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Relation between Calcium Requirement, Substrate Charge, and Rabbit Polymorphonuclear Leukocyte Phospholipase A_2 Activity†

Richard Franson* and Moseley Waite

ABSTRACT: Phospholipase A_2 isolated from rabbit granulocytes hydrolyzes liposomes of synthetic phosphatidylethanolamine and the phospholipids of autoclaved *E. coli* optimally at pH 7.4 in the presence of 1 mM of Ca^{2+}. EDTA is a potent inhibitor of phospholipase A_2 activity; Mg^{2+} does not substitute for Ca^{2+} in this reaction. Phospholipase A_2 activity in the presence of 1 mM of Ca^{2+} is optimal when the surface charge of phosphatidylethanolamine liposomes is slightly negative (−2.6). Concentrations of Ca^{2+} greater than 1 mM inhibited enzymatic activity and increased the surface charge (ζ potential) of phosphatidylethanolamine liposomes. The amphipaths, cetyltrimethylammonium bromide and diacetyl phosphate, altered the surface charge of phosphatidylethanolamine liposomes and inhibited phospholipase A_2 activity. The inhibition was partially reversed by the addition of the oppositely charged amphipath and was not due to detergent effects of the amphipaths since phospholipase A_2 activity was unaffected by similar concentrations of hexadecanol and Triton X-100. When the surface charge deviated from the proper range, maximal hydrolysis of phosphatidylethanolamine occurred at concentrations of Ca^{2+} that were strongly inhibitory in the absence of the amphipaths. These data demonstrate that the granulocytic phospholipase A_2 has an absolute catalytic requirement for calcium; higher concentrations of Ca^{2+} inhibit enzymatic activity in part by altering the surface charge of the substrate liposome.

Hydrolysis of a smectic mesophase of phospholipid by phospholipase A is influenced by the physical nature of the substrate liposome (Bangham, 1972; Brockerhoff & Jenson, 1974; Dawson, 1964), particularly the packing or spatial arrangement of phospholipids and the surface charge or ζ potential of the liposome at the lipid-water interface. Many studies have demonstrated that packing of phospholipid within bilayers or monolayers affects the susceptibility of phospholipids to hydrolysis by phospholipases. However, the question of whether phospholipases preferentially hydrolyze substrates with a given surface charge remains unanswered. Goldhammer et al. (1975) have reported that the electrokinetic characteristics of liposomes are not important determinants of susceptibility to phospholipase A attack, whereas other reports (Bangham & Dawson, 1958; Dawson et al., 1976) have shown that the electrophoretic charge of the substrate affects the hydrolysis of liposomes by lecithinase and phospholipase C. This report examines the electrokinetic substrate requirements of a highly cationic PLA_2[1] isolated from rabbit granulocytes in an attempt to separate the role played by Ca^{2+} in the catalytic event from its influence on the surface charge of the substrate liposome.

Experimental Procedure

Materials. The phospholipids of *E. coli* were labeled during growth with [1-^{14}C]oleate and the bacteria were autoclaved to destroy bacterial phospholipases and to render the bacterial phospholipids more susceptible to hydrolysis (Patriarca et al., 1972; Franson et al., 1974, 1978). 1-Acyl-2-[1′-^{14}C]linoleoyl-3-glycerophosphorylethanolamine and 1-acyl-2-[1′-^{14}C]linoleoyl-3-glycerophosphorylcholine were synthesized according to the method of Waite & van Deenen (1967). CTMB was bought from Eastman Kodak Co., Rochester, N.Y., and hexadecanol, Triton X-100, and DCP were pur-

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[1] Abbreviations used: PLA_2, phospholipase A_2; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CTMB, cetyltrimethylammonium bromide; DCP, diacetyl phosphate.

