0018–1536/85/1902–0133$02.00

Immobilized *E. coli* Alkaline Phosphatase

Its Properties, Stability, and Utility in Studying the Dephosphorylation of Proteins

KHAJA BASHEERUDDIN, VICKI ROTHMAN, AND SIMEON MARGOLIS*

Johns Hopkins University School of Medicine, 720 Rutland Avenue, Baltimore, Maryland 21205

Received August 21, 1984; Accepted November 20, 1984

ABSTRACT

We have immobilized *E. coli* alkaline phosphatase (EC 3.1.3.1) by linking it covalently to sepharose 4B. This preparation has several advantages over the soluble enzyme. The immobilized enzyme is easily separable from other constituents in incubation mixtures. The immobilized enzyme can be reused repeatedly and is more stable than the soluble enzyme to heat treatment in the presence of 10 m*M* Mg^{2+}. The insoluble and soluble phosphatases removed 75 and 77%, respectively, of the inorganic phosphorus from casein. The immobilized enzyme inactivated two enzymes believed to be active in the phosphorylated state, acyl-CoA:cholesterol acyltransferase (ACAT) by 39% and NADPH-cytochrome P-450 reductase by 89%. The utility of immobilized alkaline phosphatase for studying the phosphorylation and dephosphorylation of soluble or membrane-bound enzymes and proteins is discussed.

Index Entries: Alkaline phosphatase, immobilized; immobilized alkaline phosphatase, from *E. coli;* stability, of immobilized alkaline phosphatase; protein dephosphorylation; casein; Acyl-CoA:

*Author to whom all correspondence and reprint requests should be addressed.

cholesterol acyltransferase; dephosphorylation, immobilized alkaline phosphatase and.

INTRODUCTION

Alkaline phosphatases are widely distributed in microorganisms, plants, parasites, and mammalian tissues. These enzymes have been extensively investigated, particularly the *E. coli* alkaline phosphatase, which is often used to remove phosphate groups from proteins. Recent studies on the reversible modulation of several enzymes by a phosphorylation–dephosphorylation mechanism (1) have demonstrated the utility of a nonspecific phosphatase that can dephosphorylate enzymes. Initial steps in determining whether any suspected enzyme is regulated by phosphorylation, at least in vitro, require evidence that the catalytic activity of the enzyme can be reversibly modified by treatment with phosphatase and protein kinase. *E. coli* alkaline phosphatase has been used successfully to remove phosphate groups from several enzymes, such as HMGCoA reductase and other mammalian proteins (2). Immobilized *E. coli* alkaline phosphatase can be a useful tool to test whether the activity of an enzyme is regulated by phosphorylation. The insoluble phosphatase can be incubated with individual proteins, crude mixtures of proteins, or subcellular organelles and then easily and quickly separated from other components in the incubation. Since both inorganic and organic phosphates (pyrophosphate, ATP, adenosine 3′- or 5′-phosphate) can serve as substrates for alkaline phosphatase (3), removal of the immobilized enzyme makes it possible to assay the treated enzyme in the absence of the phosphatase, thus eliminating its continuing action on phosphorylated substrates, activators, or inactivators. Rephosphorylation of the modified protein by ATP and protein kinases can be examined without the commitant removal of phosphate groups by contaminating phosphatase. The cost of using the immobilized phosphatase is reduced because it can be reused repeatedly. Finally, the immobilized enzyme is even more stable than the soluble phosphatase.

We describe here: (*a*) a method for the immobilization of *E. coli* alkaline phosphatase by linking it covalently to an insoluble carrier (sepharose 4B), (*b*) the stability of the immobilized enzyme to heat denaturation, and (*c*) its effectiveness in removing phosphate groups from casein and from several enzymes believed to be regulated by phosphorylation.

