Adenosine 5'-triphosphatase and fructose 1,6-diphosphatase activities of thymic lymphoma and normal tissue in c57bl mice

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ADENOSINE 5'-TRIPHOSPHATASE AND FRUCTOSE 1,6-DIPHOSPHATASE ACTIVITIES OF THYMIC LYMPHOMA AND NORMAL TISSUE IN C57BL MICE

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ABSTRACT

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Adenosine 5'-Triphosphatase and Fructose 1,6-Diphosphatase Activities of Thymic Lymphoma and Normal Tissues in C57Bl Mice

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Alkaline phosphatase (APase), normally found in lymphocytes up to sixteen days gestation in C57Bl mice, has been associated with murine lymphomas. Previous studies of APase activities of lymphoma and normal tissue have shown a specific pyrophosphatase activity and a nonspecific APase activity which hydrolyzes beta-glycerophosphate, alpha-naphthylphosphate and p-nitrophenylphosphate. To establish the function of this APase activity, further biochemical characterizations of this enzyme were studied, using adenosine 5'-triphosphate (ATP) and fructose 1,6-diphosphate (FDP) as substrates. The parameters used were pH optimum, substrate concentration and substrate ratio, heat inactivation, ethylenediamine tetraacetic acid (EDTA) and phenylalanine inhibitions, and magnesium activation. The results show no significant differences in placental and lymphoma ATPase and FDPase activities with respect to pH optimum, phenylalanine inhibition, substrate ratios and heat inactivation rate. However, substrate ratios show additional...
ATPase activity in the normal adult spleen. ATPase and FDPase activities appear to be different by EDTA inhibition and magnesium activation. However, this may reflect differences in active substrate rather than different enzymes. In comparison to the nonspecific APase activity previously described, ATPase and FDPase show a greater rate of heat inactivation. However, the biphasic nature of the inactivation patterns, with the exception of lymphoma FDPase, may indicate the presence of other enzymes which show affinity to these substrates. The present data do not conclusively rule out the possibility that nonspecific APase activity may demonstrate dephosphorylation of ATP and FDP. Further elucidation of this relationship must await purification of this enzyme.
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CHAPTER I

INTRODUCTION

Alkaline phosphatase (APase), normally found in the embryonic thymus of C57Bl mice only up to 16 days of gestation (Lagerlof and Kaplan, 1967), has been associated with murine thymic lymphoma (Smith, 1961, 1962; Metcalf et al., 1962; De The, 1966).

Biochemical characterizations of APase activity in crude and partially purified extracts of thymic lymphoma have been compared to that of normal tissues using such parameters as pH optimum, heat inactivation, substrate specificity, electrophoretic mobility, inhibition and activation (Lumb and Doell, 1970; Tisdale, 1974; Yahya, 1974; Floyd, 1973). By these criteria, a specific pyrophosphatase (localized in the nucleus) and a nonspecific APase (membrane-bound) have been identified. APase has been shown to hydrolyze p-nitrophenylphosphate, beta-glycerophosphate and alpha-naphthylphosphate. These results show that there are no significant differences in the nonspecific APase activity of lymphoma, placenta, adult spleen and fetal thymus.

Although the physiological function of this APase activity is uncertain, a possible role in active transport has been suggested. In fact, murine lymphoma APase activity has been shown to transfer phosphate to nucleophilic buffers in high concentration (Prioleau, 1974). Similar phosphotransferase activity has been reported for other APases, including those of human (Amador and Urban, 1972) and chicken (Hinsberg and Laidler, 1972) intestines.
The present study was undertaken to test the hypothesis of active transport by using a nucleotide triphosphate, adenosine triphosphate, and a sugar, fructose 1,6-diphosphate, as substrates for the APase activities of murine thymic lymphoma and selected normal tissues. Several parameters have been used to determine if the activity measured is similar to the nonspecific APase activity already described (Tisdale, 1974). These parameters will include pH optimum, heat inactivation rate, substrate ratio, inhibition, activation, and kinetics.
CHAPTER II

REVIEW OF LITERATURE

Association of Alkaline Phosphatase with Mouse Leukemia

It has been well established that alkaline phosphatase (APase) is associated with murine lymphoma. The first correlation between the susceptibility to lymphoma development and the presence of APase activity was revealed by Smith (1961), comparing the thymuses of several strains of mice by histochemical techniques. She demonstrated that the lymphoid cells of the thymic cortex of adult C57Bl mice, a strain with a low incidence of spontaneous tumors, had no detectable level of APase activity. The only structures stained were the vascular endothelium and occasional reticular cells. In contrast, highly leukemic strains had detectable levels of APase activity in the non-neoplastic thymus. 

Smith (1962) further substantiated the correlation of APase activity with thymic lymphoma in the normally non-reactive thymic cortex of C57Bl mice studied at serial intervals after exposing to whole-body and thigh-shielded irradiation. By histochemical techniques, she showed that APase activity was, indeed, associated with individual lymphoid tumor cells. Metcalf et al. (1962) and Fey and Schneider (1963) later reported histochemical evidence of elevated APase activity in spontaneous thymic lymphomas of the AKR mice and in virus-induced lymphatic leukemias of the random-bred Agnes Bluhm mice, respectively. That the enzyme activity was associated with neoplastic lymphocytes, rather than a diffusion product from other structures, was indicated by the localization of enzyme activity in areas of extrathymic infiltration of the mediastinum.
(Smith, 1962), as well as by its increased levels in other organs infiltrated by tumor cells during the process of dissemination (Wilson et al., 1971).