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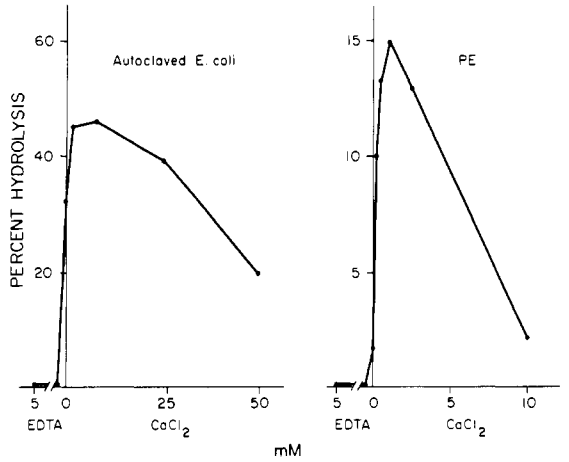

FIGURE 1: Effect of Ca^{2+} and EDTA on hydrolysis of phospholipids of autoclaved *E. coli* and liposomes of PE by granulocyte PLA_2. PLA_2 was measured as described in Methods section using 100 nmol of PE liposomes as substrate. Incubation conditions with autoclaved *E. coli* were the same as the above except that 2.5×10^8 of cells of *E. coli* labeled during growth with [1-^{14}C]oleate and then autoclaved (representing 5000 cpm and 5 nmol of phospholipid) were used as substrate. Reaction mixtures were incubated for 30 min at 37 °C in all experiments and activity is expressed as percent hydrolysis. All values are corrected for nonenzymatic hydrolysis which was less than 3% in all experiments.

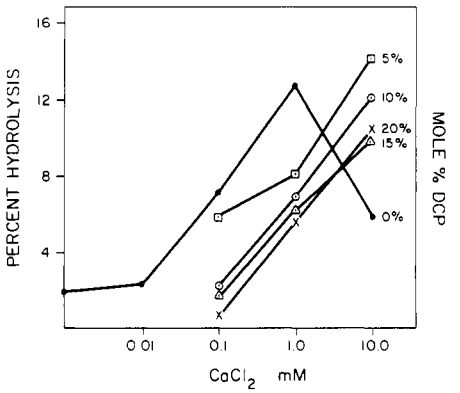

FIGURE 2: Effect of DCP on the hydrolysis of PE liposomes in the presence of Ca^{2+}. PLA_2 activity was measured as described in Methods. Percent DCP or CTMB represents the molar ratio of amphipath to phospholipid times 100.

chased from Sigma Chemical Co., St. Louis, Mo. The membrane-associated PLA_2 from rabbit granulocytes was solubilized and isolated by H_2SO_4 extraction, neutralization, and cation-exchange chromatography as previously described (Weiss et al., 1975). This method yields a PLA_2 activity enriched approximately 1000-fold over the homogenate which migrates as a single protein band at the dye front (beyond lysozyme) in polyacrylamide gel electrophoresis at pH 4.3 and as a single species in $NaDodSO_4$-polyacrylamide gel electrophoresis with a molecular weight of approximately 14 500 (unpublished data).

Methods. The phospholipase A_2 activity (EC 3.1.1.4) was measured using [1-^{14}C]oleate labeled *E. coli* or [1-^{14}C]linoleic acid labeled synthetic phospholipids as previously described (Franson et al., 1971; Waite & Van Deenen, 1967). Unless otherwise stated, reaction mixtures contained in a total of 1 mL, 10 μmol of Tris-HCl (pH 7.4), $CaCl_2$, as specified, 100 nmol of 1-acyl-2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine or 1-acyl-2-[^{14}C]linoleoyl-3-glycerophosphorylcholine, and 10 μg of purified rabbit granulocyte PLA_2. Pure phospholipid substrates were added as liposome suspensions

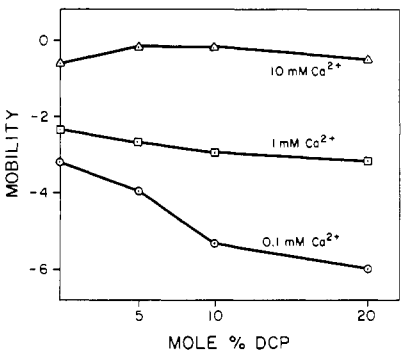

FIGURE 3: Effect of DCP on the mobility of PE liposomes in the presence of Ca^{2+}. Mobility was measured as described in Methods.