METHODS

The fixation of alkaline phosphatase to Sepharose 4B was carried out by a two-step procedure. Sepharose 4B was activated by cyanogen bro-

mide, as described by Cuatrecasas (*4*). The enzyme was coupled by using a modification of the method of Axen and Ernback (*5*). Sepharose 4B from Pharmacia was washed four times each with six volumes of deionized water. Washed gel from 6 mL of sepharose 4B was suspended in 4 mL of cyanogen bromide solution in water (35 mg/mL). The solution was stirred slowly and maintained at pH 11 by the addition of 2*N* NaOH. The temperature was also kept constant at about 22°C by adding small pieces of ice. The reaction was completed in 15 to 18 min. The activated gel was washed first with 300 mL of 0.1*M* sodium bicarbonate solution. *E. coli* alkaline phosphatase (6.5 mg) from Sigma (type III, sp. act., 45 μ/mg) was dissolved in 3 mL of 0.1*M* sodium bicarbonate (pH 7.8) or 0.1*M* Tris HCl (pH 7.8). Activated gel was added after washing with 100 mL of the respective buffers. The tube was shaken slowly at 4°C overnight, and the sepharose–enzyme complex was washed, with approximately 20 vol of the respective buffers, until phosphatase activity was not detected in the washes. Enzyme activity was determined using *p*-nitrophenol phosphate (from Sigma) as a substrate (*6*).

RESULTS AND DISCUSSION

To calculate the amount of active enzyme bound to sepharose, a known volume (1.5 mL) of the gel was washed with deionized water and Tris-HCl (100 m*M*) and dried by replacing the water of the gel with acetone. Acetone was removed under vacuum. The dried, weighed gel–enzyme complex and a known amount of the soluble enzyme (free from ammonium sulfate solution) were heated at 110°C with 6*N* HCl for 48 h and the total content of amino acids was measured with ninhydrin as described by Spies (*7*). It was found that 46 mg of enzyme (0.54 μmol) was bound to 1 g of gel. The activity of the immobilized enzyme was measured using the active-site titration method described by Ford et al. (*8*) for immobilized trypsin. A stop-flow reaction technique was used to measure the burst of enzyme activity with *p*-nitrophenol phosphate as the substrate. A small column was prepared with a measured volume (400 μL, 5.0 mg dry weight) of gel–enzyme complex. After the gel was washed with 10 mL of 1.5*M* Tris-HCl (pH 8.0; assay buffer), *p*-nitrophenol phosphate (0.003*M*) in 4 mL of the above buffer was passed through the column. Each drop was collected and OD_{410} was measured immediately after arresting the *p*-nitrophenol color by adding NaOH (0.1 mL; 1.0*N*). A total of 2 mL gave an initial burst in 100 s. Using the formula of Ford et al. (*8*), 0.29 μmol of active phosphatase was attached per gram of gel. Based on the amount of protein bound to the gel, the fractional activity of phosphatase retained by the immobilized enzyme was 0.54. Thus, 70% of the mass and 38% of the activity of the starting alkaline phosphatase was recovered in the immobilized enzyme (mean of five experiments).

It has been recommended that Tris buffers containing amino groups not be used for coupling reaction involving cyanogen bromide because Tris will couple to the gel. However, there may be some advantages to interactions between the Tris and the activated gel, provided that a sufficient number of active groups remain available to the protein. For example, coupling of the buffer to the activated gel may prevent multiple amino groups on the enzyme from interacting with the gel. Such multivalent coupling of the enzyme may mask its active site or alter its tertiary structure. In addition, as a result of interactions between Tris and activated gel, the coupled enzyme may be more spread out over the surface of the gel beads. We were, therefore, interested in determining whether useful amounts of enzyme could be coupled to the gel in the presence of Tris buffer.

