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# Immunoelectron Microscopic Analysis of the Binding of Monoclonal Antibodies to Molecular Variants of Human Placental Alkaline Phosphatase†

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ABSTRACT: Three monoclonal antibodies with distinct antigenic specificities were examined by electron microscopy for their binding to three common genetic variants (SS, FS, and FF) of human placental alkaline phosphatase. In the reaction with the monoclonal antibody H5, all three variants of human placental alkaline phosphatase preferentially formed circular immune complexes composed of two antibodies and two enzyme molecules. In separate reactions with the F11 and B2 monoclonal antibodies, the SS variant formed circular complexes and the FS variant formed Y-shaped complexes composed of one antibody and two enzyme molecules, whereas the FF variant scarcely reacted. These results confirm immunochemical data showing that H5 binds to both S and F subunits with similar affinities, whereas F11 and B2 bind the S subunit with markedly higher affinity than they do the F subunit. Furthermore, the formation of circular complexes in the reaction of the mixture of the two antibodies, F11 and B2, with FS molecules suggests that these two antibodies bind to different sites on the S subunit. Therefore, the F and S subunits differ from one another at more than one site. This is the first indication that alleles of human placental alkaline phosphatase may result from more than just single point mutations in the gene encoding them.

Human placental alkaline phosphatase [orthophosphoric monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1, PLAP[1]] is a membrane-bound enzyme composed of two subunits with molecular weights of approximately 65 000 each (Badger & Sussman, 1976; Holmgren & Stigbrand, 1976). In an electron microscopic study using negative staining, the native molecule appeared rectangular in shape with a longitudinal stain-filled groove and with each of the half-molecules (presumably each of the subunits) very often appearing to be bilobed (Takeya et al., 1984).

PLAP is a highly polymorphic protein with many more genetic variants than any other enzyme studied to date (Harris, 1980). The enzyme is coded by 3 common alleles and at least 15 rare alleles at a single autosomal locus. The most common alleles are $pl_1$ and $pl_2$ with gene frequencies of 0.62 and 0.28 among Caucasians, although these frequencies vary considerably in other population groups (Donald & Robson, 1974). Since the enzyme is a dimer, these two alleles give rise to three phenotypes expressed as SS, FS, and FF (new nomenclature 1, 2-1, and 2). Although these phenotypes are easily distinguishable on starch gel electrophoresis, the structural basis for the electrophoretic differences between the products of different alleles has not been elucidated. SS and FF types of PLAP have similar biochemical and physicochemical properties (Holmgren & Stigbrand, 1976). In an electron microscopic study, no significant difference in shape and size was observed between these two molecules (Takeya et al., 1984). They can be distinguished immunochemically if exstensive cross-absorption of polyclonal antisera is performed (Wei & Doellgast, 1980) or monoclonal antibodies (mAbs) are used (Slaughter et al., 1981; Millán et al., 1982; Jemmerson et al., 1985).

In the present study, we employed mAbs with distinct antigenic specificities for PLAP to analyze their interaction with different variants of PLAP by high-resolution electron microscopy. Immune complexes of three mAbs (H5, F11, and B2) and three types of PLAP molecules (SS, FS, and FF) were examined. The purposes of this investigation were, first, to visualize and analyze the immune complexes in the electron microscope and, second, to observe relative locations of the antigenic differences between the common allelic variants of PLAP.

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[1] Abbreviations: PLAP, human placental alkaline phosphatase; mAb, monoclonal antibody; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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## Materials and Methods

*Purification of Placental Alkaline Phosphatase.* Each variant of PLAP was purified from fresh individual placentas obtained from Scripps Memorial Hospital (La Jolla, CA). The placentas were classified according to phenotype by starch gel electrophoresis at pH 8.6 and 6.0 (Harris & Hopkinson, 1976). Homodimeric variants of SS and FF were purified as described previously (Takeya et al., 1984) including consecutive steps of 1-butanol extraction, heat treatment (56 °C, 60 min), 40–60% ammonium sulfate fractionation, and consecutive chromatography with DEAE-cellulose, octylagarose, and Sephadex G-200 columns.

