Perspectives in Alkaline Phosphatase Research

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Gene cloning and site-directed mutagenesis have had a profound effect on alkaline phosphatase research. Four distinct structural genes encoding placental, intestinal, and tissue-nonspecific isoenzymes have been cloned, sequenced, and mapped to human chromosomes. Differences in properties between the respective gene products are due to variations in primary structure involving only one, or a few, key amino acid residues. Recognition that alkaline phosphatase belongs to the category of molecules that are localized to cell membranes through a COOH-terminal glycan-phosphatidylinositol anchor provides a basis for understanding the generation of isoforms observed in plasma in disease. Isoforms produced by differential cleavage or preservation of the glycan-phosphatidylinositol anchor may offer new correlations with disease that are of diagnostic value. However, a more important contribution of alkaline phosphatase research to clinical chemistry may prove to be an increased understanding of disease processes at the molecular level.

Additional Keyphrases: isoenzymes · protein isoforms · GPI anchor

Alkaline phosphatase (EC 3.1.3.1) has been continuously and extensively investigated for more than 70 years. Throughout that period, observations of the disease-related changes in alkaline phosphatase activity in plasma provided a constant stimulus to explain the related pathology and extend the range of diagnostic applications. A vast literature accumulated, much of which reports trivial or poorly substantiated observations, or the rediscovery of facts already known. One of the reasons for this is the ease with which the activity of the enzyme can be measured, which encouraged unsystematic or superficial investigations. However, earlier work on the structure of the alkaline phosphatase molecule was hampered by its glycoprotein nature and consequent microheterogeneity and, in particular, by its binding to cell membranes. Such binding results in problems in solubilization and purification, so that most early attempts to determine the structural basis of within-tissue and between-tissue molecular variation were restricted to inferences drawn from the effects of modifications that did not abolish enzymic activity.

In spite of these difficulties, certain generalizations about human alkaline phosphatase had emerged by 1970 (1). The existence of at least three independent genetic loci had been inferred, with the clearest differences in physicochemical properties distinguishing placental alkaline phosphatase from other isoenzymes (2).

The separate identity of adult intestinal alkaline phosphatase was similarly established, while the basis of minor, though consistent, differences between alkaline phosphatases from sources other than placenta or intestine were attributed to tissue-specific differences in post-translational modification (2). The inappropriate expression of placental and placental-like alkaline phosphatases in tumors had been described (3), and the reported inability of alkaline phosphatase to act on pyrophosphate linkages was shown to have been an error resulting from the choice of inappropriate experimental conditions; this error, however, did not result in an immediate reappraisal of the function of the enzyme (4). The 1970s saw the addition of further examples of the ectopic expression of alkaline phosphatase isoenzymes in cancer, and the discovery of the normal or abnormal expression of a further putative gene product in the form of fetal intestinal alkaline phosphatase (5, 6). However, as in so many fields of biology, the advent of the techniques of molecular biology provided the means to systematize the large numbers of observations and inferences about alkaline phosphatase and to establish a sound molecular foundation for future research.

Genetics and Expression

Four structural genes encoding alkaline phosphatase have now been cloned and sequenced (7–10) and mapped to human chromosomes (11, 12) (Figure 1). The tissue-nonspecific alkaline phosphatase gene, expressed in osteoblasts, hepatocytes, kidney, early placenta, and other cells, is located on the short arm of chromosome 1, bands p36.1–p34. Adult intestinal alkaline phosphatase is encoded by a locus on chromosome 2, bands q34–q37, where also are located loci that encode the alkaline phosphatase of the mature placenta and the closely similar (placental-like) germ-cell alkaline phosphatase. The close association of these three loci presumably reflects their common, comparatively recent, evolutionary ancestry. These last three genes and their products have correspondingly similar sequences of bases and amino acids; these similarities are particularly marked in the case of placental and germ-cell alkaline phosphatases, which differ by only 7–10 amino acid residues (98% homology), depending on source. The sequence homology between the placental and intestinal isoenzymes is 86.5%, and between intestinal and tissue-nonspecific alkaline phosphatases it amounts to 56.6%.

