Kinetic Comparison of Genetically Different Acid Phosphatases of Human Erythrocytes

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SUMMARY

Two acid phosphatases were purified from homozygous, phenotypically different, human red cells. Kinetic properties of the two enzymes differed slightly, but the differences did not appear sufficient to explain the marked difference in total activity (0.65:1.00) found in homozygous human red cells.

Acid phosphatase (orthophosphoric monoester phosphohydrolase) in human red cells can be classified by electrophoresis on starch gel into several phenotypes (1-3). Family studies have shown that inheritance of acid phosphatase types follows that expected of multiple alleles. Of the homozygous enzymes, AA, BB, and CC, the AA type is most negative on electrophoresis, and CC (which is very rare) is most positive (4). Since they are multiple alleles, it is probable that the AA, BB, and CC enzymes differ in only one amino acid, which is acidic in AA, neutral in BB, and basic in CC. By assay, the red cells of individuals homozygous in AA have only 65% of the activity found in BB cells (2). As judged from AC and BC cells, CC cells should have about 50% more enzymatic activity than BB cells.

The purpose of the present study was to determine whether kinetic differences could be found in purified acid phosphatases from AA and BB individuals that would explain the marked difference in total phosphatase activity of cells from these persons.

METHODS

Electrophoresis in Starch Gel

The method devised by Hopkinson, Spencer, and Harris (2) was used with three modifications. (a) After addition of 2 parts of water to the red cells, the whole was frozen in Dry Ice-acetone.

(b) The samples were inserted in the gel as soon as it was cool, but before the gel had set completely. A water-cooled tray (E-C Apparatus Company) was used, and the samples were inserted between 12 and 20 min after the starch was poured.

(c) The gel buffer contained 0.1% 2-mercaptoethanol and 1 mM EDTA. The first modification ensures complete hemolysis and prevents tailing. The second allows the closer contact of gel and filter paper and prevents distortion. In the presence of 2-mercaptoethanol, spurious, strongly negative components are minimized, especially in samples that have been stored.

Determination of Activity

Phenyl Phosphate Method—Enzyme was added to 3 ml of solution at 30° containing 0.033 M sodium acetate (pH 6.0), 0.3 mM EDTA, and 0.22 mM phenyl phosphate, and the change in absorbance at 270 μm was measured by a recording spectrophotometer. At this wave length, phenol has an absorption maximum, while the absorption of phenyl phosphate is much less. A unit of enzyme was that amount producing a 1-unit change in absorbance per min. One such unit was equivalent to 2.97 moles of phenyl phosphate hydrolyzed per min and to 0.044 King-Armstrong unit (5). The extinction coefficients of both phenol and phenyl phosphate vary somewhat with pH. At high substrate concentration, change in absorbance at 285 μm was measured. The absorbance of hemolysates at 270 μm was too high for this method to be usable, but it was satisfactory after the first purification step.

Other Methods—If p-nitrophenyl phosphate (0.33 mM) was substituted for phenyl phosphate, change in absorption at 415 μm was measured. One absorbance unit was equal to 1.55 μmoles per min. The absorbance of both p-nitrophenol and p-nitrophenyl phosphate changes markedly with both pH and temperature.

When phenolphthalein diphosphate was used as a substrate, increase in absorbance at 274 μm was measured. One absorbance unit was equal to 2.00 μmoles per min.

For other substrates and for comparison of substrates, increase of inorganic phosphate was measured on aliquots of the reaction mixture by the method of Fiske and SubbaRow (6). Protein was estimated spectrophotometrically (7).

Purification of Enzymes

Cells from outdated blood from a blood bank were washed three times with an equal volume of buffered NaCl solution (1 volume of 0.1 M sodium phosphate, pH 7.4, plus 9 volumes of 0.9% NaCl). The type of acid phosphatase in each sample was determined by electrophoresis in starch gel, and the samples were pooled according to type and were frozen. One liter of homozygous AA cells and 1 liter of BB cells were then purified in parallel.

The initial step in purification was adsorption on calcium phosphate gel and elution therefrom. The procedure used was exactly that of Tauboi and Hudson (8), except that 0.1% 2-
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mercaptoethanol was added to all solutions used. The eluate was Fraction 2.

To Fraction 2 was added 280 g of (NH₄)₂SO₄ per liter. The precipitate was removed by centrifuging and was discarded. Then 140 g of (NH₄)₂SO₄ per liter of Fraction 2 were added. The precipitate was recovered by centrifuging and was dissolved in 10 ml of 0.1 M Tris-HCl (pH 7.5) containing 1 mM EDTA and 0.1% 2-mercaptoethanol, to give Fraction 3. This fraction was dialyzed for 5 hours at 4°C against 2 liters of a solution containing 0.01 M Tris-HCl (pH 7.5), 1 mM EDTA, and 0.1% 2-mercaptoethanol.

