Expression of NADH-cytochrome b_5 reductase during dimethyl sulfoxide-induced differentiation of Friend erythroleukemia cells

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1. INTRODUCTION

It has been shown that the enzymatic reduction of methemoglobin in mature circulating red-cells is promoted by a soluble form of NADH-cytochrome b_5 reductase (EC 1.6.2.2) [1]. Subsequent studies have demonstrated that this enzyme is related to the ubiquitous membrane-bound cytochrome b_5 reductase [2–4], and that both entities are encoded by a single gene (DIA_1) located on chromosome 22 [5,6]. In most nucleated cells ~95% of the cyto chrome b_5 reductase is bound to membranes (endoplasmic reticulum, mitochondria, nuclear and plasma membrane) [7,8]. In contrast, in the mature red cell ~60% of the cytochrome b_5 reductase activity is soluble [9]. Accumulating evidence indicates that the soluble cytochrome b_5 reductase arises from the membrane-bound enzyme by proteolytic cleavage of the hydrophobic domain of the protein [10,11]. Here, we have investigated the timing of this post-translational process during the course of erythroid differentiation. The model chosen was the Friend murine erythroleukemic cell (MELC), in which we studied the activity and distribution of the membrane-bound and soluble cytochrome b_5 reductase before and after induction by dimethyl sulfoxide (DMSO).

2. MATERIALS AND METHODS

2.1. *Cell culture and induction conditions*

Friend erythroleukemic cells (clone 707 17C, kindly donated by Dr Adrian J. Minty, Pasteur Institute, Paris) were grown in standard conditions [12]. When required for induction, dimethyl sulfoxide was added to 1.5% (v/v). Uninduced cultures in exponential phase were harvested at 7×10^5 cells/ml. A DMSO-resistant variant (clone TR 25D), non-inducible for globin mRNA hemoglobin and spectrin [13], was grown in the same conditions. Cells were harvested by centrifugation at 600 × *g* for 10 min. Pellets were washed 2 times with 0.9% NaCl and immediately treated as followed.

2.2. *Preparation of cell extracts*

2.2.1. Total extracts

The cell pellet (10^8 cells) was homogenized with a Teflon Potter homogenizer in 0.9 ml 0.25 M saccharose in a 1 mM Tris–HCl buffer (pH 7.0), 1 mM EDTA, 1 mM phenyl methyl sulfonyl fluoride. An aliquot (0.05 ml) was suspended in 0.05 ml 4% (w/v) Triton X-100 in water, vigorously stirred in a Vortex apparatus and frozen and thawed 3 times. The 30 000 × *g* supernate was used for enzyme assays.

2.2.2. Subcellular fractionation

The homogenate was centrifuged at 800 × *g* for 10 min to obtain the nuclear pellet, then at 12 000 × *g* for 15 min (mitochondrial and lysosomal pellets) and at 105 000 × *g* for 10 min (Airfuge Bechmann) to obtain the microsomal pellet and the cytosolic supernatant. The 800 × *g*, 12 000 × *g*, 105 000 × *g* pellets were suspended in 0.3 ml 4% Triton X-100, frozen and thawed 3 times, centrifuged at 30 000 × *g* and the supernates were used for enzyme assays.

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Table 1

Cytochrome b_5 reductase activity in MELC (clone 707 17C) and in erythroid and non-erythroid mouse cells

	Specific activity (nmol . min^{-1} . mg protein^{-1})	Total activity (nmol . min^{-1} . 10^8 cells^{-1})
Untreated MELC	27.2 ± 3.1 (4)	50.7
DMSO-treated MELC (4 days)	55.9 ± 11.3 (4)	161.0
Reticulocytes of DBA/2 mice	17.5	n.d.
Erythrocytes of DBA/2 mice	15.0	n.d.

n.d. = not determined

All extracts were made in the presence of 2% Triton X-100, as described in text, enabling to measure total cytochrome b_5 reductase activity (soluble and membrane-bound)

2.3. *Reticulocytes and erythrocytes of DBA/2 mice preparation*

DBA/2 mice were obtained from the Centre d'Elevage de la Source (Orléans). A reticulocyte enriched-fraction was separated on a Percoll gradient [14] after leukocyte elimination on a cellulose column. To estimate the soluble and membranous fractions of reticulocytes and erythrocytes, enzymatic assays were performed without or with 2% final (w/v) Triton X-100.

2.4. *Assay for NADH-cytochrome* b_5 *reductase activity*

Activity was assayed with the ferrocyanide–methemoglobin complex as a substrate as in [15]. The results were expressed as μmol or nmol substrate reduced/mg protein or /10^8 cells.

2.5. *Hemoglobin determination*

Hemoglobin accumulation was determined quantitatively by measuring the extinction of cytoplasmic lysates at 540 nm (11.5 E^{540}_{mM}). The extinction of untreated cells at 540 nm have been subtracted.

