Induction of δ-Aminolevulinic Acid Dehydratase in Mouse Friend Virus-Transformed Erythroleukemia Cells During Erythroid Differentiation

By Chin S. Chang and Shigeru Sassa

The activity of δ-aminolevulinic acid (ALA) dehydratase, an enzyme involved in heme biosynthesis, has been shown to increase in Friend virus-transformed murine erythroleukemia (MEL) cells during erythroid differentiation. In this study, the nature of the increase in ALA dehydratase activity in MEL cells was examined using a monospecific antibody directed to the enzyme. A sevenfold increase in ALA dehydratase activity was observed after cells had been treated with 1.5% Me2SO for 5 days. Ouchterlony double immunodiffusion analysis showed that lysates from untreated and Me2SO-treated MEL cells formed a single precipitin line with rabbit IgG directed to the normal mouse liver ALA dehydratase. A single arc of identity was also observed with the lysates from normal mouse erythrocytes, spleen, liver, and lysates from both uninduced and induced MEL cells. Rocket immunoelectrophoresis demonstrated that lysates from both uninduced and induced cells formed rockets with the IgG and that the peak height of the rocket was proportional to the ALA dehydratase activity.

Friend virus-transformed murine erythroleukemia (MEL) cells provide a useful model for the study of erythroid differentiation in culture. These cells grow continuously in culture as undifferentiated cells and can be induced to undergo erythroid differentiation by treatment with various chemicals, including dimethylsulfoxide (Me2SO).1,2 Cells undergoing differentiation exhibit a sequential increase in the activities of the enzymes in the heme biosynthetic pathway followed by the formation of hemoglobin.1-3 For example, within 36 hours after treatment, Me2SO-treated MEL cells (clone T3-C1-2) show significant increases in the activity of δ-aminolevulinic acid (ALA) synthase and ALA dehydratase and, within 48 hours, in the activity of porphobilinogen (PBG) deaminase, whereas heme concentration increases only after 96 hours.4 Our previous work suggested that the induction of heme pathway enzymes may be the result of the transcriptional activation of the genes coding for these enzymes, as both actinomycin D and 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue, blocked the enzyme induction.4 In addition, the inhibitory effect of BrdU was overcome by the simultaneous addition of thymidine,4 indicating that the inhibitory effect of BrdU on the induction of heme pathway enzymes is due to its ability to interfere with DNA replication. However, actual increases in the amount of enzyme proteins in the heme pathway have not been demonstrated directly.

The purpose of this study was to determine whether increases in the activity of enzymes in the heme biosynthetic pathway during differentiation were due to the de novo synthesis of the enzyme protein. We examined this question with respect to ALA dehydratase by using a monospecific rabbit antibody to mouse ALA dehydratase. ALA dehydratase was chosen for this study because, among the heme pathway enzymes, this enzyme is present with the greatest activity, its purification has been possible,7-9 and the early increase in its enzymatic activity closely parallels later increases in heme content in differentiated MEL cells.5 We report that both uninduced and induced MEL cells contain ALA dehydratase that is identical to that in normal mouse liver, spleen, and erythrocytes and that increased ALA dehydratase activity in MEL cells after Me2SO treatment represents transcriptional activation, leading to an increased synthesis of the normal ALA dehydratase protein.

MATERIALS AND METHODS

Cell Culture

MEL cells (clone DS-19) were maintained in culture in a modified F-12 medium supplemented with 10% heat-inactivated fetal bovine serum. Cells were grown in suspension culture by diluting the
cells every 3–4 days at 5 x 10^4 cells/mL in fresh medium to maintain a logarithmic growth rate. Cultures for experiments were inoculated at 10^4 cells/mL from cultures growing logarithmically.

**Determination of Cellular Heme Content and Enzyme Activities of the Heme Biosynthetic Pathway**

The concentration of heme in cells was determined fluorometrically as described previously. Benzidine-positive cells were assayed of mouse liver from 100 BALB/c male mice (obtained from Jackson Laboratory). Determination of Cellular Heme Content and Enzyme Activities of the Heme Biosynthetic Pathway was carried out by the method previously described. Purification of ALA Dehydratase From Mouse Liver was performed according to the method of Masters et al., using ammonium sulfate fractionation and DEAE cellulose chromatography.