Although these studies strongly suggested a correlation between the development of APase activity and the development of lymphoma, multiple interpretations have been proposed as to the nature of the correlation. APase activity might have been considered to be an attribute of malignant transformation per se or merely a reflection of the immaturity of the lymphoid tumor cells. Another interpretation might have been that the APase activity occurred as a nonspecific response to trophic disturbances, since APase activity had been detected in the normal thymic lymphoid cells of four to five week old AKR mice (Smith, 1961), which exhibited a sustained thymic hyperplasia linked to constitutional adrenocortical insufficiency (Arnesen, 1956). Still another interpretation might have been that APase activity represented a nonspecific response to involutional changes. Using a sensitive azo dye method, Lagerlof and Kaplan (1967) demonstrated slight APase activity in the thymus of seventeen day old fetal C57Bl mice, but none in the thymic lymphoid cells of newborn nor ten day old mice, which had previously been reported to contain a very high proportion of large, immature lymphoid cells. In addition, they observed that neither lymphoid hyperplasia nor acute involutional changes in the lymphoid tissues produced by adrenalectomy and corticosteroids, respectively, was accompanied by histochemically demonstrable changes in APase activity. Lagerlof and Kaplan (1967), therefore, concluded that the
occurrence of APase activity in murine thymic lymphoma was a specific response induced concomitantly with and directly related to malignant transformation per se. Lymphoma APase activity has been histochemically demonstrable on the cell membrane of lymphoid cells (De The, 1966; Lagerlof and Kaplan, 1967; Floyd, 1973).

Biochemical Characterization of Murine Lymphoma Alkaline Phosphatase

APase activity of murine thymic lymphoma has been characterized biochemically by several parameters, including pH optimum, heat inactivation rate, ratio of activity toward various substrates, electrophoretic mobility in acrylamide gel, inhibition and activation. By these parameters and histochemical localization, two distinct APase activities have been demonstrated - a specific nuclear inorganic pyrophosphatase activity and a nonspecific cell membrane activity which hydrolyzed beta-glycerophosphate, alpha-naphthylphosphate and p-nitrophenylphosphate at nearly equal rates (Tisdale, 1974; Floyd, 1973). The pH at which maximal activity occurs may vary, depending on the buffering capacity as well as the ionic strength of the assay system. Using 0.5 M 2-amino-2-methyl-1,3-propanediol, which had a better buffering capacity than 0.5 M Tris-HCl at high pH values, Lumb and Doell (1970) reported that optimum APase activity of a number of chemical- and viral-induced thymic lymphomas from C57Bl mice resulted at pH 10. With respect to electrophoretic mobility, they showed that thymic lymphoma extracts had a major band of APase activity and a minor band of slower mobility. This faster migrating APase activity has been shown to be neuraminidase sensitive (Floyd, 1973). Studies on inhibition have shown that APase
activity is inhibited by ethylenediamine tetraacetic acid, L-phenylalanine and zinc sulfate (Williams, 1971), strongest inhibitor. Moss (1969) reported that zinc, reportedly a constituent of the active center of *E. coli* APase (Schlesinger, 1966), strongly inhibited APase activity by a noncompetitive mechanism. Cory et al. (1971) demonstrated an additional "magnesium-independent" APase activity which showed less than a 0.5 fold activation by magnesium in leukemic livers of Rich virus-infected mice. Similar levels of magnesium activation of APase activity have been reported for thymic lymphomas (Williams, 1971; Floyd, 1973; Tisdale, 1974; Yahya, 1974). Williams (1971) has demonstrated that potassium and sodium also enhanced thymic lymphoma APase activity in C57Bl mice. Magnesium and potassium showed about the same level of activation; whereas, sodium activation was considerably lower. Heat inactivation studies showed that murine leukemic APase activity was heat labile. Lumb and Doell (1970) showed that about half the activity was lost in the first four minutes at 55 °C. Similar results have been reported by Cory et al. (1971) and Neumann et al. (1971).

In a comparative biochemical study of APases in thymus, spleen, liver, and kidney of C57Bl/6, SJL/J, and AKR strains of mice, Neumann et al. (1971) showed that the ratio of the rate of hydrolysis of p-nitrophenylphosphate to that of cysteamine S-phosphate was significantly greater in leukemic tissue than the corresponding ratio for the nonleukemic tissue. These observations suggested the presence of a distinct APase in murine lymphoma that was capable of distinguishing between oxygen and sulfur linkages occurring between the alcohol moiety and the phosphoryl group. A distinct APase which closely resembled
that of murine thymic lymphoma in its electrophoretic mobility and its substrate specificity was demonstrated in the serum of patients with lymphatic leukemia and infectious mononucleosis (Neumann et al., 1974). It was observed that this APase activity disappeared upon recovery from infectious mononucleosis.

Function of Alkaline Phosphatase

Although the association of APase with thymic lymphoma has been extensively documented, the physiological function of this enzyme remains uncertain. Besides involving phosphoesterase activity, this APase may also involve phosphotransferase activity, which is suggested by its localization on the cell membrane. On the basis of assays in high alcohol concentrations, it has been suggested that reactions catalyzed by APase involve a covalent phosphoryl-enzyme intermediate. This conclusion has been supported by a variety of kinetic and thermodynamic studies, including work with labeled inorganic phosphate. It has been observed (Garen and Liventhal, 1960; Harkness and Hilmore, 1962; Simpson and Vallee, 1969) that *Escherichia coli* APase catalyzed the hydrolysis of a wide range of phosphate esters, including p-nitrophenylphosphate, at about the same rate. This effect suggested the presence of a phosphoryl-enzyme intermediate, assuming that phosphorylation was more rapid than dephosphorylation. In this case, the latter would have been the rate-determining process common for all of the substrates.

Further support for the existence of a phosphoryl-enzyme has been provided by stopped-flow studies (Fernley and Walker, 1969; Reid and Wilson, 1971; Trentham and Gutfreund, 1968; Halford et al., 1969).
of *E. coli* APase. With the use of chromogenic or fluorogenic substrates, an initial burst of colored product, phenol, was observed to be followed by a slower release of product. However, this initial burst behavior was observed only at pH less than 7, which corresponded to phosphorylation (Aldridge et al., 1964; Barman and Gutfreund, 1966). The absence of this initial burst at alkaline pH, therefore, was inconsistent with the observation that at this pH, different substrates were hydrolyzed at the same rate. To overcome this discrepancy, Trentham and Gutfreund (1968) proposed that the initial burst behavior may have reflected a first-order conformational change of the enzyme-substrate complex and that this change was the rate-determining step. According to such a mechanism, a phosphoryl-enzyme may not have been required.

