Dephosphorylation of Phosphoproteins by *Escherichia coli* Alkaline Phosphatase*

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A purified commercial preparation of *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) has been shown to dephosphorylate several phosphoproteins including bovine heart glycogen synthase D, mixed phosphohistones, and rabbit skeletal muscle phosphorylase kinase but not rabbit skeletal muscle glycogen phosphorylase. Alkaline phosphatase completely removed phosphate groups previously added during the preparation of glycogen synthase D, and completely converted the enzyme into the I form. The dephosphorylation reaction was reversed by a reaction catalyzed by cyclic AMP-dependent protein kinase. The activity of alkaline phosphatase on glycogen synthase D and on p-nitrophenyl phosphate was apparently due to the same enzyme since both activities showed identical sensitivity to heat and dithiothreitol, both activities co-chromatographed on DEAE-cellulose and on Sephadex G-200 columns, and both activities co-electrophoresed on polyacrylamide gels. A second alkaline phosphatase preparation isolated from E. coli K12 also had activity on glycogen synthase and on phosphohistone as well as p-nitrophenyl phosphate. Commercial *E. coli* alkaline phosphatase selectively dephosphorylated a mixture of phosphohistones, leaving the phosphorylated sites on the H2B-H3 fraction unhydrolyzed. The resistant sites in the phosphohistone mixture were dephosphorylated in the presence of urea. Phosphorylated phosphorylase kinase was actively dephosphorylated by alkaline phosphatase and the rate and extent of hydrolysis of protein phosphate bonds was related to the extent of phosphorylation of the substrate used. All of the phosphorylase kinase-bound phosphate could be hydrolyzed from aged enzyme.

The rate of alkaline phosphatase action on heart glycogen synthase D was modulated by several substances. Activity on glycogen synthase D was specifically activated by divalent cations, especially Mn++, and by sulfate. At the same time, activity on p-nitrophenyl phosphate was related only to changes in the ionic strength of the assay medium by various additions. Glycogen was a strong inhibitor of alkaline phosphatase-catalyzed glycogen synthase D dephosphorylation while having no effect on the dephosphorylation of p-nitrophenyl phosphate. Thus, chemicals known to regulate dephosphorylation of glycogen synthase D by protein phosphatases also affected the alkaline phosphatase-catalyzed reaction, probably by substrate-mediated mechanisms.

Since the initial discovery that mammalian glycogen phosphatase (EC 2.4.1.1) and glycogen synthase (EC 2.4.1.11) may exist in two interconvertible forms (2, 3), a number of workers have examined the stoichiometry and enzymology of the interconversion of these two enzymes. The properties of the kinases and phosphatases involved in the interconversion of these enzymes have been studied extensively (4). Other phosphorylated enzymes and cellular proteins have since been discovered (5) and recent studies have indicated that some phosphoproteins with more than one phosphorylated site per protein molecule may require more than one enzyme for the phosphorylation or dephosphorylation of different sites (6-8). Thus it has become very important to find enzymes that can specifically phosphorylate or dephosphorylate one or a limited number of sites in a given phosphoprotein to be able to study the role of each phosphorylation site in the regulation of the phosphoprotein function.

Mammalian protein phosphatases with molecular weights from 40,000 to 300,000 have been found in several tissues (9-11). Three reports of highly purified protein phosphatases active on glycogen-metabolizing enzymes have appeared (12-14) and one enzyme was shown to have a broad substrate specificity (15) similar to enzymes studied in more impure states (16, 17). Thus, pure enzymes for dephosphorylation of phosphoproteins are not readily available at present. It had been known for some years that *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) obtained from commercial suppliers was able to dephosphorylate certain phosphoproteins such as histones (18), other nuclear proteins (19), and casein (20), but it was not conclusively shown in these studies that the alkaline phosphatase was responsible for the protein phosphatase activity of the preparations. Most of the mammalian protein phosphatases have been shown to be inactive on low molecular weight substrates such as p-nitrophenyl phosphate or ATP, but recently some preparations of protein phosphatase have also catalyzed hydrolysis of these low molecular weight substrates (21-23). Thus, it is clear, first, that *E. coli* alkaline phosphatase might be able to dephosphorylate phosphoproteins in a manner useful for studies on the role of phosphorylated sites in phosphoprotein substrates and second, that an...
"alkaline phosphatase-like" activity in mammalian cells might have protein phosphatase activity.