As its name suggests, the tissue-nonspecific gene is widely expressed, in contrast to the localized expression of the intestinal, placental, and germ-cell genes. In all cases except the germ-cell isoenzyme, expression gives rise to readily detectable amounts of enzymic activity, but the normal activity of germ-cell alkaline phospha-
The placental phosphatase gene exhibits a remarkable degree of allelic variation, but none of the alleles appears to be associated with disease. It is surprising that no corresponding polymorphism appears to be exhibited by other alkaline phosphatase genes, although inherited nonpathogenic overexpression of the tissue-nonspecific gene and of the intestinal gene has been reported (14-16). Congenital hypophosphatasia is the one condition in which severe or fatal consequences appear to derive from failure of normal expression of an alkaline phosphatase, in this case the tissue-nonspecific isoenzyme. The condition has recently been shown to be genetically heterogeneous (17). cDNA from four cell lines from four unrelated patients showed a different, single point mutation at each of the eight alleles; four of the mutations were present in 23 subjects with disease of varying severity (17). Compound heterozygosity seems usually to be the cause of disease. Although the degree of severity of the clinical manifestations appears to be related to the nature of specific mutations, no harmless mutations have come to light in spite of the high frequency of examination of bone alkaline phosphatase in clinical laboratories. Congenital hypophosphatasia is characterized by a generalized deficiency of the tissue-nonspecific alkaline phosphatase (18); however, no metabolic consequences in tissues such as liver or kidney have been identified.

The ectopic and increased eutopic expression of certain alkaline phosphatase genes in cancer not only has provided a new series of tumor markers, especially in germ-cell tumors (19) but, perhaps more important, has opened new opportunities for the study of changes in genetic regulation accompanying malignant transformation. Differential expression provided early evidence of the separate identities of the placental and germ-cell alkaline phosphatase genes. The occasional appearance in hepatoma and other cancers of an alkaline phosphatase (the Kasahara isoenzyme) that closely resembles fetal intestinal alkaline phosphatase (6, 20), as well as the time-dependent expression of the latter isofrom during fetal development (5), would most easily be explained by the existence of a further, separate genetic locus. However, direct comparisons of the fetal and adult intestinal isoforms have yielded equivocal results. Many of the differences between them are minor and could result from dissimilar post-translational modification. Small differences in peptide maps were observed in comparisons of post-mortem preparations (21, 22), but the adult isoenzyme (unlike the fetal form) rapidly undergoes partial proteolysis under these conditions, with a loss of molecular mass that may significantly alter maps obtained from it (23). Biochemical evidence suggests that fetal intestinal alkaline phosphatase is a heterodimer of placental and adult-intestinal subunits (24), whereas FL-amnion cells, which elaborate an electrophoretically fast isoenzyme apparently identical to the Kasahara isoenzyme, show only intestinal and placental mRNAs (25).

If fetal intestinal alkaline phosphatase is indeed a heterodimer, its temporal pattern of expression presumably reflects the coexpression of the placental (or perhaps germ-cell) gene and the intestinal gene in enterocytes during fetal development with formation of a hybrid enzyme, the amount of which declines toward term as expression of the placental gene decreases. Such an explanation would be analogous to the ontological changes in expression of creatine kinase subunits in skeletal muscle, in which the primitive expression of B monomers, forming the BB dimer, is displaced during differentiation by expression of M monomers, forming the MM dimer that is present almost exclusively at term. Coexpression of the subunits during differentiation leads to a transient appearance of the MB heterodimer, a pattern that may recur, e.g., in rhabdomyosarcoma (26).

Expression of intestinal alkaline phosphatase is normally almost entirely confined to enterocytes. However, recent work has confirmed the expression of small but significant amounts of alkaline phosphatase with the characteristics of the intestinal isoenzyme in kidney and shown it to be localized to the distal (S3) segment of the proximal tubule (27), with potential benefits in investigation of renal pathology. Trace expression of this isoenzyme has also been detected in other, non-intestinal tissues from a case of hypophosphatasia (28).

Structural Studies

The availability of primary structures of tissue-nonspecific, intestinal, placental, and germ-cell alkaline phosphatases deduced from cloned genes has rendered
obscure the long series of partial structural studies and inferences derived from selective modification of the enzyme proteins. Such studies formed the basis of earlier attempts to establish the molecular basis of the observed variations in properties among these isoenzymes. With the additional possibilities offered by site-directed mutagenesis, these sequences have provided a molecular explanation for differences in physical and catalytic properties between isoenzymes. Although human alkaline phosphatases have not yet been obtained in crystalline forms suitable for X-ray analysis, their active sites and the active site of Escherichia coli alkaline phosphatase show a high degree of conservation (29), so that key regions of the human isoenzymes can be interpreted with reference to the corresponding region of the bacterial enzyme.