Thermodynamic studies by Wilson and Dayan (1965) showed that $^{32}$P-phosphate labeled phosphoprotein was approximately $10^5$ times more stable than ordinary phosphate esters. Accordingly, Barret et al. (1969) concluded that phosphoprotein might have been found in reactions catalyzed by APase, but did not function as a kinetic mechanism. In fact, this phosphoryl-enzyme intermediate could be isolated at low pH and was shown to be a serine side chain in which one particular serine residue in the neighborhood of the active site, was phosphorylated (Lazdunski and Lazdunski, 1969; Neumann et al., 1967; Applebury et al., 1970). In this respect, APase resembled the group of enzymes known as phosphotransferases. Studies on human placental APase (Byers et al., 1972) as well as chicken intestinal APase (Hinsberg and Laidler, 1972) and murine lymphoma APase (Prioleau, 1974) have further supported
the presence of a phosphoryl-enzyme.

Prioleau (1974) has demonstrated that high concentrations of nucleophilic buffers accepted phosphate from murine placental and lymphoma APase. Heat inactivation, pH optimum and inhibition results, reportedly, were consistent with the assumption that transphosphorylase and phosphatase activities were catalyzed by the same enzyme. The difference between p-nitrophenol and phosphate concentrations was assumed to represent the concentration of phosphorylated nucleophile. The observation that an increase in APase activity paralleled the transphosphorylase activity with increasing concentrations of amino-alcohols was consistent with data obtained by Amador and Urban (1972) and Hinsberg and Laidler (1972) using human and chicken intestinal APases, respectively.

Although phosphotransferase activity could be demonstrated in nucleophilic buffers, Prioleau (1974) suggested that the $K_m$ values for these nucleophiles were too high to consider them as physiological substrates for this reaction. However, glucose, nucleosides and serine have also been used as phosphate acceptors (Anderson and Nordlie, 1967; Georgatsos, 1967; Neumann, 1969). Wilson and Dayan (1964) observed that, in order for a compound to serve as an acceptor, it had to have, in addition to the accepting hydroxyl group, a second hydroxyl group or an amino group without quarternary structure. Positive ninhydrin staining of new esters formed when amino-ethanol and serine were used as acceptors (Neumann, 1969) proved unequivocally that the phosphoryl groups of the donor are transferred exclusively to the hydroxyl groups of the acceptor.
Thus the coincidence of the ability of APase to transfer phosphate and its location on the cell membrane suggested possible functions of APase in the control of cellular proliferation. For example, APase may function as a protein kinase in cyclic AMP-related controls of cell proliferation. According to this hypothesis, APase could be phosphorylated and then could specifically transfer phosphate to a particular enzyme to activate it. Inhibition of APase activity by phenylalanine could be accounted for by suggesting that phenylalanine binds to the APase site which would normally bind the acceptor enzyme.

Byers et al. (1972), using a rapid mixing and quenching technique, have shown that phenylalanine inhibition of human placental APase activity was entirely accounted for by a stabilization of the phosphorylated-enzyme complex. Therefore, inhibition is consistent with a mechanism whereby phenylalanine competes with the acceptor enzyme in the protein kinase reaction.

Studies on the control of APase synthesis in vitro have suggested a regulatory function of APase activity in cell division. Miedema (1968) showed that APase activity decreased once confluency occurred in culture. Cyclic AMP was shown to be involved in contact inhibition and the complexities of its action at the cell surface were indicative of the effect of calcium in cyclic AMP control of thymic lymphoblast proliferation (Whitfield et al., 1973). Calcium ions have been shown to change the stimulatory effect of cyclic AMP on lymphoblast proliferation to an inhibitory one. Since calcium transport has been associated with phospholipid and pNPPase activity (Cotmore et al., 1971), it
would appear that APase may be involved in cyclic AMP-related events at the cell surface.

Adenosine Triphosphatase

The feature of transport which has become best established and currently the most investigated is the nature of the adenosine triphosphatase (ATPase) reaction catalyzed by cell membranes. There is evidence that two different independent ouabain-sensitive enzymatic sites catalyze the hydrolysis of ATP in a wide distribution of biological membranes, including calf brain (Neufeld and Levy, 1969), rat erythrocytes (Czerwinski et al., 1967), rabbit brain (Kanazawa et al., 1967), and guinea pig kidney (Hegyvary and Post, 1971). One of these sites is the sodium-potassium activated ATPase considered to be part of the mechanism for active transport of sodium and potassium across biological membranes. Kinetic analysis by Neufeld and Levy (1970) indicated that hydrolysis of ATP at this site correlated with the steady-state level of acid-stable phosphoprotein complex. Previously, Bader et al. (1968) had studied the relationship between enzymatic activity and the phosphorylated intermediate of membrane ATPases from a total of six tissues and eleven species. In each preparation, they isolated a phosphoryl-enzyme and showed that the amount of phosphoryl-enzyme was related to the enzymatic activity regardless of the range in specific activities of the ATPases. In addition, digestion of the phosphoprotein with pepsin or pronase yielded identical $^{32}$p-labeled peptides after separation by electrophoresis.

