δ-Aminolevulinate Dehydratase in Human Erythroleukemia Cells: An Immunologically Distinct Enzyme

By Chin S. Chang and Shigeru Sassa

Physicochemical and immunologic properties of δ-aminolevulinate (ALA) dehydratase in human K562 erythroleukemia cells were examined. ALA dehydratase activity was found to increase in K562 cells after treatment with butyric acid or selenium oxide. Enzyme activity in untreated K562 cells was comparable to that in normal adult erythrocytes but was increased three- to six-fold in K562 cells treated with 1.2 mmol/L butyric acid or 0.03 mmol/L selenium oxide. The Michaelis-Menten constant (Km), the inhibitor constant (Ki), and elution profile by diethylaminoethyl (DEAE) cellulose chromatography were similar for ALA dehydratase from K562 cells and normal adult and human fetal erythrocytes. However, ALA dehydratase from K562 cells did not react with a monospecific rabbit antibody against ALA dehydratase purified from normal adult erythrocytes, although the antibody reacted with the enzyme from normal adult and fetal red cells. These findings indicate that ALA dehydratase in K562 cells is immunologically distinct from the normal enzyme.

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MATERIALS AND METHODS

Cell Culture and Induction of Erythroid Differentiation

Cell cultures were maintained at 37 °C in an atmosphere of 5% CO2 and 95% air in a humidified incubator. K562 cells were a gift from Dr. L.C. Andersson, University of Helsinki, and were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Cultures were passaged every three to four days by diluting cells to 10^6 cells per milliliter to maintain a constant growth rate. For induction of erythroid differentiation, cells were maintained in the presence of 1.2 mmol/L butyric acid or 0.03 mmol/L SeO2 for five days with an initial density of 10^6 cells per milliliter.

Determination of Heme Content and the Activity of Heme Pathway Enzymes

Heme content was determined fluorometrically as described previously. The activity of ALA dehydratase was determined colorimetrically and that of porphobilinogen (PBG) deaminase by fluorometry both using 5 × 10^6 cells per assay as described previously.

Purification of ALA Dehydratase From Adult Human Erythrocytes

ALA dehydratase was purified from 3 L of outdated human adult blood according to the method of Anderson and Desnick with some modifications. Our modifications included an additional step of calcium phosphate precipitation of the enzyme before the final gel filtration chromatography, which eliminated certain other proteins and gel filtration with Sephadex S-300 (Pharmacia, Piscataway, NJ) instead of Bio-Gel A 1.5 m (Bio-Rad, Richmond, Calif). Other conditions for purification were described previously.
Analytical Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis of ALA dehydratase was performed after denaturation of the enzyme by boiling in the presence of β-mercaptoethanol (ME), using polyacrylamide gel containing 25% ethylene glycol and 0.1% SDS.\(^{14}\)

Preparation of Rabbit IgG Against Purified ALA Dehydratase

Monospecific antisera against human ALA dehydratase were raised in three rabbits using the homogeneously purified human erythrocyte ALA dehydratase as the antigen as described previously.\(^{14,15}\)

Partial Purification of ALA Dehydratase From Human Adult Erythrocytes, Fetal Erythrocytes, and K562 Cells for Immunologic Studies

Human adult or blood (1 to 3 mL) from human fetuses (16 to 20 weeks of gestation) and K562 cells (approximately 10\(^7\) cells) were centrifuged at 2,600 g for five minutes. The cell pellet was washed twice with 0.9% NaCl and then lysed by the addition of 3 vol of 10 mmol/L potassium phosphate buffer (pH 6.8) containing ME/Zn\(^{2+}\). After centrifugation at 36,000 g for ten minutes, supernatants were collected and applied to a small column (1 x 3 cm) packed with diethylaminoethyl (DEAE) cellulose. After hemoglobin was washed off the column, the enzyme was eluted using a 0 to 0.3 mol/L potassium chloride gradient in 10 mmol/L potassium phosphate buffer (pH 6.8) containing ME/Zn\(^{2+}\) and concentrated by ultrafiltration using an Amicon (Danvers, Mass) YM 10 filter membrane.

