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 phosphorylase (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 substances (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 effectively, to partially dephosphorylate 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 dramatic effects on the ability of the E. coli enzyme to act on the phophosorine esters in these proteins.

EXPERIMENTAL PROCEDURES

Materials—UDP-glucose, glucose 6-phosphate, dithiothreitol, Tris base, p-nitrophenyl phosphate, type II rabbit liver glycogen, type II calf thymus histone, and rabbit muscle phosphorylase a were all purchased from Sigma Chemical Co. Ammonium persulfate, aniline black, N,N-dimethylbenzamidamide, and N,N,N,N'-tetramethylenediamine (Temed) were from Calbiochem and high purity acrylamide was obtained from Miles Laboratories. Protosol and carrier-free 32P were from New England Nuclear. UDP-[U-14C]glucose was then removed from the reaction mixture by gel filtration, whereas from the reaction mixture to remove traces of ammonium ions on this enzyme, the suspended phosphatase was dialyzed several days against the same buffer to remove traces of ammonium ions. UDP-glucose was prepared by the method of Thomas et al. (26).

Enzymes—Escherichia coli strain C90 alkaline phosphatase, code BAPF, was purchased from Worthington. The enzyme suspension was centrifuged to collect the protein which was then dissolved in 50 mM Tris/HCl, pH 7.5. This enzyme was used at 13 units/ml in the assay described. The enzyme suspension was centrifuged to collect the protein which was then dissolved in 50 mM Tris/HCl, pH 7.5. The enzyme suspension was centrifuged to collect the protein which was then dissolved in 50 mM Tris/HCl, pH 7.5.

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Synthase D to I form and reconversion 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 substrate for phosphorylation by a bovine heart protein kinase prepared as described under "Experimental Procedures." The kinase activity of 86,000 daltons. Aliquots of the reaction mixture were assayed 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 32P-labeled glycogen synthase D was used as the substrate. The radioactive substrate had 2.1 phosphates per subunit of 86,000 daltons. Aliquot of the reaction mixture were assayed for conversion of glycogen synthase D to I activity (●) or for release of 32P (○). A control reaction incubated in the absence of alkaline phosphatase gave no reaction by either assay.

Dephosphorylation of Phosphoproteins

Panels A and B of Fig. 1 show the dephosphorylation of phosphohistone, catalyzed by Escherichia coli alkaline phosphatase. A (50 pmol of [32P]phosphohistone (0.25 mg/ml; 18 nmol of 32P/mg of histone) was dephosphorylated in 50 mM Tris/HCl, pH 7.5, 100 mM Na2SO4, and 10 mM MgCl2 with 2.0 units/ml of alkaline phosphatase. Fractions removed at the indicated times were assayed for release of 32P. After 22 h at 30°C, the reaction stopped with 4.1 nmol of 32P/mg remaining. Addition of fresh alkaline phosphatase produced no additional release of radioactivity. B, a sample of alkaline phosphatase-dephosphorylated [32P] phosphohistone containing 5.8 nmol of 32P/mg was dialyzed against 50 volumes of 50 mM Tris/ HCl, pH 7.5, 100 mM Na2SO4, 10 mM MgCl2 to remove any inhibitory products from the reaction mixture. The dialyzed material was diluted with an equal volume of either H2O (○) 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 32P was removed from the phosphohistone when urea was present (0.9 nmol of 32P/mg remaining). In the absence of urea, no release of 32P could be detected.

The dephosphorylation of 32P 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 I, 2 mM ATP, 1 μM cyclic AMP, and 5 mM MgCl2. 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 32P-labeled glycogen synthase D was used as the substrate. The radioactive substrate had 2.1 phosphates per subunit of 86,000 daltons. Aliquot of the reaction mixture were assayed for conversion of glycogen synthase D to I activity (●) or for release of 32P (○). A control reaction incubated in the absence of alkaline phosphatase gave no reaction by either assay.

The dephosphorylation of 32P phosphohistone, catalyzed by E. coli alkaline phosphatase was a completely reversible process. Moreover, additional experiments using 32P-labeled glycogen synthase D as the substrate showed that, after release from the enzyme and conversion of the enzyme to the I form occurred concomitantly (Fig. 1B). The 32P-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 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 Kₘ concentration.

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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 Mg²⁺ as was shown above for glycogen synthase.

Alkaline phosphatase-catalyzed dephosphorylation of ³²P-active muscle 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°C 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°C 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 β 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 γ³²P[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 (40, 44). The protein phosphatase activity associated with the commercial preparations 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 (40) 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, phos-
Divalent cation activation was not simply related to the ionic strength of the reaction mixture since activity in the presence of 10 mM Mg\(^{2+}\) and 150 mM Na\(^{+}\) was the same as with 10 mM Mg\(^{2+}\) alone. Addition of both Mg\(^{2+}\) and Na\(^{+}\) did not activate more than 10 mM Mn\(^{2+}\) alone, indicating that both cations may act by similar mechanisms. In contrast to the results with glycogen synthase D as substrate, the effect of the cations on p-nitrophenyl phosphate phosphatase activity was most easily correlated with ionic strength effects. A role of ionic strength on alkaline phosphatase activity under assay conditions similar to those used for p-nitrophenyl phosphate phosphatase activity.