Several studies based on the sensitivity to hydrolysis by hydroxylamine indicated that the phosphorylated intermediate was an acylphosphate.
Fahn et al. (1968), using electric organ ATPase, found that the dephosphorylating effect of hydroxylamine and its inhibitory effect of the overall ATPase reaction correlated with 30% and 25% changes, respectively. At the same time, Kahlenberg et al. (1968) devised a method for the isolation and characterization of an acylphosphate residue in ATPase preparations from pig brain. The method was devised based on the view that, if the hydroxylamine-sensitive intermediate was linked to an amino acid residue which was not C-terminal, the most likely candidates for the acylphosphate would be L-glutamyl-gamma-phosphate and L-aspartyl-beta-phosphate. Their procedure was to synthesize radioactive L-glutamyl-gamma-N-(n-propyl) and L-aspartyl-beta-N-(n-propyl) hydroxamate derivatives of digested phosphorylated and non-phosphorylated samples and to use them as carriers and markers in separation techniques. From this study, L-glutamyl-gamma-phosphate was concluded to be the phosphorylated intermediate in the ATPase reaction. This conclusion was further substantiated by Uesugi et al. (1971) who studied the enzyme activity in bovine brain.

The nature of the ATPase reaction has been interpreted in terms of conformational manifestations of the molecule according to its ionic environment. Several authors have suggested a hypothetical mechanism for ATPase activity, involving four sequential partial reactions (Post et al., 1969; Sen et al., 1969; Albers et al., 1968). The first reaction is a sodium- and magnesium-dependent phosphorylation by ATP. This represents the $E_1$-P conformation. The second reaction is a magnesium-dependent conformational change of the phosphorylated intermediate. This is referred to as the $E_2$-P conformation. The third reaction
is a potassium-dependent hydrolysis of the $E_2\cdot P$ conformation. The last reaction is a spontaneous relaxation to the original conformation, with a loss of magnesium. Hart and Titus (1973), using specific inhibitors of cation transport, have indirectly measured the degree to which the enzyme's native conformation was altered, as reflected by exposure of sulfhydryl groups to alkylation by N-ethylmaleimide. In the presence of ouabain, which is known to render the phosphorylated state ($E_2\cdot P$) resistant to potassium-dependent hydrolysis, they observed an increase in sulfhydryl group reactivity. However, the most noted exposure of sulfhydryl groups was observed in the presence of oligomycin which is known to stabilize the sodium-dependent phosphorylated state ($E_1\cdot P$). Hart and Titus (1973), therefore, concluded that the greatest extent of conformational change in the enzyme results by phosphorylation to the $E_1\cdot P$ state. This finding added support to a previous conclusion by Somogyi (1968) that the configuration of the molecule seemed to depend on whether or not it was phosphorylated.

Another structural manifestation of the membrane ATPase reaction is the involvement of phospholipids. The role of lipids as a component of the catalytic unit of ATPase is not well understood because of conflicting reports. Roelofsen et al. (1966) showed that extraction of freeze-dried red blood cell ghosts with ether had no effect on ATPase activity, even though all the sterols and 23% of the phospholipids had been removed. On the other hand, Emmelot and Bos (1968) showed that lipid extraction, phospholipase C treatment and sodium deoxycholate solubilization of plasma membranes from rat liver inactivated ATPase activity. Grishman and Barnett (1972) showed additional evidence that
an intact phospholipid bilayer was required for ATPase activity, using sheep kidneys and rabbit muscle. However, Emmelot and Bos (1968) pointed out that there was no clear evidence that phospholipids serve as cofactors in the enzymatic activity. Alternatively, they suggested that phospholipid may function to maintain the structural integrity required for the enzymatic activity of the protein.

Since it is evident that both the protein and the bilayer phospholipid structures must be intact for the functional ATPase, the determination of its actual molecular weight might vary considerably, depending on the technique employed. Nonetheless, Mizuno et al. (1968), using pig brain microsomes, estimated the smallest particle size possessing ATPase activity to be about 500,000 daltons. This estimate agreed with that of Nakao et al. (1967). However, Kepner and Macey (1968) obtained an average molecular weight of about 250,000 daltons and considered that Nakao et al. (1967) had overestimated. Still, Uesugi et al. (1971) reported an apparent molecular weight of 670,000 daltons. Kyte (1971) showed that purified ATPase from canine renal medulla contained two different polypeptide chains in equimolar concentrations. The smaller polypeptide, to which no functional significance has yet been attributed, had a molecular weight of about 57,000 daltons. The larger polypeptide which can be covalently labeled with phosphate from ATP had a molecular weight of about 84,000 to 98,000 daltons (Kyte, 1971; Atkinson et al., 1971; Hart and Titus, 1973). In contrast to Hart and Titus (1973), Atkinson et al. (1971) were able to further separate the 98,000 molecular weight ATPase monomer into smaller subunits of 25,000 or 27,000 daltons with urea and sodium dodecysulfate,
respectively. They concluded that their 280,000 molecular weight ATPase preparation probably consists of twelve subunits of 25,000 molecular weight arranged as three tetramers of 98,000 molecular weight. More recently, Stein et al. (1973) have also suggested that ATPase might be a tetrameric protein, asymmetrically disposed in the membrane.

Furthermore, it has been suggested that interactions of some of these subunits in the binding of sodium and potassium could be the basis of both the sodium and potassium activation and transport properties of ATPase. The current data indicate that the potassium-dependent p-nitrophenylphosphatase activity is another manifestation of the terminal step of the ATPase reaction (Whittam and Wheeler, 1970). As previously noted, this terminal step of the ATPase reaction is a potassium-dependent hydrolysis of the phosphorylated intermediate. Albers and Koval (1972) showed that activation of potassium-dependent p-nitrophenylphosphatase activity occurred concomitantly with inhibition of sodium- and potassium-activated ATPase activity in the presence of glycerol or dimethyl sulfoxide. They suggested that these solvents interfered with the interaction of the phosphokinase and phosphatase sites with the phosphoryl acceptor site which concurrently increased the accessibility of p-nitrophenylphosphate to the phosphatase site. The effects of ATP and sodium on the phosphatase activity of ATPase preparations have been investigated by several workers. Yoshida et al. (1969) demonstrated that the associated potassium-dependent p-nitrophenylphosphatase activity associated with ATPase preparations was stimulated by adding ATP and sodium in millimolar concentrations not
suitable for optimum ATPase activity. Besides activating the phosphatase, ATP and sodium also provided some protection against heat inactivation. The findings of Yoshida et al. (1969) and Albers and Koval (1972), therefore, support the opinion that potassium-dependent p-nitrophenylphosphatase is another conformational manifestation of the dephosphorylating step of the ATPase reaction.