Immunologic Studies

Rocket immunoelectrophoresis was performed according to the Laurell's method as modified by Grieninger et al., using an antibody containing gel supported on a polyester film sheet. Immune rockets were visualized by fixing the gel in a solution of 0.5% tannic acid and 1% acetic acid.\(^{16}\) Ouchterlony double immunodiffusion analysis was performed using plates obtained from Hyland Diagnostics, Deerfield, Ill. Western blot analysis of ALA dehydratase was performed according to Burnette\(^{17}\) using \(^{125}\)I-protein A as a probe to detect the primary antigen–antibody complex.

RESULTS

Purification of Human ALA Dehydratase and Its Antibody

The enzyme was purified about 4,000-fold with a recovery of approximately 30%. The enzyme activity was stable for at least one year in liquid N\(_2\). The final enzyme preparation appeared to be homogeneous, in that it displayed a single protein band on analytic SDS-PAGE (data not shown). The molecular weight (mol wt) of the subunit of human erythrocyte ALA dehydratase was determined to be approximately 34,000 daltons.\(^{14}\)

Both the antisera and the purified antibody (IgG) were found to precipitate ALA dehydratase, resulting in the disappearance of enzyme activity from the supernatant of the antibody–enzyme mixture. The amount of enzyme activity lost from the supernatant was entirely recovered in the immunoprecipitates, indicating that the active site of the enzyme was not the antigenic determinant and was not interfered with upon binding by the antibody. We found that 100 μL of the antisera precipitated 3.6 mU of ALA dehydratase (equivalent to 0.15 μg of enzyme). The IgG fraction prepared from the antisera had a higher titer, ie, 100 μL of IgG precipitated 14.2 mU of ALA dehydratase.

ALA Dehydratase in K562 Human Erythroleukemia Cells

Besides butyric acid, which has been reported to induce hemoglobin formation in this cell type,\(^{1,2,6}\) SeO\(_2\) was also found to be capable of inducing erythroid differentiation of K562 cells. SeO\(_2\) was examined because it had been reported to induce benzidine-positive cells in mouse Friend erythroleukemia cells.\(^{18}\)

Activity of ALA dehydratase, heme content, and the number of benzidine-positive cells in K562 cells were found to increase after the incubation for five days with butyric acid or SeO\(_2\) (Fig 1). Cells treated with butyric acid at a concentration of 1.2 mmol/L showed a sixfold increase in the activity of ALA dehydratase. The increase in the activity of ALA dehydratase was accompanied by a threefold increase in heme produc-

![Fig 1](https://example.com/image.png)

**Fig 1.** Changes in the activity of ALA dehydratase, the level of heme content, and the percentage of benzidine-positive cells in K562 cells after treatment with butyric acid or SeO\(_2\). K562 cells were incubated with 1.2 mmol/L butyric acid (BA) or 0.03 mmol/L SeO\(_2\) for five days and the activity of ALA dehydratase, heme content, and the percentage of benzidine positive cells were determined as described in Materials and Methods. The results are the mean of duplicate determinations in a representative experiment. Variations observed in a total of five experiments were approximately 50% of the mean, with gradual increases in heme content and ALA dehydratase activity probably reflecting slight increases in differentiated cells during the one-year course of study. The ratio of induced changes over the control, however, remained the same.
tion. Cells treated with SeO₂ (0.03 mmol/L) also showed an increased level in ALA dehydratase activity (threelfold) and heme content (2.5-fold).

**Chromatographic Elution Profiles of ALA Dehydratase in Lysates**

DEAE-cellulose chromatographic elution profiles of ALA dehydratase in normal adult and fetal erythrocytes as well as that in K562 cells were identical. Namely, the enzyme from the three sources was eluted as a single peak from the anion exchange column at a 0.2 mol/L KCl concentration.