In the purification of the heterodimeric FS variant from the FS placenta, the same steps up to DEAE-cellulose chromatography were employed. Then catalytically active fractions were applied to a PB 94 chromatofocusing column (Pharmacia) equilibrated with 0.025 M piperazine–HCl (pH 5.5) to separate the FS component from the accompanying SS and FF components which are also present in FS placenta. The enzyme was eluted with polybuffer 74–HCl (pH 4.0). Enzymatically active fractions were tested by starch gel electrophoresis, and those fractions which contained only the FS variant were further purified by a Sephadex G-200 column using 50 mM Tris-HCl (pH 7.4) as the running buffer. Enzyme activity was determined as described (Kottel & Fishman, 1978) using *p*-nitrophenyl phosphate as a substrate. One unit of enzyme is defined as the amount which liberates 1 μmol of *p*-nitrophenol/min at 37 °C. Protein was measured by the method of Lowry et al. (1951) or by recording the absorbance at 280 nm in column-monitoring system UV-1 (Pharmacia).

*Monoclonal Antibodies.* The mAbs were produced as previously described (Millán et al., 1982; Jemmerson & Fishman, 1982; Jemmerson et al., 1985). In brief, the spleen cells from BALB/c mice, immunized with whole HeLa TCRC-1 cells which produce placental-type alkaline phosphatase or with the SS phenotype of PLAP, were fused with P3x63-Ag8.653 myeloma cells. Positive hybrids were detected by an enzyme–antigen immunoassay (Jemmerson & Fishman, 1982) and subcloned. After being cloned, the hybridoma lines were grown intraperitoneally in pristane-primed BALB/c mice. Isolation of mAbs from ascites fluid was performed by using protein A–Sepharose as described (Chalon et al., 1979). mAbs H5 and B2, produced against PLAP of HeLa TCRC-1 cells, and mAb F11, generated against the purified SS variant of PLAP, were used in the present study.

*Electron Microscopy.* PLAP (5 μg/mL) in 10 mM ammonium acetate (pH 6.8) was mixed with an equal volume of 50 μg/mL samples of one or more of the mAbs. The molar ratio of enzyme to antibody was set at more that 1:8 so that the antigenic sites would be saturated with antibody. After 60-min incubation, the mixed solution was negatively stained with 1.5% (w/v) uranyl acetate according to the flotation method of Valentine et al. (1968) as described previously (Takeya et al., 1984). Specimens were examined in a Hitachi H-600 electron microscope at 100-kV accelerating voltage. Micrographs were taken at a magnification of 80000×.

## Results

*Purification of the FS Variant of PLAP.* Since the placentas which express the FS phenotype of PLAP contain this component as well as the SS and FF components, the chromatofocusing column was introduced to separate this heterodimeric variant from the homodimeric SS and FF products. After several purification steps described under Materials and Methods, the sample was applied to a chromatofocusing column, and the elution pattern is shown in Figure 1A. In addition to the void volume peak that showed no catalytic activity, three distinct peaks with enzyme activity were obtained. Starch gel electrophoresis identified these three peaks as SS, FS, and FF variants, in that order of elution (Figure 1B), consistent with their isoelectric points (Holmgren & Stigbrand, 1976). The ratio of the amount of SS, FS, and FF variants was 0.9:2.0:1.2 (0.56, 1.27, and 0.76 mg, respectively), essentially following Mendelian law. This result explains the early observation of Boyer (1961), who noted that the FS band gives stronger activity than the SS or FF band upon starch gel electrophoresis.

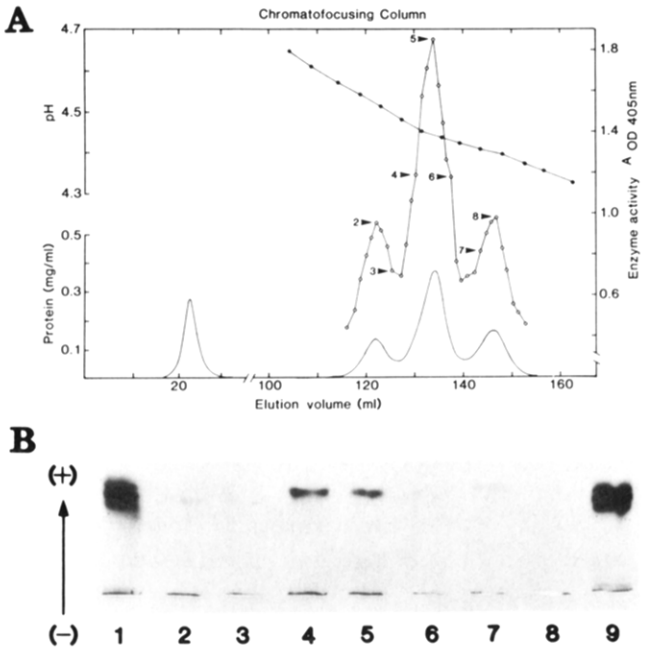


FIGURE 1: (A) Elution profile of PLAP variants on a chromatofocusing column. The numbered fractions were applied on starch gel electrophoresis which is shown in Figure 1B. (—) Protein concentration; (○) enzyme activity; (●) pH gradient. (B) Starch gel electrophoresis of fractions from the chromatofocusing column (pH 6.0). (Lanes 2–8) The number of each lane corresponds to the fraction numbered in Figure 1A; (lanes 1 and 9) butanol extract of FS placenta.

