Functional and Structural Properties of Immobilized Subunits of
Escherichia coli Alkaline Phosphatase*

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Alkaline phosphatase from Escherichia coli has been immobilized on Sepharose CL-4B using low levels of cyanogen bromide activation in order to favor the attachment of the dimer to the support through a single covalent linkage. The matrix-bound subunits obtained after dimeric preparation, sodium dodecyl sulfate gel electrophoresis of protein released from the Sepharose demonstrated the presence of sufficient amounts of dimeric structures to account for the phosphatase activity in these populations. The protein in the matrix-bound subunits was more susceptible to proteolytic digestion than that in the matrix-bound dimer, while the catalytic activity in both immobilized derivatives remained constant. These results indicate that the subunits of alkaline phosphatase are catalytically inactive, and that the low levels of phosphatase activity observed are due to the presence of trace amounts of contaminating dimers.

Titration of matrix-bound 125I-labeled subunits with soluble nascent 125I-labeled subunits restored 60% of the original phosphatase activity. The isotope ratio of 125I to 125I in the matrix-bound monomer bound 0.9 eq of Zn²⁺, while the matrix-bound dimer bound 4.1 eq of Zn²⁺; the Zn²⁺ binding sites in both immobilized derivatives had dissociation constants of 2 x 10⁻⁸ M or lower.

The preparation and characterization of immobilized subunits of alkaline phosphatase under solvent conditions optimal for renaturation should provide a valuable approach for studying the role of subunit interactions in this enzyme. Moreover, the techniques used in this investigation are generally applicable for the characterization of other immobilized enzyme derivatives.

Although many proteins form oligomeric structures, the importance of subunit interactions for enzymatic function, regulation, or conformational stability has not yet been established. The major reason for this difficulty is that the contributions of secondary and tertiary structures to the properties of the native protein cannot be distinguished from those which arise as a result of subunit interactions. Moreover, individual subunits generally cannot be maintained under conditions optimal for renaturation since they will spontaneously reform the polymeric structure.

The effects of interactions between the subunits of Escherichia coli alkaline phosphatase are of particular interest because the identical subunits of the dimer are reported to be functionally nonequivalent. Studies based on covalent phosphorylation (1-4), phosphate binding (3, 5-11), and pre-steady state kinetics (12-17) have indicated that alkaline phosphatase exhibits an unusual negative cooperativity. These negative cooperative subunit interactions have been implicated in "half-site" catalytic mechanisms in which the subunits of the dimer turn over alternately in the enzyme reaction (18-20). A different type of mechanism, in which the subunits turn over independently, has also been suggested in other studies based on pre-steady state kinetics (21), phosphate binding (22-24), and genetic or chemically modified hybrid dimers (25-27).

In order to clearly define the properties which occur as a direct result of subunit interactions in alkaline phosphatase, it is necessary to characterize the renatured subunit not only in the intact dimer, but also in the absence of subunit interactions. Although the monomeric subunits of alkaline phosphatase have been studied in different conformational states which occur during the process of refolding and renaturation, these subunits were not investigated under optimal conditions for renaturation since they exist only as transient monomers which must be maintained by acidic pH, low temperatures, metal-free conditions, or low concentrations of formamide (28-31).

A direct investigation of the properties of subunits under optimal renaturation conditions has recently been made possible through the use of immobilized enzyme derivatives (32). Immobilization to an insoluble support through a single covalent linkage; the protein is then dissociated, removing noncovalently attached subunits, to produce an immobilized subunit preparation. These monomers cannot reassociate with each other because they are attached to the rigid support; consequently, the properties of individual subunits can be characterized without the risk of spontaneous reassociation. This technique was first used to demonstrate that the subunits of aldolase are catalytically active and gain greater stability upon reassociation into an oligomeric structure (33-35). It has subsequently been applied to investigate the subunits of many other oligomeric enzymes including glycogen phosphorylase (36), avidin (37), lactate dehydrogenase (38-40), fructose diphosphatase (41), transaldolase (42), glyceraldehyde-3-phosphate dehydrogenase (43, 44), aspartate 4-decarboxylase (45), phosphoglucone isomerase (46), creatine kinase (47, 48), and arginase (49).

This approach to studying the function of subunits derived from oligomeric proteins has been used to prepare and char-

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acterize immobilized subunits of alkaline phosphatase. It has been found that preparation of a completely homogeneous subunit population immobilized on cross-linked Sepharose is extremely difficult to achieve with alkaline phosphatase, even at very low levels of activation of the support, and that the structure of immobilized enzyme derivatives must be characterized by oxidizing and analyzing interpretations of experimental data. These approaches, used to determine the structural homogeneity of immobilized derivatives of alkaline phosphatase, should prove to be generally applicable to the study of other immobilized proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**

All Tris buffer solutions were adjusted to the proper pH with HCl. Glassware and solutions were rendered metal free following the procedure described by Cospak et al. (51) containing 1 x 10^{-6} M insulin. The enzyme was resistant to the cells of osmotic shock (52) and the supernatant was applied to a DAE-Sepharose CL-6B column (4.5 x 20 cm) pre-equilibrated with 10 mM Tris-chloride, pH 8.0. After washing the column with 1 bed volume of 10 mM Tris-chloride, pH 8, 50 mM NaCl, the enzyme activity was eluted with a 26-liter linear gradient from Tris-chloride to 150 mM NaCl in 10 mM Tris-chloride, pH 8. The peak of enzyme activity was pooled and precipitated by dialysis against saturated ammonium sulfate, pH 7. The precipitate was centrifuged at 19,000 x g, 20 min, and dialyzed exhaustively against the desired buffer. Iodinated alkaline phosphatase had specific activities between 30 and 42 units/mg and a specific radioactivity of about 10^{6} cpm/mg. No loss of radiolabel iodine was observed over the course of these experiments. Samples were counted in a Packard auto Gamma spectrometer.