To 30 g of DEAE-cellulose were added 50 ml of 1.0 M Tris-HCl (pH 7.5), 5 ml of 0.2 M EDTA, and 1 ml of 2-mercaptoethanol. Water was added to give a volume of 1 liter, and a column (2.5 X 33 cm) was prepared. A slight vacuum (450-mm pressure of Hg) was applied to the lower end of the column. To the column were added successively 250 ml of 0.01 M Tris-HCl, dialyzed enzyme, 150 ml of 0.01 M Tris-HCl, and 300 ml of 0.01 M Tris-HCl containing 1% (NH₄)₂SO₄. All solutions were at pH 7.5, and all contained 1 mM EDTA and 0.1% 2-mercaptoethanol. Two brown bands formed in the column; the enzyme was eluted immediately following the second band. Fractions of 15 ml each were collected, and phosphatase activity and protein content were determined. Those fractions with specific activity greater than 0.05 were combined to give Fraction 4. The AA and BB enzymes behaved the same on the column; attempts to fractionate enzymes from heterozygous blood of types AB and AC by this method failed.

To Fraction 4 were added 350 g of (NH₄)₂SO₄ per liter; the resulting precipitate was removed by centrifuging and discarded. Then 105 g of (NH₄)₂SO₄ per liter were added and, after centrifuging, the precipitate was dissolved in 1 ml of 0.05 M Tris-

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Approximate volume</th>
<th>AA enzyme</th>
<th>BB enzyme</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td>Yield</td>
<td>Specific activity</td>
</tr>
<tr>
<td>1*</td>
<td>5000</td>
<td>1.2 X 10⁻⁴</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>1000</td>
<td>0.010</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>0.019</td>
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<td>4</td>
<td>60</td>
<td>0.12</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>2.5</td>
<td>0.14</td>
<td>9</td>
</tr>
</tbody>
</table>

* Activity determined by the method of King and Armstrong (5).

### Table II

Relative activity of 1 mM substrates at pH 6.05 and 30°C

<table>
<thead>
<tr>
<th>Substrate</th>
<th>AA</th>
<th>BB</th>
<th>Inorganic phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenyl phosphate</td>
<td>100</td>
<td>100</td>
<td>0.05</td>
</tr>
<tr>
<td>p-Nitrophenyl phosphate</td>
<td>164</td>
<td>150</td>
<td>0.02</td>
</tr>
<tr>
<td>Riboflavin 5′-phosphate</td>
<td>150</td>
<td>155</td>
<td>0.16</td>
</tr>
<tr>
<td>Phenolphthalein diphasate</td>
<td>180</td>
<td>170</td>
<td>0.03</td>
</tr>
<tr>
<td>α-Glycerophosphate</td>
<td>28</td>
<td>28</td>
<td>0.01</td>
</tr>
<tr>
<td>Ribose 5-phosphate</td>
<td>4</td>
<td>4</td>
<td>0.03</td>
</tr>
<tr>
<td>3-Phosphoglycerate</td>
<td>4</td>
<td>4</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

### RESULTS

**Electrophoresis of Purified Enzymes**—The distribution of purified AA and BB enzymes after electrophoresis in starch gel was indistinguishable from that found in AA and BB hemolysates. Each enzyme showed two components with the major fraction farthest from the origin. The position of the major band of the BB enzyme was close to but not identical with the minor band of the AA enzyme.

**Substrate Specificity**—Comparative rates of release of inorganic phosphate from several substrates are shown in Table II. Adenosine 5′-phosphate, β-glycerophosphate, glucose 1-phosphate, and glucose 6-phosphate were tested, but had no detectable (less than 1%) activity.

**Substrate Concentration**—The effect of varying the concentration of phenyl phosphate is shown in Fig. 1. In this figure, the measured rates of the AA enzyme were multiplied by a factor so that the intercepts were identical; the lines were drawn according to the usual equation,

\[
\frac{1}{v} = K_m \frac{1}{V_{max}} + \frac{1}{V_{max}}
\]

where \(v\) was initial rate and \([S]\) was substrate concentration with \(V_{max} = 0.0200\) absorbance units per min and \(K_m = 8.3 \times 10^{-4}\) M for the AA enzyme and \(7.3 \times 10^{-4}\) M for the BB enzyme. As is shown below, both \(K_m\) and \(V_{max}\) thus calculated must be corrected for the presence of inorganic phosphate in the substrate since phosphate is a competitive inhibitor.
When different substrates were compared, the maximum velocity was the same. The values of $K_m$, corrected for inorganic phosphate, are shown in Table III.

**Inhibition by Phosphate**—As shown in Fig. 1, inorganic phosphate is a competitive inhibitor of acid phosphatase. The lines in Fig. 1 were drawn from the usual equation,

$$
\frac{1}{v} = \frac{K_m}{V_{\text{max}} [S]} \left( 1 + \frac{[P_i]}{K_i} \right) + \frac{1}{V_{\text{max}}}
$$

where $[P_i]$ is molar phosphate concentration and $K_i$ was $9.2 \times 10^{-4}$ and $10.1 \times 10^{-4}$ M for the AA and BB enzymes, respectively.