**Table II**

**Correlation of protein phosphatase activity with p-nitrophenyl phosphatase activity on gel electrophoresis**

Commercial Escherichia coli alkaline phosphatase was electrophoresed at pH 9.5 as described in Fig. 6. Replicate gels were sliced into 2- to 3-mm segments and each segment was incubated with an appropriate assay mixture for determining enzyme activity on either p-nitrophenyl phosphate, phosphohistone, or glycogen synthase D. Activity on p-nitrophenyl phosphate was determined by incubating slices in standard reaction mixture for 5 min at 30°, activity with phosphohistone was determined by incubating slices with 270 μg of [\(^{32}\)P]phosphohistone for 3 h at 30°, and activity with glycogen synthase D was determined by incubating with 80 milliunits of glycogen synthase D for 20 h at 4°. Each assay was analyzed by removing aliquots for the methods described under "Experimental Procedures." The RF for each slice was calculated from the middle of the slice to the middle of a tracker dye used with each gel, i.e. bromophenol blue. The major protein band stained with Coomassie blue as in Fig. 6 had an RF of 0.32 on these gels. The minor protein contaminant had an RF of approximately 0.25.

**Table III**

**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 dihydrothiol at 30°.
Dephosphorylation of Phosphoproteins

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.

| Addition  | Relative activity on glycogen synthase D | Relative activity on p-nitrophenyl phosphate | Buffer ionic strength |
|-----------|------------------------------------------|---------------------------------------------|-----------------------|
| None      | 100                                      | 100                                         | 0.09                  |
| NaCl      |                                          |                                             |                       |
| 10 mM     | 106                                      | 108                                         | 0.10                  |
| 150 mM    | 100                                      | 134                                         | 0.24                  |
| NaNO₃    |                                          |                                             |                       |
| 10 mM     | 119                                      | 111                                         | 0.10                  |
| 150 mM    | 160                                      | 130                                         | 0.24                  |
| Na₂SO₄   |                                          |                                             |                       |
| 5 mM      | 171                                      |                                             |                       |
| 10 mM     | 194                                      | 111                                         | 0.12                  |
| 20 mM     | 206                                      |                                             |                       |
| 50 mM     | 228                                      | 124                                         | 0.24                  |
| Na₂HPO₄, 50 mM | 183                                      | 122                                         | 0.24                  |
| Na₂HPO₄, 10 mM | 0                                        | 24                                          | 0.12                  |

FIG. 3. 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 protein phosphatase on a similar substrate preparation (36). The symbols used are: O, alkaline phosphatase activity on glycogen synthase; ---, data from a previous experiment using heart protein phosphatase acting on glycogen synthase D; and ●, activity of alkaline phosphatase on p-nitrophenyl phosphate in the presence of the indicated concentrations of glycogen.

Table IV shows the effect of various anions on glycogen synthase D phosphatase and p-nitrophenyl phosphate phosphatase activities of the alkaline phosphatase. Since alkaline phosphatase had very little activity on glycogen synthase D in the absence of divalent cations, 10 mM MgCl₂ was added to all assays. With this substrate, Cl⁻ had no effect. NO₃⁻ activated only at high concentrations, and SO₄⁻ gave significant activation. Na₂SO₄ was half-saturating at approximately 5 mM and it was able to double the rate of dephosphorylation when present at near-saturating concentrations. The effects of these anions on p-nitrophenyl phosphate phosphatase activity were once again related to the ionic strength of the reaction mixture. Inorganic phosphate was an inhibitor of phosphatase activity with either substrate.

Glycogen synthase phosphatase activity of alkaline phosphatase, was inhibited by glycogen as shown in Fig. 8. For this experiment, a partially purified preparation of glycogen synthase D was used to permit comparison to experiments published previously (36). This comparison, illustrated in Fig. 8, indicates that the effect of glycogen on the dephosphorylation of partially purified glycogen synthase D with either alkaline phosphatase or a rat heart protein phosphatase gives nearly identical inhibition curves. The dephosphorylation of 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 nonspecific phosphatase catalyzed the complete dephosphorylation of bovine heart glycogen synthase D, and a selective dephosphorylation of phosphohistone and active glycogen phosphorylase 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 dephosphorylated 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-
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 that the protein-bound phosphate groups in glycogen synthase are not necessarily found in specific conformations that preclude action by nonspecific phosphatase and some caution must be used in isolating an enzyme that might normally dephosphorylate this substrate in vivo.

On the other hand, the phosphorylated site in glycogen phosphorylase was completely resistant to alkaline phosphatase.

Finally, one must consider the possibility that "alkaline phosphatase type" activities existing in mammalian tissues might function as protein phosphatases under some circumstances. The data reported in this paper show that the E. coli enzyme has protein phosphatase activity but that this activity is exceedingly low in comparison with the activity of the enzyme on low molecular weight substrates. Thus, the amounts of alkaline phosphatase necessary for activity on proteins is very high relative to the substrate concentration, i.e. 1 to 3 units/ml of alkaline phosphatase and 1.5 units/ml of glycogen synthase D. Since the specific activities of the two enzymes are nearly equal, the molar concentrations of alkaline phosphatase subunits are approximately equal to glycogen synthase subunits. These amounts of alkaline phosphatase made us initially doubtful that protein dephosphorylation was a property of the alkaline phosphatase and we have reported experiments here that substantiate the idea that alkaline phosphatase itself is responsible for protein dephosphorylation (Figs. 4, 5, 6, 7, Table II). Further studies are needed to establish if it is possible to activate alkaline phosphatase action on phosphoproteins, or to isolate other phosphatases that have good activity on both protein and low molecular weight substrates.

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