Serum Alkaline Phosphatase Isoenzymes in Lymphoproliferative Diseases

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ABSTRACT

A new isoenzyme of alkaline phosphatase (EC 3.1.3.1) has been reported to occur in sera from patients with lymphoproliferative diseases. This enzyme is characterized by an inability to hydrolyze cysteamine S-phosphate. We find that the 5,5′-dithiobis(2-nitrobenzoic acid)-coupled assay method for cysteamine S-phosphate hydrolysis is not suitable for serum, and we were unable to confirm the existence of this isoenzyme in serum by this assay method. We were unable to detect this new isoenzyme by polyacrylamide gel electrophoresis with activity stains or by the reported high sensitivity of this enzyme to inhibition by cysteamine S-phosphate when p-nitrophenyl phosphate is the substrate. We were also unable to confirm reports of a unique inhibitor of normal alkaline phosphatase in the serum of patients with infectious mononucleosis.

INTRODUCTION

There have been reports of the appearance of a new isoenzyme of APase, designated N-phosphatase, in the sera from patients with lymphoproliferative diseases (8, 10, 11). N-Phosphatase is usually the predominant APase in these sera and is characterized by an inability to hydrolyze S–P bonds as in CASP. Normal serum hydrolyzes CASP at about 0.6 of the rate at which it hydrolyzes pNPP. This same ratio is observed with purified calf intestinal APase and the Escherichia coli enzyme. The basic observation reported with sera from patients with lymphoproliferative diseases is that, in all cases and in contrast to normal serum, CASP is hydrolyzed at a rate that is very much less than 0.6 of the rate of hydrolysis of pNPP, when it is hydrolyzed at all. Another defining characteristic of N-phosphatase is that, in its hydrolysis of pNPP, it is 100 to 1000 times more readily inhibited by CASP than is normal APase.

It was also reported that sera from patients with infectious mononucleosis (which contain N-phosphatase) contain an inhibitor for normal APase (9, 11). Serum from patients with Burkitt’s lymphoma was reported not to contain this inhibitor, and the interesting speculation was made that this substance may be a reason for the self-limiting nature of mononucleosis. The basic observation was that, when infectious mononucleosis serum was added to normal serum, the APase activity measured with pNPP was decidedly less than additive.

These observations and especially the conclusions are interesting and important, and we decided to investigate them further, with the goals of purifying and characterizing N-phosphatase and of determining the chemical identity of the inhibitor.

MATERIALS AND METHODS

Varianblue [N-(4-methoxyphenyl)-1,4-benzenediamine sulfate], pNPP (Phosphatase Substrate 104), αNP, DTNB, INT, Tris (reagent grade), and calf intestinal APase (Type 7) were purchased from Sigma Chemical Co., St. Louis, Mo. 2-Amino-2-methyl-1,3-propanediol was purchased from Eastman Organic Chemicals, Rochester, N. Y. Polyacrylamide gradient gels (4 to 30%) were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Serum samples were obtained through Dr. William Robinson, the University of Colorado Medical Center, Denver, Colo., and from Dr. McCammon, the Wardenburg Student Health Center.

For measuring CASP hydrolysis we followed the procedure published by those who had discovered N-phosphatase (10). In this method the cysteamine that is released when CASP is hydrolyzed is allowed to react with the sulfhydryl reagent DTNB to produce 5-thio-2-nitrobenzoic acid, which absorbs light at 412 nm with a molecular absorbance of ε = 13,800 (4). pNPP hydrolysis was monitored by production of p-nitrophenol (3) or by production of inorganic phosphate (7).

CASP was synthesized and analyzed according to the method of Akerfeldt (1). Iodine titration gave a molecular weight of 182 compared to a calculated molecular weight of 179. Thin-layer chromatography in ethanol:pyridine:H₂O (10:5:8) showed a single black spot, indicative of CASP, with a spray of 1% CuCl₂:50% ethanol:5% NH₃.

Polyacrylamide gels were prepared according to the method of L. Fishman (5) and stained for APase activity with αNP and varianblue (5). Gels were stained for CASP-hydrolyzing ability in the same buffer [1 M 2-amino-2-methyl-1,3-propanediol (pH 9.68) with 3.0 mm magnesium and 0.233 mm zinc] with 3 mm CASP and 0.5 mg INT, as a thiol indicator (12), per ml at 37° for 30 min.

Assays were performed in 0.5 M Tris, adjusted to pH 9.0 with HCl, with 1.0 mm pNPP or CASP and 0.1 mm DTNB at 22° for the colorimetric assays, according to the method of Neumann et al. (10). Phosphate production was assayed in 0.1 M Tris (pH 8.8), at 37° with 1 mm added substrate, and compared to pNPP production under the same conditions.

