Alkaline Phosphatase Activities of 6-Thiopurine-sensitive and -resistant Sublines of Sarcoma 180

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ABSTRACT

The alkaline phosphatase activities of 6-thiopurine-sensitive and -resistant sublines of Sarcoma 180 were examined in a further study of these enzymes, which have a role in the resistance of Sarcoma 180/TG to 6-thioguanine and 6-mercaptopurine. Both neoplasms possessed particulate-bound alkaline phosphatase enzymes labeled A and B on the basis of their ease of solubilization from the particulate fraction; more Enzyme A was present in both sublines, with the total activity of Enzyme Fractions A and B being about 100-fold greater in the resistant variant. A storage-labile, sulfhydryl reagent-sensitive factor, presumably a proteolytic enzyme, was demonstrated to be involved in the release of alkaline phosphatase A from the particulate fraction of Sarcoma 180/TG. p-Chloromercuribenzoate, iodoacetamide, phenol, and ethanol were all potent inhibitors of the solubilization of alkaline phosphatase A from the particulate fraction. Both alkaline phosphatases A and B of Sarcoma 180/TG were capable of degrading 6-thioinosine 5'-phosphate to its nucleoside form at optimal and physiological pH values; the rates of hydrolysis of the 6-thioinosine nucleotide were equivalent to those of inosine 5'-monophosphate. In contrast, neither enzyme cleaved nucleoside triphosphates such as adenosine triphosphate under the conditions used. Significant differences exist in the properties of these enzymes. Thus, the optimum reaction pH of Enzymes A and B of Sarcoma 180 were considerably lower than those of the resistant variant. In addition, the thermostability and sensitivity of these enzymes to L-homoarginine inhibition differed. The findings, together with the immunological distinctions reported in the following paper (11), suggest that differences exist between the alkaline phosphatases of Sarcoma 180 and Sarcoma 180/TG.

INTRODUCTION

This laboratory has provided evidence to support the concept that in an experimental model system, a subline of the murine ascitic neoplasm Sarcoma 180 resistant to 6-thiopurines (Sarcoma 180/TG) and in acute lymphocytic leukemic cells of humans, the development of resistance to the 6-thiopurines (i.e., 6-mercaptopurine and 6-thioguanine) is at least partially attributable to an increase in the level of particulate-bound alkaline phosphatase(s) (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) present in these neoplastic cells (22, 24, 29). This catabolic activity is visualized to degrade nucleotides of the 6-thiopurines, which are the active tumor-inhibitory forms of these agents or the direct precursors of the cytotoxic form, to produce nucleosides. The nucleosides may then diffuse from the interior of the cells, a phenomenon that results in insensitivity to these tumor-inhibitory purine analogs (1, 19, 25). Wolpert et al. (29) initially demonstrated that more than 95% of the alkaline phosphatase activity of Sarcoma 180/TG was associated with insoluble particulate matter and that the cell-free sonically extracted particulate matter of this neoplasm possessed considerably more total alkaline phosphatase activity than did the parent sensitive line (Sarcoma 180). More recent work from this laboratory (12) has resulted in the development of methodology for the solubilization and partial purification from the cell-free sonically extracted particulate fraction of the drug-resistant neoplasm of 2 distinct alkaline phosphatase activities (alkaline phosphatases A and B). This paper reports investigations on the factor involved in the solubilization of Enzyme A and the use of the techniques of enzyme isolation to solubilize the alkaline phosphatase activities of the drug-sensitive parent line Sarcoma 180. Furthermore, since the alkaline phosphatase activities of the parent neoplasm have not been studied, a comparison was made of these activities with the corresponding enzymes of the 6-thiopurine-resistant variant.

MATERIALS AND METHODS

Tris, p-nitrophenylphosphate, p-chloromercuribenzoate, chelating agents, and L-phenylalanine were purchased from Sigma Chemical Co., St. Louis, Mo. L-Homoarginine and iodoacetamide were obtained from Pfaltz and Bauer, Inc., Stamford, Conn., and from Calbiochem, San Diego, Calif., respectively. Nucleotides were from P-L Biochemicals, Milwaukee, Wis. 6-Thio-IMP was obtained from P-L Biochemicals as the barium salt and was converted to the sodium form by dissolving the nucleotide with the minimum amount of sodium hydroxide solution, treating this solution with about 0.2 volume of previously washed, air-dried Dowex 50 (Na+) for 30 min, and removing the Dowex resin by centrifugation. Other chemicals were of analytical grade.