The primary structures of human alkaline phosphatases contain a sequence of 36 amino acids conformationally close to the active center that is absent from the enzyme from E. coli (30). Placental, germ-cell, and intestinal alkaline phosphatases show stereospecific, uncompetitive inhibition by compounds such as L-phenylalanine. The action of these inhibitors depends on residues located within this region (30-32). In particular, a key role is played by the residue at position 429 in determining patterns of inhibition (31, 32). This position is occupied by glutamine in placental phosphatase, by serine in intestinal phosphatase, and by histidine in the less inhibited tissue-nonspecific alkaline phosphatase (30). In germ-cell alkaline phosphatase, residue 429 is glycine (30). This is one of the few differences between the primary structures of germ-cell and placental alkaline phosphatases, yet it seems to account for the differences in the properties of the two isoenzymes. Thus, substitution of glutamine for glycine at position 429 in the germ-cell isoenzyme essentially eliminates its lower stability upon exposure to heat or EDTA as well as its greater inhibition by L-leucine, converting it to a product indistinguishable in these respects from the placental isoenzyme (32). Presumably, differences in the structure of this critical region will similarly be found to account for the specific binding of intestinal alkaline phosphatase by Reactive Yellow 13, which occurs when the latter is used as an affinity-ligand (33). Studies on inhibition by Reactive Yellow 13 in solution indicate that it can bind to the intestinal alkaline phosphatase isoenzyme in the absence of a phosphate acceptor, whereas in the case of other isoenzymes an acceptor must be present for inhibition to be observed (34).

As with many other enzymes, the inferred three-dimensional conformations of the human alkaline phosphatase isoenzymes imply that amino acid residues that are distant from each other in the linear sequence are brought into close proximity in the catalytically active enzyme conformation. Since the human enzymes, unlike the enzyme of E. coli, are membrane-bound, the question arises as to whether the conformation deduced from crystals of a soluble enzyme can be expected to be present in the membrane-bound enzyme in situ. The demonstration of typical catalytic properties of alkaline phosphatase in kidney sections (35), and similarly of activity and its inhibition in enterocyte-membrane vesicles (36), suggests that ligand-binding sites are similar in the solubilized and membrane-bound enzymes.

Since tissue-nonspecific alkaline phosphatases are the products of a single structural gene, single-residue variations in primary structure cannot account for the small, tissue-specific differences in stability to heat that were among the first differential properties of these isozymes to be identified (37). Evidence from selective modification by glycosidases suggests that the differences in properties originate in differences in carbohydrate side chains (38). It is interesting to speculate whether these apparently tissue-specific differences in glycosylation are targeted specifically toward the enzyme and, therefore, by implication, have functional significance in particular cells, or whether they reflect differences in glycosylation patterns in general in those cells.

**Anchoring of Alkaline Phosphatase to Cell Membranes**

The recognition that alkaline phosphatase belongs to the large group of proteins attached to the outer surfaces of cells by a COOH-terminal glycan-phosphatidylinositol (GPI) anchor has provided further molecular rationalization of old observations. The action of n-butanol at acid pH in solubilizing tissue alkaline phosphatase, e.g., from placenta, and the different forms observed when extraction is effected at alkaline pH are now seen to be due to the cleavage of the GPI anchor by activation of inositol-specific phospholipase A in acid conditions; the hydrophobic anchor is retained during extraction at alkaline pH (39).

The attachment of the GPI anchor to the enzyme protein during biosynthesis requires cleavage of a 29-residue, COOH-terminal peptide, exposing a new COOH-terminal residue, Asp⁴⁴, to which the anchor is attached. This process has been studied in some detail with the aid of site-directed mutagenesis with respect to the requirements for particular amino acid residues adjacent to the cleavage site (40). The presence of the GPI anchor seems to be necessary for cleavage to occur: when GPI synthesis is absent or inhibited, a proform of alkaline phosphatase accumulates that is only slowly processed and released into the medium by proteolytic removal of the COOH-terminal hydrophobic peptide. Furthermore, a single amino acid substitution, of arginine for leucine at position 500 in the COOH-terminal peptide, converts placental alkaline phosphatase expressed in COS-1 cells from a membrane-bound to a secreted protein (41).