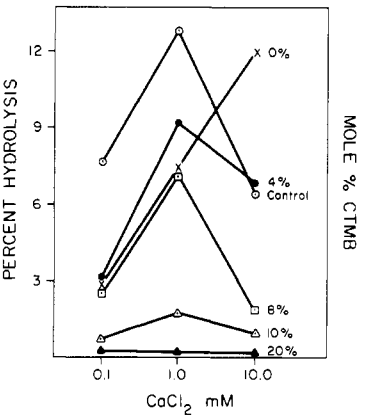

FIGURE 4: Effect on the hydrolysis of PE liposomes in the presence of $CaCl_2$ and 10% DCP. Assay conditions are described in Methods; all incubations contained 10% DCP and the indicated concentrations of CTMB and Ca^{2+}, except the control which contained only Ca^{2+}.

in water prepared by brief sonication or gentle shaking. When present, CTMB or DCP was added prior to the preparation of the liposomes.

Measurement of Surface Charge. The electrophoretic mobility of substrate particles was determined by direct observation of the particle in a horizontal electrophoresis cell as described by Bangham et al. (1958, 1962). Mobility was determined at 37 °C on the same substrate preparations and in the same buffers used to measure PLA_2 activity. The velocities of substrate particles are expressed as $\mu\ s^{-1}\ (V\ cm)^{-1}$. Mobility is directly related to the ζ potential, the charge at the plane of shear of the ionic double layer (Davies & Rideal, 1963).

Results

Hydrolysis of phospholipid of autoclaved *E. coli* and liposomes of PE by granulocyte PLA_2 is shown in Figure 1 as a function of $CaCl_2$ concentration. With either substrate, the granulocyte PLA_2 had an absolute requirement for Ca^{2+}; Mg^{2+} could not substitute for Ca^{2+} (data not shown) and no activity was noted in the presence of EDTA. The hydrolysis of phospholipids of *E. coli* was maximal in the presence of 1 to 5 mM Ca^{2+} and was inhibited by increasing concentrations of Ca^{2+}; 50 mM of Ca^{2+} inhibited phospholipase A activity by 60%. Hydrolysis of pure PE liposomes exhibited greater sensitivity to increasing concentrations of Ca^{2+}. PLA_2 activity was optimal with 1.0 mM added Ca^{2+} and was inhibited 87% by 10 mM Ca^{2+}. These data suggest that there is a catalytic requirement for low concentrations of Ca^{2+}, but do not explain the inhibition of phospholipase A activity at higher concentrations of Ca^{2+}. Since the ionic double layer is known to affect

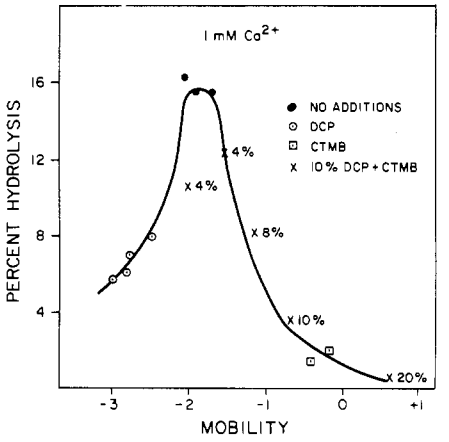

FIGURE 5: Hydrolysis of PE-liposomes as a function of mobility with 1.0 mM of Ca^{2+}. Mobility and enzyme activity were measured as described in Methods. Mobility was altered by the addition of DCP, increasing molar ratios (5, 10, 15, and 20%) from right to left, 10 or 20% CTMB; and 10% DCP in the presence of increasing concentrations of CTMB (X) as indicated.