**Irreversible Immobilization of Alkaline Phosphatase on Sepharose CL-4B**—Cross-linked Sepharose was activated following the procedure described by Axén et al. (56). A stock CNBr solution was prepared immediately prior to use by dissolving 30 mg of CNBr in 10 ml of 0.1 M NaHCO_{3}, pH 11.0. In 2 suspension (50 ml) of Sepharose CL-4B in 0.1 M NaHCO_{3}, pH 11.0, was activated by the addition of an aliquot (0.5 to 10 ml) of the CNBr solution; when the aliquot represented more than 10% of the total volume of the suspension, the concentration of Sepharose of the suspension after addition of the CNBr solution was 1 x 2. The suspension was stirred for 12 min at room temperature, the pH was maintained between 10.9 and 11.0 by addition of 2 N NaOH. An aliquot of suspension was filtered on a sintered glass funnel, washed with cold 0.5 M NaCl, 0.1 M NaHCO_{3}; pH 10, and the filtered gel was combined with an equal volume of 5 mg/ml of alkaline phosphatase in 0.5 M NaCl and 0.1 M NaHCO_{3}, pH 10. The suspension was incubated overnight at 4°C with occasional shaking.

The immobilized enzyme was rinsed and incubated in 0.1 M ethanolamine, pH 8.8, for 1 h at 0°C. The gel was then washed exhaustively with 0.1 M NaCl, 0.1 M Tris-chloride, pH 8.0, 2 x 10^{-3} M ZnCl_{2}, 4 mg MgCl_{2}, followed by 0.1 M Tris-chloride, pH 8.0, 2 x 10^{-2} M ZnCl_{2}, 4 mg MgCl_{2} on a sintered glass funnel or by resuspension and centrifugation in graduated Pyrex centrifuge tubes.

**Dissociation of Immobilized Alkaline Phosphatase**—The Sepharose-bound enzyme was transferred into 0.1 M guanidine hydrochloride, 0.1 M maleic acid, pH 2.0, as a 1 in 4 suspension by five cycles of resuspending, centrifugation, and decantation of the supernatant. The immobilized protein was then incubated for 1 h at room temperature, washed as a 1 in 4 suspension in 6 M guanidine hydrochloride, 0.1 M maleic acid, pH 2.0, 4 x 10^{-3} M ZnCl_{2}, 4 mg MgCl_{2}. The enzyme could be stored indefinitely at 4°C in this buffer, with a trace of sodium azide added.

**Alkaline Phosphatase purified using this procedure had a specific activity of 38 to 45 units/mg at 21°C.**

**Assays**—Solu ble alkaline phosphatase activity was measured by the increase in absorbance at 400 nm, with 10^{-4} M p-nitrophenyl phosphate, pH 10, containing 1 mg p-nitrophenyl phosphate/ml.

**Alkaline phosphatase immobilized on Sepharose was assayed by the addition of an aliquot of a uniform suspension of the Sepharose to 2 ml of the assay mix described above. The absorbance was continuously monitored as the assay mixture was stirred magnetically outside the spectrophotometer by a magnetic stirrer which fitted into the cuvette holder. This device was generously loaned to us by Dr. W. W. C. Chan of McMaster University. Alternatively, the assay mixture was stirred magnetically outside the spectrophotometer, and at 1- to 3-min intervals, the cuvette was transferred to the spectrophotometer where the increase in absorbance at 400 nm was recorded for a few seconds. One unit of enzyme activity is defined as the release of 1 amol of p-nitrophenol/min at 21°C, using an extinction coefficient of 17,000 for p-nitrophenol at pH 8.0 at 400 nm (18).

**Protein Determination**—Solu ble alkaline phosphatase concentrations were determined from the absorbance at 278 nm, using an extinction coefficient of 0.72 (0.1%, 1 cm) (53).

**Immobilized protein values were deter mined by counting {\textsuperscript{35}}S or {\textsuperscript{14}}C based on the specific radioactivity of the iodinated alkaline phosphatase. The presence of Sepharose did not effect the efficiency of {\textsuperscript{35}}S and {\textsuperscript{14}}C counting. The amount of Sepharose was determined from the packed volume of gel after 2 min centrifugation at 1700 x g (54). The concentration of the Sepharose CL suspensions was routinely determined by the volume of packed gel resuspended in the total volume of the suspension. Consequently, 1 ml of Sepharose CL in a total volume of 10 ml is described as a 1 in 4 suspension.**
phosphate, pH 7.5, mixed with an equal volume of 2.0 mg/ml of alkaline phosphatase in the same buffer, and incubated overnight at 4°C. An aliquot of the DTSP-Sepharose was reduced with 0.15 M mercaptoethanol in 1 mM Tris-chloride, pH 8, the mercaptoethanol removed by washing with the Tris-chloride, pH 8, and the gel then blocked with 0.1 M 5, 5'-dithiobis-(2-nitrobenzoic acid) in 1 M Tris, pH 8. The mercaptoethanol removed by exhaustive washing with the Tris-chloride, pH 8. The free sulphydryl groups produced on the gel after reduction of matrix-bound DTSP were quantitated by the absorbance at 412 nm (57) produced by the addition of mercaptoethanol to the DTNB-blocked Sepharose.