Of interest is the fact that human leukemic leukocytes have been shown to exhibit elevated sodium- and potassium-activated ATPase activity (Lichtman and Weed, 1969). On the other hand, however, Ellegaard and Dimitrov (1972) failed to demonstrate any sodium- and potassium-activated ATPase activity in lymphocytes from patients with lung carcinoma and from normal patients. However, they demonstrated an elevated oligomycin-sensitive ATPase activity in lymphocytes from patients with lung carcinoma. In contrast, patients with non-malignant diseases and normal individuals showed identical low activity. This elevated ATPase activity appears to correlate with the elevation of serum APase activity which Fishman et al. (1968) observed in patients with bronchiogenic carcinomas.

Fructose Diphosphatase

In contrast to ATPase activity, fructose 1,6-diphosphatase (FDPase) activity has been demonstrated mainly in the cytosol (Wolf and Guertler, 1972). However, Allen and Blair (1972) have shown that phospholipid appeared to be necessary for native FDPase activity. Therefore, it is possible that some FDPase activity may be membrane bound.

In terms of molecular properties, fructose 1,6-diphosphatase
(FDPase) has been found in native and altered conformations which exhibit optimal activities at neutral and alkaline pH, respectively. Sucrose gradient centrifugation studies (Byrne et al., 1971) showed that the purified enzyme migrated as a single protein component coincident with neutral FDPase activity. It has been shown to have a molecular weight of approximately 127,000 to 133,000 daltons and to contain four or more subunits. Traniello et al. (1971) showed that homogenous preparations of neutral FDPase yielded only a single species of subunit in sodium dodecylsulfate disc-gel electrophoresis. Alkaline FDPase, on the other hand, has been shown to have a lower molecular weight by about 3,000 daltons/subunit (Pontremoli et al., 1971). Pontremoli et al. (1973) have shown that the removal of the amide-terminal region of FDPase from rabbit liver by digestion with subtilisin or changes in the configuration in this region of the enzyme produced by low concentrations of urea resulted in similar alterations in catalytic and allosteric properties. These molecular changes were measured by changes in the fluorescence of the single tryptophan residue which is located near the amide-terminus. Therefore, the functional properties of the native enzyme appeared to be determined by the tryptophan-containing polypeptide.

Comparative studies of the native and altered forms of the enzyme showed upon conversion: (1) a shift in the pH optimum from neutral to alkaline pH values, (2) a loss of activation by potassium (Colombo and Marcus, 1973), (3) a diminished sensitivity to AMP inhibition, and (4) differences in the reactivity of some sulfhydryl groups with p-mercuribenzoate (Traniello et al., 1971).
In an earlier study of substrate specificity of FDPase activity, Gomori (1943) demonstrated that kidney and liver tissues contained ATPase which showed high activity toward hexose diphosphate and very little toward glycerophosphate and phenylphosphate. More recently, however, Cohen et al. (1971), using highly purified bovine hepatic FDPase showed a correlation of beta-glycerophosphatase activity with neutral FDPase activity in several preparations. In addition, these two activities exhibited similar response to the presence of 5'-AMP. Furthermore, FDP inhibited glycerol release from beta-glycerophosphate, while the latter competitively inhibited alkaline FDPase activity. Such evidence, therefore, suggested that FDPase has three definable activities which all shared a common active site: neutral and alkaline activity with FDP and the ability to dephosphorylate beta-glycerophosphate. Therefore, it would appear that FDPase is not as specific as it was believed to be.

In contrast to nonspecific APase and ATPase activities, lowered FDPase activity has been demonstrated in rat tumors (Balinsky et al., 1972; Lustig and Kellen, 1973). This phenomenon is not surprising since the latter enzyme is maximally active and favors gluconeogenesis when the AMP concentration is low and the ATP concentration is high.
CHAPTER III

MATERIALS AND METHODS

Source of Enzyme

APase was obtained from mouse tumors and from normal mouse tissues. Placentas and fetuses were removed from pregnant inbred C57Bl mice at sixteen days gestation, while spleens were removed from normal adult males. Tumors were induced by injection of newborns with urethan or by intra-thymic injection of a virus originally isolated from a 6-mercaptopurine-induced tumor (Doell and Mathieson, 1970). Crude extracts were prepared by homogenization in cold sodium chloride (0.015 M). Extracts were then filtered through 2 x 2 gauze and stored at -4 C.

Assay for p-Nitrophenol

When p-nitrophenylphosphate (pNPP) was used as a substrate, APase activity was determined by measuring p-nitrophenol (p-NP). The reaction mixture contained 0.8 ml of 0.05 M ammediol buffer (2-amino-2-methyl-1, 3-propanediol) with 5 mM magnesium chloride adjusted to pH 10, 0.1 ml of tissue extract, and 0.1 ml of 33.8 mM pNPP. The reaction was stopped after 30 min at 37 C by adding 2 ml of 0.1 M EDTA/in 0.5 N NaCH (EDTA/NaCH). Blanks were prepared as above, except that EDTA/NaCH was added before addition of substrate. The concentration of pNP was determined at 400 nm on a Turner colorimeter with a Chemputer digital readout attachment (Mediconic International).