The work reported here is an initial study on the use of alkaline phosphatase for dephosphorylation of proteins. *E. coli* alkaline phosphatase is shown to dephosphorylate glycogen synthase D, partially dephosphorylate phosphohistone and glycogen phosphorylase kinase, and to be inactive on glycogen phosphorylase. The conformation of the protein around certain of the phosphorylated sites in these proteins apparently has a dramatic effect on the activity of the *E. coli* enzyme to act on the phosphorylser esters in these proteins.

**EXPERIMENTAL PROCEDURES**

Materials—UDP-glucose, glucose 6-phosphate, dithiothreitol, Tris base, p-nitrophenol, type II rabbit liver glycogen, type II-A calf thymus histone, and rabbit muscle phosphorylase a were all purchased from Sigma Chemical Co. Ammonium persulfate, aniline blue black, N,N'-methylenebisacrylamide, and N,N',N'-tetramethylethylenediamine (Temed) were from Canalo and high purity acrylamide was obtained from Miles Laboratories. Protocol and carrier-free $^{32}$P, were from New England Nuclear. UDP-$^{1-4}$Clglucose was prepared as described (16) and dialyzed against the same buffer to remove traces of ammonium sulfate. Alkaline phosphatase from *E. coli* strain K12 was prepared by the procedure of Simpson et al. (25) from cells grown to late log phase. A unit catalyzes the transfer of 1 pmol of glucose from UDP-glucose to glycogen per min under standard assay conditions.

Enzymes—*Escherichia coli* strain C90 alkaline phosphatase, code BAPP, was purchased from Worthington. The enzyme suspension was centrifuged to collect the protein which was then dissolved in 50 mM Tris/HCl, pH 7.8. This stock enzyme solution was stored at 4°C between experiments. In experiments designed to study effects of ions on this enzyme, the suspended phosphatase was dialyzed several days against the same buffer to remove traces of ammonium sulfate. Alkaline phosphatase from *E. coli* strain R12 was prepared by the procedure of Simpson et al. (25) from cells grown to late log phase in medium containing salts, glucose, and supplemented with 0.5% Bacto peptone (26).

Bovine heart glycogen synthase was purified by a method to be published elsewhere (27) that is similar in the initial stages to that reported by Brown and Lurker (27). The enzyme usually contained greater than 95% glycogen synthase I after chromatography on 8-aminobutyrolagarose and it could be converted to 99% glycogen synthase D by incubation with cyclic AMP, Mg$^{2+}$, and ATP (or Iy-32P)ATP. The enzyme was centrifuged through 2 ml of 50% sucrose at 165,000 $\times g$ after conversion and dissolved in 50 mM Tris, pH 7.5. This preparation was such a preparation used in this study was 13 units/mg of protein and there was no synthase phosphatase activity detectable under our assay conditions. The radioactive enzyme used in Fig. 2 had a specific activity of 32 units/mg of protein and the glycogen synthase D used for the experiment shown in Fig. 9 was phosphoprotein medium containing salts, glucose, and supplemented with 0.5% Bacto peptone (26).

Bovine heart cyclic AMP-dependent protein kinase was purified by slight modifications of a published method (29). The catalytic subunit of this enzyme was prepared according to the method of Schiender and Reimann (30). Active fractions of the catalytic subunit from a hydroxylapatite column were pooled, and concentrated by dialyzing against 60% glycerol, 0.35 M potassium phosphate buffer (pH 6.8), and 0.1 mM dithiothreitol. The dialyzed enzyme containing glycerol was stored at -20°C. It was stable for at least 6 months under these conditions. The preparation contained 9.8 units (nanomoles of P$_i$ incorporated per min/mg of histone kinase activity per ml) when assayed by the method of Reimann et al. (30).