Reversibly immobilized alkaline phosphatase was blocked, washed, and dissociated under the same conditions described for irreversibly immobilized alkaline phosphatase derivatives.

Cross-linking, Release, and SDS Gel Electrophoresis of Reversibly Immobilized Alkaline Phosphatase—The Sepharose derivatives were washed in 0.5 M NaCl, 0.1 M NaHCO₃, pH 10, as 1 in 2 suspensions and cross-linked with 1.5 mg/ml of dimethyl suberimidate (58) for 1 h before the protein was released from the support by incubation as a 1 in 2 suspension in 60 mM mercaptoethanol in 2 mM Tris-chloride, pH 8, for 1 h. The suspensions containing the released protein were then reacted with 0.2 M iodoacetamide and 0.01 M Tris-chloride, pH 8, overnight at 21°C. The Sepharose was removed by filtration; then sodium deoxycholate (0.015%) was added to the filtrate and incubated for 2 h. The protein and deoxycholate carrier (59) were precipitated by addition of 70% trichloroacetic acid to a final concentration of 7%.

The samples were then analyzed by SDS-gel electrophoresis as described by Weber and Osborn (60) using gels containing 7% acrylamide and 2.0% bisacrylamide. The gels were prerun for 3 h with 0.01% (v/v) thioglycolic acid before application of the samples (~10 μg of protein/gel). Gels were stained and destained according to the procedure described by Fairbanks et al. (61). The staining intensity of the bands in the gels was determined using a Gilson gel scanner.

Proteolytic Digestion of Immobilized Alkaline Phosphatase Derivatives—Proteolytic digestion was initiated in matrix-bound alkaline phosphatase derivatives, prepared as 1 in 2 suspensions in 0.1 mM Tris-chloride, pH 8, 2 × 10⁻⁵ M ZnCl₂, and 4 mM MgCl₂, by adding pronase (10 mg/ml) to a final concentration of 0.07 mg/ml. At points throughout the course of the digestion, samples of the supernatant were taken to determine the counts released from the gel; the suspension was centrifuged 1 min before sampling the supernatant and was immediately resuspended. After 1 h at room temperature, the samples were washed with 1 M NaCl, 0.1 M Tris-chloride, pH 8, 2 × 10⁻⁵ M ZnCl₂, 4 mM MgCl₂, followed by 0.1 M Tris-chloride, pH 8, 2 × 10⁻⁵ M ZnCl₂, 4 mM MgCl₂, and the enzyme activity and protein remaining on the gel were determined.

Complementation of ¹²⁵I-Labeled Matrix-bound Subunits with Soluble ¹²⁵I-Labeled Subunits—Immobilized subunit alkaline phosphatase was stored slowly as a 1 in 100 suspension in 0.5 M Tris-chloride, pH 8.0, 2 × 10⁻⁵ M ZnCl₂, 4 mM MgCl₂. At 15-min intervals, an aliquot of 7 ml excess of ¹²⁵I-labeled alkaline phosphatase (4 mg/ml; <0.1 unit/mg) in 6 mM guanidine hydrochloride was added. The course of the titration was monitored by taking a 1-ml aliquot of the suspension before adding the next aliquot of protein, washing the sample with 1 M NaCl, 0.1 M Tris-chloride, pH 8, 2 × 10⁻⁵ M ZnCl₂, and 4 mM MgCl₂ (six times with 15 ml), followed by 0.1 M Tris-chloride, pH 8.0, 2 × 10⁻⁵ M ZnCl₂, and then counting. After 25 aliquots were added, the entire suspension was filtered, washed exhaustively with the high and low salt buffers as described above, assayed, and counted. After 12 h at 4°C, further additions of denatured ¹²⁵I-labeled alkaline phosphatase to a small aliquot of the washed gel continued the following sample procedure.

Zinc Binding in Immobilized Alkaline Phosphatase Derivatives—Matrix-bound alkaline phosphatase derivatives and a control Sepharose sample were washed six times as 1 in 5 suspensions and incubated for 40 h in 10 mM 8-hydroxyquinoline-5-sulfonic acid, pH 8.0, at room temperature to remove all bound metal ions. After removing the choice, the samples were washed six times as a 1 in 5 suspension with a 10 mM Tris-chloride, pH 8.0, 50-μl aliquots (0.25 nmol) of a 5.0 × 10⁻⁶ M ZnCl₂ stock solution (2.94 × 10⁻⁶ M NaCl) were added. The amount of Zn⁺⁺ bound specifically to the protein in the immobilized alkaline phosphatase derivatives was obtained by subtraction of the total amount of Zn⁺⁺, the Zn⁺⁺ that was free in solution as well as Zn⁺⁺ bound nonspecifically to the Sepharose or the glass tube. This value is equal to the total amount of Zn⁺⁺ added to the Sepharose control that had the same concentration of free Zn⁺⁺ as the respective immobilized alkaline phosphatase derivative.

Determination of Alkaline Phosphatase Activity—Experimental values at very low or very high Zn⁺⁺ concentrations were not used in the Scatchard plots since, on one hand, the amount of free Zn⁺⁺ was too low for accurate determination and, on the other hand, the corrections for free Zn⁺⁺ and nonspecifically bound Zn⁺⁺ were too large relative to the total amount of added Zn⁺⁺. Therefore, the Scatchard plots were prepared using only samples where the counts from free Zn⁺⁺ were 5-fold greater than the background and when Zn⁺⁺ bound specifically to the protein was at least 40% of the total Zn⁺⁺ added.