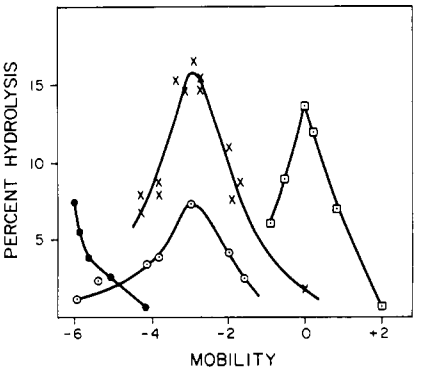

FIGURE 6: Hydrolysis of amphipath-altered PE liposomes as a function of surface charge and Ca^{2+} concentration. Mobility and enzyme activity were measured as described in Methods. Mobility was altered at each concentration of Ca^{2+} [(●) 0.01 mM; (⊙) 0.1 mM; (X) 1.0 mM; (⊡) 10.0 mM] with DCP or CTMB alone as described in Figures 2-5.

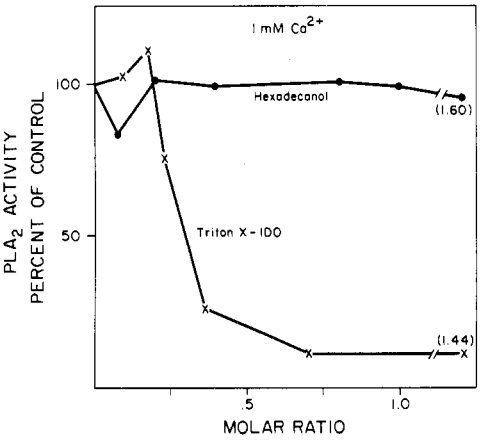

FIGURE 7: Effect of hexadecanol and Triton X-100 on the hydrolysis of PE liposomes. Enzyme activity is measured as described in Methods and expressed as percent of control. Concentrations of hexadecanol and Triton X-100 are expressed as the molar ratio of detergent to phospholipid.

the electrophoretic properties of phospholipids, we examined the effect of Ca^{2+} on the surface charge of PE liposomes.

In the absence of added Ca^{2+}, PE liposomes migrate with a −3.5 charge (not shown); with increasing Ca^{2+}, the liposomes become less negatively charged. In the presence of 1 mM Ca^{2+}, liposomes exhibited a −2.5 charge and were maximally hydrolyzed (Figure 1), whereas in the presence of 10 mM of Ca^{2+}, the charge on the liposomes was −0.9 and hydrolysis was greatly inhibited. These data indicate that Ca^{2+} alters the surface charge of PE liposomes and suggest that the granulocyte PLA_2 may preferentially hydrolyze a negatively charged substrate liposome. Therefore, the effect of charged amphipaths, CTMB (cationic) and DCP (anionic), on the mobility and susceptibility to hydrolysis of PE liposomes was examined.

The hydrolysis of PE liposomes by PLA_2 at various concentrations of Ca^{2+} in the presence of the indicated percentages of DCP is shown in Figure 2. PLA_2 activity was inhibited by DCP in a dose-dependent manner in the presence of 0.1 mM and 1.0 mM of Ca^{2+}. By contrast, in the presence of inhibitory concentrations of Ca^{2+} (10 mM), phospholipid hydrolysis was stimulated by DCP at all concentrations tested. We also did similar experiments using PC liposomes. Under all conditions tested, no hydrolysis of PC liposomes was observed. In the presence of the lowest level of Ca^{2+} used, 0.1 mM, the addition of DCP to the liposomes of PE caused an increase in the negative charge (Figure 3). Increasing the concentration of Ca^{2+} to 1.0 mM reduced the surface charge of the PE liposomes and the addition of DCP caused little, if any, increase in the negative mobility. Ten millimolar of Ca^{2+} essentially neutralized the liposomes and the charge was unaffected by 20% DCP. The data presented in Figures 2 and 3 suggest that, whereas a negative charge favors catalytic activity, too great a negative charge is inhibitory.