Female CD-1 mice, 9 to 12 weeks old, were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. The development and transplantation of the neoplastic cell lines were described previously (28).

Alkaline phosphatase activities were measured spectrophotometrically at 25° according to a previously used procedure (12), except that the reaction was conducted at pH 8. The abbreviations used are: 6-thio-IMP, 6-thioinosine 5'-phosphate; 6-thio-GMP, 6-thioguanosine 5'-phosphate.
p-nitrophenylphosphate as the substrate. The reaction was initiated by addition of an appropriate amount of enzyme to the reaction mixture at 37°, and aliquots of the reaction mixture were removed at 5-min intervals for determination of liberated P. The definition of an activity unit and specific activity for both acid and alkaline phosphatase activities were the same as described earlier (12). Protein was measured by the method of Lowry et al. (14).

The procedures used for the preparation of sonic extracts, solubilization of phosphatase activities and isolation of alkaline phosphatases A and B from both Sarcoma 180 and Sarcoma 180/TG cell lines were essentially the same as those reported earlier (12). Because of the relatively low activities of alkaline phosphatases A and B of Sarcoma 180 cells, crude extracts of these solubilized enzymes were further purified for some studies by fractionation with ethanol as described (12); the specific activities of the 2 enzymes from Sarcoma 180 were both increased about 8-fold by such treatment. The same degree of purification was also achievable for Enzyme A of Sarcoma 180 by storing at 4° for more than 2 months and removal of denatured nonenzymatic protein by centrifugation. The concentrated Enzyme A of Sarcoma 180, partially purified with such a procedure, was used in the experiments throughout this investigation.

Measurement of the degree of inhibition of enzymatic activities by the organ-specific alkaline phosphatase inhibitors, L-phenylalanine and L-homoarginine, was carried out by incubating an appropriate amount of enzyme with various concentrations of inhibitors at room temperature for 15 min. p-Nitrophenylphosphate was then added to initiate the reaction, and activity was determined spectrophotometrically.

RESULTS

The alkaline phosphatase activities obtained by solubilizing sonic extracts of Sarcoma 180 and Sarcoma 180/TG ascites cells are listed in Table 1. Approximately 4.6 times more total alkaline phosphatase A activity than phosphatase B activity was present in Sarcoma 180/TG cells; in a like manner, 3.7-fold more Enzyme A than Enzyme B activity was present in the parent subline, Sarcoma 180. Furthermore, in 4 experiments, including the one shown in Table 1, the average specific activities of alkaline phosphatases A and B were about 130 and 160 times greater, respectively, in the resistant variant than in Sarcoma 180.

In a similar manner in the fraction containing alkaline phosphatase A, an average increase in acid phosphatase activity of 2.1-fold over that occurring in Sarcoma 180 (Table 2) was present in the 6-thiopurine-resistant variant. No significant acid phosphatase activity was detected in the fraction containing alkaline phosphatase B in either cell line.

We reported earlier (12) that some labile factor, possibly a sulfhydryl-dependent enzyme, was involved in the solubilization of Enzyme A from the particulate matter of Sarcoma 180/TG. To gain more information on the properties of this factor, its stability as a function of storage at 4° was measured; the results are shown in Chart 1. Total apparent alkaline phosphatase activity of the particulate matter increased with time of storage at 4° reaching a plateau value of about 190% of the initial total activity by 7 days. Incubation of particulate matter at 37° and at pH 7.6 for 23 hr markedly increased total enzyme activity up to 7 days of storage at 4°. Storage of the sonically treated particulate matter at 4° for up to 7 days did not affect the solubilization of alkaline phosphatase A following incubation at 37°; however, prolonged storage at 4° for 13 days resulted in a pronounced decrease in the capacity of this isoenzyme to be released from the particulate matter.

