bically with the protein side chains while enhancing the net ionic character of the substrate to reduce this diffusional limitation by enhancing water mobility and to provide an attractive ionic force for the oppositely charged enzyme. If such effects were coupled with even subtle conformational effects induced in the substrate by hydrophobic ligands, the degree of enzymatic susceptibility could well be considerable.

In summary, the present study demonstrates that the elastolytic process is readily modifiable by a variety of small molecules which can bind to elastin. Therefore, it does not seem unreasonable to propose that ligands with the appropriate chemical characteristics could exert an effect on the metabolism of elastin fibers *in vivo*. For example, fraying of elastic fibers has been observed in lipid-associated aortic plaques. Whereas the majority of lipids which interact with elastin and which are implicated in the pathology of aortic tissues are electrostatically neutral (*i.e.*, cholesterol, cholesterol esters, phospholipids, and triglycerides (Kramsch *et al.*, 1971)), the presence of free fatty acids has also been demonstrated in proximity to elastic fibers in the fatty streak (Chobanian and Manzur, 1972). The cumulative influence on even small amounts of free fatty acids might be considerable over an extended length of time. The possibility of fatty acids playing a regulating role in elastolysis seems even more important in light of the present observation that these ligands can reverse the marked inhibition of elastolysis which occurs at physiological ionic strengths.

Acknowledgment

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Stimulation of Alkaline Phosphatase by Analogs of Inorganic Pyrophosphate†

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ABSTRACT: Hydrolysis of low concentrations of 4-nitrophenyl phosphate by nonspecific alkaline phosphatases from *Escherichia coli* and from bovine intestine is stimulated by low concentrations of methylene diphosphonate or imidodiphosphate, which are analogs of inorganic pyrophosphate (PP_i). Under similar conditions PP_i, P_i, inorganic phosphite, and EDTA have little or no effect. The observed stimulations are inconsistent with the "flip-flop" mechanism; the results favor a model involving cooperativity between the two subunits of the enzyme, but are also consistent with a model involving two isomeric forms of the enzyme, in which the PP_i analogs shift the equilibrium toward the form which has a higher affinity for substrate.

We have recently reported that inorganic pyrophosphate (PP_i) and its analogs in which the bridge oxygen atom is replaced by either an imido or methylene group competitively inhibit the hydrolysis of 4-nitrophenyl phosphate by nonspecific alkaline phosphatases of *Escherichia coli* and bovine intestine (Kelly *et al.*, 1973). We observed in the course of this investigation that under some conditions the PP_i analogs (but not PP_i itself) had a small but reproducible stimulatory effect upon these enzymes. In this communication we present these observations and their interpretation with respect to the mechanism of action of these enzymes.

Materials and Methods

The source of all materials, including enzymes (both of

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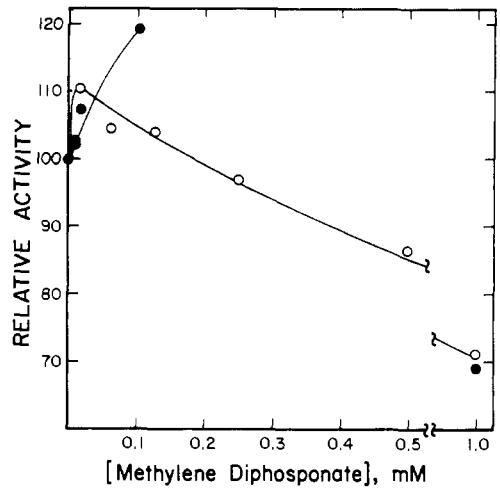

FIGURE 1: Effect of methylene diphosphonate on the rate of hydrolysis of 4-nitrophenyl phosphate by bovine intestinal alkaline phosphatase. Assay conditions were (●) 5 μM and (○) 10 μM 4-nitrophenyl phosphate in 1 M Tris (pH 8.0), 30°. Velocity is expressed relative to samples without methylene diphosphonate.

