Studies on Myeloperoxidase Activity
I. Spectrophotometry of the MPO-H₂O₂ Compound

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Myeloperoxidase, MPO, forms a dissociable hydrogen peroxide compound with an apparent dissociation constant $K = 1 \times 10^{-4}$ M.

The hydrogen peroxide compound formed at peroxide concentrations with a numerical value below the one found for the dissociation constant contains only one perhydroxyl per molecule. This compound is stable and may be used for the oxidation of suitable substrates.

Each of the two iron atoms in the MPO molecule combines at increased hydrogen peroxide concentrations with separate perhydroxyls. The latter are most probably held by the iron atoms in a favourable position for a mutual exchange of electrons, which process initiates reactions resulting in a disintegration of the peroxide into water and molecular oxygen.

Hydrogen peroxide concentrations above $2 \times 10^{-4}$ M destroy the iron porfyrin structure and denature the enzyme.

The majority of the white blood cells, i.e. the myelocytes or granulocytes, enclose in their granules large quantities of the green coloured protein originally named verdoperoxidase and later renamed myeloperoxidase, MPO. Besides the protein moiety, which has a basic character — I. P. $> pH 10$ —, the MPO molecule contains two identical iron porfyrin structures. MPO was originally termed a peroxidase because of its capacity to form a spectroscopically demonstrable peroxide compound and to use the peroxide in this complex in a subsequent oxidative reaction when suitable substrates were added.

The peroxidative activity of the MPO was early found to differ in many respects from those of peroxidases of other origin, especially peroxidases from the vegetable kingdom. Its activity could not be checked by the usual peroxidase activity tests. MPO was inhibited and even destroyed by the prescribed peroxide concentrations. Some recent investigations have even more strongly emphasised the importance of an adequate control of the hydrogen peroxide concentration when the catalytic activity of MPO is to be tested. It has been found that MPO can utilize hydrogen peroxide for peroxidation most effectively, if the hydrogen peroxide concentration is kept extremely low. Certain reactions require a con-
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tinuous addition at a rate even as slow as 5–10 moles per mole of MPO per min if they are to be brought into effect at all.

It has for these reasons been found important to make a thorough study of the properties of the MPO-hydrogen peroxide compound at various hydrogen peroxide concentrations. The present investigation has been restricted mainly to spectrophotometry of these compounds.

EXPERIMENTAL

Material. The MPO used in these investigations was prepared according to the procedure published in 1958. The analytical data for this preparation were almost identical with those given in that report: Fe-content 0.074 % and an absorbancy value of 1.2 at 427 mm for the concentration 1 mg MPO per ml.

Technique. Light absorption measurements have been carried out using a Zeiss PMQ 11 recording spectrophotometer, and 10 mm optical cells. As the light passes the optical cells twice in this instrument the recorded values have been divided by two in order to refer the experimental data to an absorbing path of 10 mm.

Evolution of molecular oxygen from hydrogen peroxide has been measured by the use of Warburg-apparatus "V 85", B. Braun Co. The test solution were prior to the experiments freed from dissolved oxygen and kept under argon. The evolution of oxygen was measured by the usual technique and the total amount calculated as the sum of the gaseous and the dissolved oxygen. The amount of MPO used was calculated to be large enough for complete disintegration of the hydrogen peroxide.

Experiments. Measurements of the absorbancy of the MPO·H₂O₂ compound in equilibrium with the remaining MPO have been carried out by recording:

1. the values within the whole spectral region 700 – 400 mm, Figs. 1 and 2, or the values at wavelengths for the maximal increase and decrease, 452 and 427 mm, as soon as possible after the addition of H₂O₂ and the formation of the MPO·H₂O₂ compound, Fig. 6.
2. the differences between the absorbancy values for MPO and the values for the MPO – MPO·H₂O₂ mixture at specified H₂O₂ concentration, Fig. 3.
3. the absorbancy at 452, 443 and 427 mm during the thirty min period following the establishment of the equilibrium state between MPO and its H₂O₂ compound, Fig. 4. Fig. 5 reproduces values estimated for the hydrogen peroxide concentration during this 30 min period.