Assay for Phosphate

APase activity upon the substrates ATP and FDP was determined by measuring phosphate. The standard reaction mixture contained 0.8 ml of
0.05 M ammediol buffer with 5 mM magnesium chloride adjusted to pH 10, 0.1 ml of tissue extract and 0.1 ml of 50 mM ATP or 100 mM FDP. Ammediol buffer was adjusted to pH 8 to 11 in experiments to determine optimum pH. The concentrations of ATP were varied from 0.1 mM to 10 mM in kinetic experiments. The reaction was stopped after 30 min at 37 C by adding 2 ml of molybdo-vanadate reagent III (Lecocq and Inesi, 1966). Tubes were immediately cooled on ice before reading. Controls were prepared in the following manner: The substrate control contained 0.1 ml of the appropriate substrate, 0.1 ml of deionized water, and 0.8 ml of buffer. The extract control contained 0.1 ml of extract, 0.1 ml of deionized water, and 0.8 ml of buffer. The blank contained 0.1 ml of the appropriate substrate and 0.8 ml of buffer. The reaction was stopped as above, at which time 0.1 ml of extract was then added to the blank. Phosphate concentration was determined at 375 nm on a Turner colorimeter with a UV attachment in addition to the Chemputer. The blank was subtracted from the experimental. In most cases, the blank represented the additive effect of the substrate and extract controls.

Heat Inactivation

Tubes were prepared for assay and were heated at 55 C for appropriate times. The tubes were allowed to cool on ice. Controls prepared for assay were kept on ice for the appropriate times. Tubes were allowed to preincubate at 37 C for 5 min before adding the substrate. The heat inactivation rate is described in the equation \( A = A_0 e^{-k_H t} \) where \( A_0 \) is the unheated activity, \( A \) is the activity remaining after time heated \((t)\) and \( k_H \) is the heat inactivation rate constant.
Activation and Inhibition

Using the appropriate substrate, activation of phosphatase activity by 4 mM magnesium chloride was determined by comparing the activities in the presence and absence of activator. EDTA and L-phenylalanine were used as inhibitors in final concentrations of 0.1 mM and 10 mM, respectively. Extracts were preincubated with buffer containing EDTA for 5 to 10 min before adding substrate. Percent inhibition was determined by comparison with the appropriate control.

Statistical Analysis

Analysis of variance was performed on data from different tumor and normal tissue extracts to determine significance. Computation of data was done at the Atlanta University Center Computer Center, using the TSSST subroutine of the Scientific Subroutine Package with an IBM (International Business Machines) 1130 computer.
CHAPTER IV

EXPERIMENTAL RESULTS

Optimum pH

Effects of pH on normal and leukemic tissue extracts, using ATP and FDP as substrates, are shown in Fig. 1. It can be seen that the optimal activity for lymphoma and placental ATPase activities and lymphoma FDPase activity is a broad peak between pH 9 and 10. On the other hand, placental FDPase activity shows a distinct peak at pH 10.

Heat Inactivation

Typical heat inactivation patterns are demonstrated for alkaline ATPase and FDPase activities (Fig. 2). Although the points are not exactly linear, Least Squares analysis was used to determine the slope, which is the heat inactivation rate. Table 1 shows the means of several experiments for comparison. The Student's t-test (p 0.01) shows no significant differences in heat inactivation rates from tumor and placental extracts with either substrate.

Kinetics of Alkaline ATPase Activity

Figure 3 shows a Lineweaver-Burk plot of kinetic experiments on placental extracts with ATP as a substrate. A K_m value of 0.27 ± 0.16 mM represents the mean and standard deviation of at least three experiments. The saturating concentration of ATP appears to be around 2.5 mM.

Inhibition

Tables 2 and 3 show inhibition of normal and leukemic tissue extracts by EDTA and L-phenylalanine, respectively, with ATP and FDP as
Fig. 1. The pH optimum of alkaline phosphatase activities of placenta and lymphoma using adenosine-5'-triphosphate and fructose-1,6-diphosphate as substrates.
Fig. 2. Heat inactivation of alkaline phosphatase activities of placenta and lymphoma using adenosine-5'-triphosphate and fructose-1,6-diphosphate as substrates. The natural logarithm of the activity remaining is plotted against the time incubated at 55°C in order to determine the heat inactivation rate.
Table 1. Heat inactivation rate of alkaline phosphatase activities.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Adenosine 5'-triphosphate</th>
<th>Fructose 1,6-diphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placenta</td>
<td>0.30 ± 0.04</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>0.34 ± 0.03</td>
<td>0.27 ± 0.03</td>
</tr>
</tbody>
</table>

*Heat inactivation rate is the slope of the line obtained by plotting the natural logarithm of the remaining activity against the time heated at 55 C (Fig. 2).
Fig. 3. Lineweaver-Burk plot of the alkaline phosphatase activity of placenta using adenosine-5'-triphosphate as substrate.
\[ \frac{1}{V_{ATP}} \] (nMoles Pi⁻¹ minutes⁻¹)

\[ \frac{1}{[ATP]} \] (mMoles⁻¹)

[Diagram with data points and trend line]
Table 2. Inhibition of alkaline phosphatase activities by ethylenediamine tetraacetic acid (EDTA).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Adenosine 5'-triphosphate</th>
<th>Fructose 1,6-diphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placenta</td>
<td>87.0 ± 5.5</td>
<td>17.0 ± 11.2**</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>91.5 ± 16.9</td>
<td>11.5 ± 14.3**</td>
</tr>
</tbody>
</table>

*The mean and standard deviation of at least three experiments given as percent inhibition by 0.1 mM EDTA.

**Significantly different from the inhibition of alkaline adenosine triphosphatase activity by the Student's t-test (p < 0.01).
Table 3. Inhibition of alkaline phosphatase activities by L-phenylalanine.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Adenosine 5'-triphosphate</th>
<th>Fructose 1,6-diphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placenta</td>
<td>35.7 ± 27.0**</td>
<td>23.0 ± 4.9</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>35.5 ± 6.3</td>
<td>34.5 ± 21.9</td>
</tr>
</tbody>
</table>

*The mean and standard deviation of at least three experiments given as percent inhibition by 10 mM L-phenylalanine.