Phosphorylated Proteins—[32P]Histone was prepared as described previously from Sigma mixed histones, type II-A (16). Both types of bovine heart protein kinase described above were used at times for phosphorylation of the histones and no differences were observed in the total incorporation of radioactive phosphate into the histone. Our phosphokinase preparation was further purified by elution of radioactive phosphate mg of histone protein. (Protein determinations were based on the Lowry method (32)).

$^{32}$P-labeled active phosphorylase kinase was prepared from inactive calf skeletal muscle phosphorylase kinase purified by published methods (33). The enzyme (1.0 mg/ml) was incubated with 0.1 mm (Iy-32P)ATP (1 mCi/mmol), 8.0 mm MgCl$_2$, and 37 units/ml of catalytic subunit from cyclic AMP-dependent protein kinase in 50 mm B-glycerol-P, 1.0 mM EDTA, 0.4% mercaptoethanol, and 10% sucrose at pH 6.8 and 30°C. Radioactive ATP was removed by gel filtration using a small Sephadex G-50 column equilibrated with 50 mm Tris/HCl, 0.4% mercaptoethanol, and 50 mm KF at pH 7.5.

Glycogen synthase D containing radioactive phosphate was prepared as described under "Enzymes."

Phosphohistone assays—Alkaline phosphatase activity was assayed by the activity on p-nitrophenol phosphate (34, 35). In most cases, the assay was modified by using an assay buffer of 50 mm Tris/HCl, pH 7.5, and a temperature of 30°C. This permitted comparison with protein substrates that were dephosphorylated under these same conditions.

Glycogen synthase phosphatase activity of the *E. coli* alkaline phosphatase was assayed by conversion of the D form of glycogen synthase to the I form or by following the release of radioactive phosphate from the substrate. The reaction was incubated at 30°C in 50 mm Tris/HCl, pH 7.5, 5 mm dithiothreitol, 10 mm MgCl$_2$, and 100 mm Na$_2$SO$_4$, when added. Alkaline phosphatase was added to start the reaction, giving a total reaction volume of either 25 or 50 µl. With a glycogen synthase D concentration of 1.5 units/ml (1 synthase unit catalyzes the transfer of 1 µmol of glucose from UDP-glucose to glycogen per min under standard assay conditions), the alkaline phosphatase concentration required in the reaction mixture was 1 to 2 units/ml. A unit of alkaline phosphatase was defined as the amount of enzyme that catalyzes the hydrolysis of 1 µmol of p-nitrophenyl phosphate/min in 50 mm Tris/HCl, pH 7.5. After incubation for 10 min or less, aliquots of reaction mixtures were withdrawn and diluted 10- or 20-fold in 50 mm Tris/HCl, 4 mM EDTA, 20 mm dithiothreitol, 100 mm KF, and 0.1% rabbit liver glycogen at pH 7.8. The samples were then assayed for glycogen synthase I and total glycogen synthase activity as previously described (24, 36). Phosphatase activity was measured as the increase in the glycogen synthase I activity. Release of $^{32}$P, from radioactive glycogen synthase D by phosphatase was measured as previously described (16).

Phosphohistone phosphatase was assayed by release of silicotungstic acid-soluble $^3P$, from $^{32}$P$^3P$histone as previously described (16).

To determine the activity of alkaline phosphatase on rabbit skeletal muscle glycogen phosphorylase a, the substrate was dialyzed for 2 days at 5°C against 50 mm Tris/HCl, pH 7.5, 5 mm dithiothreitol, and then preincubated with the same buffer plus any additions at 30°C. The phosphatase reaction was started by the addition of alkaline phosphatase, and aliquots of reaction mixtures were withdrawn and diluted with 10- or 20-fold in 50 mm Tris/HCl, pH 6.0, 1 mg/ml of bovine serum albumin at the appropriate intervals. Phosphatase activity was then measured colorimetrically (37) in the absence and presence of AMP to determine the extent of conversion of phosphorylase a to phosphorylase b. Alkaline phosphatase did not interfere with the phosphorylase assay.

