Control of Oxygen Affinity of Hemoglobin in K562 Cells Induced by Hemin

By Yongji Wu, Ann Dean, William Egan, and Alan N. Schechter

The oxygen affinity of hemoglobin in K562 cells induced by hemin and the relationship between levels of 2,3-diphosphoglycerate (2,3-DPG) and hemoglobin have been investigated. Absorption spectra of induced cells indicate that the hemoglobin is oxygenated; oxygen dissociation curves are symmetric, with a P50 of 20 ± 0.9 mm Hg, Hill coefficient of 2.5, and a normal temperature dependence. The intracellular pH measured by phosphorus 31 nuclear magnetic resonance (NMR) was 7.3. The amount of 2,3-DPG was determined by an enzymatic method and by 31P NMR. The level of 2,3-DPG in uninduced K562 cells, containing 0.5 pg of hemoglobin per cell, was low (5 ± 0.5 μmole/10⁸ cells), but increased to 64 ± 5 μmole/10⁸ cells upon induction of hemoglobin accumulation (to a final level of 20 pg hemoglobin/cell). For several experiments, there was a closely coordinated relationship between 2,3-DPG and hemoglobin levels, at about 1:1 stoichiometry of the two molecules. The time course of induction of hemoglobin, and of 2,3-DPG levels, are very similar; both processes are reversible. These data suggest that induction of hemoglobin synthesis in K562 cells by hemin results in hemoglobin-containing cells with normal oxygenation properties and that 2,3-DPG and hemoglobin levels are coordinately controlled in these cells. Elucidation of the mechanism of this effect should be of importance in understanding the erythroid-like differentiation of these cells.

The K562 HUMAN LEUKEMIA cell line originated from a patient with chronic myelogenous leukemia in terminal blast crisis, and possesses the capability to synthesize fetal and embryonic hemoglobins, but not adult hemoglobins. High levels of hemoglobin (20–26 pg/cell), of which about 9% is HbF, 33% Gower I (α2γ2), and 58% Portland (α2γ2), can be achieved under certain culture conditions, for example, 20 μM hemin in glucose-deficient medium. Furthermore, the process of hemoglobin accumulation is reversible. Thus, this cell line provides a useful tool to study coordinate control of the expression of functionally related gene systems during induction of hemoglobin synthesis and may also be useful for studying the physiologic properties of human embryonic hemoglobin, about which relatively little is known.

The present studies primarily concern aspects of the factors that control oxygen affinity in induced K562 cells. We have measured oxygen equilibria of intact K562 cells, the effect on the oxygen affinity, the intracellular pH, as well as 2,3-diphosphoglycerate (2,3-DPG) levels in these cells. We have particularly studied the relationship between 2,3-DPG and hemoglobin levels in these cells during the induction of hemoglobin synthesis. We found that induced K562 cells had an oxygen affinity very similar to that of human fetal red cells and that accumulation of hemoglobin was accompanied by coordinate increase of 2,3-DPG levels in these cells.

MATERIALS AND METHODS

Cell Line and Culture Conditions

K562 cells were cultured in suspension in RPMI 1640 medium containing 2 mM glutamine, 10 mM HEPES, 10% fetal calf serum, penicillin, and streptomycin in a humidified incubator containing 5% CO₂ in air at 37°C. For the induction of hemoglobin synthesis, 20 μM hemin (GIBCO, Grand Island, NY) was added to K562 cells at a density of 5 × 10⁵ cells/ml in medium containing no glutamine. The cells were grown for 5 days to a density of 0.3 × 10⁶ cells/ml and harvested by centrifugation. Viable cells were counted with a hemocytometer using the trypan blue exclusion test.

Hemoglobin Level

A quantity of 1.5 × 10⁶ cells was collected, washed 3 times with 0.15 M NaCl, and lysed in 1 ml 0.15 M NaCl/0.02 M Tris (pH 7.4)/0.1% NP-40 (Shell, Tulsa, OK), and the hemoglobin in the lysates was measured spectroscopically from absorption bands at 576, 540, and 414 nm for oxyhemoglobin, and at 630 nm for methemoglobin (limit of detection was about 5% methemoglobin).

Methemoglobin Reductase

Aliquots of 2 × 10⁶ induced and uninduced K562 cells and human mature red cells were collected, washed 3 times with phosphate-buffered saline (PBS, without magnesium and calcium, Biofluids, Rockville, MD), pH 7.4, and resuspended in 1 ml distilled water. Levels of the enzyme activity in these cells were determined by using the method reported previously, except that sonication was employed for making lysates of K562 cells.