RESULTS

E. coli alkaline phosphatase was immobilized on Sepharose CL with low levels of cyanogen bromide (1 mg of CNBr/ml of packed gel) in order to favor the attachment of the dimer to the solid support through one covalent linkage. When the matrix-bound (MB) dimer was dissociated and renatured, the MB-"monomer" obtained exhibited 20% of the phosphatase activity with 30% of the specific activity of the original MB dimer. This result raised the possibility that the subunit of alkaline phosphatase might be catalytically active with a much less efficient turnover than the native dimer. Since previous investigations produced conflicting reports on whether a monomeric form of the enzyme might be catalytically active (90, 31, 52), experimental studies were conducted to establish the functional and structural properties of immobilized alkaline phosphatase subunits.

Fig. 1 shows that the extent of immobilization of ¹²⁵I-labeled alkaline phosphatase protein increased as the amount of CNBr used for activation of the support varied from 0.1 to 2.0 mg of CNBr/ml of packed gel. A plot of enzyme activity in MB-alkaline phosphatase versus the protein content (Fig. 2) demonstrates that the specific activity of MB-alkaline phosphatase varies between 15 and 30 units/mg for these different immobilized alkaline phosphatase subunits.

![Fig. 1 (left). The dependence of the immobilized protein content on the extent of cyanogen bromide activation. The cyanogen bromide activation of Sepharose CL, immobilization, and protein determinations were performed following the procedures described under "Methods.

![Fig. 2 (right). Phosphatase activity as a function of the immobilized protein content in the MB-alkaline phosphatase. Immobilizations, enzyme assays, and protein determinations were performed as described under "Methods."](http://www.jbc.org/Downloadedfrom/fig1.jpg)
preparations; an average value of 22 units/mg can be obtained from the linear region of the curve.

When the MB-dimer alkaline phosphatase, prepared on Sepharose CL activated with different levels of CNBr, was dissociated with acidic guanidine hydrochloride, the level of catalytic activity in the MB-monomer relative to the activity of the MB-dimer from which it was derived showed a strong dependence on the extent of activation of the Sepharose CL (Fig. 3). As the activation varied from 0.1 to 2.0 mg of CNBr/ml of gel, the enzyme activity in the MB-monomer increased from less than 5% to more than 20% of the activity observed in the MB-dimer. This evidence indicated that phosphatase activity is not an intrinsic property of the MB-monomer since enzymatically active MB-monomers should have a constant activity relative to the MB-dimer that is independent of the level of CNBr activation. Because MB-alkaline phosphatase in acidic guanidine hydrochloride had less than 0.15% of its original activity and repeated washing of the MB-enzyme with denaturant failed to remove any additional protein (Fig. 4), the presence of enzyme activity in the MB-monomer arises from the interaction of subunits attached to the support in close enough proximity to each other to reform a dimeric structure upon removal of denaturant. Consequently, it is necessary to lower the level of CNBr activation of Sepharose CL to 0.3 mg/ml of gel in order to reduce the contaminating dimer molecules to 5% of the MB-monomer population (Fig. 3).

The presence of contaminating dimeric structures in a MB-monomer population can be directly demonstrated if the protein is attached to the support through a cleavable linkage (63–65). Reversibly immobilized monomer populations can be cross-linked, released from the support, and then analyzed for the presence of polymeric structures by SDS-gel electrophoresis.

Since recent studies using reversibly immobilized alkaline phosphatase did not establish whether a decrease in the level of activation of this type of support resulted in a decrease in either the relative activity or the amount of dimer contaminating the MB-monomer population (65), alkaline phosphatase was reversibly immobilized on DTSP-Sepharose CL activated to different extents at relatively low levels. This reversible support was obtained by reacting ethylene diamine with CNBr-activated Sepharose CL-4B and then incubating the resulting amino-Sepharose with an excess of the bifunctional reagent, dithiobis (succinimidyl propionate) (DTSP) (66) to produce a reactive, lysyl-specific spacer arm which can be cleaved by reduction with mercaptoethanol. The extent of reaction of the amino-Sepharose with DTSP was determined from the free sulphydryl content of the reduced DTSP-Sepharose.

The protein content and enzyme activity of two samples of alkaline phosphatase reversibly immobilized on Sepharose containing different levels of DTSP are shown in Table I. A decrease in the level of Sepharose activation from 51 to 27 nmol of DTSP/ml of gel reduces by 2-fold the amount of immobilized protein and activity in the MB-dimer with no change in the specific activity (19 units/mg). Moreover, the drop in extent of activation of the support results in a decrease in the catalytic activity of the MB-monomer from 10% to 4.5% of that of the corresponding MB-dimer sample, consistent with the studies performed on irreversibly immobilized alkaline phosphatase (Fig. 5).