**Electrophoresis Methods**—Polyacrylamide gels for phosphohistone separations were prepared and run according to the method of Fanym and Chalkley (38). Ten to twenty micrometers of phosphohistone were run on each gel and gels were stained with 0.1% aniline blue black, in acetic acid-ethanol-H$_2$O (7:43:83). Bands corresponding to histone fractions were excised (2-mm slices) and incubated at 37°C for 20 h in the presence of 10 ml of 3% Protosol in scintillation counting fluid (PermaFluor I in toluene, Packard Instruments). Virtually all radioactive associated with the stained histone bands was present in the 2-mm slices cut out of each gel.

Phosphorylase kinase electrophoresis was carried out in 5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate as described by Weber and Osborn (39). Gels were stained, destained, and counted as described above for phosphohistone preparations.

**RESULTS**

Action of *Escherichia coli* Alkaline Phosphatase on Phosphoproteins—Bovine heart glycogen synthase D incubated with alkaline phosphatase under the conditions specified under "Experimental Procedures," was effectively converted into the I form of the enzyme (Fig. 1A). If the alkaline phosphatase was then removed from the reaction mixture by gel filtration,
Dephosphorylation of Phosphoproteins

The dephosphorylation of glycan synthase D by alkaline phosphatase was a completely reversible process. Moreover, additional experiments using P-labeled glycogen synthase D as the substrate showed that release of the enzyme and conversion of the enzyme to the I form occurred concomitantly (Fig. 1B). The P-labeled glycogen synthase D used in this experiment had approximately two phosphate groups per subunit of enzyme. Since both serine phosphate groups were removed by the alkaline phosphatase, the reaction specificity of alkaline phosphatase does not permit a differentiation between these phosphate groups in glycogen synthase D. The rate of conversion of glycogen synthase D to the I form was proportional to the concentration of alkaline phosphatase with 0.22 to 1.8 units/ml of alkaline phosphatase when the glycogen synthase D concentration was 1.5 units/ml. Dephosphorylation of a glycogen synthase D by alkaline phosphatase gives saturation kinetics with half-maximal activity at 0.25 unit/ml of glycogen synthase D. Therefore, the concentration of glycogen synthase D used in these studies is 6-fold higher than the K_m concentration.

The dephosphorylation of P-labeled phosphohistone, catalyzed by E. coli alkaline phosphatase, is shown in Fig. 2. Panel A of the figure shows that most of the radioactive phosphate could be released from the phosphohistone in this reaction, but that there was a residual amount of radioactivity that was not released. Even after incubation of the reaction mixture for 22 h with active alkaline phosphatase, approximately 20% of the radioactive phosphate was protein-bound. Panel B (Fig. 2) shows that a sample of histone that was extensively dephosphorylated by the E. coli alkaline phosphatase could be treated with urea and the remaining radioactivity could be released by the phosphatase. In the absence of urea, no dephosphorylation occurred. Thus, at least one phosphorylated site in the mixed phosphorylated histone substrate was buried in the protein structure and inaccessible to the alkaline phosphatase unless it was uncovered by urea treatment. In order to discover the site of the unreactive phosphorylated site in the reaction mixture contained the dephosphorylated glycogen synthase D, 2 mM ATP, 1 mM cyclic AMP, and 5 mM MgCl_2. Conversion of the glycogen synthase to the D form was followed by glycogen synthase assays as described previously (36). B, correlation of conversion of glycogen synthase D to I with the release of protein-bound phosphate. This incubation conditions used in this experiment were the same as in A, except that P-labeled glycogen synthase D was used as the substrate. The radioactive substrate had 2.1 phosphates per subunit of 86,000 daltons. Aliquote of the reaction mixture were assayed for conversion of glycogen synthase D to I activity (○) or for release of P (○). A control reaction incubated in the absence of phosphatase gave no reaction by either assay.

