration: 5 mg protein/ml. Sedimentation velocity experiments were performed in a Spinco model E ultracentrifuge, at 20° and 59780 rpm. Alkaline phosphatase activity was measured before and after ultracentrifugation, and after reactivation.

<table>
<thead>
<tr>
<th>Alkaline phosphatase</th>
<th>Additives</th>
<th>pH</th>
<th>$s_{20,\text{w}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>0.01 M Tris-HCl</td>
<td>8.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Reactivated</td>
<td>0.1 M KNO$_3$ + 0.1 M HCl</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Native</td>
<td>0.01 M KNO$_3$ + 0.1 M NaOH</td>
<td>12.7</td>
<td>2.6$^a$</td>
</tr>
<tr>
<td></td>
<td>13.0</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.5</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>Reactivated</td>
<td>0.01 M Tris-HCl</td>
<td>8.0</td>
<td>6.0$^b$</td>
</tr>
<tr>
<td>Apophosphatase</td>
<td>0.1 M KNO$_3$ + 5.0 x 10$^{-3}$ M</td>
<td>12.7</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>+ 2 M HCl</td>
<td>8.0</td>
<td>2.6</td>
</tr>
</tbody>
</table>

$^a$ In one experiment performed under the same conditions $s_{20,\text{w}} = 1.2$ was obtained.

$^b$ Only one experiment gave a single sedimentation peak with $s_{20,\text{w}} = 6.0$; in the other cases two sedimentation peaks appeared, one with $s_{20,\text{w}} = 6.0$ (containing about 40% of the material) and one with $s_{20,\text{w}} = 2.3$ (about 60% of the material).

It seems probable that the loss of phosphatase activity at high pH, like the loss of activity at low pH, is accompanied by the dissociation of the protein molecule into inactive subunits. The reactivation occurs through the reassociation of these subunits into active dimers as the pH is lowered.

The stimulation by zinc on the reactivation of the high pH inactivated enzyme indicates the importance of the metal ion in the reactivation process. The ultracentrifugation analysis by Schlesinger et al., and experiments carried out at pH 8.0 on Sephadex G-75 show that the apoenzyme is a dimer, and that no dissociation to monomers occurs after removal of zinc only. However, as the present communication shows, the data obtained in the experiments performed on metal-free enzyme indicate that reactivation and reassociation of the high pH inactivated enzyme do not occur in the absence of zinc ion.

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That EDTA prevents the reactivation of acid treated alkaline phosphatase has been shown previously.4

The recovery of the activity of the alkali incubated enzyme under the experimental conditions used is rather low. The rate and extent of reactivation of acid denatured enzyme is affected by protein concentration, ionic strength, pH, metal ions, and temperature.4 The reactivation of alkali inactivated phosphatase may also be dependent on ionic strength, protein concentration, temperature, and other factors. By finding the best conditions for reactivation, probably the recovery of the enzymatic activity can be increased.

Acknowledgements. I wish to express my sincere thanks to Professor Bo G. Malmström for his continuous interest. The excellent technical assistance of Miss G. Alm is much appreciated.

This investigation has been supported by research grants from the Swedish Natural Science Research Council, the Institute of General Medical Sciences, U.S. Public Health Service (GM 12280-03), and the Agricultural Research Service, U.S. Department of Agriculture (FG-Sw-107).

REFERENCES


Received October 28, 1971.

Acta Chem. Scand. 26 (1972) No. 6