Manometric measurements of the effect of MPO upon hydrogen peroxide, experiment 7, have, when tested by Warburg technique, given the following results:

<table>
<thead>
<tr>
<th>M₄O₂ added</th>
<th>O₃ evolved</th>
</tr>
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<tbody>
<tr>
<td>3 ml 2 × 10⁻⁸ M; 6 × 10⁻⁶ moles</td>
<td>3.4 × 10⁻⁸ moles</td>
</tr>
<tr>
<td>3 ml 1 × 10⁻⁶ M; 3 × 10⁻⁶ &quot; &quot;</td>
<td>1.2 × 10⁻⁸ &quot; &quot;</td>
</tr>
<tr>
<td>3 ml 0.5 × 10⁻⁶ M; 1.5 × 10⁻⁶ &quot; &quot;</td>
<td>0.4 × 10⁻⁸ &quot; &quot;</td>
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RESULTS

The light absorption spectrum for MPO has within the visible part of the spectrum, 400–700 mm, two well-defined absorption maxima at 565 and 427 mm, and three minor ones at 675, 620 and 490 mm.

The addition of hydrogen peroxide results in the formation of an MPO·H₂O₂ compound, which spectrophotometrically is characterized by increased absorbancy in the wave-length regions 675–550, max. 622 mm and 490–433, max. 452 mm. Figs. 1 and 2, dotted lines.

Fig. 1 demonstrates the absorbancy for the MPO·H₂O₂ compound at an initial hydrogen peroxide concentration of 2 × 10⁻⁴ M and after various intervals of time. The hydrogen peroxide complex formed is fairly stable. The hydrogen peroxide disintegrates slowly. Twentyfour hours after the addition of the peroxide the absorbancy curve has returned to its original appearance. In the experiment demonstrated in Fig. 2 the hydrogen peroxide concentration is initially ten times higher than in the one discussed above. During the first 10 min there

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Fig. 1. Spectra of MPO, 0.46 mg/ml (Fe-conc. $6.14 \times 10^{-4}$ M), after addition of $H_2O_2$, $2 \times 10^{-4}$ M:
- immediately
- after 30 min
- after 90 min
- after 24 h

Fig. 2. Spectra of MPO, 0.46 mg/ml (Fe-conc. $6.14 \times 10^{-4}$ M), after addition of $H_2O_2$, $2 \times 10^{-3}$ M:
- immediately
- after 5 min
- after 20 min
- after 24 h

Fig. 3. Difference spectra between MPO and its $H_2O_2$-compound, MPO-conc. 0.92 mg/ml (Fe-conc. $12.8 \times 10^{-4}$ M):
- $1.96 \times 10^{-4}$ M $H_2O_2$
- $1.28 \times 10^{-4}$ M $H_2O_2$
- $6.4 \times 10^{-4}$ M $H_2O_2$
- $3.2 \times 10^{-4}$ M $H_2O_2$
- $1.6 \times 10^{-4}$ M $H_2O_2$

is a continuous disappearance of absorbancy over the whole spectral region, while at the same time the shape of the absorption curve, characteristic for the peroxide complex, is retained throughout. This indicates a saturation of the MPO with peroxide and a destruction of the enzyme during that period of time, when hydrogen peroxide was bound to the iron porphyrin structures. Not until the amount of hydrogen peroxide exceeding the level of about $1 \times 10^{-4}$ M has become disintegrated the spectrum will adopt the characteristics of the non-complex-bound enzyme.

The absorption spectrum of MPO becomes completely destroyed and its protein moiety denatured in experiments with an initial hydrogen-peroxide concentration of $2 \times 10^{-3}$ M and higher.