RESULTS

Although the APase activities of normal and abnormal sera are low, we could measure the rate of hydrolysis of pNPP without difficulty (see Table 1). However, the measurement of the enzymic hydrolysis of CASP with DTNB was...
complicated by increases in optical density which arose from other sources, and we were unable to make satisfactory measurements although we tried 23 different normal and abnormal sera. CASP hydrolysis can be easily measured with purified calf intestinal enzyme or E. coli enzyme, and we find that the rate of hydrolysis of this substrate under the specified conditions (see "Materials and Methods") is about 0.7 of the rate of hydrolysis of pNPP. This confirms the 0.6 value given by Neumann et al. (10).

There are several reasons why we could not make reliable measurements of CASP hydrolysis with sera. Corrections that are relatively large compared to the APase activity have to be made for the spontaneous hydrolysis of CASP and of DTNB in alkaline solution. The correction for the reaction of sulfhydryl-containing proteins in the sera with DTNB is very difficult to assign rates to either the control or the serum. This increase arises from the hydrolysis of DTNB, which is not possible to measure the enzymic hydrolysis of CASP in serum unless the APase activity is unusually high. From our experience we conclude that it is not possible to measure the enzymic hydrolysis of CASP in serum unless the APase activity is unusually high.

In the serum we had from a patient with Hodgkin's disease, the APase activity was about 5 times higher than normal. The DTNB reduction due to CASP hydrolysis in these cases was much greater than the reduction due to serum proteins, and we were able to measure CASP hydrolysis. In this sample CASP hydrolysis was about 0.6 of pNPP hydrolysis (Table 1).

The problem of measuring CASP hydrolysis in diluted serum cannot be solved by using more serum because that also increases the concentration of sulfhydryl-containing proteins as well as phosphate and phosphate esters.

We examined the APase isoenzymes by acrylamide gel electrophoresis with gradient gels and the αNP and CASP activity stains, coupled with variamine blue and INT, respectively. We find that CASP hydrolysis by APase can be visualized on polyacrylamide gels but that the method is not as sensitive as that with αNP hydrolysis. With normal sera from 2 people, we found 2 very close prominent bands with αNP and 1 diffuse band at the same position with CASP as substrate. We obtained the same results with sera from 1 patient with acute lymphatic leukemia, 1 patient with chronic lymphatic leukemia, 2 patients with lymphosarcoma, and 1 patient with infectious mononucleosis. We also obtained the same results with sera from 1 patient with chronic myeloid leukemia and 1 patient with Hodgkin's disease.

The gel electrophoresis work, we were looking for the possible appearance of a new band in sera from patients with lymphoproliferative diseases which would show up in the αNP activity staining procedure but not in the CASP activity staining method. Such a band would fit the definition of N-phosphatase. We saw no such band. Possibly, N-phosphatase was not resolved but appeared at the same position as one of the normal isoenzymes. However, if N-phosphatase were the major isoenzyme, as previous reports of 90 to 100% N-phosphatase would indicate (11), we should have observed a much lesser intensity of the CASP activity stain; this did not occur. In all cases we applied equal amounts of APase activity, as determined by pNPP hydrolysis, to the gels. As expected, there were no apparent differences in the αNP activity stains, but there were also no apparent differences in the CASP activity stains. Thus we find no evidence for the existence of a large percentage of N-phosphatase in the sera that we tested from patients with lymphoproliferative diseases. If it were present at all, it was not present as a dominant isoenzyme.

We also examined the effect of CASP as an inhibitor of the hydrolysis of pNPP with sera from a normal person, from 1 patient with chronic lymphatic leukemia, and from 2 patients with lymphosarcoma (Table 2). The percentage of inhibition with 1 mM CASP and 1 mM pNPP was 37, 38, 35, and 28%, respectively. Thus we did not find that the APase activity of sera from patients with lymphoproliferative diseases, as measured with pNPP, is more sensitive to inhibition by CASP.