In these experiments, we have manipulated the surface charge with a negatively charged amphipath and the positive counterion Ca^{2+} in the double layer. To more accurately assess the influence of charge within the liposome, it was necessary to manipulate the charge with two oppositely charged amphipaths.

The inhibition of PLA_2 activity by 10% DCP in the presence of 1 mM of Ca^{2+} was partially relieved by the addition of 4% CTMB, a cationic amphipath (Figure 4). Increasing concentrations of CTMB (>4%) resulted in marked inhibition which was most pronounced at 10 mM of Ca^{2+}. No enzymatic activity was detectable in the presence of 20% CTMB regardless of Ca^{2+} concentration. These data indicate that the amphipaths are not inhibitory in the presence of the proper mixtures, and that their influence is exerted through their charge characteristics.

The hydrolysis of PE liposomes as a function of substrate surface charge in the presence of 1 mM of Ca^{2+} is shown in Figure 5. PLA_2 preferentially hydrolyzes liposomes within a narrow range of negative surface charge at approximately −2.0 units. The addition of either DCP (5 to 20%) or CTMB (10 and 20%) alters the surface charge and inhibits enzymatic activity. Inhibition by either amphipath alone is at least partially relieved by the addition of the oppositely charged amphipath. The decreased activity seen in the charge range (−1.6 to −2.2) could be the result of surface dilution since 14% (mol/mol) of the liposomes is DCP and CTMB.

The relationship between amphipath-induced alterations of substrate surface charge and susceptibility to hydrolysis as a function of Ca^{2+} is shown in Figure 6. With 0.1 or 1.0 mM of Ca^{2+}, liposomes with a negative charge near −2.0 are hydrolyzed maximally; deviation of the surface charge from this range results in loss of enzymatic activity. Surprisingly, liposomes in the presence of 0.01 mM of Ca^{2+} were appreciably hydrolyzed only when the surface charge was −6.0, whereas at 10 mM of Ca^{2+}, PLA_2 activity was optimal with neutral

liposomes. This demonstrates that, although the charge induced by the amphipath exerts a major influence on enzymatic activity, the double layer effect of Ca^{2+} can exert an equal effect.

The effect of Ca^{2+} and charged amphipaths on the susceptibility of liposomes to PLA_2 attack could be due to detergent alterations of phospholipid packing within the liposome. Therefore, PLA_2 activity was measured in the presence of increasing concentrations of the long chain alcohol, hexadecanol, and the nonionic detergent, Triton X-100, compounds known to influence the spatial arrangement of phospholipids (Figure 7). PLA_2 activity was unaffected by molar ratios of hexadecanol to PE up to 1.6 in the presence or absence of 1 mM of Ca^{2+}. By contrast, Triton X-100 inhibited PLA_2 activity 75–90%, but only when the molar ratio of Triton to PE exceeded 0.2, the maximal ratio of amphipath to phospholipid used in these experiments. The results are consistent with the proposal that the changes observed upon the addition of DCP and CTMB are not due to detergent effects on the substrate or enzyme.

Discussion

Previous studies concerning the relation between electrokinetic characteristics of phospholipid liposomes and their susceptibility to hydrolysis by phospholipases have led to conflicting reports. Thus, Bangham et al. (1958, 1962) concluded that the activities of lecithinase from *Penicillium notatum,* and phospholipase C from *Clostridium perfringens* are controlled by the electrophoretic charge at the substrates surface by comparing electrophoretic mobilities of liposomes and their susceptibility to hydrolysis. On the other hand, Goldhammer et al. (1975), studying the effects of ionic surfactants on phospholipase activity, concluded that liposomal surface charge was not a crucial factor in determining susceptibility of liposomes to hydrolysis by PLA_2 from bee and snake venoms and by phospholipase C from *Clostridium welchii.* More recently, Dawson et al. (1976) demonstrated that the phospholipase C of *Clostridium welchii* indeed has an absolute requirement for Ca^{2+} ions and attacks pure PC liposomes only when their ζ potential is made positive by the addition of cations, such as Ca^{2+}. However, in confirmation of earlier studies, the same enzyme also attacked individual phospholipid molecules in the presence of detergents without any requirement for a net positive surface charge, suggesting that spatial arrangement of phospholipids as well as electrostatic potential could influence hydrolysis of liposomes by phospholipase C.

