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Kinetic Parameters for the Cleaved Substrate, and Enzyme and Substrate Stability, Vary with the Phosphoacceptor in Alkaline Phosphatase Catalysis

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Nine different isoenzymes and (or) isoforms of alkaline phosphatase (ALP; EC 3.1.3.1) from human tissue were studied with respect to K_m and V_{max} values for *p*-nitrophenyl phosphate (*p*-NPP) in seven different potential phosphoacceptors/buffers. Generally, the phosphoacceptors/buffers with the lowest affinity for *p*-NPP (highest K_m values) gave the highest V_{max} values; for the nine enzyme forms in this study, the mean K_m and V_{max} values were greatest in 2-(ethylamino)ethanol (EAE). The two aminopropanol buffers gave the lowest K_m and V_{max} values. The phosphoacceptors/buffers *N*-methyl-D-glucamine (MEG), diethanolamine, and Tris had intermediate K_m and V_{max} values. Hydrophilic liver ALP retained >90% of its activity after 24 h at 30 °C in both 1.0 and 0.3 mol/L Tris and 2-amino-2-methyl-1,3-propanediol and in 0.3 mol/L MEG. This isoenzyme showed greatest inactivation upon prolonged exposure to 1.0 and 0.3 mol/L EAE, the activity at 24 h being ~50–66% of that at zero time. *p*-NPP underwent the greatest spontaneous degradation, ~2.5 times that of baseline levels, in 1 mol/L MEG. There was little degradation in all of the buffers tested at 0.3 mol/L or in Tris, EAE, and 2-amino-2-methyl-1-propanol at 1.0 mol/L.

Indexing Terms: *isoenzymes · kinetic enzyme analysis · enzyme activity · phosphotransferase*

Cleavage of *p*-nitrophenyl phosphate (*p*-NPP) by alkaline phosphatase (ALP; EC 3.1.3.1) in the presence of phosphoacceptors showed that the formation of both alcohol (*p*-nitrophenol) and new phosphoesters could be saturated as the concentration of acceptor increased (*1*).[1] The data followed Michaelis–Menten kinetics, indicating a binding site on the enzyme for the phosphoacceptor, probably competing with water. However, the three isoenzymes expressed in placenta, adult intestine, and liver differ in their transphosphorylation abilities. With seven phosphoacceptors, the liver enzyme was the most efficient (highest V_{max}/K_m ratio) catalyst of transphosphorylation, followed by the placental enzyme. The ratio of phosphotransferase:phosphohydrolytic activity at V_{max} varied little among the isoenzymes. However, there was obvious preference for phosphoacceptor (*1*). Excluding ethanolamine, which was a very poor phosphoacceptor, 2-(ethylamino)ethanol (EAE) had the lowest K_m values, followed by 2-amino-2-methyl-1-propanol (AMP) and *N*-methyl-D-glucamine (MEG). The highest V_{max} value was obtained in the presence of diethanolamine (DEA), followed by EAE and MEG (*1*).

These basic kinetic considerations are important when selecting a phosphoacceptor/buffer for the routine assay of ALP (*2*). Currently, the International Federation of Clinical Chemistry (IFCC) Reference Method has AMP for a phosphoacceptor (*3*). This compound has been plagued with purity problems that affect assay results (*4*); it is also not a particularly good phosphoacceptor when compared with several others (*1*). Several reports recommend MEG for the routine assay of ALP (*5, 6*), and two thorough evaluations have been published (*7, 8*). Also, the German Society of Clinical Chemistry has published a proposed standard method for the determination of catalytic concentrations of ALP in serum or plasma with MEG as phosphoacceptor/buffer (*9*); the Japanese Society of Clinical Chemistry has recommended a method with EAE (*10*).

The stability of the *p*-NPP in the phosphoacceptor/buffer is also important for the routine assay of ALP. Instability of the substrate may produce a high baseline absorbance during storage, resulting in a short shelf life for the reagent and increased costs and (or) wastage. The phosphoacceptor/buffer may also be used for dilution of ALP samples, and it is therefore important to know if the buffer significantly inactivates the ALP, especially if the diluted sample is to be stored before assay.