When the reversibly immobilized protein was irreversibly cross-linked with dimethyl suberimidate, released from the Sepharose with mercaptoethanol, and analyzed by SDS-gel electrophoresis, dimeric structures could be observed in both the MB-dimers and MB-monomers (Fig. 5). Although the contaminating dimers in both the MB-monomers gave very faint bands even on heavily loaded SDS gels, the amount of protein in the band could be quantitatively determined by densitometric scans on a sensitive absorbance scale (Fig. 6, b and c). After correcting for the 49% efficiency in cross-linking with dimethyl suberimidate, evaluated from gel scans of the MB-dimer (Fig. 6a), as well as for the different molecular weights of the dimer and monomer, it could be calculated that 3.3% and 6.8% of the molecules in the two MB-monomer samples (27 and 51 nmole of DTSP/ml of gel, respectively) are dimers (Table II). These amounts of contaminating dimer correspond closely to the relative levels of enzyme activity in the MB-subunit samples (4.5 and 10%, respectively), indicating that the enzyme activity observed in these MB-monomer preparations can be accounted for by the presence of contaminating dimers, and that the MB-monomer of alkaline phosphatase is catalytically inactive.

Further support that the enzyme activity in the MB-monomer is not due to a low functional activity of the subunit is shown by proteolytic digestion of irreversibly immobilized MB-dimer and MB-monomer alkaline phosphatase (Fig. 7). Approximately 30% of the MB-dimer protein was removed

![Table 1](http://www.jbc.org/)

**Table 1**

<table>
<thead>
<tr>
<th>Sample</th>
<th>DTSP content</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific activity</th>
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<td></td>
<td>units/ml gel</td>
<td>%</td>
<td>µg/ml gel</td>
<td>units/mg</td>
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<tr>
<td>MB-dimer</td>
<td>27</td>
<td>2.4</td>
<td>100</td>
<td>125</td>
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<tr>
<td>MB-monomer</td>
<td>27</td>
<td>0.11</td>
<td>4.5</td>
<td>68</td>
</tr>
<tr>
<td>MB-dimer</td>
<td>51</td>
<td>4.7</td>
<td>100</td>
<td>265</td>
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<tr>
<td>MB-monomer</td>
<td>51</td>
<td>0.48</td>
<td>10</td>
<td>136</td>
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* Based on nmol of free sulphydryl/ml of gel, determined after reduction of the gel as described under "Experimental Procedures."

* Based on 3H.

* Samples were prepared as described under "Experimental Procedures," using 2.0 (c) and 7.5 mg of CNBr/ml of gel (d) for activation.
Fig. 5. SDS-gel electrophoresis of reversibly immobilized alkaline phosphatase derivatives after cross-linking and release from the DTSP-Sepharose. Samples are (1) MB-dimer, 27 nmol of DTSP/ml of gel; (2) MB-monomer, 27 nmol of DTSP/ml of gel; (3) MB-dimer, 51 nmol of DTSP/ml of gel; (4) MB-monomer, 51 nmol of DTSP/ml of gel. The enzyme activities and protein contents for these samples are shown in Table I. All procedures are described in detail under "Methods." Approximately 15 µg of protein were applied to the MB-dimer SDS gels (samples 1 and 3), while about 10 µg of protein were applied to the MB-monomer SDS gels (samples 2 and 4) so that the intensity of the lower molecular weight band was similar in all samples. Migration proceeds from the top to the bottom of the gel.

Fig. 6. Densitometric scans of the SDS gels shown in Fig. 5 of the reversibly immobilized alkaline phosphatase derivatives. Scans are of (a) MB-dimer, 27 nmol of DTSP/ml of gel; (b) MB-monomer, 27 nmol of DTSP/ml of gel; (c) MB-monomer, 51 nmol of DTSP/ml of gel. The broken traces in scans b and c show scans at 4 times the sensitivity to clarify the differences in the minor peaks migrating at the dimer molecular weight. Migration proceeds from left to right. The scan of the MB-dimer, 51 nmol of DTSP/ml of gel, was identical with that of the MB-dimer, 27 nmol of DTSP/ml of gel shown in a. Scans were performed on a Gilson gel scanner.

Table II

<table>
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<tr>
<th>Sample</th>
<th>DTSP content</th>
<th>% Dimer a</th>
<th>% Dimer b (by weight)</th>
<th>% Dimer c (by molarity)</th>
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<td>MB-dimer</td>
<td>27</td>
<td>51</td>
<td>49</td>
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<td>MB-dimer</td>
<td>51</td>
<td>51</td>
<td>49</td>
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<td>MB-monomer</td>
<td>51</td>
<td>94</td>
<td>5.6</td>
<td>13</td>
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</table>

a See Table I.

Table II

Distribution on SDS gel electrophoresis of protein released from cross-linked reversibly immobilized alkaline phosphatase derivatives.

The ability of the MB-monomer to reassociate with soluble subunits to form a dimer was demonstrated by a titration of during proteolytic digestion (Fig. 7) without a concomitant decrease in enzyme activity (Table III), indicating that the MB-dimer contains a minor proportion of catalytically inactive molecules that are susceptible to pronase. Native alkaline phosphatase has previously been shown to be resistant to proteolytic digestion by both pronase and trypsin (29). Although the MB-monomer lost 70% of its immobilized protein as a result of pronase digestion, its catalytic activity was not affected (Table III), indicating that phosphatase activity does not arise from the majority of the MB-monomer protein population. The specific activity of the MB-monomer has increased 4-fold after pronase digestion, compared to only a 1.4-fold increase in the specific activity of the MB-dimer. Although it might be expected that 85 to 90% of protein (based on 7% contaminating dimers) in the MB-monomer would be digested, raising the specific activity to the same level as the MB-dimer (43 units/mg), complete proteolytic digestion of the inactive subunits may be partially obstructed by the Sepharose matrix.