By the symmetrically shaped curves and the well-defined isosbestic points, 490 and 443 m$\mu$, Fig. 3, the differences in absorbancy for test solutions of which one contains MPO only and the other a mixture of MPO and MPO · $H_2O_2$ in proportions determined by the concentration of hydrogen peroxide, demonstrates that the two iron atoms in each MPO molecule most probably form identical peroxide compounds, and also that each iron atom forms only one type of hydrogen peroxide complex. This statement is valid for hydrogen peroxide concentrations below the level of $2 \times 10^{-4}$ M. At concentrations above this value the absorbancy increases at 622 m$\mu$ more than expected. At $1 \times 10^{-3}$ M, for instance, there is a continuous increase at 622 m$\mu$ during the initial 30–40 sec, while at 452 m$\mu$ the absorbancy value at the same time decreases as a consequence of the destruction of the enzyme. This increase in absorbancy at 622 m$\mu$ seems, despite the fact that part of the iron-porphyrin structures is destroyed, to indicate the formation of a reaction product which can provoke an analogous and more pronounced light absorption then the perhydroxyls themselves.

Fig. 4 demonstrates absorbancy values at 452 m$\mu$ (max. increase for the $H_2O_2$ compound), 443 m$\mu$ (isosbestic point), and 427 m$\mu$ (max. decrease) during a thirty min period following the addition of hydrogen peroxide. The initial hydrogen-peroxide concentrations were $2 \times 10^{-4}$, $2.5 \times 10^{-4}$, $5 \times 10^{-4}$ and $10 \times 10^{-4}$ M. The curves for the absorbancy values at 452 and 427
Fig. 4. Absorbancy readings for the MPO-H$_2$O$_2$ compound during a 30 min period after admixture of H$_2$O$_2$ in amounts giving the concentrations indicated in the figure. MPO-conc. 0.46 mg/ml = 3.07 x 10$^{-4}$ M. (Fe-conc. 6.14 x 10$^{-4}$ M). Absorbancy at 452 μm –——– ; 443 μm ———— ; and 427 μm ———–. The lines intersect when the H$_2$O$_2$-conc. is 0.75 x 10$^{-4}$ M.

μm are only slightly convergent for the experiment where the initial hydrogen peroxide concentrations is 2 x 10$^{-4}$ M. The absorbancy values after a thirty min period correspond to a concentration of about 1.5 x 10$^{-4}$ M. An increase in the initial H$_2$O$_2$ concentration from 2 x 10$^{-4}$ to 2.5 x 10$^{-4}$ M results in a significantly faster hydrogen peroxide disintegration. The concentration decreases during the thirty min period from 2.5 x 10$^{-4}$ to 0.75 x 10$^{-4}$ M. The hydrogen peroxide concentration values decrease in the other two experiments in Fig. 4 with a pronounced increased rate. The initially high values, 5 x 10$^{-4}$ and 10 x 10$^{-4}$ M, reach the 0.75 x 10$^{-4}$ M level after 10 and 12 min respectively.

The absorbancy values as well as the ratio between them in these experiments depend upon the hydrogen peroxide concentrations. Thus, knowledge of the values for the absorbancy at 452 and 427 μm makes it possible to calculate the hydrogen peroxide concentrations at any time during the experiment. Fig. 5 summarizes calculations made for six different experiments. It is quite evident that the hydrogen peroxide decomposes most rapidly in the experiment with the highest initial hydrogen peroxide concentration. It is also clear that the fast initial disintegration does not decline when the concentration reaches a certain lower level. It continues even after the concentration has decreased below the value reached in experiments with a low initial hydrogen peroxide concentration. A theory to explain these facts will be discussed below. According to this theory it seems most likely that one peroxide molecule attaches to each of the two iron atoms and that these two molecules react with each other. The product formed might thereafter react with excess of hydrogen peroxide and disintegrate into water and molecular oxygen. It seems likely that this intermediate may have a structure able to give the increased absorbancy at 622 μm, discussed above.

The absorbancy readings at 452 and 427 μm from twenty-four separate experiments with varying initial hydrogen peroxide concentrations have been plotted versus the negative logarithm for the hydrogen peroxide concentration in Fig. 6. The absorbancy values indicate the ratio of iron atoms forming a peroxide complex to non-complex-bound atoms. It has therefore been possible to use these experimental values for a calculation of this ratio and to plot these

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Fig. 5. Decrease of \( \text{H}_2\text{O}_2 \) conc. during the first 30 min period following the addition of \( \text{H}_2\text{O}_2 \) to MPO, calculated from absorbancy values found in experiments with known \( \text{H}_2\text{O}_2 \) concentration. MPO-conc.: 0.46 mg/ml = 3.07 \times 10^{-4} \) M. Initial conc. of \( \text{H}_2\text{O}_2 \) is indicated by the intercent on the axis of ordinates.

