Enzymes of oxygen metabolism during erythrocyte differentiation

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During differentiation of rabbit bone-marrow erythroblasts, the activities of superoxide dismutase and catalase increase in parallel with haemoglobin, suggesting a balance between the degradation and formation of superoxide by these systems. On the other hand, the decline in glutathione peroxidase and reductase activities as erythroid cells differentiate indicates that these enzymes are of greater importance in immature erythroblasts and may play only a minor role in relation to the removal of hydrogen peroxide in mature cells.

The superoxide anion radical (O$_2^-$) is produced by all aerobic cells as a normal metabolic intermediate of molecular oxygen utilization during oxidative processes. This radical and the product of its dismutation, H$_2$O$_2$, are precursors of the other active forms of oxygen (most probably OH$^-$ or O$_2^+$) which damage biological membranes. Thus, enzymes utilizing O$_2^-$ and H$_2$O$_2$ protect the cell against the toxic action of oxygen (Fridovich, 1974, 1975; Michelson et al., 1977).

Erythrocytes are very suitable for investigating a possible correlation between the production of O$_2^-$ and the levels of enzymes utilizing O$_2^-$ and H$_2$O$_2$. In the mature erythrocyte, haemoglobin is the main producer of O$_2^-$, and about 2-3% of circulating haemoglobin is oxidized per day (Winterbourn et al., 1976). Therefore we may expect some correlation between the haemoglobin content and the levels of enzymes of oxygen metabolism.

In comparative investigations of erythrocytes from various birds and mammals, whose haemoglobin content ranged from 6 to 22 g/100 ml of blood, it was established that the values of superoxide dismutase per g haemoglobin varied less than 2-fold, whereas catalase and glutathione peroxidase activities showed 150- and 57-fold differences respectively (Maral et al., 1977). Since these differences could be due to species peculiarities, we decided to investigate the levels of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase during erythrocyte differentiation in one and the same species.
Methods

Treatment of animals

Rabbits were made anaemic by five daily injections of 0.3 ml of neutralized 2.5% (w/v) phenylhydrazine/kg body weight (Arnstein et al., 1964). The animals were killed on the fifth day after the last injection by bleeding from the heart.

Cell separation

Bone marrow was scraped from the femur and humerus, and the cells were disaggregated in ice-cold phosphate-buffered saline (0.145 M NaCl in 0.02 M sodium phosphate buffer, pH 7.4; approx. 10 vol.) by gentle pipetting. The suspension was filtered through a double layer of muslin, and the cells were collected by centrifugation at 500 g for 10 min and washed twice in the same medium. Bone-marrow erythroid cells were fractionated by velocity sedimentation in a Ficol-bovine-serum-albumin gradient in a chamber designed by Denton and Arnstein (1973). As mammalian erythroid cells differentiate, they become smaller, and the change in size is sufficient to allow efficient fractionation (Clissold et al., 1977). Disaggregated marrow at a density of 3 x 10^6 cells/ml was allowed to sediment for 4-5 h, 30 fractions were collected, and appropriate consecutive fractions were pooled to give the following cell fractions: proerythroblasts, basophilic cells, polychromatic cells, early orthochromatic cells, and late orthochromatic cells. Details of the separation achieved have been reported previously (Denton & Arnstein, 1973; Clissold et al., 1977). Cell numbers were determined with a Coulter counter.

Blood erythrocytes from normal rabbits were separated from the plasma by centrifugation at 1000 g for 10 min, and the buffy coat of white cells at the top was removed by aspiration. The cells were washed twice with phosphate-buffered saline.

Bone-marrow cells and circulating erythrocytes were lysed in 10 mM Tris-HCl buffer, pH 7.4, and the membrane fraction was sedimented by centrifugation at 12 000 g for 30 min and resuspended in 1 ml of the same buffer.

Protein content

Protein concentrations in the lysates and sediments were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Enzyme assays

Superoxide dismutase (SOD) (EC 1.15.1.1). SOD activity was assayed in lysates by measuring the reduction of Nitroblue-Tetrazolium (NBT) and the increase of optical density at 560 nm according to the method of Beauchamp and Fridovich (1971). A unit of SOD activity is the amount of enzyme giving 50% inhibition of NBT reduction.

Catalase activity (EC 1.11.1.6). Catalase activity was determined in lysates by measuring the decrease of optical density at 240 nm during enzymic decomposition of H_2O_2 (Aebi, 1970). Enzyme activity was expressed as velocity constant k per mg of protein.
Glutathione peroxidase (EC 1.11.1.9). Glutathione peroxidase activity was measured in sediments of cell fractions with H$_2$O$_2$ as a substrate (Günzcer et al., 1972). The enzyme activity was expressed as nmol oxidized NADPH/min/mg protein.

Glutathione reductase (EC 1.6.4.2). Glutathione reductase activity was assayed in lysates of cell fractions by the method of Pinto and Bartley (1969). The enzyme activity was expressed as nmol oxidized NADPH/min/mg protein.