The purpose of this study was to investigate the effect of phosphoacceptors on the kinetic parameters of *p*-NPP cleavage. I found that these compounds can significantly change the apparent K_m and apparent V_{max} values for *p*-NPP. Moreover, these compounds have differential effects on the stability of *p*-NPP and ALP.

Materials and Methods

Chemicals. Phenylmethylsulfonyl fluoride, leupeptin, pepstatin, 2-*N*-morpholinoethanesulfonic acid (MES), Triton X-100, Tris, *p*-NPP, AMP, and 2-amino-2-methyl-1,3-propanediol (AM-1,3-P) were obtained from Sigma Chemical Co., St. Louis, MO. Sepharose CL-6B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and DEA and EAE ("Gold Label") were from Aldrich Chemical Co., Milwaukee, WI. All other chemicals were of the highest grade available from Fisher Scientific Co., Fair Lawn, NJ.

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[1] Nonstandard abbreviations: ALP, alkaline phosphatase; *p*-NPP, *p*-nitrophenyl phosphate; EAE, 2-(ethylamino)ethanol; AMP, 2-amino-2-methyl-1-propanol; MEG, *N*-methyl-D-glucamine; DEA, diethanolamine; IFCC, International Federation of Clinical Chemistry; mAb, monoclonal antibody; MES, 2-*N*-morpholinoethanesulfonic acid; and AM-1,3-P, 2-amino-2-methyl-1,3-propanediol.

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Enzymes. The forms and sources of ALP were purified amphiphilic (membrane anchor of diacylglycerol still attached) from liver and placenta; purified hydrophilic (membrane anchor removed) from liver and placenta; purified hydrophilic from adult intestine; and partially purified from a lymphoma cell line (L428), an osteosarcoma cell line (Saos-2), meconium, and bone.

Hydrophilic dimeric ALP from liver was purified with an immobilized monoclonal antibody (mAb) as described (*11*). For purification of the amphiphilic form from liver, the mAb was also utilized but in a different protocol (*12, 13*). A placenta, secured at the time of delivery, washed, cleaned of membranes, and frozen at −70 °C, was the starting material for the purification of hydrophilic enzyme from placenta. The tissue (100 g) was thawed and homogenized for two 3-min periods in a blender at 4 °C in buffer consisting of 1 g/L phenylmethylsulfonylfluoride, 10 μmol/L leupeptin, 20 μmol/L pepstatin, and 250 mmol/L MES, pH 6.5. Triton X-100 was added to a final concentration of 10 mL/L, and the mixture was stirred for 15 min at 4 °C. An equal volume of cold *n*-butanol was added slowly and mixing continued for 15 min. Centrifugation at 17 000 × *g* for 30 min allowed recovery of the aqueous layer. This layer was diluted to 400 mL/L with cold acetone and mixed for 15 min at 0 °C. The pellet recovered from centrifugation at 17 000 × *g* for 20 min was dissolved in 20 mL of buffer containing 300 mmol/L NaCl, 1.0 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, 1 g/L phenylmethylsulfonylfluoride, 10 μmol/L leupeptin, 20 μmol/L pepstatin A, and 10 mmol/L Tris, pH 7.5. This solution was dialyzed for 18 h against phosphonate buffer: 100 mmol/L NaCl, 1.0 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, and 10 mmol/L MES, pH 6.0. After centrifugation at 17 000 × *g* for 20 min, the supernate was pumped onto a 2.5 × 8.0 cm column of immobilized *p*-aminobenzylphosphonic acid coupled via an azo linkage to tyraminylSepharose (*14*). The column was washed with phosphonate buffer and the ALP eluted in this same buffer plus 50 mmol/L *p*-NPP. The eluate was concentrated to 5 mL in an Amicon stirred cell and pumped onto a 3.0 × 80.0 cm column of Sepharose CL-6B equilibrated in buffer: 300 mmol/L NaCl, 1.0 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, and 10 mmol/L Tris, pH 7.6. Fractions containing ALP were pooled and concentrated as above. Amphiphilic enzyme from human placenta was purified as previously described (*15*).