The ability of the MB-monomer to reassociate with soluble subunits to form a dimer was demonstrated by a titration of
the $^{125}$I-labeled MB-monomers with soluble $^{131}$I-labeled sub-units. The ratio of the $^{125}$I-labeled protein to $^{125}$I-labeled protein serves as a very sensitive indication of the extent of reassociation throughout the titration (Fig. 8). The complementation of $^{125}$I-MB-monomer was complete after 20 additions of $^{131}$I-soluble protein, resulting in an isotope ratio of 1.0. After 25 additions, the sample was washed and stored at 4°C for 12 h. Further additions of soluble protein to an aliquot of the MB-renatured dimer failed to increase the ratio of $^{125}$I to $^{131}$I-labeled protein above 1.1.

**Control experiments** in a similar titration have shown that Sepharose binds less than 0.1 μg of $^{131}$I-labeled protein/ml of gel (0.07 μg/ml of gel). A MB-dimer titrated with $^{131}$I-subunits showed no increase in the protein content, but 10% of the total protein was labeled with $^{131}$I, suggesting that a finite equilibrium may exist between monomer and dimer resulting in subunit exchange. However, such a conclusion is premature without obtaining conditions that allow a much higher percentage of subunit exchange and thus establishing that this exchange is a property representative of all the MB-dimer molecules on the support. If a finite monomer-dimer equilibrium does exist, it appears that it can only be detected when a large excess of soluble subunits is present since both the activity and protein in the MB-dimer are stable for several days in buffer. It is not necessary to consider the possibility of subunit exchange when analyzing the MB-renatured dimer since all $^{125}$I-subunits are covalently attached to the Sepharose.

The activity and protein contents of the MB-renatured dimer are compared to those of the MB-dimer and MB-monomer alkaline phosphatase in Table IV. The original MB-dimer contained 90 μg of protein/ml of gel at a relatively high specific activity of 31 units/mg. After dissociation and renaturation, the MB-monomer contained 54% of the original protein, but only 6% of the enzyme activity observed in the MB-dimer, resulting in a 9-fold drop in specific activity. When the MB-monomer was complemented with soluble $^{131}$I-subunits, 97% of the original MB-dimer protein content was restored, with a ratio of $^{131}$I-protein to $^{125}$I-protein of 1.06, indicating that the MB-subunit had been fully complemented. However, the restoration of catalytic activity in the MB-renatured dimer was not quite so efficient; 64% of the phosphatase activity in the MB-dimer was observed in the MB-renatured dimer. This represents a 6-fold increase in the specific activity upon complementation of the MB-dimer with soluble subunits. Other complementation experiments have resulted in restoration of up to 80% of the activity. This efficiency of renaturation is consistent with the extent of renaturation of the soluble protein reported by Schlesinger and Barrett (28) who observed 70 to 100% renaturation of acid-dissociated soluble alkaline phosphatase.

When the MB-renatured dimer was reconstituted with acidic guanidine hydrochloride, the enzyme activity dropped to 7% of the MB-renatured dimer while the isotope ratio was decreased 4-fold to 0.26, confirming that a dimeric structure is responsible for phosphatase activity. Theoretically, the ratio should have dropped to zero after dissociation; the reason for incomplete release of $^{131}$I-protein is not known.

The zinc binding properties of the MB-alkaline phosphatase derivatives were investigated because of the importance of Zn$^{2+}$ for the catalytic activity and structural stability in the native soluble enzyme (67–72). Bound Zn$^{2+}$ can be completely

**Fig. 7** (left). Decrease in immobilized protein content during proteolytic digestion. The release of $^{125}$I-labeled protein from the MB-dimer ($) and MB-monomer ($) was determined by counting the $^{125}$I-protein in the supernatant during treatment with 0.07 mg/ml of pronase. The amount of remaining protein in the samples is expressed as per cent of the original protein on the Sepharose. The last points, taken after 60 min, correspond to the direct determination of the insoluble protein in the MB-alkaline phosphatase derivatives after removal of the pronase and soluble protein by washing with high and low salt buffers. A detailed protocol is described under "Methods." The percentage of sample before digestion with pronase for 1 h (see "Experimental Procedures").

**Fig. 8** (right). Titration of $^{131}$I-labeled MB-monomer alkaline phosphatase with soluble denatured $^{131}$I-labeled subunits. The ratio of $^{131}$I-labeled protein to $^{131}$I-labeled protein bound to the Sepharose is plotted as a function of the number of additions of soluble denatured subunits. The titration was performed following the procedure described under "Methods." After 25 additions, the gel was washed and stored 12 h. Subsequent additions were made following the same procedure.

**TABLE III**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activity $^a$</th>
<th>Protein $^b$</th>
<th>Specific activity $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/ml gel</td>
<td>μg/ml gel</td>
<td>units/mg</td>
</tr>
<tr>
<td>MB-dimer</td>
<td>2.55 90</td>
<td>59 66</td>
<td>43 139</td>
</tr>
<tr>
<td>MB-monomer</td>
<td>0.18 100</td>
<td>12 25</td>
<td>15 428</td>
</tr>
</tbody>
</table>

$^a$ MB-dimer was prepared as described under "Experimental Procedures" using 0.3 mg of CNBr/ml of gel. MB-monomer was prepared by dissociation and renaturation.

$^b$ Percentage of sample before digestion with pronase for 1 h (see "Experimental Procedures").