**Variable levels of activation also observed.
substrates. Both tumor and placental alkaline ATPase activities are inhibited approximately 90% by the presence of EDTA, while alkaline FDPase activity is inhibited less than 20% by the action of this metal chelator. L-phenylalanine partially inhibits APase activities of tumor and placenta with either ATP or FDP as substrate. However, variable levels of activation of alkaline ATPase activity by L-phenylalanine were observed only in placenta, ranging from 1.4 to 2.6 times the level of activity in the absence of L-phenylalanine. Further experiments are in progress to explain this inconsistency.

Activation

Table 4 illustrates the activation of normal and leukemic tissue by magnesium. While magnesium activation of lymphoma alkaline ATPase activity is approximately twice that of placental alkaline ATPase, there is no significant difference in activation of alkaline FDPase activities in these tissues (p > 0.01).

Substrate Ratio

Table 5 indicates substrate ratios of normal and leukemic tissue extracts in the presence of ATP, FDP, and pNPP. The Student's t-test (p > 0.01) shows that there are no significant differences in the ratios of alkaline ATPase and FDPase activities within or between placenta and lymphoma. Both alkaline ATPase and FDPase activities in placenta and lymphoma are significantly different from those activities in the 16-day fetus. The Student's t-test (p < 0.01) shows that the spleen has a significantly greater alkaline ATPase activity than that of the placenta, lymphoma or the 16-day fetus. Alkaline FDPase activity of the spleen, however, does not differ significantly from that of placenta and lymphoma.
Table 4. Activation of alkaline phosphatase activities by magnesium.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Adenosine 5'-triphosphate</th>
<th>Fructose 1,6-diphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placenta</td>
<td>3.42 ± 1.29</td>
<td>1.25 ± 0.12</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>7.72 ± 1.52**</td>
<td>1.32 ± 0.25</td>
</tr>
</tbody>
</table>

*The mean and standard deviation of at least three experiments given as activity in the presence of 4 mM magnesium chloride divided by activity in the absence of magnesium chloride.

**Significantly different from the activation of placental alkaline adenosine-triphosphatase activity by the Student's t-test (p < 0.01).
Table 5. Distribution of alkaline phosphatase activities in normal and leukemic tissues.

<table>
<thead>
<tr>
<th>Substrate Ratio*</th>
<th>Substrate</th>
<th>Placenta</th>
<th>Spleen</th>
<th>Fetus</th>
<th>Lymphoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP(^1)</td>
<td>1.65 ± 0.84</td>
<td>9.75 ± 2.23**</td>
<td>0.39 ± 0.18**</td>
<td>2.30 ± 0.36</td>
</tr>
<tr>
<td></td>
<td>FDP(^2)</td>
<td>1.18 ± 0.36</td>
<td>0.68 ± 0.45</td>
<td>0.77 ± 0.11***</td>
<td>1.69 ± 0.46</td>
</tr>
</tbody>
</table>

*The mean and standard deviation of at least three experiments given as the ratio of activity toward the indicated substrate to the activity toward p-nitrophenylphosphate.

**Significantly different from lymphoma and placental adenosine triphosphatase activities by the Student's t-test (p < 0.01).

***Significantly different from lymphoma and placental fructose diphosphatase activities by the Student's t-test (p < 0.01).

\(^1\)ATP represents adenosine 5'-triphosphate.

\(^2\)FDP represents fructose 1,6-diphosphate.
Table 6. Summary of alkaline phosphatase activities in murine lymphoma and placenta.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Nonspecific*</th>
<th>Lym$^3$</th>
<th>Pla$^4$</th>
<th>Lym$^3$</th>
<th>Pla$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH optimum</td>
<td>10</td>
<td>9 - 10</td>
<td>9 - 10</td>
<td>9 - 10</td>
<td>10</td>
</tr>
<tr>
<td>Heat inactivation rate</td>
<td>0.13</td>
<td>0.34</td>
<td>0.30</td>
<td>0.27</td>
<td>0.35</td>
</tr>
<tr>
<td>Inhibition by EDTA (%)</td>
<td>87.7</td>
<td>91.5</td>
<td>87</td>
<td>11.5</td>
<td>17</td>
</tr>
<tr>
<td>Inhibition by L-phe (%)</td>
<td>31.6</td>
<td>35.5</td>
<td>35.7</td>
<td>34.5</td>
<td>23</td>
</tr>
<tr>
<td>Activation by magnesium</td>
<td>1.3</td>
<td>7.7</td>
<td>3.4</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Substrate ratio</td>
<td>1.3</td>
<td>23</td>
<td>1.7</td>
<td>1.7</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*No significant difference was shown in any of these criteria between activities of placenta, lymphoma, and adult spleen (Tisdale, 1974; Yahya, 1974).

$^1$ATPase represents alkaline adenosine 5'-triphosphatase activity.

$^2$FDPase represents alkaline fructose 1,6-diphosphatase activity.

$^3$Lym represents lymphoma.

$^4$Pla represents placenta.
CHAPTER V

DISCUSSION AND CONCLUSIONS

The data presented on the properties of murine lymphoma and placental alkaline phosphatase activities in the presence of adenosine 5'-triphosphate (ATP) or fructose 1,6-diphosphate (FDP) show no significant differences in heat inactivation rate, L-phenylalanine inhibition or pH optimum. There is also no significant difference in alkaline ATPase and FDPase activities with tissues by the above parameters. Substrate ratios indicate no significant differences between levels of alkaline ATPase and FDPase activities in placenta or in lymphoma. There are also no significant differences in the levels of either activity between the two tissues.

However, alkaline ATPase and FDPase activities of placenta and lymphoma are different by parameters of EDTA inhibition and magnesium activation. Both lymphoma and placental alkaline ATPase activities are strongly inhibited by EDTA, while alkaline FDPase activities of these tissues are relatively resistant to the action of the metal chelating agent. Lymphoma alkaline ATPase activity is more highly activated by magnesium than placental ATPase activity. At the same time, however, there is no significant difference in magnesium activation of alkaline FDPase activities in these tissues.