Intestinal ALP from fresh autopsy ileum was purified as described (*16*). The lymphoma cell line L428 was established from the pleural effusion of a patient with Hodgkin disease. Cells were grown in suspension in RPMI 1640 medium containing 50 mL/L fetal calf serum. The cultures were maintained at 37 °C in 50 mL/L CO_2. Saos-2 cells were cultured similarly to confluency. Cells were washed three times in 2 mL of buffer containing 1 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, 150 mmol/L NaCl, and 10 mmol/L Tris, pH 7.6. They were resuspended in this same buffer without the NaCl and sonicated for 30 s at low setting in a Beckman (Fullerton, CA) Sonic 2000 sonicator. This was followed by addition of Triton X-100 to 10 mL/L and stirring for 90 min. The supernate recovered after centrifugation was mixed with a solution of 5 μmol/L leupeptin, 25 μmol/L pepstatin A, and 0.1 g/L phenylmethylsulfonylfluoride. This solution was frozen at −20 °C until use.

Meconium was collected and frozen immediately. Purification of ALP from this material has been described (*17*). The enzyme from human bone was purchased from Calzyme Laboratories (San Luis Obispo, CA) as an impure preparation.

Enzyme kinetic assay. The standard assay for ALP has been described (*18*). All enzymes were diluted with 50 mmol/L Tris, 1.0 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, pH 7.5, and standardized to 500 U/L in an assay medium that contained 1.0 mol/L EAE, 1.0 mmol/L $MgCl_2$, 15 mmol/L *p*-NPP, and 0.1 mmol/L $ZnCl_2$, pH 10.3. For all ALPs, 1 U of activity will convert 1 μmol of substrate to product in 1 min. For the purpose of this investigation, the chemical nature of the phosphoacceptor/buffer was varied but was always used at 1.0 mol/L, pH 10.3, and 30 °C. The seven phosphoacceptors/buffers evaluated were AMP, AM-1,3-P, DEA, EAE, ethanolamine, MEG, and Tris. For K_m determinations, *p*-NPP was varied over a minimum 10-fold concentration range, with a minimum of five concentration points. Kinetic parameters were determined by computer (*19*).

ALP stability. The stock solution of liver hydrophilic ALP (activity 1450 U/L) was in a 50 mmol/L bicarbonate buffer containing 1 mmol/L $MgCl_2$ and 0.1 mmol/L $ZnCl_2$, pH 10.3. One part stock solution was added to nine parts of the buffers tested and incubated at 30 °C for 24 h. Each buffer, after addition of stock, was at a concentration of 1.0 or 0.3 mol/L, pH 10.3 (all contained 1 mmol/L $MgCl_2$ and 0.1 mmol/L $ZnCl_2$). All ALP assays were done at 30 °C in a 1.0 mol/L EAE assay mixture that contained 15 mmol/L *p*-NPP, 1 mmol/L $MgCl_2$, and 0.1 mmol/L $ZnCl_2$, pH 10.3. The release of *p*-nitrophenol over time was monitored as an increase in absorbance at 405 nm, and ALP activity was expressed in U/L. Assays were done at least in triplicate for each buffer at the following times: immediately after addition of stock (0 h), at 8 h, and at 24 h. Triplicate activity values were used to calculate a mean and SD for each buffer at each time (five values were used when the ALP exhibited inconsistent activity).

Stability of p-*NPP.* The stock *p*-NPP concentration was 150 mmol/L. One part stock solution was added to nine parts of each buffer tested and incubated at 30 °C for 30 min. Each buffer, after addition of stock, was at a concentration of 1.0 or 0.3 mol/L, pH 10.3 (all contained 1 mmol/L $MgCl_2$ and 0.1 mmol/L $ZnCl_2$). The final *p*-NPP concentration in each buffer was 15 mmol/L. The accumulation of *p*-nitrophenol via nonenzymatic *p*-NPP breakdown was monitored continuously at 405 nm. Results (mean and SD) were based on triplicate runs in each buffer.

Results

Seven compounds that could serve as phosphoacceptors and buffers for the assay of ALP were evaluated with respect to their effect on the K_m and V_{max} values for

the substrate *p*-NPP. There was profound inhibition of all tissue forms of the enzyme when ethanolamine was included in the assay at 1.0 mol/L; therefore, the data collected for this potential phosphoacceptor are not reported here.