$^c$ Activity and protein content of MB-alkaline phosphatase derivatives after proteolytic treatment

**TABLE IV**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activity $^a$</th>
<th>$^{131}$I-protein $^b$</th>
<th>$^{131}$I-protein $^c$</th>
<th>Specific activity $^d$</th>
<th>Isotope ratio $^e$</th>
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<td>μg/ml gel</td>
<td>%</td>
<td>μg/ml gel</td>
<td>%</td>
</tr>
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<td>MB-dimer</td>
<td>2.8 100</td>
<td>90 100</td>
<td>31 100</td>
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<td></td>
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<td>49 54</td>
<td>3.5 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MB-renature dimer</td>
<td>1.8 64</td>
<td>42 47</td>
<td>45 50</td>
<td>21 67</td>
<td>1.06</td>
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<tr>
<td>MB-renature dimer after dissociation</td>
<td>0.12 4</td>
<td>36 40</td>
<td>9.5 11</td>
<td>3 9</td>
<td>0.26</td>
</tr>
</tbody>
</table>

$^a$ MB-dimer was prepared using Sepharose CL-4B activated with 0.3 mg of CNBr/ml of gel. MB-renatured dimer was prepared by complementation of MB-monomer with soluble subunits. MB-renatured dimer was dissociated with acidic guanidine hydrochloride using the same procedure for dissociation of the original MB-dimer. All procedures are described in detail under "Experimental Procedures."

$^b$ Ratio of micrograms of $^{131}$I-labeled protein to micrograms of $^{131}$I-labeled protein.

$^c$ Percentage of that observed in MB-dimer.
removed by treatment with the chelating agent, 8-hydroxyquinoline-5-sulphonic acid, to produce an inactive dimeric enzyme (68). Catalytic activity can be restored by the addition of Zn$^{2+}$; although it is generally agreed that the dimeric enzyme binds four equivalents of Zn$^{2+}$ (68-75), there is conflicting evidence concerning the number of Zn$^{2+}$ ions required to restore most of the catalytic activity (6, 7, 68-77).

Titration of the MB-apodimer and MB-apomonomer in metal-free buffer produced the binding curves shown in Fig. 9. A linear plot of free Zn$^{2+}$ (as counts per min) versus nanomoles of Zn$^{2+}$ added was observed over the entire range of Zn$^{2+}$ concentrations for a reference solution of buffer containing no added Sepharose (Fig. 9). The plots of free Zn$^{2+}$ versus added Zn$^{2+}$ for the MB-apodimer, MB-apomonomer, and a control Sepharose are not linear, and the relative amounts of free Zn$^{2+}$ are lower than in the reference solution, demonstrating that Zn$^{2+}$ binds to the different Sepharose suspensions. There is essentially no free Zn$^{2+}$ (counts per min) in solution for either the MB-apomonomer or MB-apodimer suspensions at low Zn$^{2+}$ concentrations, indicating the presence of high affinity binding sites for Zn$^{2+}$ in both of these MB-alkaline phosphatase derivatives. Extrapolation of the linear region of the plots at higher Zn$^{2+}$ concentrations indicates that these sites are saturated after the addition of 1.7 and 6.8 nmol of Zn$^{2+}$ to the MB-apomonomer and MB-apodimer, respectively. In contrast, there appears to be relatively weak Zn$^{2+}$ binding to the control Sepharose which contains no bound alkaline phosphatase. Even at the lowest Zn$^{2+}$ concentrations, a large part of the Zn$^{2+}$ does not bind to the control Sepharose, showing that it does not contain the high affinity sites that are present in both the MB-apomonomer and MB-apodimer.

Scatchard plots for the Zn$^{2+}$ specifically bound to the protein in the MB-apomonomer and MB-apodimer are shown in Fig. 10. The plot of MB-dimer is linear at higher Zn$^{2+}$ concentrations and extrapolates to a value of 4.1 g atoms of Zn$^{2+}$/mol of MB-apodimer. The upward curvature in the plot suggests the existence of sites with different affinities for Zn$^{2+}$, with a limiting slope corresponding to a dissociation constant of 1.5 x 10$^{-8}$ M for Zn$^{2+}$ sites with the lowest affinity. These results are consistent with the studies on soluble dimeric alkaline phosphatase which have shown the presence of two classes of Zn$^{2+}$ sites (68) with dissociation constants of less than 3 x 10$^{-8}$ M (74). The plot in Fig. 9 for the MB-dimer also indicates the existence of two distinct classes of Zn$^{2+}$ binding sites since very little free Zn$^{2+}$ can be detected in solution until after 50% of the total Zn$^{2+}$ sites (~3 nmol) have been saturated.

The recovery of enzyme activity in the MB-dimer is almost complete after binding of Zn$^{2+}$ to only 50% of the total sites (Fig. 10), whereas further additions of a large molar excess of Zn$^{2+}$ (125 eq) caused only an additional 10% increase in activity. This suggests that the addition of 50% of the total Zn$^{2+}$ bound is sufficient to restore catalytic activity.

The Scatchard plot for the MB-monomer shown in Fig. 10 extrapolates to a value of 0.9 g atoms of Zn$^{2+}$/mol of monomeric protein. This amount of tightly bound Zn$^{2+}$ cannot be accounted for by the presence of contaminating dimers in the MB-monomer since the phosphatase activity of the MB-monomer was only 6% of that observed in the MB-dimer. The scatter of points in the Scatchard plot for the MB-monomer, as well as in the upper region of the Scatchard plot for the MB-dimer, precludes the assignment of definitive values for the dissociation constants for Zn$^{2+}$ derived from the slopes in these regions, although the data do suggest that the affinity for Zn$^{2+}$ is greater than that observed in the lower region of the Scatchard plot for the MB-dimer ($K_d < 1.5 \times 10^{-8}$ M).