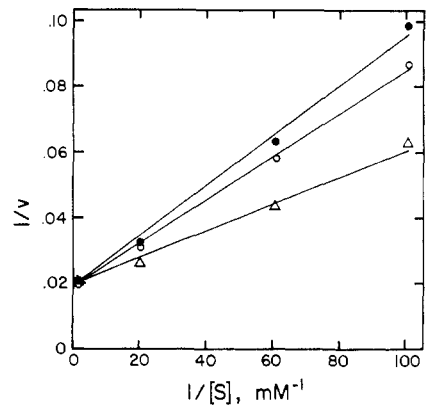

FIGURE 2: Effect of imidodiphosphate on the rate of hydrolysis of 4-nitrophenyl phosphate by *E. coli* alkaline phosphatase. Assay conditions were 0 (●), 10^{-5} M (○), and 10^{-4} M (△) imidodiphosphate and varied 4-nitrophenyl phosphate in 1 M Tris (pH 8.0), 30°. Velocity is expressed as μmol of 4-nitrophenol released per min per mg of protein.

which were purified to near homogeneity) and PP_i analogs, was as previously described (Kelly *et al.*, 1973). Conditions for enzyme activity assays are described in the figure legends. In all assays, the measured product was 4-nitrophenol produced enzymatically from 4-nitrophenyl phosphate, monitored and recorded continuously using a Beckman DB-G spectrophotometer and Sargent SRLG recorder. This procedure ensured that the results obtained were actually initial rates.

Results

The effect of the methylene analog of PP_i, methylene diphosphonate, on the rate of hydrolysis of low concentrations of 4-nitrophenyl phosphate by intestinal alkaline phosphatase is shown in Figure 1. As previously described (Kelly *et al.*, 1973), methylene diphosphonate at relatively high concentrations (mM range) behaves as a competitive inhibitor; under these conditions the observed value for K_I is 1.5 mM. However, at low concentrations methylene diphosphonate appears to stimulate, rather than inhibit, the hydrolysis of 4-nitrophenyl phosphate.

Similar stimulation of 4-nitrophenyl phosphate hydrolysis has been observed with *E. coli* alkaline phosphatase, utilizing both methylene diphosphonate and imidodiphosphate, the imido analog of PP_i. As shown in Figure 2 for the stimulation of the *E. coli* enzyme by imidodiphosphate, maximum activation is observed only at low substrate concentrations, well below the K_m (K_m is 0.04 mM in this experiment). The stimulation appears to be competitive with substrate; the maximum observable degree of stimulation and the concentration of analog which gives maximum stimulation are dependent upon the substrate concentration.

Note that the stimulation of 4-nitrophenyl phosphate hydrolysis was monitored by the production of 4-nitrophenol; thus the hydrolysis of imidodiphosphate or the chemically and enzymically stable methylene diphosphonate (Kelly *et al.*, 1973) cannot itself be the source of the increased product. Stimulation could be due to chelation by the analogs of small concentrations of inhibitory metal ions, but Cd(II), Cu(II), Ca(II), Mg(II), Zn(II), Ni(II), Mn(II), Ba(II), or Sr(II) added in 1 mM concentration did not significantly inhibit the initial rate. The addition of these metal ions did obliterate the stimulatory effect of 15 μM methylene diphosphonate, possibly through the formation of a metal ion–methylene diphosphonate complex which does not stimulate the hydrolysis of 4-nitrophenyl phosphate; this is consistent with the previously observed inhibition of pyrophosphatase activity by excess metal ions which complex with PP_i (Eaton and Moss, 1967; Fernley and Walker, 1967). Further, addition of EDTA, which chelates metal ions much more strongly than does PP_i (Sillen and Martell, 1964) (and, presumably, PP_i analogs), had no effect at concentrations equivalent to the concentrations of PP_i analogs which gave maximum stimulation. Although interpretation of the effects of EDTA or other metal binding agents on metalloenzymes such as alkaline phosphatase (Simpson and Vallee, 1968) can be complicated by the possibility of inactivation due to chelation of essential metal ions, it appears that the stimulatory effect of low concentrations of imidodiphosphate and methylene diphosphonate is not due simply to chelation of inhibitory metals.

Discussion

Analogs of PP_i which are themselves enzymatically stable or only very slowly hydrolyzed can at very low concentrations in the absence of added metal ion stimulate the production of 4-nitrophenol from 4-nitrophenyl phosphate as catalyzed by intestinal or *E. coli* alkaline phosphatases. The observed stimulations appear to be competitive with substrate; at high concentrations of substrate these analogs have little effect. The simplest explanation for this phenomena is positive cooperativity: at low substrate concentrations the analogs bind to one site of the enzyme dimer and enhance the binding of the 4-nitrophenyl phosphate to the other active site; at sufficiently high concentration the 4-nitrophenyl phosphate competes with the activator for the second site, producing the same maximum velocity in the presence and absence of activator.