The kinetic parameters for nine different tissue sources or isoforms of ALP were determined. Apparent K_m values and the mean values for all enzyme forms with each phosphoacceptor are shown in Table 1. There is considerable variability in K_m values among the phosphoacceptors, with a sixfold difference between the highest in EAE and the lowest in AM-1,3-P. The K_m values for *p*-NPP in MEG were intermediate between these extremes. A comparison of values reveals that the phosphoacceptor is more important in determining the *p*-NPP K_m values than is the tissue source or the ALP isoenzyme.

Apparent V_{max} values and the mean values for all enzyme forms with each phosphoacceptor are listed in Table 2. All stock enzyme solutions were standardized to 500 U/L in 1.0 mmol/L EAE; thus, the extrapolated V_{max} values for all the enzyme forms in this phosphoacceptor should be 500 U/L. The fact that the mean is only 416 U/L is probably a reflection of the error associated with the extrapolation of several assays under non-zero-order conditions to those of saturating substrate. In contrast to their effect on *p*-NPP K_m values, the phosphoacceptors have considerably less influence on V_{max} values; a possible exception may be the two intestinal ALPs, where the velocities are low in AMP and DEA. There is only a twofold difference in the mean velocity for the nine enzyme forms with AMP and EAE.

When the hydrophilic liver ALP was initially diluted 10-fold (undiluted activity = 1450 U/L) into the buffers at 1.0 and 0.3 mol/L, there was an increase in its activity in all of the buffers except EAE (Figure 1). In both concentrations of AM-1,3-P and Tris, the average ALP activity was essentially unchanged from its zero time activity at both 8 and 24 h. The same was true for the 0.3 mol/L MEG. However, in the 1.0 mol/L MEG, there was a moderate decrease in activity when ALP was assayed after 8 and 24 h. This was also true for both concentrations of AMP and DEA at 24 h. Only EAE contributed to a substantial decrease in ALP activity at 8 and 24 h, the loss being ~25–50% of that of the zero time activity. Prolonged incubation in 0.3 mol/L Tris

Table 1. Apparent K_m Values of ALPs for *p*-NPP Measured in Six Phosphoacceptor Buffers

	K_m, mmol/L					
Enzyme source	AMP	AM-1,3-P	DEA	EAE	MEG	Tris
Amphiphilic liver	0.92	0.67	1.9	2.9	1.6	3.0
Hydrophilic liver	1.1	0.52	1.2	2.7	1.4	1.9
Amphiphilic placenta	0.72	0.47	2.1	1.7	1.0	2.1
Hydrophilic placenta	1.8	0.51	2.9	2.1	0.88	1.4
Lymphoma cells	1.1	0.43	1.7	2.7	1.1	1.4
Osteosarcoma cells	0.74	0.42	0.91	2.8	1.4	1.5
Meconium	0.92	0.44	1.5	3.2	0.93	1.2
Hydrophilic intestine	0.78	0.35	1.1	4.3	1.0	0.91
Bone	1.1	0.77	1.7	4.3	1.3	1.8
Mean	1.02	0.51	1.7	3.0	1.2	1.7
SD	0.33	0.13	0.59	0.88	0.24	0.61

Table 2. Apparent V_{max} Values of ALPs for *p*-NPP Measured in Six Phosphoacceptor Buffers

	V_{max}, U/L[a]					
Enzyme source	AMP	AM-1,3-P	DEA	EAE	MEG	Tris
Amphiphilic liver	256	233	297	381	460	268
Hydrophilic liver	280	266	233	478	398	310
Amphiphilic placenta	201	210	287	430	335	393
Hydrophilic placenta	293	259	587	610	325	379
Lymphoma cells	206	213	265	494	297	250
Osteosarcoma cells	239	278	205	324	387	319
Meconium	99	151	113	365	277	261
Hydrophilic intestine	49	176	91	354	276	351
Bone	136	346	226	305	149	449
Mean	195	237	256	416	322	331
SD	84	58	143	98	89	68

[a] Stock enzyme solutions were standardized to 500 U/L in 1.0 mol/L EAE, 1 mmol/L $MgCl_2$, and 0.1 mmol/L $ZnCl_2$, pH 10.3.