**Purification of ALA Dehydratase From Mouse Liver**

Purification of ALA dehydratase from mouse liver was performed according to the method of Anderson and Desnick, with some modifications. All purification procedures were carried out at 4°C using buffers containing 7 mmol/L β-mercaptoethanol and 0.1 mmol/L ZnCl_2, unless otherwise stated. One hundred eighty grams of mouse liver from 100 BALB/c male mice (obtained from Jackson Laboratory, Bar Harbor, Me) were homogenized in ~3 vol of 10 mmol/L potassium phosphate buffer (pH 6.8). The homogenate was centrifuged at 26,000 g for 20 minutes to remove particulate matter. The supernatant was removed and was subjected to ammonium sulfate fractionation. Precipitates between 30% and 60% ammonium sulfate saturation were collected by centrifugation at 5,000 g for 20 minutes and dissolved in a small volume of the same buffer. The solution was then heated at 70°C for 5 minutes. After centrifugation at 10,000 g for 10 minutes, the supernatant was applied onto a column of DEAE cellulose (2.6 x 40 cm) and eluted using a linear KCl gradient between 0 and 0.5 mol/L. Fractions containing ALA dehydratase activity were collected and loaded onto a column of octyl-Sepharose (2.6 x 20 cm) equilibrated with 20 mmol/L phosphate buffer (pH 6.8) containing 0.25 mol/L KCl. ALA dehydratase activity was recovered in the void volume and was then loaded onto a phenyl-Sepharose column (2.6 x 40 cm) equilibrated with 20 mmol/L phosphate buffer (pH 6.0), and the enzyme was eluted with a linear gradient of 0%–80% ethylene glycol. Fractions containing enzyme activity were loaded onto a hydroxyapatite column (2.2 x 12 cm) equilibrated with 20 mmol/L phosphate buffer (pH 6.0), and enzyme was eluted with a linear gradient of potassium phosphate buffer between 20 mmol/L (pH 6.0) and 300 mmol/L (pH 7.5). ALA dehydratase was collected and precipitated with ammonium sulfate (60% saturation), and the precipitate was dissolved in 5 ml of 50 mmol/L potassium phosphate buffer (pH 6.8) containing 0.25 mol/L KCl. After dialysis against 100 vol of the same buffer for 2 hours, the enzyme solution was loaded onto a Sepharose S-300 column (2.5 x 100 cm) equilibrated with the same buffer. The enzyme eluted from the S-300 column was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for the determination of the molecular weight of the enzyme subunit. SDS-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn. Molecular weight standards used included: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

**Preparation of Rabbit IgG Directed to Purified Mouse Liver ALA Dehydratase**

Three New Zealand rabbits were injected intradermally and intramuscularly with 1 mg of the purified ALA dehydratase mixed with an equal volume of Freund’s complete adjuvant. This treatment was repeated once a week for three weeks. Booster injections of 0.5 mg of enzyme were administered on the fifth and seventh week. Rabbits were bled from an ear vein weekly to collect 25–50 mL of blood per bleeding. Bloods were obtained each week until the tenth week. Animals were then killed, and all blood was collected by heart puncture. IgG fractions against mouse ALA dehydratase was prepared according to the method of Masters et al., using ammonium sulfate fractionation and DEAE cellulose chromatography.

**Titration of Immune Titers of Rabbit Antisera and IgG Directed to Mouse Liver ALA Dehydratase**

Homogeneously purified mouse liver ALA dehydratase was diluted in 0.1 mol/L phosphate buffer (pH 7.4) containing 100 μg/mL of bovine serum albumin. ALA dehydratase (0.2 μg/mL) was mixed with 10 μL of purified IgG in serial dilution. Mixtures were stored at 4°C overnight and centrifuged at 700 g for 5 minutes. ALA dehydratase activity remaining in the supernatant after incubation was determined by the method of Sassa, using 5 μL of supernatant.

**Rocket Immunoelectrophoresis**

Rocket immunoelectrophoresis was performed according to the method of Laurell, as described by Grienginger et al. Electrophoresis was performed with an antibody-containing agarose gel supported on a film sheet, 70 x 100 mm, cut from a roll of polyester film. Six milliliters of a 1% agarose solution in electrophoresis buffer (sodium barbital 44 mmol/L, pH 8.6), maintained at 54°C, was mixed with 50 μL of rabbit IgG to mouse ALA dehydratase. This mixture was then cast over the film sheet, 12 wells of 2.4-mm diameter were punched at 4-mm intervals in the middle of the gel. Serially diluted lysates of both untreated and MeSO-treated MEL cells, which were prepared by freeze-thawing cell pellets three times in liquid nitrogen followed by centrifugation at 26,000 g for 20 minutes, were applied into each well. Electrophoresis was carried out at 10 V/cm for 90 minutes, after which the gel was placed directly in a solution of 0.5% tannic acid–1% acetic acid for visualization of immunoprecipitates.