Two lines of evidence, the amplitude of the burst transient at acid pH indicating only one molecule of substrate is hydrolyzed per enzyme molecule before the steady state is reached (Halford, 1971) and anticooperative binding of P_i with 1 mol per enzyme bound with a dissociation constant in the micromolar range and additional P_i bound much more loosely (Lazdunski *et al.*, 1970; Simpson and Vallee, 1970), have prompted previous suggestions of subunit interactions in the function of alkaline phosphatase (Lazdunski *et al.*, 1970; Simpson and Vallee, 1970).

This apparent interaction between the two active sites has been viewed either as a coupled conformation change of the

dimer in which the two sites alternate in a catalytically active form, *i.e.*, the "flip-flop" model (Lazdunski *et al.*, 1970), or as an example of conventional cooperativity (Simpson and Vallee, 1970). The presently observed activation is more consistent with the latter view, for it permits activator to remain bound to one subunit while stimulating multiple turnovers at the other site. The enzyme can bind ligands at both sites simultaneously; catalytic activity at one site is not abolished by binding competitive inhibitor at the other site (Halford, 1971). The "flip-flop" model requires, for stimulation of 4-nitrophenyl phosphate hydrolysis at one site, prior association of activator with, and subsequent dissociation from, the other site. These additional steps would be required each time the enzyme turns over. It is difficult to conceive how doubling the number of association–dissociation steps could lead to a faster overall rate for the reaction.

Recently, Bloch and Schlesinger (1973) have reported the presence in purified preparations of *E. coli* alkaline phosphatase of up to 2 mol of endogeneous phosphate which directly affects the amplitude of the burst transient at acid pH and which should affect the apparent dissociation constant for added phosphate. Accordingly, they suggest that previously cited evidence indicating subunit cooperativity may instead be due to endogenous phosphate. In models involving independence of subunits, subtle kinetic anomalies have been accounted for (Reid and Wilson, 1971; Halford *et al.*, 1972; Halford, 1972) by a mechanism involving isomerization of the enzyme between two forms, only one of which binds substrate strongly. Stimulation of activity of PP_i analogs at low substrate concentration as reported here may be explained in terms of such models by a mechanism in which the analogs shift the equilibrium of the isomerization in favor of the form which has the higher affinity for substrate.

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Purification and Kinetic Mechanism of Rat Liver Glycogen Synthase†

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ABSTRACT: Glycogen synthase has been purified from rat liver by a rapid and unique procedure involving the reversible precipitation of the glycogen-free enzyme in the cold. The final enzyme was purified 1400-fold over crude tissue extract and has a specific activity of 22 μmol of glucose incorporated into glycogen min^{-1} mg of $protein^{-1}$ at 37° in the presence of 20 mM glucose-6-P. The purified synthase appeared homogeneous when subjected to polyacrylamide gel electrophoresis. Synthase subunit molecular weight was determined by electrophoresis in sodium dodecyl sulfate to be 77,000–80,000. Analysis of substrate and inhibition kinetics indicate the synthase reaction involves the formation of a ternary complex of enzyme and substrates with random binding of substrates. No significant exchange between UDP and UDPglucose was detected in the absence of glycogen. 1,5-Gluconolactone was found to be an inhibitor of the reaction suggesting an intermediate with the glucosyl moiety in a half-chair conformation exists during the reaction sequence.

Uridine diphosphate glucose:glycogen α-1,4-glucosyltransferase (EC 2.4.1.11), the rate-limiting enzyme for glycogen synthesis, is present in most tissues in two forms which are interconvertible by phosphorylation and dephosphorylation reactions (Larner and Villar-Palasi, 1971). The phosphorylated enzyme, which is dependent on glucose-6-P[1] for activity (synthase D) is thought to have little activity under physiological conditions, so that dephosphorylation to a glucose-6-P independent form (synthase I) turns on glycogen synthesis *in vivo* (Mersmann and Segal, 1967). These interconversions are mediated by a protein kinase and phosphatase which in turn may be influenced by various hormonal (Blatt and Kim, 1971) and metabolic states (Kato and Bishop, 1972). Synthase activity in rat liver also displays a diurnal rhythm due to control of its synthesis (McVerry and Kim, 1972a) and is regulated by cellu-

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[1] Abbreviations used are: UDP, uridine 5′-diphosphate; glucose-6-P, glucose 6-phosphate.