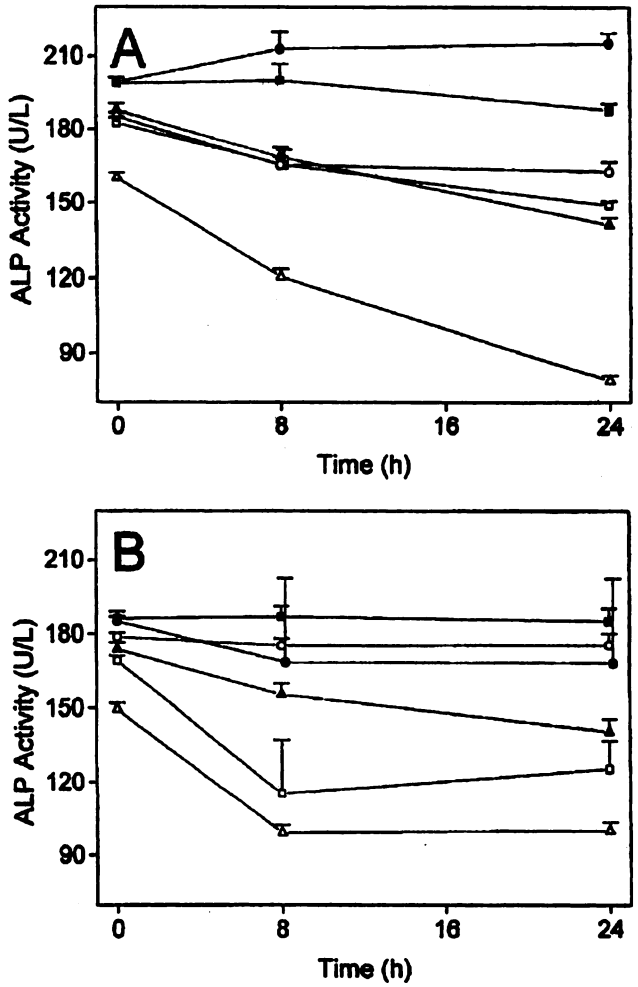

Fig. 1. Stability of hydrophilic liver ALP in phosphoacceptors at 1.0 mol/L (*A*) and 0.3 mol/L (*B*): AM-1,3-P, ■; Tris, ●; AMP, ▲; DEA, □; MEG, ○; EAE, △

Enzyme at 1450 U/L in bicarbonate at pH 10.3 was diluted 1:9 in the appropriate concentration of phosphoacceptor, pH 10.3, and incubated at 30 °C (see *Materials and Methods*). *Bars* are 1 SD

and DEA resulted in erratic enzyme activity, reflected by large SD values. This effect was not evident in 1 mol/L Tris and DEA.

Spontaneous degradation of *p*-NPP was greatest in 1.0 mol/L MEG (Figure 2*A*) and was ~150% greater than the baseline degradation found in all of the 0.3 mol/L buffers tested (Figure 2*B*). Of the remaining 1.0 mol/L buffers, AMP, EAE, and Tris showed baseline *p*-NPP degradation, whereas in AM-1,3-P and DEA, *p*-NPP exhibited moderate degradation.

Discussion

In selecting a phosphoacceptor/buffer for the routine assay of ALP, many aspects must be considered, and previous publications have addressed some of these for possible candidates (*5–9*). The major concern with the present IFCC-recommended method for ALP assay (*3*) is that the purity of the AMP used as a phosphoacceptor/buffer is inconsistent and may inhibit the catalytic activity (*4*).