The figure shows that most of the radioactive phosphate could be released from the phosphohistone in this reaction, but that there was a residual amount of radioactivity that was not released. Even after incubation of the reaction mixture for 22 h with active alkaline phosphatase, approximately 20% of the radioactive phosphate was protein-bound. Panel B (Fig. 2) shows that a sample of histone that was extensively dephosphorylated by the E. coli alkaline phosphatase could be treated with urea and the remaining radioactivity could be released by the phosphatase. In the absence of urea, no dephosphorylation occurred. Thus, at least one phosphorylated site in the mixed phosphorylated histone substrate was buried in the protein structure and inaccessible to the alkaline phosphatase unless it was uncovered by urea treatment. In order to discover the site of the unreactive phosphorylated site in the

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** A, alkaline phosphatase-catalyzed conversion of glycogen synthase D to I form and reversion to the D form by phosphorylation. Glycogen synthase D was incubated at a concentration of 1.5 units/ml under conditions described under "Experimental Procedures." Escherichia coli alkaline phosphatase was present at 5 units/ml. The time course of the conversion of the substrate into glycogen synthase I was followed by assays of the activity of the glycogen synthase at the times indicated. After 2 h, the incubation mixture was chromatographed on a small Sepharose 6B column to remove alkaline phosphatase and the glycogen synthase was used as the substrate for phosphorylation by a bovine heart protein kinase prepared as described under "Experimental Procedures." The kinase reaction mixture contained the dephosphorylated glycogen synthase I, 2 mM ATP, 1 mM cyclic AMP, and 5 mM MgCl_2. Conversion of the glycogen synthase to the D form was followed by glycogen synthase assays as described previously (36). B, correlation of conversion of glycogen synthase D to I with the release of protein-bound phosphate. The incubation conditions used in this experiment were the same as in A, except that P-labeled glycogen synthase D was used as the substrate. The radioactive substrate had 2.1 phosphates per subunit of 86,000 daltons. Aliquote of the reaction mixture were assayed for conversion of glycogen synthase D to I activity (○) or for release of P (○). A control reaction incubated in the absence of alkaline phosphatase gave no reaction by either assay.

![Figure 2](https://example.com/fig2.png)

**Fig. 2.** Dephosphorylation of phosphohistone by Escherichia coli alkaline phosphatase. A, P-phosphohistone (0.25 mg/ml; 18 nmol of P/mg of histone) was dephosphorylated in 50 mM Tris/HCl, pH 7.5, 100 mM NaSO_4, and 10 mM MgCl_2 with 2.0 units/ml of alkaline phosphatase. Fractions removed at the indicated times were assayed for release of P. After 22 h at 30°C, the reaction stopped with 4.1 nmol of P remaining. Addition of fresh alkaline phosphatase produced no additional release of radioactivity. B, a sample of alkaline phosphatase-dephosphorylated P-phosphohistone containing 5.8 nmol of P/mg was dialyzed against 50 volumes of 50 mM Tris/HCl, pH 7.5, 100 mM NaSO_4, 10 mM MgCl_2, to remove any inhibitory products from the reaction mixture. The dialyzed material was diluted with an equal volume of either H_2O (○) or 8 M urea (○). Additional alkaline phosphatase was added to give an increment of 2 units/ml of this enzyme. After a 48-h incubation, 85% of the resistant P was removed from the phosphohistone when urea was present (0.9 nmol of P/mg remaining). In the absence of urea, no release of P could be detected.

![Graph](https://example.com/graph.png)

![Graph](https://example.com/graph.png)
phosphohistones, we examined the original phosphohistone and the alkaline phosphatase-dephosphorylated histone by gel electrophoresis using established methods (38). Table I shows that radioactivity was found in all fractions of the original phosphohistone substrate and after phosphatase treatment, the only fraction with substantial radioactivity remaining was the H2B-H3 fraction. Dephosphorylation of phosphohistone could be reversed by added bovine heart protein kinase, ATP, and Mg++. As was shown above for glycogen synthase.

Alkaline phosphatase-catalyzed dephosphorylation of 32P-active phosphorylase kinase prepared as described under "Experimental Procedures" is shown in Fig. 3. The substrate was partially dephosphorylated by alkaline phosphatase and the extent of removal of the kinase-bound phosphate was related to the amount of phosphate originally present. These reactions were carried out in the presence of 30 mM KF and contaminating protein phosphatases were inhibited while alkaline phosphatase was not affected by this concentration of KF. The reaction was terminated at 2 h because there was a continuous very slow release of substrate-bound phosphate that took up to 1 day to complete. This slow release of phosphate was most likely a result of substrate denaturation during the reaction since assay of phosphorylase kinase activity indicated that approximately half the activity was lost after 1 to 2 h. When phosphorylase kinase was aged at 4° for 1 week, the rate of dephosphorylation was increased and after 3 h, no protein-bound phosphate was detectable.