**RESULTS**

**Purification of ALA Dehydratase From Mouse Liver**

ALA dehydratase from mouse liver was purified approximately 1,200-fold with a 50% yield (Table 1). The precipitate from 30%–60% saturation with ammonium sulfate contained more than 90% of the original enzyme activity. Enzyme activity was stable for at least 2 weeks at 4°C in the presence of ammonium sulfate or for longer time periods in liquid nitrogen. Heat inactivation at 70°C for 5 minutes removed most of the contaminating proteins and resulted in a tenfold purification. ALA dehydratase did not bind to octyl-Sepharose, but this step removed other proteins more hydrophobic than the enzyme itself. In contrast, the enzyme was avidly bound to the less hydrophobic phenyl-Sepharose, and this step provided an additional 14-fold purification. The final gel filtration step yielded another 1.5 purification. The enzyme solution was concentrated by ultrafiltration (Amicon YM-10 membrane) and stored in liquid nitrogen.
Table 1. Purification of δ-Aminolevulinic Acid Dehydratase From Mouse Liver

<table>
<thead>
<tr>
<th>Step</th>
<th>Vol (mL)</th>
<th>Total Activity (U)</th>
<th>Specific Activity (U/mg Protein)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>520</td>
<td>220</td>
<td>0.02</td>
<td>100</td>
<td>1</td>
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<tr>
<td>Ammonium sulfate</td>
<td>190</td>
<td>201</td>
<td>0.03</td>
<td>91.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>130</td>
<td>146</td>
<td>0.25</td>
<td>66.4</td>
<td>12.5</td>
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<tr>
<td>Octyl-Sepharose</td>
<td>96</td>
<td>132</td>
<td>0.34</td>
<td>60.0</td>
<td>17</td>
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<tr>
<td>Phenyl-Sepharose</td>
<td>131</td>
<td>116</td>
<td>4.92</td>
<td>52.7</td>
<td>246</td>
</tr>
<tr>
<td>Hydroxypatite</td>
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<td>101</td>
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<td>45.9</td>
<td>788</td>
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<tr>
<td>Sephacryl S 300</td>
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<td>86</td>
<td>23.8</td>
<td>39.1</td>
<td>1,164</td>
</tr>
</tbody>
</table>

The results represent values for purification of the enzyme from 100 BALB/cJ mouse livers. The purification procedures are described in Materials and Methods.

After gel filtration chromatography, the enzyme appeared to be homogeneous, as indicated by the presence of a single protein band on analytical SDS-PAGE, having a molecular weight of 36,000 (Fig 1). This value is in good agreement with the value reported by others for this enzyme from DBA/2J mouse liver and for the enzyme from other sources.

Characterization of Rabbit IgG Anti-Mouse Liver ALA Dehydratase

Both rabbit antisera and the purified IgG fraction precipitated the mouse liver ALA dehydratase (Fig 2). IgG directed toward mouse liver ALA dehydratase precipitated 28.7 μg of the purified enzyme per 100 μL of IgG (Fig 2). The enzyme activity lost from the supernatant of the antigen–antibody mixture after incubation was entirely recovered in the precipitate.

ALA Dehydratase Activity in Untreated and Me2SO-Treated MEL Cells

The basal level of ALA dehydratase activity was low but detectable in untreated MEL cells. The level of the enzyme remained unchanged in untreated cells in culture for many passages. In contrast to the untreated cells, cells treated with Me2SO showed a significant increase in ALA dehydratase activity. A maximal increase (sevenfold) in the enzyme activity was observed in cells treated with 1.5% Me2SO for 5 days. The level of enzyme induction was found to be proportional to the concentration of Me2SO used up to 2.0% (Fig 3).

Cross-reactivity of Rabbit IgG Anti-Mouse Liver ALA Dehydratase With ALA Dehydratase in MEL Cells

Ouchterlony double immunodiffusion analysis showed that lysates from untreated and Me2SO-treated MEL cells formed precipitin lines with IgG directed to the normal mouse liver ALA dehydratase. A single arc of identity was formed at the intersections of all the enzyme preparations examined, i.e., ALA dehydratase from liver, spleen, and erythrocytes from normal mice (BALB/c), as well as lysates from untreated and Me2SO-treated MEL cells (Fig 4).