Oxygen Dissociation Curves

Induced K562 cells (0.2 × 10⁹) were pelleted and resuspended in 0.08 ml of PBS, pH 7.4. The oxygen dissociation curves of the whole induced K562 cells were determined using a Hem-O-Scan (Hemo-Scan, Travenol Laboratories, Savage, MD) at 37°C with a P50 of 40 mm Hg, according to the method recommended by the manufacturer. The influence of temperature on the oxygen dissociation curves was measured with the same equipment adjusted to required temperature.

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Size of K562 Cells

Diameters of K562 cells were measured with a ZB1 Coulter Counter integrated with a Coulter Channelizer (Coulter Electronics Inc., Hialeah, FL), according to the procedure recommended by the manufacturer. Microspheres with known diameters were used as standard references.

Magnesium Concentration

Aliquots of 5 × 10⁷ induced and uninduced K562 cells were collected, washed 3 times with PBS, and packed by centrifugation. The cell pellet was resuspended with PBS at a ratio of 0.45 volume of cells and 0.55 volume of liquid, similar to the ratio in whole blood. The cell suspensions were frozen, thawed, and sonicated. The lysates, kept on ice, were sent to Galbraith Laboratories, Inc. (Knoxville, TN) for measurement of magnesium concentration.

2,3-DPG and Sugar Phosphate Levels

Induced and uninduced K562 cells (3 × 10⁷) were collected, washed 3 times with ice-cold 0.15 M NaCl, deproteinized with 1.0 ml of ice-cold 0.6 M perchloric acid, and then neutralized with 0.10 ml of ice-cold 2.5 M potassium carbonate. 2,3-DPG in the neutralized supernatant was measured spectrophotometrically by disappearance of NADH upon enzymatic conversion of 2,3-DPG to glyceraldehyde-3-phosphate using commercial 2,3-DPG test kits (Boehringer, Mannheim, W. Germany).

³¹P NMR spectra of K562 cells and fresh human blood [anticoagulated with standard acid-citrate-dextrose (ACD, National Institutes of Health formula A)] were obtained with a Bruker WM-300 spectrometer. A quantity of 4 × 10⁷ induced or uninduced K562 cells was collected, washed twice with 0.15 M NaCl, and resuspended in fresh human plasma to a density of 2 × 10⁸ cells/ml. Some studies were performed on cellular extracts from 10⁶ induced and uninduced K562 cells, which were deproteinized by addition of 4.0 ml of ice-cold 0.6 M perchloric acid, and the clarified supernatants were neutralized by 0.40 ml of ice-cold 2.5 M potassium carbonate. The extracts were lyophilized and dissolved in 2.0 ml of a 0.1 M Tris buffer at pH 7.6. The Tris buffer contained approximately 20% D₂O as an internal lock signal. Two milliliters of each sample was analyzed in a 10-mm diameter standard NMR tube (Wilmad, Buena, NJ).

Intracellular pH

Intracellular pH of induced and uninduced K562 cells in fresh human plasma and human mature red cells was measured by means of ³¹P NMR spectroscopy (Bruker WM-300 spectrometer operating at 121 MHz). Intracellular pH was deduced from the chemical shifts of intracellular adenosine triphosphate (ATP).

RESULTS

Oxygen Affinity

Absorption spectra of induced K562 cells (not shown) indicated that virtually all of the hemoglobin was in the oxygenated form and no methemoglobin was detected. The oxygen dissociation curve of induced K562 cells containing 20 pg hemoglobin/cell is shown in comparison to that of human mature red cells (Fig. 1A). The curves were reproducible, reversible, and symmetric. The P₅₀ of the induced K562 cells was 20 mm Hg (20 ± 0.9 mm Hg), and the P₅₀ of adult whole blood was 26 mm Hg. The Hill coefficients of the induced K562 cells and the adult whole blood, calculated using the Hill equation, were 2.5 and 2.7, respectively.

The influence of temperature on the P₅₀ value of the induced K562 cells was very similar to its effect on the P₅₀ of mature red cells and on hemoglobin A₇₅, i.e., a decrease of temperature from 30°C to 20°C decreased the P₅₀ value about twofold, from 14 mm Hg to 7.5 mm Hg (Fig. 1B).

Level of Methemoglobin Reductase Activity

The level of the enzyme activity was taken to be the rate of dye (2,6-dichlorobenzenoneindophenol) reduction expressed as absorbance change per minute × 10⁴. The induced K562 cells had a mean rate of 45, with a standard deviation (SD) of 10, and the unin-
duced K562 cells had a mean rate of 43 with a SD of 8; the red cells had a mean rate of 38 ± 8. Thus, there was no significant statistical difference in the level of the enzyme activity between induced and uninduced K562 cells.