In a separate experiment, it was shown that an incompletely phosphorylated substrate (approximately 1.25 32P/337,000-dalton subunit) having a ratio of 0.65 radioactive phosphate groups in α subunits for every phosphate in the β subunits was 13% dephosphorylated after incubation with 3 units/ml of alkaline phosphatase at 30° for 1 h. The ratio of α to β phosphate groups was decreased to 0.37 with an apparent selective dephosphorylation of the α subunit.

Attempts to dephosphorylate glycogen phosphorylase with *E. coli* alkaline phosphatase were unsuccessful. Using the methods described under "Experimental Procedures" and with alkaline phosphatase (8 units/ml) incubated with either 0.2 or 2.0 mg/ml of skeletal muscle phosphorylase α, there was no detectable conversion of the enzyme to the b form after 2 h. Addition of glucose (29 mM), theophylline (2.5 mM), or MgCl₂ (10 mM) had no effect on the reaction. Thus, neither very high concentrations of alkaline phosphatase, nor additives that might stimulate dephosphorylation were able to produce dephosphorylation by *E. coli* alkaline phosphatase.

The preceding studies indicate that both glycogen synthase, and phosphohistones can be substrates for *E. coli* alkaline phosphatase. They are extremely poor substrates for this enzyme, however, when compared to low molecular weight phosphate esters such as p-nitrophenyl phosphate or ATP. Using glycogen synthase D, phosphohistone, and γ-32P-ATP at a concentration of 1 µM [32P]-phosphoester or phosphoanhydride bond, the rates of release of radioactive P, relative to that with glycogen synthase D were glycogen synthase D = 1, phosphohistone = 3.2, and ATP = 826.

**Evidence that *E. coli* Alkaline Phosphatase is Also Glycogen Synthase Phosphatase** — The evidence that *E. coli* alkaline phosphatase preparations can catalyze dephosphorylation of glycogen synthase D, phosphohistones, and active phosphorylase kinase prompted an investigation of the characteristics of
coli produce three forms of alkaline phosphatase that can be separated on DEAE-cellulose chromatography, both enzyme activities appeared as a single peak when eluted with a linear salt gradient. Fig. 5 shows that attempts to separate the enzyme activities on Sephadex G-200 resulted likewise in only one identical peak of activity when the fractions were assayed with either substrate. Since it was known that some strains of E. coli produce three forms of alkaline phosphatase activity, it was important to determine whether the protein phosphatase activity was a property of the alkaline phosphatase itself or a contaminating protein phosphatase.

Heat inactivation of both the glycogen synthase phosphatase and the p-nitrophenyl phosphatase activities associated with the E. coli phosphatase preparation is shown in Fig. 4A. After the phosphatase was incubated at 89° for the indicated times, aliquots were removed and allowed to incubate at 30° for at least 30 min to eliminate any reversible heat denaturation of the alkaline phosphatase which might interfere with the assays. The time course of heat denaturation was the same for either substrate. The extreme stability to heat treatment is a well documented property of E. coli alkaline phosphatase (40), while protein phosphatases are generally heat labile (14, 41, 42). There was also a loss of both glycogen synthase phosphatase and p-nitrophenyl phosphatase activity when the alkaline phosphatase preparation was incubated at 30° in the presence of 20 mM dithiothreitol (Fig. 4B). The time course of dithiothreitol denaturation of the phosphatase was identical for both substrates. Denaturation of E. coli alkaline phosphatase by reducing agents has been described previously (43), while protein phosphatases are generally stable in the presence of reducing agents.