As shown in Table II, all substrates studied contained inorganic phosphate. Inspection of Equation 2 shows that phosphate added as a constant fraction, $F_\text{i}$, of substrate will give a value of

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (M)</th>
<th>$V_{\text{max}}$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenyl phosphate</td>
<td>$8.7 \times 10^{-4}$</td>
<td>$7.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>p-Nitrophenyl phosphate</td>
<td>$1.4 \times 10^{-4}$</td>
<td>$1.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>Phenolphthalein diphosphate</td>
<td>$1.8 \times 10^{-5}$</td>
<td>$1.4 \times 10^{-5}$</td>
</tr>
<tr>
<td>α-Glycerophosphate</td>
<td>$5.8 \times 10^{-3}$</td>
<td>$5.5 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

The optimum pH at high substrate concentration was near pH 6.0, while at low concentration the optimum approached pH 5.0. The AA enzyme was relatively less active at low pH than the BB enzyme.

**Effect of Temperature**—As shown in Fig. 3, the apparent activation energy of $V_{\text{max}}$ was 11,000 cal per mole for both enzymes, and that of $K_m$ was 8,000 cal per mole. Inorganic phosphate inhibited the AA enzyme more than the BB enzyme at higher temperatures, but this difference disappeared below 20°C. As was noted by Tsuboi and Hudson (9), an Arrhenius plot of data obtained with this enzyme does not give a straight line at any finite substrate concentration. This is necessarily the case if both $V_{\text{max}}$ and $K_m$ give straight lines of differing slope in an Arrhenius plot.

**Stability**—Enzyme was diluted 20-fold in 0.1 M Tris-acetate, pH 7.4, and placed in a bath at temperatures between 38°C and 50°C; residual activity was determined at intervals. Under these conditions, the enzyme was stable.
conditions, the first order rates of inactivation were 0.19 min\(^{-1}\) at 45\(^\circ\) and 0.11 min\(^{-1}\) at 38\(^\circ\). The rates of inactivation of AA and BB enzymes were indistinguishable.

**DISCUSSION**

Since \(V_{\text{max}}\) is the same for all substrates of acid phosphatase, it seems reasonable to conclude that all substrates react through a single step that is independent of substrate concentration. The simplest formulation of the reaction is

\[
ROPOH^- + E \cdot H \xrightarrow{K_i} ROH + E'POH^-
\]

\[
E'POH^- + H_2O \xrightarrow{K_2} E \cdot H + HPO_4^-
\]

This scheme, an ester interchange followed by hydrolysis, does not include substrate binding, since such binding does not appear to be obligatory for a nonspecific enzyme. Since two steps are involved, the reaction should follow Michaelis-Menten kinetics formally, but the significance of the derived constants is different from that in the case in which substrate binding is assumed. If initial rates of reaction are considered, \(V_{\text{max}}\) is \(K_2\), the maximal rate of hydrolysis of the enzyme-phosphate ester; \(K_m = K_2/K_1\); and \(K_i\) is \(K_2/K_{-2}\), the equilibrium constant for hydrolysis of the enzyme-phosphate ester.

The AA and BB enzymes differed only slightly in kinetic behavior, and the differences observed were only detectable in parallel experiments. The differences can be summarized as follows.

1. The rate of the BB reaction was less dependent on substrate concentration than was the AA reaction. This was true with all substrates tested and at all pH values.
2. Phosphate inhibited the AA enzyme more than the BB enzyme.
3. The maximum velocity of the AA enzyme was lower relatively at lower pH.

There is certainly no obvious explanation in these small kinetic differences for the difference found in assay of the two enzymes in red cells. The assay was performed at high substrate concentration (0.02 M p-nitrophenyl phosphate at pH 6.0 and 37\(^\circ\) (2)), where differences due to differing \(K_m\) would be minimized. Furthermore, the small difference in \(K_m\) values for the two enzymes is in the wrong sense.

Since the life span of the human erythrocyte is over 100 days, a difference in stability of the two enzymes could conceivably account for the difference in assay. No such difference was found under the test conditions.

The difference in activity of the two enzymes may be due to some property not disclosed by studies of this type, such as a difference in susceptibility of the two enzymes to proteolysis, or there may be, in fact, less of the AA enzyme synthesized by the cell. If less is synthesized, either the rate of synthesis is lower or the control mechanism which stops synthesis does so at a different point. No sure knowledge is available on these possibilities.

An analogous example can be found in the abnormal hemoglobins, where glutamic acid in HbA is replaced by valine to give HbS or by lysine to give HbC. Cells of the heterozygotes, AS and AC, contain about 60% HbA and 40% HbS or HbC. In the case of the hemoglobins, the most negative protein is most abundant, while, with acid phosphatases, the most negative is least prevalent as judged by enzymatic activity.

**REFERENCES**

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