In a previous publication, my colleagues and I reported on the affinity and velocity of three isoenzymes of ALP for seven different phosphoacceptors (*1*). During the course of these studies it became evident that not only do the isoenzymes have widely differing affinities (K_m values) for phosphoacceptors, but also the nature of the phosphoacceptors can affect the K_m value for the phosphoryl donor, in this case, *p*-NPP. This is not unreasonable when one realizes that the ALPs probably have a binding site for the phosphoacceptor (*1*), and thus this second substrate could alter the affinity for the first substrate (*p*-NPP). Here I document these effects with nine different tissue sources or isoforms of ALP. If this were the only consideration, then one would select the phosphoacceptor that resulted in the lowest K_m value and highest V_{max} for *p*-NPP. However, the data show that the phosphoacceptors resulting in the greatest V_{max} values also result in the highest K_m values (MEG resulted in intermediate values for both K_m and V_{max}). For example, *p*-NPP K_m values are highest with EAE and so are V_{max} values. A high K_m can be compensated for with a higher substrate concentration to assure zero-order kinetics, but this could result in higher costs. Why liver ALP exhibited differential stability in the phosphoacceptors is not obvious, but these compounds do have a binding site on the enzyme and thereby may influence stability; the presence of impurities may also be a contributing factor (*4*).

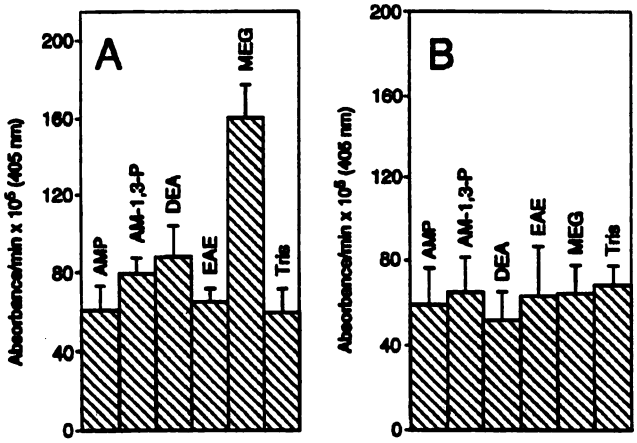

Fig. 2. Spontaneous degradation of 15 mmol/L *p*-NPP in phosphoacceptors at 1.0 mol/L (*A*) and 0.3 mol/L (*B*)
Incubation was at 30 °C for 30 min; bars are 1 SD

Spontaneous degradation of *p*-NPP in 1.0 mol/L MEG has been noted previously (*8*), but is not considered significant if the phosphoacceptor is used at 30 °C at a concentration of 0.3–0.4 mol/L (*8*). The increased degradation (about threefold) at 37 °C may preclude the use of MEG at this higher temperature (*8*). In addition to the K_m and V_{max} parameters, the effect of prolonged exposure of enzyme and of substrate to these buffers should be taken into account when choosing a phosphoacceptor/buffer.

Several reports have proposed the use of MEG as a phosphoacceptor for ALP assays, and certain of the attributes of this compound have been documented (*5–9*). However, it is not always apparent that these are disadvantages of other possible candidates. DEA is a better phosphoacceptor than MEG and exhibits considerably less nonenzymatic degradation of *p*-NPP (*8* and Figure 2), but it may also contain impurities that inhibit ALP catalytic activity and it shows differential activation (bias) of the isoenzymes (*9*). Recently, Masson and Holmgren (*20*) reported on the assay of sera from numerous animal species and from humans in AMP and in DEA. Expression of the enzyme activities as a ratio revealed large differences among some of the species during activation by these two phosphoacceptors (*20*). Thus, the nature of the phosphoacceptor will also be important for laboratories doing animal work.

The assessment of a new phosphoacceptor/buffer for clinical ALP assays should include an evaluation of the kinetic parameters associated with both substrate (*p*-NPP) and phosphoacceptor. Further, all reasonable phosphoacceptors should be included in a comparison in which assay variables are examined. The conditions, particularly the phosphoacceptor/buffer, for the assay of ALP catalytic activity in normal sera (containing predominantly hydrophilic liver enzyme) may not be the best conditions with which to assay other fluids or sera (or dilutions of sera) that contain significant amounts of other isoforms of the enzyme. It is also important to understand the role and necessity of added $ZnCl_2$ and $MgCl_2$. The resulting ions may not be necessary if serum is assayed but should be considered for the assay of other biological fluids, dilutions of serum, and animal specimens.

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