**Cell Size and Magnesium Concentration**

There was heterogeneity in the size of both uninduced and induced K562 cells. The average diameter of uninduced K562 cells was 14.5 μ, with a range from 10.2 μ to 17.9 μ, and the average diameter of induced K562 cells was 14.1 μ, with a range from 9.9 μ to 17.9 μ. Assuming that K562 cells are spherical, the average volume of both induced and uninduced K562 cells would be 1,460–1,600 cu μ, i.e., at least 16 times larger than that of mature human red cells.

Magnesium concentration in uninduced and induced K562 cells was 36 μg/ml and 37 μg/ml, respectively, within the range for mature human red cells (34–56 μg/ml).12

**Relationship Between 2,3-DPG and Hemoglobin Levels**

Levels of 2,3-DPG in K562 cells and mature red cells, determined by the enzymatic method, are shown in Table 1. Uninduced K562 cells, containing 0.5 pg hemoglobin/cell, contained 5 n mole of 2,3-DPG per 10⁸ cells, similar to the level found in uninduced Friend leukemia cells;11 however, after induction of hemoglobin synthesis by hemin, the hemoglobin level increased to 20 pg/cell, and the level of 2,3-DPG concomitantly increased to 64 n mole/10⁸ cells.

The time course of 2,3-DPG increase was very similar to that of hemoglobin accumulation over a 5-day period. The cells had a doubling time of about 24 hr and an almost linear growth over 5 days. For several experiments, there was a linear relationship between 2,3-DPG and hemoglobin levels at about 1:1 stoichiometry of the two molecules. Cells removed from hemin-containing medium and resuspended in fresh medium showed diminishing levels of both hemoglobin and DPG, which, after 5 days, approached the control level (Fig. 2).

<table>
<thead>
<tr>
<th>Table 1. 2,3-DPG Levels in K562 Cells</th>
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<td><strong>Cell Type</strong></td>
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<tr>
<td>Uninduced K562 cells</td>
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<tr>
<td>Induced K562 cells</td>
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<td>Culture day 1</td>
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<td>Culture day 2</td>
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<td>Culture day 4</td>
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<td>Culture day 5</td>
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<td>Normal human red cells</td>
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**31P NMR Spectra of K562 Cells and Cellular Extracts**

31P NMR spectra of suspensions of induced and uninduced K562 cells are shown in Fig. 3; for comparison, the spectrum of normal red blood cells is provided. The spectrum from the red blood cells is relatively simple, consisting primarily of the two resonances from 2,3-DPG and the γ, α, and β resonances from ATP; since whole blood was used, plasma phospholipid signals are also seen. The most readily identifiable peaks on spectra of the K562 cells are the γ, α, and β resonances from ATP, and they are also the most prominent resonances. The chemical shift difference between the α and β, as well as the β and γ, resonances of ATP in induced and uninduced K562 cells was the same as in red blood cells, indicating a similar intracellular pH. The Mg²⁺ concentration, which is known to affect the titration properties of ATP, was the same in the K562 cells as in red blood cells (see above). The intracellular pH of human red blood cells, measured by 31P NMR, has been reported to be 7.29 ± 0.0814 and 7.20 ± 0.034;15 therefore, we expect the K562 intracellular pH to be in this range.

A relatively broad resonance, or set of resonances, was observed in the K562 cells in the region of the spectrum where 2,3-DPG absorbs energy; however, this is also the region of the spectrum where sugar phosphates, in general, absorb energy. In order to determine whether the signals at 4 ppm (Fig. 3) derive primarily from 2,3-DPG, a perchloric acid extract of induced K562 cells was examined; this is shown in Fig. 4. Several peaks appeared in the region of 3.5–5.25 ppm. By means of addition of authentic materials, six different sugar phosphates, including fructose-6-phos-
phosphate, fructose-1-phosphate, fructose-1,6-phosphate, glyceraldehyde-3-phosphate, glucose-6-phosphate, and 2,3-DPG, were identified. Because a part of one of the 2,3-DPG peaks overlapped with the resonance from fructose-6-phosphate, it was not possible to measure 2,3-DPG quantitatively.

Although the 31P spectrum of the extract of uninduced K562 cells (Fig. 4) was similar to that of the induced cells, the sugar phosphate levels, including 2,3-DPG level, were considerably lower. This observation is in accord with whole cell spectra (Fig. 3), in which sugar phosphate levels in the uninduced cells are lower than those in induced cells.

**DISCUSSION**

During the last few years, the human leukemia K562 cell line has been used in many laboratories as a model system for the study of human erythroid differentiation and globin gene expression. Therefore, it is important to know the physiologic properties of hemoglobin synthesized in these cells and the relationship between hemoglobin and some factors related to hemoglobin function, such as 2,3-DPG, during the induction of hemoglobin synthesis.