The pooled fractions of the FS variant were applied on Sephadex G-200 for further purification and separation from the polybuffer. The specific activity of the purified FS variant was 807 units/mg, which compared well with published values for SS and FF variants (756 and 715 units/mg, respectively; Takeya et al., 1984).

*Monoclonal Antibodies to PLAP.* Three mAbs with distinct reaction properties to each variant of PLAP were chosen for the present study. mAb H5 reacts equally with both SS and FF variants, whereas two mAbs, F11 and B2, react strongly with the SS variant but poorly with the FF variants (Jemmerson & Fishman, 1982; Jemmerson et al., 1985). Figure 2 shows the reaction patterns of two mAbs, F11 and H5, with the SS, FF, and FS phenotypes on starch gel electrophoresis at pH 6.0. H5 retarded all SS, FF, and FS phenotypes. On the other hand, F11 retarded SS but did not retard the FF phenotype, thus showing the same specificity observed in the enzyme-linked immunoabsorbent assay (Millán et al., 1982). In the F11 + FS reaction, the SS and FS bands were retarded, but the FF band was not. Of special interest is the fact that in the reaction of F11 + FS, two retardation bands were observed, suggesting that two different immune complexes were formed, whereas there were single retardation bands in the other reactions. mAb B2 gave essentially the same reaction patterns as mAb F11 (data not shown). These results are

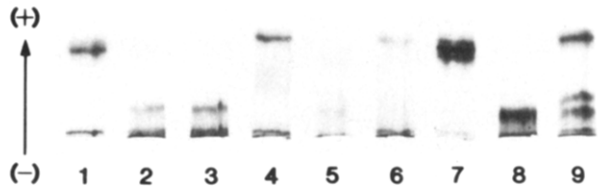


FIGURE 2: Reaction patterns of mAbs H5 and F11 with the SS, FF, and FS phenotypes of PLAP as seen by starch gel electrophoresis (pH 6.0). Butanol extracts of three phenotypes of PLAP were diluted to equivalent enzyme activity. Ten microliters of antibody (100 $\mu$g/mL) was mixed with 20 $\mu$L of enzyme solution. After 60-min incubation at room temperature, 15 $\mu$L of sample was applied to each lane. Normal mouse serum served as the control. (Lane 1) SS + normal serum; (lane 2) SS + H5; (lane 3) SS + F11; (lane 4) FF + normal serum; (lane 5) FF + H5; (lane 6) FF + F11; (lane 7) FS + normal serum; (lane 8) FS + H5; (lane 9) FS + F11.

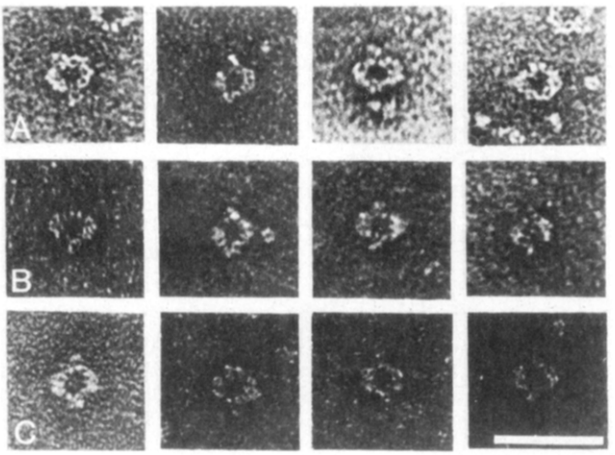


FIGURE 3: Electron micrographs of the immune complexes produced by mAbs H5 with each of three PLAP variants. (A) H5 + SS; (B) H5 + FS; (C) H5 + FF. Bar = 50 nm.

consistent with other immunochemical data showing that B2 and F11 bind the F variant with markedly decreased affinity relative to the S variant (Jemmerson & Fishman, 1982; Jemmerson et al., 1985).