We were concerned that the amount of enzyme detected in an immobilized form might be caused by nonspecific absorption of the enzyme to the gel or a failure to wash the gel–enzyme complex under sufficiently rigorous conditions. Therefore, a control reaction was carried out where sepharose gel and enzyme in Tris buffer (0.1*M*, pH 7.8) were shaken slowly overnight at 4°C to monitor nonspecific binding of the enzyme to the gel. When the gel–enzyme mixture was washed with 0.1*M* Tris, 80% of the enzyme activity was recovered in 10 vol of the wash. The gel itself had no trace of phosphatase activity. The gel–enzyme complex, obtained by coupling of the enzyme to activated gel, was washed with an additional 20 vol of Tris-HCl buffer (2*M*; pH 7.8). A total of 3% of the activity was released in the washes. These results suggest that phosphatase was coupled to the gel by specific interactions that were not broken under rigorous washing conditions. This coupling demonstrated the feasibility of using Tris buffer. However, when coupling was carried out in the presence of sodium bicarbonate buffer, 22% more enzymatic activity was recovered on the gel. As shown in Table 1, less than 3% of the total alkaline phosphatase activity was found in the supernatant after 60 min at 37°C. Further, there was no loss of total enzymatic activity during incubations in Tris-HCl for 30 min or KCl (0.5*M*) for 60 min. However, small loses of total enzymatic activity, perhaps by inactivation of the enzyme, were observed (14.5 and 7.6% respectively) when the gel–enzyme complex was incubated in Tris-HCl at 37°C for 60 min or heated at 55°C in 0.5*M* KCl for 30 min. Under the latter conditions almost 5% of the enzymatic activity was released into the supernatant. These results suggest that higher salt concentrations may protect against the loss of phosphatase activity during longer incubations. When gel–enzyme complex was incubated with 100 m*M* sodium bicarbonate buffer, a small percentage of the enzyme activity was found in the supernatant as compared to Tris buffer (Table 1). More importantly, this release of the enzyme from gel–enzyme complex into supernatant in either Tris or sodium bicarbonate buffers was the same for 1 or 2 h. When gel–enzyme complex was incubated for 1 h at 37°C in the presence of 100 m*M* Tris-HCl buffer, pH 7.8, 3.2% activity

TABLE 1
Stability of Immobilized Alkaline Phosphatase Linked to Sepharose 4B

Incubation conditions[a]		Phosphatase activity,[b] % of control	
Buffer and time, min	Temp., °C	Gel	Supernatant
(a)	0	100	0
(a) 30	37	97	1.5
60	37	83	2.5
120	37	81	2.5
(b) 30	37	98	1.9
60	37	98	1.3
30	55	88	4.4
(c) 60	37	95	1.0
120	37	94	1.0

[a]Washed gel–enzyme complex (see Methods) was incubated with equal volumes of (a) 20 m*M* Tris HCl buffer, pH 7.8 or (b) 0.5*M* KCl at 37 or 55°C for 30, 60, 120 min, and (c) 100 m*M* sodium bicarbonate buffer, pH 7.8 at 37°C for 60 or 120 min. Phosphatase activity was determined in the gel and the supernatant after pelleting the gel–enzyme complex by centrifugation for 1 min at 1000 g.

[b]The activity of the gel–enzyme complex on ice is expressed as 100%. The control activity for (b) was same as the control without KCl.

was found in the supernatant. The protein loss into the supernatant was measured by the method of Bradford (*15*); 3.9% protein was found to be released into the supernatant after incubation, suggesting that the actual loss of the enzyme was not greater than the loss of the enzyme activity in the supernatant. Taken together, gel–enzyme coupling carried out either in the presence of Tris or bicarbonate buffer, was stable to 2*M* Tris-HCl buffer wash and to incubations at higher temperatures with 0.5*M* KCl. The data provide evidence that alkaline phosphatase was coupled to the gel by a covalent bond.

The gel–enzyme complex was stable at 4°C for at least 2 mo in the presence of 0.02% azide (12% of initial activity was lost after 3 mo) and can be stored at room temperature, although we do maintain it at 4°C.

Garen and Levinthal (*6*) observed no appreciable loss of *E. coli* phosphatase activity when it was heated at 85°C in the presence of 10m*M* Mg^{2+}. However, we found that alkaline phosphatase activity declined by about 85 and 100% when the soluble enzyme was heated at 85–87°C for 30 min in the presence and absence of Mg^{2+}, respectively (Table 2). When the immobilized enzyme was heated at 85–87°C for 30 min, 80% of its activity was recovered in the presence of 10 m*M* Mg^{2+} and about 32%

TABLE 2
Heat Inactivation of Soluble and Immobilized Alkaline Phosphatase

Conditions[a]	Alkaline phosphatase activity, % of control	
	Soluble enzyme	Insoluble enzyme
Control	100	100
Heating	<1	32.5[b]
Heating + $MgCl_2$	16	80.3

[a]Soluble and immobilized enzyme (100 μg protein/mL) were heated at 85–87°C for 30 min with occasional shaking in 100 m*M* Tris-HCl, pH 7.8, with or without $MgCl_2$ (10 m*M*). Control tubes were left on ice for the same time period. Phosphatase activity was determined as described in Methods. Data are averages of two separate experiments carried out in duplicate.