Immunologic Quantitation of ALA Dehydratase in MEL Cells

The rabbit IgG directed to mouse liver ALA dehydratase was used to quantitate ALA dehydratase in...
INDUCTION OF ALA DEHYDRATASE

Fig 2. Immunotitration of mouse ALA dehydratase by rabbit antiserum and IgG. Immunotitration was carried out as described in Materials and Methods. Based on these findings, 100 μL of antiserum (C) and IgG (D) were calculated to precipitate 86 mU (or 3.6 μg) and 688 mU (or 28.7 μg) of ALA dehydratase, respectively. The enzyme activity lost from the supernatant of the enzyme-antibody mixture was entirely recovered in the precipitate.

MEL cells undergoing differentiation. Figure 5 shows the result of rocket immunoelectrophoresis of lysates from uninduced and Me2SO-induced MEL cells. Both lysates formed rockets with the IgG directed to the liver enzyme. The peak height of the rocket was proportional to the amount of ALA dehydratase activity from each source. The slope of linear plots of rocket peak heights vs ALA dehydratase activity was identical for both lysates from uninduced and Me2SO-induced cells (Fig 6).

Succinylacetone Inhibition of ALA Dehydratase in Me2SO-treated MEL Cells

Succinylacetone is a potent inhibitor of ALA dehydratase in human erythrocytes,9 chick embryo liver,9,16 rat liver,13 and Friend cells.18 In this study, we examined the question of whether succinylacetone also interferes with the synthesis of the enzyme. When added with Me2SO (1.5%) to MEL cell cultures for 5 days, succinylacetone decreased ALA dehydratase activity to 6% of the level of Me2SO controls at 10^{-7} mol/L. This level of enzyme activity was even lower than in untreated cells. At higher concentrations of succinylacetone, ie, from 10^{-6} to 10^{-3} mol/L, the activity of ALA dehydratase was almost completely abolished. However, rocket immunoelectrophoresis of cell lysates from cultures treated with succinylacetone and Me2SO demonstrated that there were similar amounts of ALA dehydratase protein in all cultures treated with various concentrations of succinylacetone (Fig 7). When both the enzyme activity and the concentration of the enzyme were plotted as a function of succinylacetone concentration, it became clear that the observed loss of ALA dehydratase activity after the chemical treatment was strictly due to the inhibition of enzyme activity and not to decreased synthesis of the enzyme (Fig 8).
Fig 6. Relationships between the enzyme activity and the peak height of immune rockets of ALA dehydratase in MEL cells. Lysates used were from untreated (○) and Me2SO-treated cultures (●) (1% for 5 days). Enzyme assay and rocket immunoelectrophoresis was performed as described in Materials and Methods.

BrdU Inhibition of ALA Dehydratase Induction in Me2SO-treated Cells and the Reversal of the Inhibition by Thymidine

We reported earlier that Me2SO induction of ALA dehydratase activity can be completely prevented by the simultaneous treatment of MEL cells with BrdU, a thymidine analogue. The BrdU inhibition was reversed by the treatment with thymidine (TdR), indicating that intact DNA synthesis is necessary for the increase of ALA dehydratase activity. When cell lysates were examined for the presence of immunoreactive ALA dehydratase protein, it was found that cells treated with Me2SO and BrdU had 36 ng ALA dehydratase/10⁶ cells, compared with 133 ng enzyme protein/10⁶ cells in Me2SO-treated control cells (Fig 9). The addition of TdR to the BrdU-inhibited culture restored the level of ALA dehydratase to 93 ng protein/10⁶ cells. These changes in the immunoreactive ALA dehydratase protein were proportional to the enzyme activity found in these cells. Namely, ALA dehydratase activity in these cells was 3,290, 890, and 2,290 pmol PBG formed/10⁶ cells, for Me2SO, Me2SO + BrdU, and Me2SO + BrdU + TdR treatment, respectively.

DISCUSSION

The results of the present study demonstrate that the levels of ALA dehydratase activity, as well as the enzyme protein, are increased in murine Friend erythroleukemia cells after induction of erythroid differentiation. Because ALA dehydratase activity does not show appreciable decay in MEL cells after actinomycin D treatment, the observed increase in ALA dehydratase activity after Me2SO treatment cannot be due to a decreased enzyme degradation. These findings have thus provided for the first time direct evidence for de novo synthesis of a heme biosynthetic pathway enzyme in erythroid precursor cells.