The effects of several inhibitory agents on the release of Enzyme A from the particulate fraction of Sarcoma 180/TG

Table 1

<table>
<thead>
<tr>
<th>Neoplasm Fraction</th>
<th>Vol-ume (ml)</th>
<th>Total activity (units)</th>
<th>Protein (mg)</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcoma 180/TG</td>
<td>57</td>
<td>409</td>
<td>23336</td>
<td>34</td>
</tr>
<tr>
<td>Enzyme A</td>
<td>46</td>
<td>712</td>
<td>32752</td>
<td>7.8</td>
</tr>
<tr>
<td>Enzyme B</td>
<td>25</td>
<td>285</td>
<td>7120</td>
<td>0.48</td>
</tr>
<tr>
<td>Sarcoma 180</td>
<td>57</td>
<td>7.6</td>
<td>430</td>
<td>36</td>
</tr>
<tr>
<td>Enzyme A</td>
<td>46</td>
<td>7.1</td>
<td>328</td>
<td>5</td>
</tr>
<tr>
<td>Enzyme B</td>
<td>25</td>
<td>3.6</td>
<td>89</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Sarcoma 180/ TG (units/mg)</th>
<th>Specific activity (Sarcoma 180/TG: Sarcoma 180)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.9</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>20.5</td>
<td>2.3</td>
</tr>
</tbody>
</table>
is shown in Table 3. p-Chloromercuribenzoate, iodoacetamide, phenol, and ethanol were potent inhibitors of the solubilization of alkaline phosphatase A, while NaN₃ and CaCl₂ were less potent in this regard. None of these agents significantly decreased total alkaline phosphatase activity of the sonic extract after the incubation process at 37° for 21 hr. The temperature dependence of the release of Enzyme A from the particulate matter of Sarcoma 180/TG is shown in Chart 2. Maximum solubilization of Isoenzyme A occurred at 32-37°.

The substrate specificities of highly purified alkaline phosphatases A and B of Sarcoma 180/TG have been partially examined previously (12); we have expanded this comparison to include IMP and 6-thio-IMP as substrates at acidic, optimum, and physiological pH values. With highly purified enzymes (see Table 4), ATP and CTP were also included as substrates to examine the capabilities of Enzymes A and B of Sarcoma 180/TG to hydrolyze nucleoside triphosphates. The results of these studies, comparing activities with nucleotide substrates to that of p-nitrophenylphosphate as the standard, are shown in Table 4. As previously reported with other nucleotides (12), alkaline phosphatase B had significantly more activity with 5'-nucleoside monophosphates as substrates than it did with p-nitrophenylphosphate at the optimum reaction pH of 9.5; in contrast, Isoenzyme A exhibited a distinct preference for p-nitrophenylphosphate at the optimum reaction pH of 9.5; in contrast, Isoenzyme A exhibited a distinct preference for p-nitrophenylphosphate at the optimum reaction pH of 9.5; in contrast, Isoenzyme A exhibited a distinct preference for p-nitrophenylphosphate at the optimum reaction pH of 9.5; in contrast, Isoenzyme A exhibited a distinct preference for p-nitrophenylphosphate at the optimum reaction pH of 9.5; in contrast, Isoenzyme A exhibited a distinct preference for p-nitrophenylphosphate at the optimum reaction pH of 9.5; in contrast, Isoenzyme A exhibited a distinct preference for p-nitrophenylphosphate at the optimum reaction pH of 9.5; in contrast, Isoenzyme A exhibited a distinct preference for p-nitrophenylphosphate at the optimum reaction pH of 9.5; 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activities of Enzymes A and B of Sarcoma 180 are expressed at pH 8.3 and 8.8, respectively, which are considerably lower than those observed for the comparable enzymes of Sarcoma 180/TG (about pH 9.8 for both Enzymes A and B).

The comparative heat stabilities of these enzymatic activities were determined (Chart 4). The findings indicate that Enzyme A of Sarcoma 180 is the most stable catalyst to heat inactivation under the conditions used. Thus, no decrease in the activity of this enzyme occurred when it was exposed to 56° for 10 min, and the enzyme retained about 60% of its activity when it was exposed to 65° for the same period of time. In contrast, phosphatases A and B of Sarcoma 180/TG lost their entire activities when they were treated at the same temperature for 2.5 min. At 56°, however, about 40 to 50% of the original activities of Enzymes A and B of Sarcoma 180/TG were maintained. Similarly, alkaline phosphatase B of Sarcoma 180 was relatively more heat tolerant than was Enzyme B of the resistant subline Sarcoma 180/TG, maintaining about 28% of its initial activity even after exposure to 65° for 10 min.