[b]When Mg^{2+} (10 m*M*) was added after the heat treatment, alkaline phosphatase activity was 36% of control.

of its activity was retained in the absence of Mg^{2+}. However, when Mg^{2+} was added immediately before assaying the soluble or immobilzed enzymes heated without Mg^{2+}, neither enzyme showed an appreciable increase in activity.

We are unable to explain the discrepancy between our results and those of Garen and Levinthal (*6*) because of the lack of experimental detail in their paper. However, thermal inactivation of alkaline phosphatase depends on the source of the enzyme, the ionic strength, the presence of activators, the pH, and the concentration of enzyme (*9*). Clearly, the insoluble enzyme was more stable to heat treatment than was the soluble phosphatase.

To test whether the gel–enzyme complex could remove phosphate groups from large proteins, a solution of the phosphoprotein casein (approximate molecular weight of 375,000 daltons) was incubated with the soluble and immobilized enzymes (Table 3). The soluble and immobilized enzymes released 77 and 75% of the phosphorus, respectively. TCA supernatants from casein alone, soluble enzyme, and casein plus sepharose 4B gel contained no detectable phosphorus (Table 3).

When the gel–enzyme complex, which had already been incubated with casein, was washed three times with 5 vol of 100 m*M* Tris-HCl, pH 7.8, and reincubated with casein, 1.06 μg phosphorus was measured in the TCA supernatant. Similar amounts of phosphorus were detected in the TCA supernatant when casein was treated with immobilized gel that was washed as described above after a second incubation with casein. These findings indicate that the insoluble enzyme can be used repeatedly.

The activities of microsomal ACAT, which esterifies cholesterol and purified NADPH–cytochrome P-450 reductase (P-450 reductase) from

phenobarbitol-treated rat liver, were inhibited when microsomes or purified P-450 reductase were incubated with soluble alkaline phosphatase. This loss was dependent upon the incubation time and phosphatase concentration (data not shown). These results are consistent with the hypothesis that these enzymes are less active in their dephosphorylated form (*11,12*). We compared the effects of soluble and immobilized phosphatases on the activities of these two enzymes. The insoluble and soluble phosphatases reduced the activity of ACAT by 39 and 20%, respectively, and P-450 reductase by 89 and 74%, respectively. These losses in enzymatic activity can not be attributed to contamination of the phosphatase with proteases since no proteolytic activity was detected by the method of Nelson et al. (*16*) when large amounts of the soluble enzyme were incubated with casein.

Though the immobilization of alkaline phosphatase on insoluble materials, such as porous glass (*13*), has been previously reported, the present method is rapid, simple, and reproducible. The amount of active alkaline phosphatase bound to sepharose-4B is 32-fold higher than reported for glass. Barimina et al. (*14*) immobilized the enzyme on DEAE-cellulose, but did not report the amount of alkaline phosphatase bound. We can not compare the stability of their immobilized phosphatase with our own product because they studied the stability of the enzyme under different conditions.

This gel–enzyme complex also served our primary purpose of removing phosphate groups from large proteins, for example casein, ACAT, and cytochrome P-450 reductase. Therefore, this immobilized enzyme is an effective and useful tool for studying the dephosphorylation of soluble or membrane-bound enzymes.

TABLE 3
Treatment of Casein with the Gel–Enzyme Complex[a]

	Phosphorus content in TCA filtrate,[e] %
Casein[b]	0
Casein + sepharose (gel)	0
Casein + gel–enzyme	0.27[c]
Casein + phosphatase (soluble)	0.28[d]
Soluble phosphatase	0

[a]A casein solution (440 μg containing 1.56 μg phosphorus) was incubated with the soluble and immobilized enzymes in 1 mL Tris-HCl buffer (20 m*M*, pH 7.8) for 30 min at 37°C. The suspension was centrifuged (1000*g* for 1 min) and inorganic phosphorus content was measured in the TCA supernatant as described by Chen et al. (*10*).

[b]Total measured phosphorus content was 1.56 μg (0.35%).

[c,d]1.176 μg and 1.214 μg phosphorus, respectively, were obtained in TCA filtrate.

[e]Amount of phosphorus (μg) × 100/total amount of protein used (μg).

ACKNOWLEDGMENTS

We gratefully acknowledge the expert technical assistance of Carol Doherty.

These studies were supported by US Public Health Service Grant AM 28298 and a fellowship from the American Heart Association, Maryland Affiliate.

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