Several attempts to separate the activities of the commercial alkaline phosphatase preparation on p-nitrophenyl phosphate and on glycogen synthase D were unsuccessful. On DEAE-cellulose chromatography, both enzyme activities appeared as a single peak when eluted with a linear salt gradient. Fig. 5 shows that attempts to separate the enzyme activities on Sephadex G-200 resulted likewise in only one identical peak of activity when the fractions were assayed with either substrate. Since it was known that some strains of E. coli produce three forms of alkaline phosphatase that can be separated on DEAE-cellulose chromatography (40, 44), a preparation containing these forms was examined for both the phosphatase activities in question. The alkaline phosphatase purified from E. coli strain K12 as described under "Experimental Procedures" was chromatographed on DEAE-cellulose to separate the three forms of this enzyme (Fig. 6). There are three distinct p-nitrophenyl phosphatase activity peaks, but only the two peaks eluting at higher salt concentration show significant synthase phosphatase and histone phosphatase activities. When the low salt peak was concentrated by ultrafiltration in an Amicon system with a PM-10 membrane and subjected to Sephadex G-100 gel filtration, the resulting alkaline phosphatase gave histone phosphatase and glycogen synthase phosphatase activity equivalent to the high salt DEAE-peaks. Most likely some inhibitor of protein phosphatase activity in the low salt peak was removed by the gel filtration. Thus, the separation of E. coli strain K12 alkaline phosphatase activity into three fractions on DEAE-cellulose showed that all three forms of the enzyme had activity on low molecular weight phosphate esters as well as on phosphoprotein.

The purity of the commercial alkaline phosphatase preparations used in these studies was checked by gel electrophoresis in two different gel systems. The gels obtained when the electrophoresis was carried out at pH 9.5 or pH 4.5 are shown in Fig. 7. In both gels, there is evidence of only a single contaminating protein and in no case was it possible to find more than two bands on these gels. The activity of the protein bands separated by pH 9.5 electrophoresis using both p-nitrophenyl phosphate and phosphoproteins as substrates is shown in Table II. The activities on p-nitrophenyl phosphate, phospho-
Cation effects on alkaline phosphatase activity with glycogen synthase D and p-nitrophenyl phosphate as substrates

Alkaline phosphatase was assayed with glycogen synthase D and p-nitrophenyl phosphate as substrates under the conditions described under "Experimental Procedures." Glycogen synthase D concentration was 2 units/ml, and p-nitrophenyl phosphate was 2 mM. Alkaline phosphatase was used at 1.5 units/ml for glycogen synthase D and 3 milliunits/ml with p-nitrophenyl phosphate. All assays were run in 50 mM Tris/HCl, pH 7.5, 5 mM dihydroxyacetone at 30°C.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Relative activity on glycogen synthase D</th>
<th>Relative activity on p-nitrophenyl phosphate</th>
<th>Buffer ionic strength</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>-40</td>
<td>79</td>
<td>0.04</td>
</tr>
<tr>
<td>CaCl₂, 10 mM</td>
<td>119</td>
<td>103</td>
<td>0.09</td>
</tr>
<tr>
<td>MgCl₂, 10 mM</td>
<td>100</td>
<td>100</td>
<td>0.09</td>
</tr>
<tr>
<td>MnCl₂, 10 mM</td>
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<td>0.09</td>
</tr>
<tr>
<td>MgCl₂, 10 mM +</td>
<td>270</td>
<td>103</td>
<td>0.14</td>
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<tr>
<td>NaCl, 150 mM</td>
<td>75</td>
<td>137</td>
<td>0.19</td>
</tr>
<tr>
<td>NaCl, 150 mM +</td>
<td>100</td>
<td>134</td>
<td>0.24</td>
</tr>
<tr>
<td>MgCl₂, 10 mM</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
TABLE IV

Anion effects on alkaline phosphatase activity with glycogen synthase D and p-nitrophenyl phosphate as substrates

All assays were run as described under Table III except that 10 mM MgCl₂ was added to each sample.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Relative activity on glycogen synthase D</th>
<th>Relative activity on p-nitrophenyl phosphate</th>
<th>Buffer ionic strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
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<td>100</td>
<td>0.09</td>
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<tr>
<td>NaCl</td>
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<tr>
<td>10 mM</td>
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<td>108</td>
<td>0.10</td>
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<tr>
<td>150 mM</td>
<td>100</td>
<td>134</td>
<td>0.24</td>
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<tr>
<td>NaNO₃</td>
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<tr>
<td>10 mM</td>
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<td>111</td>
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<tr>
<td>150 mM</td>
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<td>130</td>
<td>0.24</td>
</tr>
<tr>
<td>Na₂SO₄</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5 mM</td>
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<tr>
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<td>183</td>
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<td>Na₂HPO₄</td>
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<td>24</td>
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</table>