Our data demonstrate that the highly purified rabbit granulocyte PLA_2 has an absolute Ca^{2+} requirement for catalyzing the hydrolysis of phospholipids of autoclaved *E. coli* and PE liposomes. The phospholipids of the biomembranous substrate, autoclaved *E. coli,* are hydrolyzed extensively in the absence of added Ca^{2+} (probably due to endogenous Ca^{2+}) and are less sensitive than PE liposomes to inhibition of hydrolysis by increasing concentrations of Ca^{2+}. The differences in the inhibition by Ca^{2+} of the hydrolysis of the two structures presumably are due to the heterogeneity of the cell envelope and membrane of *E. coli.* Calcium functions, at least in part, to alter the ζ potential of PE liposomes which in turn affects the susceptibility of substrate to hydrolysis. Thus, PE liposomes, within a narrow range of negative charge (-2.2 ± 0.5) and concentrations of Ca^{2+} (1 to 2.5 mM; Figures 1 and 3), are hydrolyzed maximally by the granulocytic PLA_2. Increasing concentrations of Ca^{2+} (5 and 10 mM) inhibited enzymatic activity and produced less negatively charged PE liposomes. Inhibition of PLA_2 activity by 10 mM of Ca^{2+} could be due to previously described Ca^{2+}-induced alterations of the size and morphology of PE liposomes (Bangham & Horne, 1964; Waite, unpublished observations). The addition of DCP in the presence of 10 mM of Ca^{2+} enhanced PLA_2 activity at all concentrations tested (5–20%), but had no effect on the mobility of PE liposomes (Figures 4 and 5). There are at least two reasons for this: the enzyme could be altering the physical structure of the liposome, or alternatively the enzyme may differentiate between the charge of the liposome and that produced in the double layer. At present, we cannot differentiate between the two, but the experiments in which we used neutral detergents and combinations of DCP and CTMB would favor the latter interpretation. The importance of the environment at liposomal surface in hydrolysis is further demonstrated by the inactivity of the enzyme when the quaternary amine of PC is present.

Interestingly, Mg^{2+} does not support granulocytic PLA_2 activity (Franson et al., 1974), even though it alters the ζ potential of phospholipid liposomes similarly (Kamo et al., 1974, and unpublished data). As indicated by Dawson et al. (1976), this suggests that Ca^{2+} acts as an essential coenzyme in the substrate–enzyme interaction and is independent of the role for Ca^{2+} in producing the correct ζ potential. Similar conclusions were drawn from the studies by Wells (1972) and Pieterson et al. (1974) implicating catalytic roles for Ca^{2+} in the snake venom and pancreatic phospholipases A_2. More recent studies by de Haas et al. (1978) demonstrate that Ca^{2+} also has an important effect on the conformation and, therefore, the enzymic activity of the pancreatic PLA_2. An examination of such alterations of the granulocytic PLA_2 awaits purification to the point of homogeneity.