**Physicochemical Properties of ALA Dehydratase**

Table 1 summarizes the physicochemical properties of ALA dehydratase from normal adult, fetal erythrocytes, and K562 cells. Equal numbers of adult erythrocytes and K562 cells contained comparable levels of ALA dehydratase activity, while, comparatively, fetal erythrocytes had approximately twice the enzyme activity. Since the protein content of K562 cells (340 μg/10⁶ cells) is considerably greater than that in normal adult red cells (35 μg/10⁶ cells), the K562 enzyme activity is substantially lower than that in normal adult erythrocytes on a protein per milligram basis. The Michaelis-Menten constant (Km) of the enzyme for ALA, determined using a Hanes-Woolf plot, was similar for adult, fetal, and K562 ALA dehydratase (Fig 2). Succinylacetone was found to be a potent competitive inhibitor of ALA dehydratase in all three enzyme preparations, as was the case with the enzyme in other tissues. Namely, the inhibitor constants (Ki's) of the enzyme for succinylacetone were 0.18 μmol/L, 0.11 μmol/L, and 0.18 μmol/L for adult, fetal, and K562 ALA dehydratase, respectively (Table 1).

**Immunologic Studies**

Rocket immunoelectrophoresis of partially purified ALA dehydratase from human adult erythrocytes, fetal erythrocytes, and K562 cells demonstrated that both the adult and fetal erythrocyte enzymes formed rockets with the antibody directed toward adult erythrocyte ALA dehydratase (Fig 3). When increasing amounts of ALA dehydratase activities were added, proportional increases in the heights of rocket peaks were observed for both adult and fetal enzymes. For a given amount of enzyme protein, fetal ALA dehydratase was significantly more active than the adult enzyme (Fig 3). In contrast to ALA dehydratase in normal adult and fetal erythrocytes, the enzyme in K562 cells failed to form a rocket with the rabbit IgG anti-human adult erythrocyte ALA dehydratase, even using samples containing high levels of ALA dehydratase activity (approximately 700 pmol/h) (Fig 3). An extremely wide range of concentration of K562 ALA dehydratase was also examined (50 to 1,600 pmol/h), but no rocket formation was observed (data not shown).

Ouchterlony double-immunodiffusion analysis of ALA dehydratase in human erythroid cells using rabbit IgG anti-human erythrocyte ALA dehydratase (Fig 4) showed a linked arc of identity with ALA dehydratase purified from human adult erythrocytes (as expected), and with ALA dehydratase partially purified from human adult and fetal erythrocytes (Fig 4, wells 1, 2, and 3). In contrast, no precipitin line was