3. RESULTS

3.1. *Total NADH-cytochrome* b_5 *reductase in non-induced and DMSO-treated MELC*

When MELC (clone 707 17C) are exposed to 1.5% DMSO over 4 days, they exhibit an ~ 2-fold increase of the specific activity of cytochrome b_5 reductase (table 1). When expressed per 10^8 cells the increase of activity was 3-fold. The enzymatic activity of uninduced MELC was comparable (although not identical) to that found in reticulocytes and erythrocytes from DBA/2 mice. The activities of other enzymes such as glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were not modified by DMSO treatment.

3.2. *Time course of induction of total cytochrome* b_5 *reductase activity in DMSO-treated MELC*

The enzyme activity was studied in MELC (clone 707 17C) harvested at 2, 4 and 6 days in culture media containing 1.5% (v/v) DMSO and in control cultures without DMSO. The medium was not changed during the 6 day period. A difference in enzyme specific activity of Friend cells (+ 21%) was detected after 2 days of DMSO treatment, when the cells were still actively proliferating (fig.1). This increase became more pronounced (+ 76%) after 4 days, when the culture entered stationary phase. After 6 days, when the majority of the cell population was synthesizing hemoglobin, the total cytochrome b_5 reductase specific activity was increased ~ 2-fold in DMSO-treated cells. The kinetics of this increase follows the kinetics of hemoglobin production and shows a good correlation between the two processes. The enzyme activities were also measured in a DMSO-resistant variant (clone TR 25D) non-inducible for hemoglobin and spectrin

[13]. After 2, 4 and 6 days of DMSO treatment of these cells the increase of cytochrome b_5 reductase specific activity was 12%, 18% and 24%, respectively.

3.3. *Subcellular distribution of cytochrome* b_5 *reductase activity in DMSO-treated MELC*

The 12 000 × *g* pellet exhibited marked increase of the specific activity after 2, 4 and 6 days of DMSO treatment of MELC (clone 707 17C) (fig.2). The 800 × *g* and 105 000 × *g* membranous fractions also displayed a rise in enzymatic specific activities. In contrast, only a minor increase of the soluble cytosolic enzyme was noted; this soluble

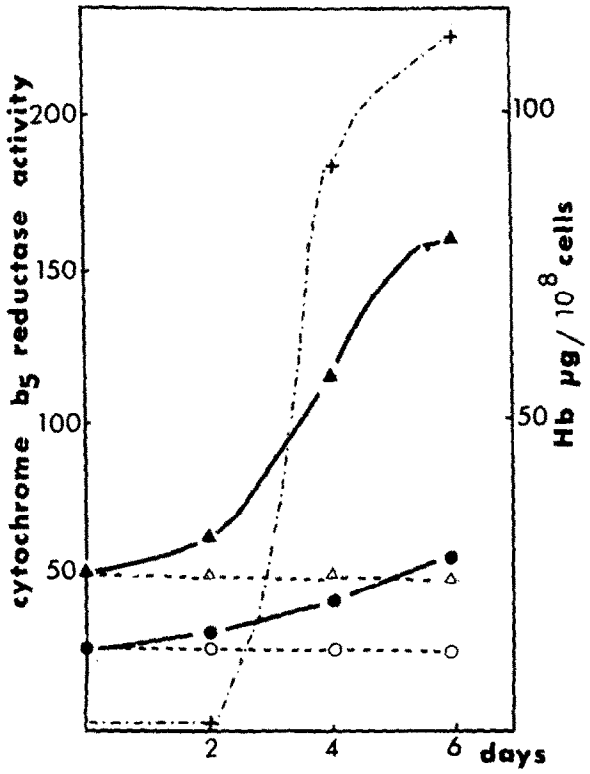

Fig.1. Cytochrome b_5 reductase activity during DMSO-treatment of MELC. Friend cells (clone 707 17C) were grown in presence of 1.5% DMSO (v/v) and pelleted after 2, 4 and 6 days culture: (▲) total activity (nmol . min^{-1} . 10^8 cells^{-1}) after induction by DMSO; (△) total activity (nmol . min^{-1} . 10^8 cells^{-1}) without DMSO; (●) specific activity (nmol . min^{-1} . mg protein^{-1}) after DMSO induction; (○) specific activity (nmol . min^{-1} . mg protein^{-1}) without DMSO; (+) hemoglobin induction.