Results and Discussion

The data in Table 1 show an increase of the catalase and SOD activities expressed per mg of haemoglobin-free soluble protein. Allowing for the increase in haemoglobin as a percentage of total cell proteins during bone-marrow erythroid-cell differentiation (Denton et al., 1975; Arnstein, 1976), the results of catalase activity agree with the earlier data of Denton et al. (1975). It seems that the activities of SOD and catalase increase in parallel with the level of haemoglobin, which is a primary producer of O$_2^-$ in erythrocytes. Because haemoglobin synthesis increases sharply in non-dividing cells, the high

Table 1. Enzyme activities of separated developing erythroblasts

The activities of superoxide dismutase (units/mg protein), catalase (k/mg protein), and glutathione reductase (nmol oxidized NADPH/min/mg protein) were assayed in 12 000-g supernatant after hypo-osmotic lysis of the cells. The values were recalculated per mg haemoglobin-free soluble protein, using the data of Denton et al. (1975) and Arnstein (1976) for haemoglobin content (as % of the total soluble protein in the cells). The activity of glutathione peroxidase (nmol oxidized NADPH/min/mg protein) was assayed after centrifuging cell lysates at 12 000 g. Experimental procedures are described in the text.

<table>
<thead>
<tr>
<th>Cell fractions</th>
<th>Hb (%)</th>
<th>SOD</th>
<th>Catalase</th>
<th>Peroxidase</th>
<th>Reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proerythroblasts</td>
<td>10</td>
<td>15.0</td>
<td>0.18</td>
<td>793</td>
<td>34.6</td>
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<tr>
<td>Basophilic erythroblasts</td>
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<td>15.1</td>
<td>0.19</td>
<td>593</td>
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<tr>
<td>Polychromatic erythroblasts</td>
<td>30</td>
<td>15.1</td>
<td>0.28</td>
<td>597</td>
<td>42.9</td>
</tr>
<tr>
<td>Orthochromatic erythroblasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early</td>
<td>60</td>
<td>20.8</td>
<td>0.36</td>
<td>481</td>
<td>40.0</td>
</tr>
<tr>
<td>Late</td>
<td>70</td>
<td>22.3</td>
<td>0.40</td>
<td>392</td>
<td>29.4</td>
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<tr>
<td>Erythrocytes</td>
<td>85</td>
<td>37.7</td>
<td>0.46</td>
<td>260</td>
<td>15.9</td>
</tr>
</tbody>
</table>
levels of SOD and catalase in the mature erythrocytes compared with
those of the late orthochromatic cells suggest that synthesis of these
enzymes may increase late in erythroid-cell development, thus
protecting cells against the increased formation of O$_2^-$.

Another enzyme which is involved in the degradation of H$_2$O$_2$

is glutathione peroxidase. This enzyme is localized mainly in
erythrocyte membranes (Maral et al., 1977), and for this reason its activity was
measured and expressed per mg of membrane protein. The glutathione peroxidase
activity decreased markedly during differentiation. This
enzyme reaction depends on the reduction of glutathione by glutathione
reductase and NADPH, and the efficiency of glutathione reductase thus
depends on that of NADP-reducing enzymes. It was found that the
activity of glutathione reductase also decreases at the late ortho-
chromatic-cells stage. Because it is known that enzyme stability may
be influenced by the substrate concentration, the decrease of the
glutathione reductase activity might be due to a decrease in NADPH
production. It has been established that glucose-6-phosphate dehy-
drogenase and 6-phosphogluconate dehydrogenase activities decrease
sharply in non-dividing orthochromatic cells, possibly as a result of an
active controlled process (Denton et al., 1975). Taken together, the
present data and earlier results suggest that the efficiency of the
whole glutathione peroxidase system decreases during erythrocyte
differentiation.

The velocity constants of catalase and glutathione peroxidase for
H$_2$O$_2$-degradation in erythrocytes are approximately equal (Chance et
al., 1979). However, catalase degrades only H$_2$O$_2$, and its increase
during cell differentiation suggests a function in protecting cells
against non-enzymic attack by H$_2$O$_2$. Glutathione peroxidase degrades
both H$_2$O$_2$ and organic peroxides, and its decrease during differentia-
tion, as well as its localization in plasma membranes, suggests that
this enzyme protects the cell mainly against lipid peroxides.

The above data indicate that SOD and catalase protect mature
erythrocytes against O$_2^-$ and H$_2$O$_2$, whereas the glutathione peroxidase
system is of greater importance in the early stages of erythrocyte
differentiation and its role in removing H$_2$O$_2$ decreases in mature
cells. However, the glutathione peroxidase system has an important
function in protecting cells from lipid peroxides, which are the main
haemolytic factors in the case of oxidative stress (Chance et al.,
1979). Probably, this enzyme also compensates for the loss of
function of catalase in patients with acatalasaemia.

References

Aebi H (1970) Katalase in Methode der Enzymatischen Analyse