Differences in activation levels of lymphoma and placental ATPase activities by magnesium may have been due to differences in endogenous levels of magnesium present in the assay system. Alternatively,
lymphoma extracts may have additional magnesium-dependent ATPases. However, effects on the substrate and not the enzyme may account for differences in alkaline ATPase and FDPase activities by EDTA inhibition and magnesium activation. Marcus et al. (1973) showed that bovine hepatic FDPase activity was competitively inhibited by high concentrations of both its substrate and magnesium. In addition, the $K_i$ for magnesium inhibition was the same as the $k_{diss}$ for the magnesium-substrate complex. This indicated that magnesium may have inhibited FDPase activity by complexing with the free substrate. From this study, they suggested a sequential mechanism for magnesium and free FDP binding. In this mechanism, either the former or the latter could bind to the enzyme first. On the other hand, the "true" substrate for ATPase activity was thought to be the magnesium-ATP complex (Ulrich, 1964). However, the free substrate and free magnesium could react with the enzyme in an inhibitory manner (Horgan, 1974). Therefore, one might expect differences in EDTA inhibition and magnesium activation of APase activity when ATP and FDP are used as substrates.

In comparison to lymphoma or placenta, spleen shows greater alkaline ATPase activity. There are no significant differences in levels of alkaline FDPase activities. These observations suggest that spleen may also have additional ATPases. Substrate ratios also reveal that alkaline ATPase and FDPase activities observed in the 16-day fetus differ significantly from those activities in placenta and lymphoma. Substrate ratios of either FDPase or ATPase activities for lymphoma, placenta, and 16-day fetus show a similar relationship to that demonstrated by non-specific APase activity for these tissues (Floyd, 1973).
However, the relationship between substrate ratios of ATPase activities for lymphoma, placenta, and spleen is inconsistent with substrate ratio data reported for nonspecific APase activity in these tissues. Tisdale (1974) showed that there was no significant difference between substrate ratios for lymphoma, placenta, and spleen non-specific APase activities.

Therefore, a relevant question is whether ATPase and FDPase activities in murine lymphoma and placenta are the same as the non-specific APase activity described by others (Tisdale, 1974; Yahya, 1974). Magnesium activation indicates that ATPase activities of placenta and lymphoma are distinct from non-specific APase activity. However, as previously mentioned, differences by this parameter may reflect effects on substrate rather than on enzyme. Heat inactivation rate is the only other parameter which shows any difference in the non-specific APase, ATPase, and FDPase activities in lymphoma and placenta. However, the apparent biphasic nature of ATPase and FDPase heat inactivation patterns, with the exception of lymphoma FDPase, suggests that there may be other enzymes showing affinity to these substrates. Therefore, differences in heat inactivation rates alone are not sufficient to conclude that these activities represent different enzymes. Two independent studies relevant to the proposed question have demonstrated potassium-dependent p-nitrophenylphosphatase activity of ATPase (Albers and Koval, 1972) and beta-glycerophosphatase activity of FDPase (Cohen et al., 1971). Therefore, it is possible that ATPase and FDPase activities in murine placenta and lymphoma result from conformational changes of the non-specific APase in these tissues.
Similarities in murine lymphoma, placental, and 16-day fetal thymus APase activities (Floyd, 1973) appear to be consistent with reports of fetal-like APase activity in HeLa (human cervical carcinoma) cells. Elson and Cox (1969) have shown that a HeLa cell APase isozyme, which differed from APase in normal adult tissues with respect to chemical and physical characteristics (Cox and Griffin, 1967), resembled a fetal form of the enzyme isolated from human placenta (Robson and Harris, 1965; 1967) by parameters of electrophoretic mobility, immunodiffusion, pH optimum, pH stability, inhibition by L-phenylalanine, and molecular weight determination. Like HeLa cell APase, human placental APase also has been shown to differ in several of its properties from that occurring in other tissues (Aleem, 1971; Hindriks et al., 1973; Posen et al., 1969). Both HeLa cell (Singer et al., 1974) and human placental (Aleem, 1971) APase isozymes have been localized on the cell membrane.

APase activity in human placenta (Posen et al., 1969) or HeLa cells (Spencer and Macrae, 1971) and APase activity in corresponding murine tissue (Floyd, 1973) appear to be similar with respect to pH optimum and inhibition by L-phenylalanine. A preliminary kinetic study of murine placental APase activity in the presence of ATP shows a $K_m$ value which is similar to that for highly purified human placental APase in the presence of ATP. However, human placental and HeLa cell APase isozymes have been shown to be more heat stable than murine lymphoma and placental APases.

The present data do not conclusively rule out the possibility that non-specific APase may hydrolyze ATP and FDP. Since alkaline
ATPase and FDPase activities within and between lymphoma and placenta differ only by parameters of EDTA inhibition and magnesium activation, which may reflect properties of the substrate rather than of the enzyme, it is possible that these activities are of the same enzyme rather than of distinct enzymes. Further elucidation of the relationship of these activities must await purification of the APase enzyme.
CHAPTER VI

SUMMARY

1. Murine lymphoma and placental alkaline ATPase and FDPase activities show no significant difference with respect to parameters of heat inactivation rate, L-phenylalanine inhibition and pH optimum.

2. Alkaline ATPase and FDPase activities in both tissues differ by parameters of EDTA inhibition and magnesium activation. However, these differences may reflect properties of the active substrates rather than the enzyme itself.

3. In comparison to lymphoma and placenta, spleen shows additional alkaline ATPase activity, while no significant differences in alkaline FDPase levels in these tissues were observed.

4. Alkaline ATPase and FDPase activities appear to be distinct from nonspecific APase activity with respect to heat inactivation rate.

5. Present data do not conclusively eliminate the possibility that nonspecific APase may hydrolyze ATP and FDP.


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