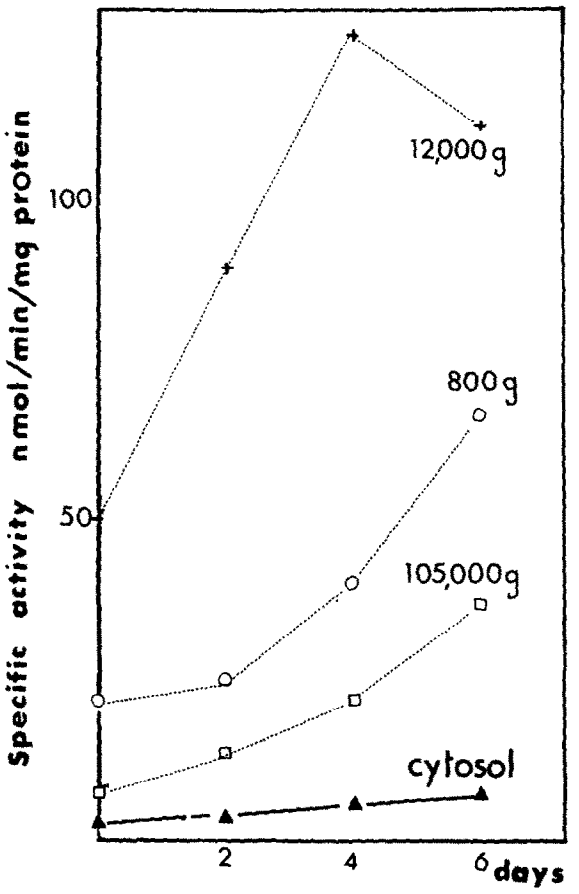

Fig.2. Subcellular distribution of cytochrome b_5 reductase in MELC during DMSO-treatment. MELC (clone 707 17C) were homogenized and fractionated as in section 2: (○) 800 × *g* pellet; (+) 12 000 × *g* pellet; (□) 105 000 × *g* pellet; (▲) cytosolic fraction.

fraction corresponds to a very small fraction of the total activity (only ~ 6% of the total activity/10^8 cells). To study the changes occurring during later stages of erythroid maturation, reticulocytes and erythrocytes of DBA/2 mice were prepared and enzyme activities measured as in section 2. The soluble and membranous forms of the enzyme in reticulocytes were found to be 43% and 57% of the enzymatic activity, respectively. In erythrocytes, the soluble enzyme corresponds to 69% and the membrane-bound enzyme to 31%. Thus, in reticulocytes and in adult erythrocytes, the proportion of soluble enzyme activity was much higher than in uninduced and DMSO-treated MELC.

4. DISCUSSION

MELC are a favorable material to investigate the relationship between the ubiquitous membrane-bound cytochrome b_5 reductase and its soluble counterpart which is characteristically elevated in circulating mature erythrocytes. Indeed, DMSO-treated MELC are known to progress from non-specialized erythroid cells analogous to pro-erythroblasts to specialized cells resembling orthochromatic erythroblasts. They express some of the properties of differentiating erythroid cells, such as synthesis of adult-type hemoglobin and some erythroid proteins [16–20]. Membrane-bound redox proteins, particularly cytochrome b_5, were shown in the T_3L_{1-2} MELC [21].

Our results provide evidence that MELC (clone 707 17C) exhibit a cytochrome b_5 reductase activity, which is increased ~ 3-fold (on a cellular basis) after erythroid differentiation in the presence of DMSO. This induction concerns exclusively the membrane-bound enzyme. The level of induction is comparable to that of other enzymes [16–20], while spectrin, hemoglobin and enzymes of the heme synthetic pathway are induced to a higher level. The timing of induction is similar to that of hemoglobin, therefore corresponding to a late program [19]. In [21], no increase of the membrane-bound cytochrome b_5 was shown during the induction of another Friend cell line, clone T_3L_{1-2}. In non-induced MELC, we found that the membrane-bound cytochrome b_5 reductase activity was present in nuclear, mitochondrial and microsomal fractions, whereas very little activity was detected in the cytosol. Induced MELC exhibited the same distribution, which indicates that the induction concerns only the membrane-bound enzyme. Moreover, during the induction, there is no release of the soluble form. This result agrees with [21], where no increase of soluble cytochrome b_5 in induced MELC was found. We conclude that in immature erythroid cells, cytochrome b_5 reductase is essentially bound to membranes. DMSO-stimulated differentiation induces the membrane-bound enzyme activity and does not result in the 'solubilization' of cytochrome b_5 reductase. We found that the induced MELC contain hemoglobin in the reduced state (not shown), while the soluble methemoglobin reduction system (soluble cytochrome b_5 and its reductase) is not yet present. This suggests that the membrane-bound system is functional with regard to the reduction of methemoglobin. In an acellular system, we also observed that methemoglobin is reduced by the endogenous membrane-bound enzyme and cytochrome b_5 of MELC (not shown). Thus, the two membrane-bound proteins may constitute an active reducing system of methemoglobin in MELC.

One of the limits of MELC model is that these cells rarely proceed in vitro to the non-nucleated stage of differentiation characteristic of normal erythropoiesis. Therefore, to determine the exact stage of solubilization of the membrane-bound cytochrome b_5 reductase, the last step of erythroid maturation was studied. Reticulocytes and erythrocytes were prepared from DBA/2 mice, from which Friend cells originated. Our results show that the respective values of the soluble enzyme were 6% of the total activity in Friend cells, 43% in reticulocytes and 69% in erythrocytes.

We conclude that cytochrome b_5 reductase is synthesized as a membrane-bound form during the proliferative phase of erythroid differentiation and that the soluble form is released by proteolysis occurring at a late stage of differentiation when most of the subcellular organelles of the late orthochromatophilic erythroblasts are degraded. Proteolytic activities have been reported in red cell [11,22] and reticulocyte [22] membranes. The nature of the protease(s) involved in this process remains to be elucidated.

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