We have previously described the granule localization and purification of the membrane-associated PLA_2 from rabbit granulocytes (Franson et al., 1974; Weiss et al., 1975). On $NaDodSO_4$–polyacrylamide gel electrophoresis and electrophoresis at pH 4.3, the purified PMN–PLA_2 migrates as a single species of apparent molecular weight of 14 500 and appears more cationic than lysozyme. Recent studies suggest that the PLA_2 is localized on the cytosol surface of PMN granules since maximal PLA_2 (equivalent to sonicated or detergent-treated granule preparations) activity is obtained by incubating intact granules that exhibit otherwise maximal enzymatic latency for typical intragranular marker enzymes during a 15-min incubation at 37 °C (unpublished observation). The ectopic localization of the granule PLA_2 and its absolute catalytic requirement for Ca^{2+} suggest that this PLA_2 could be uniquely controlled by the ionic, and perhaps the Ca^{2+}, microenvironment to initiate membrane disruptive events. In addition, the unusual cationic charge, as well as the Ca^{2+} requirement and localization of the granulocyte PLA_2 may be of special interest because of recent reports that indicate that basic PLA_2 is more cytotoxic than anionic PLA_2 (Kawauchi et al., 1971; Belori et al., 1973). Indeed, we have previously demonstrated that the granulocyte PLA_2 participates in the Ca^{2+}-mediated breakdown of phospholipids of intact *E. coli* which is coincident with an increase in microbial permeability and loss of viability (Weiss et al., 1975). Snake venom (*Crotalus adamanteus*) PLA_2 had no effect in this regard.

Acknowledgments

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Supplementary Material Available

Figure illustrating the electrophoretic behavior of the isolated PMN-PLA_2 used in these experiments (1 page). Ordering information is available on any current masthead page.

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Nuclear Magnetic Resonance Studies of Inorganic Phosphate Binding to Yeast Inorganic Pyrophosphatase†

Donald J. Hamm and Barry S. Cooperman*

ABSTRACT: Yeast inorganic pyrophosphatase is a dimer of identical subunits. Previous work (Rapoport, T. A., et al. (1973) *Eur. J. Biochem. 33,* 341) indicated the presence of two different Mn^{2+} binding sites per subunit. In the present work, the binding of inorganic phosphate to the Mn^{2+}-inorganic pyrophosphatase complex has been studied by ^1H and ^{31}P nuclear magnetic resonance. Two distinct phosphate sites have been found, having dissociation constants of 0.24 mM and 18 mM. The Mn^{2+}-^{31}P distance from tightly bound Mn^{2+} to phosphate bound in the low affinity site (6.2 Å) is consistent with outer sphere binding. Binding to both phosphate sites can be simultaneously inhibited by the pyrophosphate analogue, hydroxymethanebisphosphonate, providing evidence for the physical proximity of these two sites. The weaker Mn^{2+} site is apparently far from both phosphate sites. From the magnitudes of the dissociation constants found for both phosphate and analogue binding and the recent work of P. D. Boyer and his co-workers (private communication) on enzyme-catalyzed phosphate-water exchange, it appears unlikely that the hydrolysis of enzyme-bound pyrophosphate is the rate-determining step in the overall enzymatic catalysis of pyrophosphate hydrolysis, at least when Mn^{2+} is the required divalent metal ion cofactor.

Phosphoryl transfer is among the most widespread reactions of biochemical importance. The enzymes catalyzing reactions of this type have in common a general requirement for divalent metal ions for activity. Yeast inorganic pyrophosphatase (EC 3.6.1.1, pyrophosphate phosphohydrolase) shares in this requirement and displays several properties which make it attractive as a model for phosphoryl transfer enzymes (Cooperman et al., 1973). The early work on this enzyme was reviewed by Butler (1971), at which time comparatively little was known. Since then our understanding of the enzyme has increased considerably. The enzyme is a dimer made up of identical monomers of molecular weight 33 000-35 000 (Ridlington et al., 1972; Heinrikson et al., 1973). The primary sequence has been determined (Cohen et al., 1978) and an X-ray crystallographic determination of its structure is well underway (Bunick et al., 1978). In addition, important information has been obtained on the binding of divalent metal ions and of pyrophosphate and pyrophosphate analogues (Ridlington & Butler, 1972; Cooperman & Chiu, 1973a; Rapo-

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