**DISCUSSION**

Immobolized enzyme derivatives have been used to characterize the properties of subunits of several oligomeric proteins in order to gain insight into the role of the quaternary structure in these proteins. However, when this approach was first applied to study immobilized subunits of alkaline phosphatase, preliminary observations were misleading since relatively high levels of catalytic activity were observed in the MB-subunit derivatives. These initial preparations of "monomeric" MB-alkaline phosphatase were obtained from native
enzyme immobilized on cross-linked Sepharose CL-4B, activated with 1 mg of CNBr/ml of gel; previous studies with other oligomeric enzymes have indicated that this level of activation should be sufficiently low to ensure attachment of the oligomer to Sepharose through a single covalent linkage. The enzyme activity observed in the MB-monomer was dependent on the level of CNBr activation of the support, indicating that the activity arose from the presence of contaminating dimers covalently attached to the Sepharose CL.

Low levels of contamination of MB-subunit preparations cannot be easily detected by the protein content alone since the low amounts of immobilized protein used in these studies make analysis difficult, and a variability of 10% in protein determinations could "mask" a contaminating dimer content of up to 20%. Furthermore, protein content alone is insufficient evidence for homogeneity in subunit populations because, even after complete dissociation, MB-subunits might still interact with each other due to their close proximity on the support as well as some flexibility in the matrix itself.

In previous investigations of MB-subunits, additional evidence for a monomeric structure was provided by demonstrating that the functional properties of the MB-subunit differed from the MB-oligomer (33-35, 37, 41, 42, 48), although such evidence in itself only shows that the structure is not identical with the native oligomeric form. In those cases where distinct differences in functional properties cannot be detected, the dependence of the relative level of enzyme activity in the MB-subunit on the extent of CNBr activation of the support is a means to demonstrate that the activity arises from covalent attachment of more than one subunit of an oligomer to the solid support.

The studies performed on alkaline phosphatase have shown that the levels of CNBr which were required to ensure attachment of alkaline phosphatase to the Sepharose CL by a single bond are much lower than those previously used on other enzymes. This might be due to the use of cross-linked Sepharose since Sepharose cross-linked with either divinylsulfone or epichlorhydrin has been shown to result in a much higher efficiency of protein immobilization (37). Although higher levels of CNBr activation (5 mg/ml of gel) have been shown to cause attachment of more than one subunit of aldolase to Sepharose (34), the effect observed with alkaline phosphatase immobilized on Sepharose CL is much more pronounced. Nonetheless, the use of cross-linked Sepharose is beneficial for this type of study since permanent cross-links in the matrix render it more stable to denaturants and pH changes (78), and the increased rigidity of the support severely limits reassociation of MB-subunits due to mobility of the agarose matrix (37).

Direct evidence for a homogeneous monomeric preparation can be obtained by using reversibly immobilized enzyme derivatives, in which the extent of the subunit interactions can be evaluated by cross-linking and analysis of the protein on SDS gel electrophoresis. This type of approach has been used to produce exclusively intramolecular cross-links in the monomeric proteins trypsin and papain (65) with dimethyl suberimidate and has also been applied to examine subunit interactions in G-actin (64) and alkaline phosphatase (65). In this investigation, the studies on reversibly immobilized alkaline phosphatase were extended in order to demonstrate the presence of levels of contaminating dimers as low as 3% of a MB-monomer population and to establish that the level of catalytic activity is dependent on the concentration of contaminating dimers which, in turn, increases with the extent of activation of the support. This approach has been shown to be sufficiently sensitive to demonstrate even traces (<3%) of oligomeric contamination and, consequently, can serve as a valuable method to test the homogeneity of immobilized subunit preparations.

These studies have also shown that alkaline phosphatase requires a dimeric structure for catalytic activity and resistance to proteolytic digestion and that the MB-monomer binds 0.9 equivalent of Zn2+, while the MB-dimer binds 4.1 equivalents of Zn2+. This conclusion is consistent with the Zn2+ binding stoichiometry for the soluble dimeric alkaline phosphatase (71) as well as the report by Falk (31) that inactive soluble monomers of alkaline phosphatase, prepared in 5% formamide, bind one Zn2+ per monomer.

Since previous work has indicated that soluble alkaline phosphatase should be exhaustively dialyzed in order to remove any protein-bound 8-hydroxyquinoline-5-sulfonic acid, which may alter the Zn2+ binding stoichiometry of the apoprotein (5), the possibility that a small amount of chelator could be bound to the MB-protein was considered. However, the direct and extensive washing process performed on the dilute suspensions of immobilized protein should be equally effective in removing chelator as dialysis of a very concentrated sample of soluble protein. Moreover, the metal-binding stoichiometries obtained in these titrations concur with those reported by Falk (31) for soluble alkaline phosphatase monomers dissociated in 5% formamide, bind one Zn2+ per monomer.

Although alkaline phosphatase subunits bind Zn2+ and are capable of reassociating with soluble subunits to reform a dimer, subunit interactions are required for catalytic activity. The exact mechanism by which these interactions confer phosphatase activity is still unknown; however, further investigations using immobilized enzyme derivatives to characterize subunits of chemically modified forms of alkaline phosphatase with altered catalytic properties as well as to prepare hybrid dimers should provide further evidence concerning the exact contribution of the quaternary structure to the properties of alkaline phosphatase.

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