Alkaline phosphatase A of Sarcoma 180 was found to have a sensitivity to metal-chelating agents that was similar to that reported earlier for Sarcoma 180/TG (12). L-Phenylalanine had little effect on the isoenzymes of the drug-sensitive and -resistant tumors; however, the organ-specific inhibitor of alkaline phosphatase, L-homoarginine, caused pronounced inhibition of both alkaline phosphatase activities from Sarcoma 180/TG. In contrast, both catalysts from Sarcoma 180, particularly Enzyme B, were relatively insensitive to inhibition by L-homoarginine (Chart 5).

**DISCUSSION**

The 6-thiopurines are useful agents, primarily in combination with other drugs, in the treatment of human acute leukemias. Considerable evidence exists to indicate that for these purine antimetabolites to exert their cytotoxic action they must be converted to the nucleotide level, a process catalyzed by the enzyme hypoxanthine-guanine phosphoribosyltransferase. In both transplanted neoplasms and microorganisms, the most frequent biochemical change that leads to the acquisition of insensitivity to these agents is the deletion or marked decrease in the activity of this enzymatic activity (2). The loss of hypoxanthine-guanine phosphoribosyltransferase activity, however, appears to be an infrequent mechanism in the development of resistance to 6-mercaptopurine and 6-thioguanine by human leukemic cells (3, 22-24, 27). An alternate mechanism, described by this laboratory as being involved in the attainment of resistance of both the murine neoplasm Sarcoma 180/TG and acute lymphocytic human leukemic cells to the 6-thiopurines (1, 12, 22, 24, 26, 29), consists of a constitutive increase in particulate alkaline phosphatase activity, which rapidly catabolizes the active 6-thiopurine nucleotide form to the nucleoside level which is envisioned to be lost through diffusion from the neoplastic cell.

In a previous study (29) we reported that the levels of acid phosphatase activity of Sarcoma 180 and Sarcoma 180/TG were identical, whereas total alkaline phosphatase activity was markedly greater in the particulate fraction of the resistant variant. More than 95% of the alkaline phosphatase activity of Sarcoma 180/TG is particulate bound, and we have found that alkaline phosphatases from both sensitive and resistant lines can be solubilized. Since such solubilization markedly increases the apparent total activity of these enzymes in both sensitive and resistant sublines, it was conceivable that a significant quantity of the enzymatic activity had not been accurately measured in these earlier studies. For this reason we compared in this investigation the levels of activity of alkaline phosphatase isoenzymes A and B following the extraction and separation procedures...
previously developed (12). The results of these studies demonstrated that cells of the resistant variant contain about 100 times the total alkaline phosphatase activity of Sarcoma 180; furthermore, approximately 2-fold more acid phosphatase activity existed in solubilized extracts of Sarcoma 180/TG compared to the 6-thiopurine-sensitive parent line.

Our previous findings (12) also suggested that some factor was involved in the solubilization of alkaline phosphatase A from the particulate fraction of Sarcoma 180/TG cells. The data presented in Table 3 indicate that the sulfhydryl reagents p-chloromercuribenzoate and iodoacetamide at concentrations of $5 \times 10^{-3}$ M almost entirely inhibit the release of Enzyme A. Phenol and ethanol were also found to be significant inhibitors of this process. In addition storage of sonic extracts of Sarcoma 180/TG at 4°C for up to 2 weeks prior to the solubilization procedure resulted in less than 15% of the alkaline phosphatase activity being released into the supernatant fraction; this occurred with no decrease in total alkaline phosphatase activity during the storage period. These phenomena are consistent with the concept that the factor involved in the release of alkaline phosphatase A from the particulate fraction of these neoplastic cells may be a sulfhydryl-dependent proteolytic enzyme that decays during storage at 4°C.

6-Thio-GMP has been shown to be an extremely poor substrate for the enzyme ATP-GMP phosphotransferase (15, 16). This finding provides an explanation for the relatively large quantities of 6-thio-GMP that accumulate in neoplastic cells following exposure to 6-thioguanine (15, 17, 19, 25). In a like manner evidence is available to indicate that 6-thio-IMP is not readily phosphorylated to the di- and triphosphate levels (21). This investigation indicates that these mononucleotide forms of the 6-thiopurines are susceptible to the increased levels of alkaline phosphatases present in resistant neoplastic cells at acidic, optimal, and physiological pH values; studies by this laboratory (1) and by others (19) have supported these enzymatic findings by demonstrating that the half-life of acid-soluble 6-thio-GMP in Sarcoma 180/TG is considerably shorter than that of Sarcoma 180.