*Electron Microscopy of PLAP–mAb Complexes.* Figure 3 displays a gallery of immune complexes of mAb H5 and each type of PLAP molecule. The majority of immune complexes formed by H5 appeared to be circular in shape and composed of two PLAP molecules and two antibodies for all three PLAP variants examined. There was no recognizable difference between the immune complexes formed by the three different variants with mAb H5. On the other hand, in the reactions with mAb F11, dissimilar reaction patterns were observed with the different variants. SS molecules and mAb F11 formed the same circular complexes (Figure 4A) as in the case of H5. In the reaction with FS molecules, mAb F11 preferentially formed Y-shaped complexes composed of two enzyme molecules and one antibody, whereby antibody is attached to only one site on each dimer (Figure 4B). Very few immune complexes were observed in the reaction of mAb F11 with FF molecules (Figure 4C). The mAb B2 formed essentially the same type of immune complexes with each of the PLAP variants as mAb F11 did. Figure 5 illustrates representative immune complexes formed by mAbs F11 or B2 with SS or FS molecules. As shown in the schematic drawings, both mAbs seemed to bind two of four electron-transparent regions. With the FS molecule, both mAbs seem to bind only one of four regions.

Table I shows the number of nonreactive enzyme molecules and each type of immune complex formed from the variant–mAb reaction. Thus, with H5, the predominant shape was

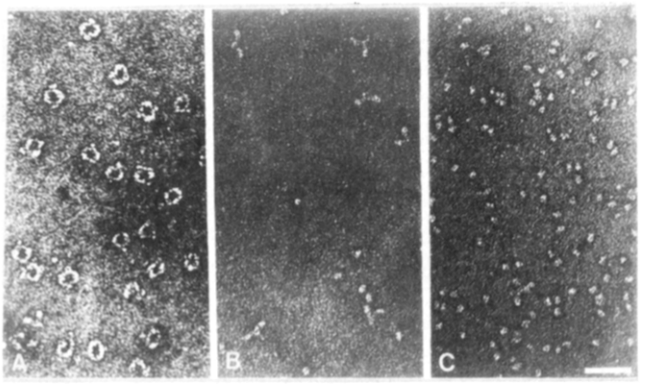


FIGURE 4: Electron micrographs of the mAb F11 reaction with each of three PLAP variants. (A) F11 + SS; (B) F11 + FS; (C) F11 + FF. Bar = 50 nm.

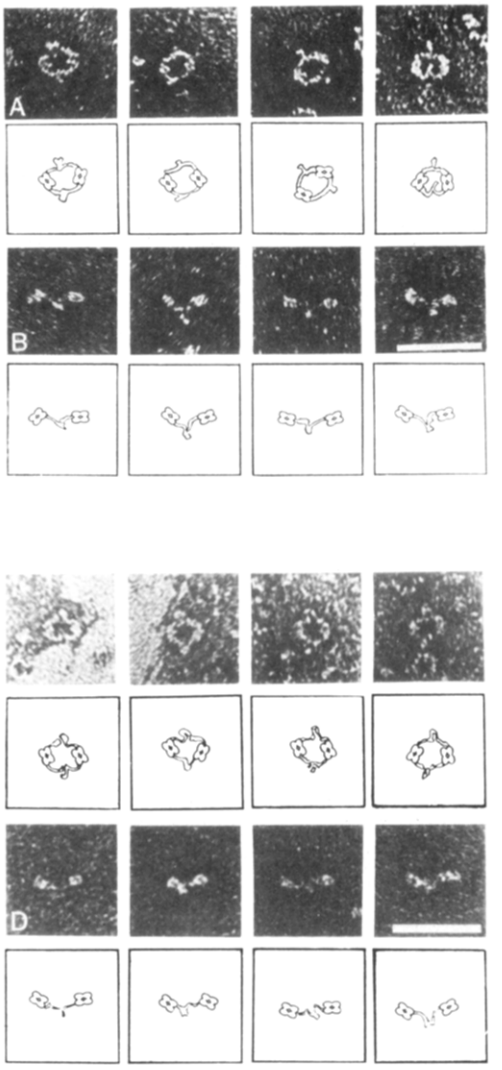


FIGURE 5: Electron micrographs and schematic drawings of representative immune complexes of F11 + SS (A), F11 + FS (B), B2 + SS (C), and B2 + FS (D). Bar = 50 nm.

circular for all three variants. In the reaction with F11 or B2, SS molecules predominantly formed circular complexes, and FS molecules preferentially formed Y-shaped complexes, whereas both mAbs scarcely bound to the FF molecules. No individual PLAP molecule attached by more than two antibodies was observed in any combination. These results provide supportive evidence that both mAbs F11 and B2 bind epitopes in the S subunit which differ from corresponding regions in the F subunit.