### Table 1

<table>
<thead>
<tr>
<th>Serum</th>
<th>Diagnosis</th>
<th>pNPP hydrolysis (\text{nmol} \ \text{p-nitrophenol per min per ml serum at } 22° \text{in } 0.5 \text{ M Tris (pH 9.0)} \text{ with } 1 \text{ mM pNPP.})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>6.8</td>
</tr>
<tr>
<td>2</td>
<td>Infectious mononucleosis</td>
<td>3.7</td>
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<tr>
<td>3</td>
<td>Infectious mononucleosis</td>
<td>5.4</td>
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<tr>
<td>4</td>
<td>Chronic lymphatic leukemia</td>
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<td>5</td>
<td>Acute lymphatic leukemia</td>
<td>6.4</td>
</tr>
<tr>
<td>6</td>
<td>Chronic lymphatic leukemia</td>
<td>24</td>
</tr>
<tr>
<td>7</td>
<td>Hodgkin's disease</td>
<td>36 (\text{b})</td>
</tr>
<tr>
<td>8</td>
<td>Normal</td>
<td>6.8</td>
</tr>
<tr>
<td>9</td>
<td>Malignant melanoma</td>
<td>7.1</td>
</tr>
<tr>
<td>10</td>
<td>Acute granulocytic leukemia</td>
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<td>11</td>
<td>Chronic myeloid leukemia</td>
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<td>12</td>
<td>Lymphosarcoma</td>
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<td>13</td>
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</tr>
<tr>
<td>23</td>
<td>Hodgkin's disease</td>
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</tr>
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</table>

\(\text{b} \) \text{CASP hydrolysis was } 21 \text{ nmol } 5\text{-thio-2-nitrobenzoic acid per min per ml serum, as in Footnote a, with 1 mM CASP and 0.1 mM DTNB.}\)
Before testing infectious mononucleosis serum for an inhibitor of normal APase, we tested normal serum as an inhibitor of normal serum. We found that the rate of hydrolysis of pNPP by normal serum is less than additive with increasing amounts of the same serum in the same volume of 1 mM pNPP at 22°. We also found that the activities of 2 different normal sera were not additive. The inhibitory effect of serum can be demonstrated more dramatically with purified calf intestinal APase or the E. coli enzyme with about 10 times the activity contained in the initial addition of serum and a lower concentration of substrate (3.3 x 10^-8 M). The very marked inhibition is shown in Chart 1. Serum that was passed through a Sephadex G-25 column to remove small molecules such as P, esters still produced a large amount of inhibition. Purified human serum albumin yielded comparable inhibition. Inhibition of APase by serum albumin has been previously noted (2).

We examined several sera from patients with infectious mononucleosis for an especially potent inhibitor with normal serum and the 2 purified enzymes, but we did not find any important differences in the degree of inhibition. Thus we find no evidence for the existence of a special or unique inhibitor of normal APase in infectious mononucleosis serum.

The assay of APase in serum is complicated by the presence of phosphate, phosphate esters, and serum albumin. In 6-fold dilution of normal serum with 1 mM pNPP assay medium, we found that more than twice as much phosphate was produced as p-nitrophenol. This discrepancy has been previously reported (3). The extra phosphate arises from the phosphate esters of serum, serving as substrates and thereby necessarily acting as inhibitors of pNPP hydrolysis. These interferences can be substantially eliminated in APase assays by using 10 mM pNPP, as has been previously noted (6).

**DISCUSSION**

Our studies show that it is extremely difficult to measure CASP hydrolysis by serum under the conditions described for the identification of N-phosphatase. We were unable to make satisfactory measurements of CASP hydrolysis in diluted serum, except in a sample from Hodgkin’s disease, which had a high APase activity. In the latter case the interference from sulfhydryl proteins was manageable. The difficulty of measuring CASP hydrolysis in diluted serum raises serious doubts concerning the experimental basis for determining the existence of N-phosphatase. Our gel electrophoresis studies with activity stains indicate that N-phosphatase definitely does not occur as a dominant phosphatase in our samples of sera from patients with the lymphoproliferative diseases acute lymphatic leukemia, chronic lymphatic leukemia, lymphosarcoma, and infectious mononucleosis. We cannot rule out the possibility that it occurs as a minor component although we have no evidence that it does. We have examined only a few samples of sera from patients with lymphoproliferative diseases. Thus, although we can say that N-phosphatase definitely does not occur in all cases of these diseases, we cannot say that it may not occur in some cases.

We were also unable to confirm reports of a unique inhibitor of APase in infectious mononucleosis serum. We find that some substances that are normal components of serum, especially phosphate esters that total about 10 mM and serum albumin (40 mg/ml), inhibit APase under the assay conditions of 1 mM pNPP. We do not observe a larger effect with serum from 3 patients with infectious mononucleosis than we do with normal serum. Thus we conclude that the existence of a special inhibitor in all or any cases of infectious mononucleosis serum is doubtful.

The present study shows that N-phosphatase does not occur as a dominant APase in the sera from all patients with lymphoproliferative diseases and casts doubt upon the existence of this reported isoenzyme. The present study also shows that there is no special inhibitor of APase in the sera from patients with infectious mononucleosis serum tested and casts doubt upon the significance and existence of this reported inhibitor.

**REFERENCES**

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