Although alkaline phosphatase enzymes from both bacterial (10, 30) and mammalian (9) sources are capable of degrading nucleoside di- and triphosphates, alkaline phosphatase A of Sarcoma 180/TG did not exhibit activity towards ATP and CTP, and phosphatase B from the same neoplasms did not hydrolyze ATP. These findings provide an explanation for why intracellular pools of physiological nucleotides, which exist primarily as triphosphates (20), are protected from the relatively high alkaline phosphatase activities in Sarcoma 180/TG, as suggested by labeling experiments conducted earlier by this laboratory (26). These labeling experiments also suggest that physiological purine mononucleotides such as IMP are not extensively catabolized by the alkaline phosphatases of Sarcoma 180/TG in intact cells. The reason for this apparent lack of catabolism is not clear; however, it is possible that the intracellular distribution of IMP is different from that of the 6-thiopurine nucleotides.

We reported earlier (12) that Enzyme A of Sarcoma 180/TG was a more classical type of alkaline phosphatase in its substrate preferences, whereas phosphatase B, although clearly an alkaline phosphatase, was more active towards 5'-nucleotides than p-nitrophenylphosphate. On the basis of these findings conducted at the optimum enzymatic pH, we suggested that Enzyme B was the prime candidate as the biocatalyst involved in the increased rate of dephosphorolation of the 6-thiopurine nucleotides in Sarcoma 180/TG. Experiments described in Table 4 of this report indicate, however, that both alkaline phosphatases A and B exhibit preference for 5'-nucleotides as substrates at a physiological pH, making it difficult to sustain the earlier conclusion.

Dissimilarities exist in the enzymatic properties of alkaline phosphatases A and B of Sarcoma 180 and Sarcoma 180/TG. As reported earlier (12), alkaline phosphatases A and B of Sarcoma 180/TG differ at least in their substrate specificity, degree of magnesium activation, and electrophoretic mobility on polyacrylamide gels.

Significant differences exist in the susceptibility of the enzymes of Sarcoma 180 and Sarcoma 180/TG to $L$-homoarginine. $L$-Homoarginine is an organ-specific inhibitor of alkaline phosphatase, being active against enzymes isolated from human bone and liver (6, 13) and noninhibitory towards alkaline phosphatases of human intestine and placenta (7). The alkaline phosphatases of Sarcoma 180/TG were sensitive to the inhibitory effects of this agent, whereas the enzymes of Sarcoma 180 were only minimally affected.

Considerable differences in heat stability were also observed between enzymes of Sarcoma 180 and Sarcoma 180/TG. Enzyme A of Sarcoma 180 was considerably more heat tolerant than was either Enzyme A of Sarcoma 180/TG or Enzyme B from both resistant and sensitive cell lines. In this respect Enzyme A of Sarcoma 180 more closely resembles alkaline phosphatases of human placenta (28) and of patients with certain neoplasms (4, 5, 18), whereas Enzyme A of Sarcoma 180/TG is similar to that of human liver alkaline phosphatase (28). On comparison of the heat stability of Enzyme B of Sarcoma 180 to that of Sarcoma 180/TG, phosphatase B of Sarcoma 180 was also more tolerant of heat. However, since the specific activities of the Sarcoma 180 enzymes are significantly lower than those of Sarcoma 180/TG, the difference in thermostability as well as in sensitivity to $L$-homoarginine may be influenced by the presence of nonenzymatic protein.

The pK's for optimum activity of cell-free extracts of Enzymes A and B of both cell lines were also remarkably different, with the alkaline phosphatases of Sarcoma 180 having a considerably lower optimum reaction pH than did the comparable enzymes of Sarcoma 180/TG. The optimum reaction pH of the impure Enzymes A and B of Sarcoma 180/TG was not significantly different from those of the corresponding highly purified enzymes (12). These findings, together with immunological evidence presented in the following paper (11), suggest that alkaline phosphatases A and B of Sarcoma 180 and Sarcoma 180/TG are different enzymes.

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