The ability of hemoglobin to load and unload oxygen is usually determined by measuring the equilibrium between hemoglobin and oxygen, i.e., oxygen dissociation curve. The induced K562 cells measured under physiologic conditions displayed sigmoid, symmetric oxygen dissociation curves similar to those of human fetal and mature red cells. The P50 value of induced K562 cells, 20 ± 0.9 mm Hg, is nearly the same as that of human embryonic red cells (18–20 mm Hg), a little higher than that of human fetal red cells (17.5–18 mm Hg), but much lower than that of mature red cells (26 ± 1.0 mm Hg). The effect of temperature on the oxygen affinity of induced K562 cells is almost the same as on those of human mature red cells and hemoglobin A. The intracellular pH in induced and uninduced K562 cells, measured by 31P NMR, is similar to the intracellular pH in human mature red cells. The value of the Hill coefficient of the cells, 2.5, is very close to that of human fetal and adult hemoglobin (2.7–3.1), and indicates that there is a normal heme–heme interaction of the embryonic and fetal hemoglobins within K562 cells. No methemoglobin was detected in induced K562 cells, probably due to the fact that, like human red cells and most nonerythroid cells, K562 cells also contain a normal level of methemoglobin reductase activity (identical to cytochrome b5 reductase). Induction by hemin does not result in any change in the enzyme activity. These data suggest that the induction of hemoglobin synthesis in K562 cells leads to the appearance of cells with normal oxygenation properties.
HEMOGLOBIN OXYGEN AFFINITY IN K562 CELLS

Fig. 4. (A) $^{31}$P NMR spectrum (121.5 MHz) of the extract of uninduced K562 cells. Spectral conditions were as in Fig. 3 except that 65,000 transients were averaged, and a 0.5 Hz line-broadening was applied. The chemical shift scale is in ppm and is relative to inorganic phosphate, the value of which was set to 2.8 ppm (see ref. 16). (B) $^{31}$P NMR spectrum (121.5 MHz) of an extract of induced K562 cells. Spectral conditions were as above, except that 175,000 transients were collected. For purposes of presentation, the $\beta$-phosphorus resonance ($\delta = -19.6$; broadened triplet) is not shown. The inset region ($-5.25$ - $3.5$ ppm) displays the sugar phosphate resonances. The $^{31}$P resonances, identified by the addition of authentic materials, are indicated as follows: 1. fructose-6-phosphate; 2. fructose-1-phosphate; 3. fructose-1,6-diphosphate (a. C-1 phosphate and b. C-6 phosphate); 4. glyceraldehyde-3-phosphate; 5. glucose-6-phosphate; 6. 2,3-DPG (the "b" peak overlaps with the resonance from fructose-6-phosphate).

The function of human embryonic hemoglobin is relatively unknown because of the difficulty in obtaining enough suitable material for study. Induced K562 cells contain about 90% of total hemoglobin as embryonic hemoglobins and have an oxygen affinity much higher than that of mature red cells, but close to that of fetal red cells. This result is consistent with previously reported data on embryonic hemoglobins.19,25 The physiologic significance of a low oxygen affinity of embryonic hemoglobin may be related to the fact that the oxygen consumption per weight is higher in the early embryonic stage compared to the late fetal period.26

2,3-DPG is a potent modifier of hemoglobin function in mammalian red cells. Because induced K562 cells have normal oxygenation properties, it is important to determine the levels of 2,3-DPG in these cells. We found that the levels of 2,3-DPG concomitantly increase with the accumulation of hemoglobin at about 1:1 stoichiometry of the two molecules during the induction of hemoglobin synthesis by hemin. A similar phenomenon has recently been found in Friend leukemia cells27 and also in development of rabbit erythroid cells.28

$^{31}$P NMR spectra of induced and uninduced K562 cells and cellular extracts showed that upon the induction of hemoglobin synthesis, sugar phosphate metabolism in induced K562 cells became much more active than that in uninduced K562 cells. Besides 2,3-DPG, other sugar phosphates such as fructose-1,6-phosphate, fructose-1-phosphate, fructose-6-phosphate, glyceraldehyde-3-phosphate, as well as glucose-6-phosphate, also increased in induced K562 cells. Induction of K562 cells by hemin does not lead to terminal differentiation, and induced K562 cells retain nuclei and other cytoplasmic organelles. Therefore, the
characteristics of metabolism in induced K562 cells are likely to differ from, and be more complicated than, metabolism in mature human red cells.

The coordinate increase in hemoglobin and 2,3-DPG levels suggests the possibility of either coordinate expression of genes controlling 2,3-DPG synthetase and hemoglobin synthesis or modulation of specific enzyme activities in these cells. We are presently examining the enzymatic and genetic basis of this effect. The human leukemia K562 cell line should be of value in studying the molecular basis of coordinate control of the synthesis or expression of macromolecules during erythroid cell differentiation.

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REFERENCES

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