Fig. 6. Upper curves: Absorbancy readings for MPO — MPO \( \cdot \text{H}_2\text{O}_2 \) at 427 \( \bigcirc \) and 452 nm \( \bigotimes \) in solutions with known \( \text{H}_2\text{O}_2 \) concentrations. MPO-conc. 0.46 mg/ml = 3.07 \times 10^{-4} \) M. (Fe-conc. 6.14 \times 10^{-4} \) M).

Lower curve: The percentage of iron atoms not combined with \( \text{H}_2\text{O}_2 \) as a function of \( \text{H}_2\text{O}_2 \) conc. \( \bigcirc \) : Evaluated values from the experimental points in the upper curves.

Dotted line: Theoretically calculated dissociation curve on the assumption of a p\( K_{\text{app}} \) value of 4.0.

results on a diagram in the same figure. The distribution of the experimental points agree with a theoretical, simple dissociation curve with an apparent dissociation constant of 1 \times 10^{-4} \) M.

The results from the manometric measurements in experiment 7 show that the disappearance of hydrogen peroxide in the experiments demonstrated by Fig. 4 is followed by a formation of molecular oxygen. Thus, MPO acts in these experiments as a catalase. The ratio between the number of moles of oxygen formed per mole of hydrogen peroxide varies in the different experiments. Therefore, the results obtained can not at present be used for a true estimation of the number of hydrogen peroxide molecules used up in the reactions resulting in its disintegration into oxygen and water.

**DISCUSSION**

Each MPO molecule contains two identical iron-porphyrin structures, and each of these enters, on the addition of hydrogen peroxide, into a dissociation equilibrium:

\[
\begin{align*}
\text{Fe}^{3+} + \text{H}_2\text{O}_2 & \rightleftharpoons \text{Fe} \cdot \text{H}_2\text{O}_2 \\
\text{Fe}^{3+} & \rightleftharpoons \text{Fe} \cdot \text{H}_2\text{O}_2
\end{align*}
\]

where the MPO molecule with its two iron atoms is described by the

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\[
\begin{array}{c}
\text{symbol} \\
\text{symbol}
\end{array}
\begin{array}{c}
\text{Fe}^3+ \\
\text{Fe}^3+
\end{array}
\]

The value for the apparent dissociation constant, \(1 \times 10^{-4}\) M, has been determined.

The complex formed is fairly stable when the hydrogen-peroxide concentration is kept below the value for the dissociation constant, \(i.e\), when on an average not more than one of the two iron atoms is occupied by peroxide.

Both iron atoms take up hydrogen peroxide at higher peroxide concentration. Formation of the compound with two perhydroxyls in the same molecule is followed by a decomposition of hydrogen peroxide. If the formation and consumption of intermediates are neglected, the reaction may be written:

\[
\left[ \begin{array}{l}
\text{Fe} \cdot \text{H}_2\text{O}_2 \\
\text{Fe} \cdot \text{H}_2\text{O}_2
\end{array} \right] \leftrightarrow \left[ \begin{array}{l}
\text{Fe}^3+ \\
\text{Fe}^3+
\end{array} \right] + 2 \text{H}_2\text{O} + \text{O}_2 \] (2)

Provided that its concentration does not exceed a limit of about \(2 \times 10^{-3}\) M, hydrogen peroxide decomposes faster, the higher the initial hydrogen peroxide concentration. At and above this concentration the iron-porphyrin structures are destroyed and the enzyme denatured by intermediates formed and accumulated at the increased decomposition rate.