Several mechanisms of release of GPI-anchored alkaline phosphatase molecules can be envisaged, with the subsequent generation of different isoforms depending on the presence or absence of the GPI anchor (Figure 2). The products of the action of various specific phospholipases are often only slightly different: e.g., the products of cleavage of the GPI anchor by phosphatidylinositol-specific phospholipases C or D (Figure 2) would differ only by the presence or absence of a terminal phosphate
group in the released enzyme molecules. Alterations in hydrophobicity resulting from the loss of the GPI anchor of alkaline phosphatases, and even the small differences between the products of the actions of phospholipases C and D, can, however, be detected by partitioning in two-polymer aqueous-phase systems (42), permitting the changes accompanying enzyme release to be studied relatively easily. These methods confirm the differences in behavior between the alkaline phosphatases of serum and bile and, if one compares these isoenzymes with anchor-retaining preparations of placental alkaline phosphatase, the data support the presence of the anchor in the alkaline phosphatase of bile and its absence from most serum alkaline phosphatase molecules (Raymond FD, Moss DW, Fisher D, unpublished data). The presence of a phosphatidylinositol-specific phospholipase D in plasma (43–45) probably accounts for the appearance of the nonaggregated, dimeric form of hepatocyte-derived alkaline phosphatase in plasma, whereas the absence of this activity from bile appears to account for the aggregated form of alkaline phosphatase in that fluid (46). However, it is not yet apparent whether release of alkaline phosphatase into plasma by phospholipase action is a rate-limiting step, or, if it is, whether changes in phospholipase activity contribute to altered concentrations of alkaline phosphatase in plasma in disease.

Pathology and Clinical Chemistry

The processes by which concentrations of alkaline phosphatases in plasma come to reflect disease processes are now known in considerable detail. Increased osteoblastic activity is the source of the raised levels of the bone-derived isoenzyme in bone disease; similarly, the initial change in hepatobiliary disease is an increased production of hepatic alkaline phosphatase (47, 48), probably as a result of accumulating bile acids due to localized or generalized cholestasis. The site of synthesis is the hepatocytes (49) in which increased translation of alkaline phosphatase mRNA occurs (50). Whereas in these two instances increased enzyme production is the primary cause of the increased concentrations in plasma, changes in rate of removal account for the much rarer increases in adult intestinal alkaline phosphatase. This isoenzyme enters the circulation in large amounts by way of the thoracic duct lymph (51), but, as an asialoglycoprotein, it is very rapidly cleared by hepatocyte receptors; this rapid clearance is the determinant of the normally low concentrations of intestinal phosphatase in serum. Therefore, a reduced rate of clearance, e.g., in chronic liver disease such as portal cirrhosis, is more likely than intestinal disease to increase serum intestinal alkaline phosphatase concentrations, as is indeed observed (52). Fetal intestinal alkaline phosphatase is sialylated and therefore persists longer in the plasma of premature infants in whom it occurs (20).

The nature of the anchorage of alkaline phosphatases to cell membranes, together with the presence of inositol-specific phospholipase D activity in plasma and its apparent absence from bile, provides an explanation for the isozymes of alkaline phosphatase detectable in plasma, especially in hepatobiliary disease (46). Cleavage of the phosphatidic acid moiety by plasma phospholipase would release a hydrophilic, nonaggregating alkaline phosphatase from hepatocytes into plasma. Since no cleavage of the anchor would occur in bile, release of GPI-containing alkaline phosphatase molecules (e.g., by detergent action) would be expected to lead to the formation in that fluid of higher-molecular-mass aggregates of the enzyme, or complexes with other lipid-rich molecules. These would enter the plasma in cholestasis, probably by way of increasingly permeable tight junctions between hepatocytes, where they could undergo further complex formation, e.g., with lipoprotein X.

Isozymes of alkaline phosphatase with the properties predicted by this hypothesis are present in bile and plasma in hepatobiliary disease. Hydrophilic alkaline phosphatase of the expected relative molecular mass, retaining the tissue-specific properties conferred on it by the specific glycosylation patterns of its cells of origin, is indeed the predominant form of alkaline phosphatase in plasma from patients with liver disease (53). This isozyme has the same behavior in aqueous, two-phase, partition systems as alkaline phosphatase prepared in ways that remove the GPI anchor (42; Raymond et al., unpublished data). A corresponding isozyme with appropriate tissue-specific properties can be obtained from cells of an osteoblast-derived line, suggesting that similar mechanisms underlie the release from osteoblasts of bone-type alkaline phosphatase into the plasma in bone disease (42; Raymond et al., unpublished data). The apparently critical dependence of the addition of the GPI anchor on the presence of the appropriate COOH-terminal peptide in the nascent alkaline phosphatase molecule, as well as other requirements for this step, has prompted the suggestion that incorrectly processed, and therefore secreted, forms may contribute to alkaline phosphatase in extracellular fluids (41). However, the close similarities between alkaline phosphatase isozymes in serum or plasma and those released from cells in vitro...
by phospholipases or by incubation with serum imply that release of incorrectly processed molecules may make a minor contribution, if any, to the population of alkaline phosphatase isoforms in plasma. Such in vitro experiments also support other observations that the phospholipase of plasma has D, rather than C, specificity (43, 44). They further suggest that the substrate of the plasma phospholipase may be alkaline phosphatase initially released in a macromolecular form, rather than membrane-linked enzyme, because in one series of experiments hydrophilic alkaline phosphatase released by incubating osteosarcoma-derived SAOS-2 cells in serum was always accompanied by an increase in the hydrophobic, macromolecular isoform (46).