Table I: Numbers of Each Type of Immune Complex in Each Set[a]

| type of immune complex | [b] | | | free enzyme | unrecognizable |
|---|---|---|---|---|---|
| reaction with H5 | | | | | |
| SS | 3 | 203 | 19 | 525 | 11 |
| | (1) | (91) | (8) | | |
| FS | 2 | 140 | 22 | 459 | 18 |
| | (1) | (86) | (13) | | |
| FF | 2 | 145 | 14 | 371 | 19 |
| | (1) | (90) | (9) | | |
| reaction with F11 | | | | | |
| SS | 8 | 201 | 27 | 64 | 37 |
| | (3) | (85) | (11) | | |
| FS | 0 | 9 | 149 | 588 | 6 |
| | (0) | (6) | (94) | | |
| FF | 0 | 7 | 14 | 1281 | 5 |
| reaction with B2 | | | | | |
| SS | 10 | 225 | 31 | 499 | 9 |
| | (4) | (84) | (12) | | |
| FS | 0 | 22 | 159 | 562 | 10 |
| | (0) | (12) | (88) | | |
| FF | 0 | 2 | 9 | 1470 | 6 |

[a] The values in parentheses are the percentages of the total number of complexes for each interaction. For each series of experiments, at least 150 immune complexes were examined requiring from 3 to 6 microphotographic fields, except the reaction of F11 + FF and B2 + FF where three unselected entire micrographic fields were examined. [b] Including larger circular complexes.

Table II: Numbers of Circular and Y-Shaped Immune Complexes in the Reaction of mABs F11 and B2 and the FS Variant of PLAP

| reaction | circular | Y shaped | percent[a] |
|---|---|---|---|
| FS + F11 | 9 | 148 | 5.7 |
| FS + B2 | 22 | 159 | 12.2 |
| FS + F11 + B2 | 233 | 49 | 82.6 |

[a] The percentage of the circular complexes in the total number of complexes. Only interpretable immune complexes were counted in 4–6 entire micrographic fields containing at least 150 complexes in each series of experiments.

To determine if mAbs F11 and B2 bind to the same site on the S subunit, the two different mAbs were mixed together with FS molecules, which essentially have only one binding site for each mAb, and the mixture was subjected to negative staining (Figure 6). If both mAbs bind to the same site, the Y-shaped complex will predominate as was observed with each mAb alone. However, if the two mAbs bind different sites, then circular immune complexes should be formed. The experimental results proved the latter case. Table II shows a greatly increased number of circular complexes in the reaction of the two mAbs with FS molecules compared to the reaction of a single mAb and the FS molecules. This increased formation of circular complexes was further confirmed by starch gel electrophoresis (Figure 7). The faster migrating retardation band, presumably composed of the Y-shaped complex in F11 + FS and B2 + FS, is not seen in the reaction of F11 + B2 + FS, and only the slowest migrating retardation band, presumably composed of circular complexes, is observed. These results imply that the two mAbs, F11 and B2, bind different epitopes on the S subunit which are not present on the F subunit. Furthermore, this suggests that the F and S subunits differ structurally at more than one site on the polypeptide chain.

## Discussion

In the present study, FS molecules were separated from SS and FF molecules by chromatofocusing column chromatography. Since each variant has a different isoelectric point

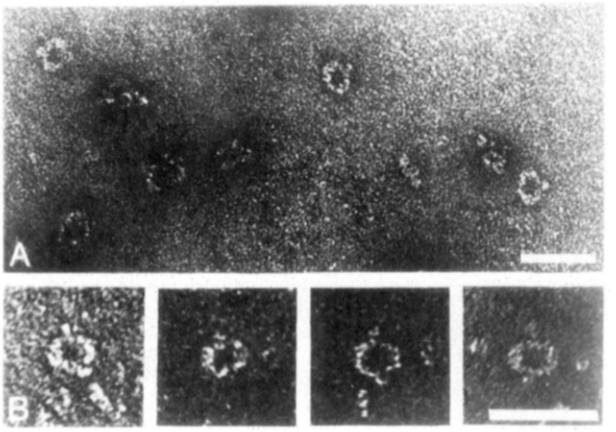


FIGURE 6: General field (A) and selected (B) electron micrographs of immune complexes observed after mixing two mAbs, F11 and B2, with the FS variant of PLAP. In addition to the circular complexes, some large complexes are formed probably because of low-affinity reactivity of F11 and B2 with the F subunit. Bars = 50 nm.