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**Table 1. Physicochemical Properties of ALA Dehydratase From Normal Adult Erythrocytes, Normal Fetal Erythrocytes, and K562 Cells**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>ALA Dehydratase Activity (pmol/10⁶ cells · h)</th>
<th>Km (mmol/L)</th>
<th>Ki (Succinylacetone) (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal adult erythrocytes</td>
<td>75</td>
<td>0.55</td>
<td>0.18</td>
</tr>
<tr>
<td>Normal fetal erythrocytes</td>
<td>130</td>
<td>0.60</td>
<td>0.11</td>
</tr>
<tr>
<td>K562 cells</td>
<td>85</td>
<td>0.59</td>
<td>0.18</td>
</tr>
</tbody>
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ALA dehydratase activity was assayed as described in Materials and Methods. Km and Ki were determined by Hanes-Woolf plot of [S]/V vs [S]. Variations observed for Km and Ki in three experiments were within 10% for adult and fetal erythrocytes. Identical results were obtained for K562 cells in two experiments. ALA dehydratase activity was the mean of three experiments for adult fetal red cells and two experiments for K562 cells, respectively (variations less than 15%).
Fig 3. Rocket immunoelectrophoresis of partially purified ALA dehydratase from K562 cells, normal fetal erythrocytes, and adult erythrocytes. Three hundred microliters of rabbit IgG anti-human erythrocyte ALA dehydratase were used in 6 ml of 1% agarose solution for the preparation of an electrophoretic gel film. The amount of ALA dehydratase applied to each well was as follows: well 1, K562, 0.35 mU; well 2, K562, 0.70 mU; well 3, fetal, 0.10 mU; well 4, fetal, 0.21 mU; well 5, fetal, 0.42 mU; well 6, fetal, 0.83 mU; well 7, fetal, 1.67 mU; well 8, adult, 0.07 mU; well 9, adult, 0.14 mU; well 10, adult, 0.27 mU; well 11, adult, 0.54 mU; well 12, adult, 1.08 mU. One milliunit of the enzyme activity was defined as 1 nmol PBG produced per hour at 37°C. Formed with ALA dehydratase of K562 cells (Fig 4, well 4). These data demonstrate that K562 ALA dehydratase is immunologically distinct from the ALA dehydratase from normal human adult and fetal erythrocytes. Purified mouse liver ALA dehydratase used as a reference control did not form an immunoprecipitin line with anti-human ALA dehydratase IgG (Fig 4, well 5).

Western blot analysis of ALA dehydratase was performed, and the results are shown in Fig 5. Immune complexes from both normal adult and fetal erythrocyte lysates formed a radioactive band with 125I-protein A corresponding to a mol wt of 34,000 daltons identical to that of the purified enzyme, while K562 lysates did not show any detectable radioactive band. These findings are consistent with those observed with rocket immunoelectrophoresis and Ouchterlony double-immunodiffusion analysis, and these findings substantiate our conclusion that K562 ALA dehydratase is immunologically distinct from the enzyme in normal red cells.

DISCUSSION

The results in this study demonstrate an increase of ALA dehydratase activity in human K562 erythroleukemia cells after treatment for five days with butyric acid and SeO₂. Induction of ALA dehydratase was accompanied by increases in the activity of PBG deaminase (data not shown), heme content, and the number of benzidine-positive cells (Fig 1). Our results also confirm an earlier finding on the induction of ALA dehydratase in K562 cells. K562 cells had been demonstrated to exhibit certain embryonic or fetal characteristics such as the synthesis of embryonic and fetal hemoglobin.
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antigens,¹⁰ and a fetal isozyme of lactate dehydrogenase.⁷ Nevertheless, ALA dehydratase in K⁵⁶² cells showed the same elution profile on DEAE-cellulose chromatography as with ALA dehydratase in normal adult or fetal erythrocytes, indicating the identity of their net ionic charges. The plot of the substrate concentration [S]/[the velocity [V] vs [S] of the three enzyme preparations [Fig 2] also showed that they all had similar Kₘ values. The value of Kᵢ using succinylacetone as the inhibitor was comparable with that of the three enzyme preparations. Thus, the physicochemical properties of ALA dehydratases from K⁵⁶² cells, normal human adult erythrocytes, and fetal erythrocytes were closely similar or indistinguishable.

Notwithstanding the kinetic similarities, there were immunologic differences between ALA dehydratase of K⁵⁶² cells and that of normal adult or fetal erythrocytes. A monospecific rabbit antibody directed to ALA dehydratase of normal adult human erythrocytes reacted with both adult and fetal erythrocyte ALA dehydratase; however, it did not react with K⁵⁶² ALA dehydratase in both Ouchterlony double-immunodiffusion and rocket immunoelectrophoresis. Because ALA dehydratase preparation partially purified from K⁵⁶² cells contained significantly more protein than the adult and fetal enzyme preparations, Western blot analysis of ALA dehydratase was performed in order to eliminate possible interference with other proteins in immunologic analysis.¹⁷ The results of Western blot analysis also indicated that adult and fetal ALA dehydratase, but not the K⁵⁶² enzyme, can be immunologically identified using an antibody against normal adult ALA dehydratase. These findings indicate that K⁵⁶² ALA dehydratase is antigenically distinct from that of normal adult or normal fetal erythrocytes. The lack of cross-reactivity between ALA dehydratase of K⁵⁶² cells and that of adult or fetal erythrocytes contrasts with the situation in the murine erythroleukemia model in which an antibody directed to ALA dehydratase from normal mouse liver has been shown to cross-react with the enzyme in Friend cells.³² It is known that essentially all the induced phenotypes in Friend cells are similar or identical to those observed in normal erythroid cells.³³