The results obtained highly emphasizes the close relationship between peroxidative and hydrogen-peroxide decomposing effects for catalysts containing two or more structures able to form weakly dissociated hydrogen peroxide compounds. The compound formed by attaching only one hydrogen peroxide per molecule is stable and may be used for the oxidation of substrates. The formation of a compound with two hydrogen peroxide molecules bound to favourably located active centres with adaptable charge might result in a mutual exchange of electrons and the generation of a highly reactive derivative, presumably an ozonide. This might then decompose rapidly in the presence of an excess of hydrogen peroxide through the over-all reaction:

\[
\text{H}_2\text{O}_3 + 2 \text{H}_2\text{O}_2 \rightarrow 3 \text{H}_2\text{O} + 2 \text{O}_2 \] (3)

Hydrogen peroxide has complete electron shells and its electrons have a symmetrical distribution. Acceptance of electrons from donors and a mutual exchange between two hydrogen-peroxide molecules within the same MPO structure necessitate a shift of charge, a break in the state of equilibrium and even disruption of the molecule. This is most probably brought into effect by the charge capacity of the tri-valent porphyrin iron.

The results described do not permit a definite decision as to whether hydrogen peroxide forms its complex with the iron atoms in competition with some other group whose concentration may be dependent upon the actual hydrogen ion concentration, \(e.g\), hydroxyl. Variations in pH values from 5 to 9 in experiments with an equal hydrogen-peroxide concentration do not have any influence upon the spectral reading indicating the ratio between complex bound to non-complex bound iron. However, as will be described and discussed in a forthcoming report, the formation of a chloride compound is governed not only by the chloride ion,

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but also by the hydroxyl ion concentration in conformity with the law of mass action. These differences in the two types of experiment may well be explained by the fact that hydrogen peroxide is a weak acid, \( K = 1.5 \times 10^{-12} \). The per-hydroxyl ion concentration is therefore dependent upon the actual pH value, while the chloride ion concentration is independent of this. A shift in the hydrogen ion concentration will influence the dissociation of hydrogen peroxide to the same extent but in opposite direction as it influences a tri-valent iron-hydroxyl group of the MPO. The probability of a competitive mechanism operating in the formation of the hydrogen peroxide compound supports the idea that the complex is established through an attraction between the tri-valent iron and the negatively charged perhydroxyl ions, and not as result of juxtaposition with molecular hydrogen peroxide.

Spectrophotometry registers changes in absorbancy only and gives no information concerning the character of the exposed reactions or concerning the physico-chemical properties of the reaction products. Complementary investigations with other techniques will be required for a more complete understanding of the reactions discussed. However, even with our present partial knowledge it may be permissible to summarize the experimental results and considerations in the foregoing in a schematic formula indicating the processes initiated by MPO in a milieu containing hydrogen peroxide together with substances acting as hydrogen donors, \( \text{DH}_2 \), or hydrogen-peroxide only.

\[
\begin{align*}
\text{Fe}^{3+}\text{OH}^+ & \quad \text{Fe}^{3+}\text{O}_2\text{H}^- \\
\text{Fe}^{3+}\text{OH}^+ & \quad \text{Fe}^{3+}\text{O}_2\text{H}^- \\
\text{Fe}^{3+}\text{Oh}^+ & \quad \text{Fe}^{3+}\text{O}_2\text{H}^- \\
\text{Fe}^{3+}\text{OH}^+ & \quad \text{Fe}^{3+}\text{O}_2\text{H}^- \\
+ & \quad + \\
n\text{H}_2\text{O}_2 & \quad (n-1)\text{H}_2\text{O}_2 & \quad (n-2)\text{H}_2\text{O}_2 & \quad \text{H}_2\text{O}_3 & \quad \text{H}_2\text{O}_3 & \quad \text{H}_2\text{O}_3 & \quad \text{H}_2\text{O}_3
\end{align*}
\]

\[
\text{DH}_2 \rightarrow \text{Fe}^{3+}\text{O}_2\text{H}^- + (n-1)\text{H}_2\text{O}_2 + 2\text{H}_2\text{O} + \text{D}
\]

\[
\begin{align*}
\text{H}_2\text{O}_3 + (n-2)\text{H}_2\text{O}_2 & \rightarrow 3\text{H}_2\text{O} + 2\text{O}_2 + (n-4)\text{H}_2\text{O}_2
\end{align*}
\]

Acknowledgement. This investigation has been supported by a grant from Statens Medicinska Forskningsråd.

REFERENCES

4. Unpublished observations.

Received April 17, 1963.

Acta Chem. Scand. 17 (1963) Suppl. 1