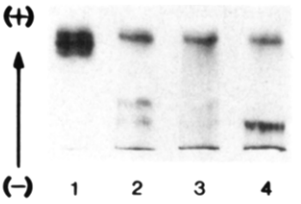


FIGURE 7: Reaction of mAbs F11 and B2 with the FS phenotype of PLAP seen by starch gel electrophoresis (pH 6.0). Ten microliters of antibody or a mixture of two antibodies (100 μg/mL) was added to 20 μL of enzyme solution. After 60-min incubation at room temperature, 15 μL of sample was applied to each lane. (Lane 1) FS + normal serum; (lane 2) FS + F11; (lane 3) FS + B2; (lane 4) FS + F11 + B2.

(Holmgren & Stigbrand, 1976), this technique was very useful in separating FS molecules. Furthermore, by monitoring the protein concentration continuously, the amount of each variant was quantified. The ratio of the SS, FS, and FF variants was 0.9:2.0:1.2, essentially following Mendelian law. This method can be applied to separate each component of other oligomeric proteins which show heterogeneities owing to subunit combination. By comparing the amount of each component, one might be able to suggest the basis of the regulation mechanism of heterogeneity, whether Mendelian or not.

mAbs against PLAP have become a very valuable tool to explore the structural differences between its genetic variants. Thus, Slaughter et al. (1981) reported that 6 of the 18 mAbs produced against PLAP showed significantly reduced binding to one or another of the products of the 3 common alleles. Also, Millán et al. (1982) reported that the mAb F11 was found to be reactive with SS but not with FF, and the heterodimeric phenotypes of FS revealed intermediate reactivities in enzyme-linked immunoabsorbent assay. Studies by Jemmerson et al. (1982, 1985) of F11 and a second mAb, B2, that also distinguished between FF and SS variants indicated that both mAbs bound the SS variant with much higher affinities than they bound the FF variant. In the present study, we were able to determine the number of binding sites of each variant with each of three different mAbs, using immunoelectron microscopy which enabled direct visualization of the immune complexes. SS, FF, and FS molecules possess two binding sites for H5. SS molecules have two binding sites for F11 and B2, whereas the FF molecules showed practically no binding to either F11 or B2. Consistent with these binding observations, heterodimeric molecules (FS) have only one binding site for F11 or B2. Furthermore, the mixing experiment of FS

molecules with F11 and B2 was performed to determine whether these two mAbs bind the same site on the S subunit or not. The increased formation of circular immune complexes in the mixture implied that F11 and B2 bind different sites on the S subunit. As these two mAbs recognize S subunit specific determinants, the result means that the F and S subunits differ structurally at more than one site. This isssue can now be more thoroughly addressed through gene sequencing since PLAP cDNA has recently been cloned and sequenced (Kam et al., 1985; Millán, 1986).

Although the dimeric character of PLAP molecules is widely accepted by the biochemical data and the genetical surveys, PLAP molecules observed in the electron microscope (Takeya et al., 1984) were apparently composed of four electron-transparent regions. However, the application of mAbs in immunoelectron microscopy clearly demonstrated the dimeric nature of this enzyme. As for the circular immune complex, the mAbs seemed to bind two of four electron-transparent regions, suggesting that each subunit is composed of two adjacent regions mimicking a dumbbell shape.

Direct visualization of the immune complexes in the electron microscope has proven to be a very useful method to locate the antibody binding site on macromolecules, such as ribosomal protein (Wabl, 1974), fibrinogen (Price et al., 1981; Norton & Slayter, 1981), or proteoglycan (Buckwalter et al., 1982). The application of mAbs to immunoelectron microscopy was reported by several authors (Kirchhausen et al., 1983; Lünsdorf et al., 1981). Since the mAbs recognize only one epitope on the antigen, if the antigen has a repeating structure or is composed of subunits possessing the same antigenic structures such as PLAP molecules, it is possible to determine the number of binding sites by counting the number of antibody molecules in the antigen–antibody complex.

The application of mAbs to immunoelectron microscopy facilitates a better understanding of subunit arrangements of oligomeric enzymes. If one can now produce mAbs against functionally interesting regions of the molecule, such as the enzyme catalytic site or the membrane-insertion site, the immunoelectron microscopic approach can be expected to provide even more detailed structural information of the enzyme molecule.

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**Registry No.** Alkaline phosphatase, 9001-78-9.

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