An additional finding of interest is that, for a given amount of enzyme activity, adult ALA dehydratase formed a larger immune rocket than fetal ALA dehydratase (cf, well 4 vs well 10 in Fig 3), indicating that, for a given amount of the immunotitratable protein, fetal ALA dehydratase is more active than the adult enzyme. Higher specific activity of the fetal enzyme as compared with the adult enzyme has also been reported for ALA dehydratase prepared from mouse liver.²⁴ An understanding of the basis for the increased catalytic activity of the fetal enzyme in the liver and in erythrocytes will probably require complete sequencing of amino acids from both the fetal and the adult enzymes.

Several possibilities can be considered for the distinctive immunologic nature of K⁵⁶² ALA dehydratase. First, different cells or tissues in the same animal may possess isoforms, i.e., different gene products with an apparently same enzymologic property but with distinctive antigenicity. Such examples are seen in the case of fructose 1,6-biphosphatases and lactate dehydrogenases from various tissues. The possibility that the K⁵⁶² ALA dehydratase is a normal tissue variant is not likely, however, because ALA dehydratase in a variety of tissues was shown to be identical and no isoforms were recognized for this enzyme.²⁷

Second, K⁵⁶² ALA dehydratase might be a product of the same gene coding for normal ALA dehydratase, but the enzyme protein may have been altered by a posttranslational modification such as glycosylation. It is known that glycosylation of an enzyme frequently alters the immunologic property but not necessarily the catalytic property of the enzyme.²⁸

Third, ALA dehydratase from K⁵⁶² cells might be an embryonic form of the enzyme, which could be the product of a different gene from the adult gene, and expressing itself only during very early stages of embryonic development. For example, this cell line is known to synthesize embryonic hemoglobins, e.g., Hb Gower I, and Hb Portland, which are normally synthesized only prior to eight-week gestation.²⁹ If ALA dehydratase synthesis is under a genetic control similar to that of hemoglobin formation in K⁵⁶² cells, the enzyme in K⁵⁶² cells in culture might be a distinct enzyme protein from the adult or the fetal enzyme. Embryo-specific expression of heme pathway enzymes is not known; however, it is an interesting problem to pursue. Another possibility may be that the K⁵⁶² enzyme may be an extremely prototypical enzyme even compared with the fetal enzyme. Although such a possibility cannot be excluded, it is rather unlikely, since K⁵⁶² cells express the synthesis of HbF.²⁹

Fourth, since the K⁵⁶² cell line was originally established from cells obtained from the pleural effusion of a patient in the blastic phase of chronic myelocytic leukemia, ALA dehydratase from K⁵⁶² cells might represent an abnormal isozyme secondary to the malignant process. Therefore, the enzyme may be produced by a specific gene activated as the result of transformation of cells, and thus it may be different from the enzyme in normal adult or normal fetal erythrocytes.
In order to distinguish between these possibilities, complete purification of K562 ALA dehydratase is necessary for amino acid analysis as well as amino acid sequence studies. Such studies would require a large number of cells, which was not possible in this study. However, ALA dehydratase in K562 cells is the first example of an aberrant heme pathway enzyme demonstrated in transformed erythroleukemia cells and thus warrants a further study of its full structural characteristics.

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