Quantitation of ALA dehydratase was accomplished by rocket immunoelectrophoresis using a monoclonal antibody raised in the rabbit by immunization with ALA dehydratase purified from the liver of BALB/c mice. The purified enzyme was homogenous, as indicated by the presence of a single protein band with a molecular weight of 36,000 in SDS-PAGE, which is comparable to that reported previously for
Fig 9. ALA dehydratase protein in MEL cells treated with Me2SO, Me2SO + BrdU, and Me2SO + BrdU + TdR. Cells were incubated with chemicals for 5 days. ALA dehydratase protein was quantitated by rocket immunoelectrophoresis, as described in Materials and Methods. Lysates used were prepared from (A) 2 x 10⁶ cells treated with 1.5% Me3SO; (B) 2 x 10⁶ cells treated with 1.5% Me2SO + 10⁻⁴ mol/L BrdU; and (C) 2 x 10⁶ cells treated with 1.5% Me2SO + 10⁻⁴ mol/L BrdU + 7 x 10⁻⁴ mol/L TdR.

this enzyme purified from DBA/2 mouse liver. The antibody raised in the rabbit was shown to cross-react with mouse liver ALA dehydratase, as expected, and also with the enzyme in normal spleen and erythrocytes (Fig 4). In Ouchterlony double immunodiffusion analysis, the antibody produced a single immunoprecipitin line not only with the purified enzyme, but also with the enzyme in the crude extract from erythrocytes, spleen, liver, and MEL cells. The fact that there was a single arc of identity with precipitin lines from different tissue preparations, including that of MEL cells, indicates that our antibody preparation recognized strictly ALA dehydratase and not other proteins in the crude enzyme preparations.

The antibody directed to mouse liver ALA dehydratase was also shown to cross-react with the enzyme in both untreated and Me2SO-induced MEL cells. These data demonstrate that ALA dehydratase in both uninduced and induced cells is identical and that increased enzyme activity in induced cells is due to an increase in the same enzyme protein that was present in uninduced cells. The increase in the enzyme activity also closely paralleled the increase in the enzyme protein (Fig 6).

Similar quantities of ALA dehydratase protein were demonstrated in Me2SO-treated cells after succinylacetone treatment as compared with the Me2SO-induced controls. Succinylacetone, a structural analogue of ALA, is a potent inhibitor of ALA dehydratase activity, having an approximately 1,000-fold greater affinity for the catalytic site of the enzyme than does the natural substrate, ALA. Despite the almost complete inhibition of ALA dehydratase activity (~99%), succinylacetone-treated cells did not show appreciable changes in PBG deaminase activity and heme content (data not shown) up to a succinylacetone concentration of 10⁻¹ mol/L. These findings indicate that these cells retained a sufficient level of heme biosynthetic capacity, until the activity of ALA dehydratase reached an extremely low level (< 2%). These data also suggest that succinylacetone is an extremely specific inhibitor of ALA dehydratase and that it may be useful in the study of the role of ALA dehydratase in cellular differentiation in other systems as well.

BrdU has been shown to inhibit the Me2SO-mediated increases in globin mRNA, heme, hemoglobin, and activities of ALA dehydratase and PBG deaminase. The BrdU inhibition is probably due to the result of the chemical’s inhibition of TdR incorporation into DNA, as the inhibition by BrdU can be reversed by treatment with excess TdR, but not with uridine. In our present study, we demonstrated that Me2SO induction of ALA dehydratase activity, as well as the immunologically reactive enzyme protein, were both inhibited to a comparable degree by BrdU and that the BrdU-mediated inhibition was partially overcome by TdR treatment. These findings indicate that the increases in ALA dehydratase in MEL cells undergoing erythroid differentiation are due to de novo synthesis of the enzyme protein. Our data also suggest that the induction of ALA dehydratase in these cells by Me2SO is dependent on intact DNA synthesis and probably involves transcriptional activation of the structural gene coding for ALA dehydratase. To our knowledge, this is the first demonstration of de novo synthesis of a heme pathway enzyme protein during cell differentiation. Availability of the rabbit antibody to ALA dehydratase would make it possible to examine the mode of synthesis of ALA dehydratase in a cell-free system and to quantitate the amount of mRNA in MEL cells undergoing erythroid differentiation.

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Induction of delta-aminolevulinic acid dehydratase in mouse Friend virus-transformed erythroleukemia cells during erythroid differentiation

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