FIG. 8 Glycogen inhibition of alkaline phosphatase activity on glycogen synthase D. A preparation of bovine heart glycogen synthase (2 units/ml) prepared as previously described (28) was incubated with 3 units/ml of alkaline phosphatase at various concentrations of rabbit liver glycogen. The activity of the reaction is compared to that obtained earlier with a partially purified preparation of heart glycogen synthase D and p-nitrophenyl phosphate by alkaline phosphatase is shown to be insensitive to glycogen. It is apparent that the glycogen inhibition of alkaline phosphatase action on glycogen synthase D cannot be a result of a direct effect on the phosphatase but must involve participation of the substrate in the inhibition complex.

**DISCUSSION**

The results reported here show that E. coli alkaline phosphatase is able to dephosphorylate selected phosphorylated sites in specific proteins. This non-specific phosphatase catalyzed the complete dephosphorylation of bovine heart glycogen synthase D and a selective dephosphorylation of phosphohistone and active glycogen phosphatase kinase, but did not attack the single phosphorylated site in glycogen phosphorylase a. Thus, alkaline phosphatase may be a useful tool for studying the role of certain protein-bound phosphate esters in the activities of these proteins and may, in fact, be tested for the study of other proteins that were not included in this study.

There are at least two advantages in using alkaline phosphatase to study protein dephosphorylation. First, since alkaline phosphatase has activity on both low molecular weight substrates and on proteins, enzyme activity with both types of substrates may be compared under equivalent conditions to determine whether regulatory effects are the result of substrate-directed regulation or phosphatase-directed regulation. In the present study, it is shown that the dephosphorylation of glycogen synthase D is regulated by certain ions and by glycogen in a manner that may best be explained by substrate-directed regulation. The specificity of cation activation of the alkaline phosphatase-catalyzed glycogen synthase dephosphorylation (Table III) closely resembled the activation of bovine heart phosphatase acting on the same substrate (16). Likewise, the anion activation of both reactions showed a similar specificity (Table IV; Ref. 16). Alkaline phosphatase action on glycogen synthase was also inhibited by glycogen just as had been shown previously with a heart protein phosphatase (36). Secondly, since both phosphohistones and active phosphorylase kinase were partially dephosphorylated by alkaline phosphatase it may be possible to use this enzyme to further study the role of specific phosphorylatable sites in these proteins by selective dephosphorylation of fully phosphorylated molecules. Both of these protein substrates were phosphorylated with purified heart protein kinase, and partial dephosphorylation by alkaline phosphatase may suggest a difference in the substrate specificity of the kinase and phosphatase. However, both substrates were completely dephosphorylated when either exposed to urea or aged, indicating that the selectivity of alkaline phosphatase may be a result of conformational changes in the substrates after phosphorylation by the kinase. Further studies on the site specificity of alkaline phosphatase for these substrates will have to be carried out with detailed attention to reaction conditions to prevent changes in sub-

![Graph showing glycogen inhibition of alkaline phosphatase activity on glycogen synthase D.](http://www.jbc.org/)

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strate conformation that may result in the hydrolysis of phosphate esters in protected conformations.

Data presented in this report suggest a note of caution on designating the substrate specificity of a protein phosphatase that might have the characteristics of glycogen synthase D phosphatase. Since an enzyme as nonspecific as E. coli alkaline phosphatase shows many of the properties of known glycogen synthase phosphatases from mammalian sources, one might isolate such an enzyme from some cell or tissue and erroneously designate it a glycogen synthase phosphatase. This, it can be concluded, is exceedingly low in comparison with the activity of the enzyme on low molecular weight substrates.

Finally, one must consider the possibility that "alkaline phosphatase-type" activities existing in mammalian tissues might be an intermediate case since it could be partially dephosphorylated by alkaline phosphatase.

Dephosphorylation of phosphoproteins by Escherichia coli alkaline phosphatase.
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