Characterization of alkaline phosphatase in bile, and of that released from cells by incubation with bile in vitro, shows the predicted presence of a high-molecular-mass, hydrophobic form (46; Raymond et al., unpublished data). Similar forms are also present in plasma, normally in small amounts, where they form part of the high-molecular-mass "fast-liver" fraction separated by conventional techniques that also includes enzyme-bearing membrane fragments (koinzymes) (54).

Recognition of glycans-phosphatidylglycerol by glycoprotein is a contributing factor to the observed increase in volume of data (55). However, it seems probable that, because of the steady advance of imaging and biopsy techniques, practical application of intricate biochemical analysis of this kind will remain confined to centers with a particular interest and expertise in the field. Therefore, the most valuable contribution of alkaline phosphatase isoform studies is likely to remain in furthering the understanding of pathological processes at cellular and molecular levels.

Similar considerations probably account for the relatively small impact that the ability separately to identify and measure bone- and liver-derived alkaline phosphatases has had on clinical chemistry practice. This distinction played a significant part in the 1960s in the emerging understanding of changes in plasma alkaline phosphatase accompanying hepatobiliary disease; yet, in spite of advances in methodology, the vast numbers of total alkaline phosphatase measurements made daily on clinical samples generate relatively few requests for isoenzyme analysis. More than half of these additional requests concern the identification of the tissue of origin of an unexpectedly elevated serum alkaline phosphatase activity, and they can be answered by a qualitative technique such as the inspection of an alkaline phosphatase zymogram (56). The indications for quantitative analysis of bone or liver alkaline phosphatase have also been debated. One application of the measurement of bone phosphatase that has been shown to be valuable is in the detection and monitoring of metastases to bone. Especially when interpreted together with other tests, measurements of bone alkaline phosphatase in serum give good indications of a favorable response to treatment (57).

Physiological Function of Alkaline Phosphatase

The ability to manipulate alkaline phosphatase genes and to express them in various cells promises to shed new light on this perennially perplexing topic. As is so often the case in alkaline phosphatase research, recent developments in this area include the resurrection and reassessment of older ideas.

The strongest inference about the function of alkaline phosphatase comes from the universal association of the presence of the enzyme and mineralization of bone and, conversely, the deficiency of both enzyme and mineralization in hypophosphatasia. It has been recognized for many years that the striking hydrolysis of orthophosphates by alkaline phosphatase at strongly alkaline pH in vitro is probably irrelevant to its function in vivo: the comparable rates of hydrolysis of pyrophosphates and orthophosphates at physiological pH was noted nearly 30 years ago (4), supporting suggestions that osteoblastic alkaline phosphatase might favor bone mineralization by removal of inhibitors of crystallization such as inorganic pyrophosphate (58). Altered metabolism of phosphoethanolamine and pyridoxal 5'-phosphate, as well as of inorganic pyrophosphate, has also been observed in hypophosphatasia (59). These possibilities have recently been reappraised with respect to alkaline phosphatase in fibroblasts from normal individuals and patients with hypophosphatasia (60). A generalized function of ecto-alkaline phosphatases in facilitating, by dephosphorylation, the entry into cells of metabolites to which the cell membrane would otherwise be impermeable seems a reasonable hypothesis. It is also possible that the active site of alkaline phosphatase, and also other specific modifier-binding sites, indicate a receptor function for membrane-bound alkaline phosphatase. Tissue-nonselective alkaline phosphatase is able to bind extracellular matrix proteins, such as collagen (61). This may provide a mechanism for the attachment of osteoblasts to cartilage, promoting calcification.

References

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