# THE PEROXIDASE REACTION OF HUMAN METHAEMOGLOBIN: CHARACTER OF THE AMINO ACID ELECTRONDONOR AND THE INFLUENCE OF OXYGEN

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*Dedicated to Professor Otto Wichterle on the occasion of his 80th birthday.*

The chemical modification of tyrosyl residues in methaemoglobin led to the decreased peroxidase activity and thus confirmed the role of tyrosyl residues in the catalytic process. The ESR spectra of methaemoglobin modified by tetranitromethane showed on the participation of tyrosine in the generation of superoxide anion radical. After repeated catalytic cycles the rapid decreasing of superoxide anion radical content was observed. This fact indicated, that the generation of superoxide anion radical is connected with tyrosyl residues. These residues with high probability are the source of the second electron in the peroxidase catalytic process. For the generation of superoxide anion radical during the peroxidase reaction the dissolved oxygen in the reaction mixture is necessary. This oxygen is also responsible for tyrosine destruction and thus for the decreasing of peroxidase activity of methaemoglobin.

Methaemoglobin ($Hb^+$)*, the ferric state of haemoglobin (Hb), is activated by $H_2O_2$, producing a short-lived intermediate with one oxidizing equivalent on the haem and one on the globin[1,2]. Studies of $H_2O_2$-activated $Hb^+$ show that although it is not identical with compound *I* or *II* of horse radish peroxidase, it has some structural features in common with both[3]. Although this intermediate has been considered a model for the peroxidase compounds, haemoglobin does not complete the reaction cycle and is not a true peroxidase[4]. It was important to find that $Hb^+$ itself looses its peroxidase activity (PA) after several catalytic cycles induced by repeated addition of substrate and $H_2O_2$.

* Abbreviations: $Hb^+$ methaemoglobin, TNM tetranitromethane, Tiron 4,5-dihydroxy-1,3-benzene disulfonic acid, PA peroxidase activity.

In the case of true peroxidases it is generally accepted that one oxidizing equivalent comes from the haem iron resulting in $Fe^{4+}$ state. The second electron is then supplied either by the porphyrine system with formation of $\pi$ cation radical (in the case of horseradish peroxidase) or by amino acid residue with formation of the corresponding amino acid radical[5,6] (in the case of cytochrome c peroxidase).

It was probable that radicals will play role also in the catalytic peroxidase mechanism of $Hb^+$.

Our studies[4] showed that for peroxidase system of $Hb^+$–ascorbic acid–$H_2O_2$ an asymmetrical ESR signal with $g_\perp \sim 2$ has been obtained which is interpreted as the perpendicular region of anisotropic spectrum of superoxide anion radical.

It was suggested that the second electron comes from the porphyrine ring. The short lived $\pi$ cation radical of porphyrine immediately accepting an electron, the charge being transferred to the proximal histidin residue and probably to some further amino acid residue. This amino acid residue obviously undergoes irreversible destruction after the first catalytic cycles. It looses its ability to act as electrondonor, whereby the PA of $Hb^+$ decreased. It was observed that tyrosine content in $Hb^+$ decreased as much as to 40% of the original value during the catalytic reaction. It can be concluded that tyrosine residues act as the internal electrondonor in the $Hb^+$ peroxidase action.

The aim of the present work was to confirm the role of tyrosine residues in peroxidase catalytic mechanism of $Hb^+$ by their chemical modification. ESR measurements should made possible to derive a hypothesis about the role of oxygen in the superoxide production and tyrosine destruction.

## EXPERIMENTAL

The human methaemoglobin ($Hb^+$) was prepared by oxidation of human oxyhaemoglobin[7] with potasium hexacyanoferrate(III). The pure $Hb^+$ was separated on a Sephadex G-25 column.

Tyrosyl residues were modified[8] using tetranitromethane (TNM) (Fluka AG, Basel). The reaction was carried out in phosphate buffer pH 7.2 at room temperature with tenfold molar excess of the reagent. The reaction of TNM with tyrosine produces 3-nitrotyrosine, nitroformate and two protons. The monitoring of the formation of nitroformate at 350 nm is sensitive method for the study of the time course of the reaction of TNM with tyrosyl residues.

Peroxidase activity was measured according to Connel and Smithies[9]. Absorbance of tetraquaiacol at 470 nm, recorded 90 s after the addition of $H_2O_2$ to the reaction mixture was used as a measure of relative peroxidase activity (PA).

### The ESR Spectra

ESR spectra were recorded on a ESR-220 spectrometer (Academy of Sciences, East Berlin, Germany), magnetic field was measured on a H-NMR magnetometer (Radiopan, Poland), microwave frequency on a frequency counter C3-54 (C.I.S.) with following conditions: microwave power 50 mW, modulation amplitude 0.2 mT, time constant 0.005 s, scan range 10 mT, scan speed 3 min, calibration standard Mn(II)/ZnS.

## RESULTS AND DISCUSSION

The relative peroxidase activity of $Hb^+$ was measured in the course of chemical modification of its Tyr residues with TNM. The time course of chemical modification expressed by absorbance at 350 nm and the corresponding time dependence of relative peroxidase activity (PA) of $Hb^+$ are represented in Fig. 1. As it is seen, the modification of Tyr residues in $Hb^+$ led to decreasing of PA. It was found that the chemical modification of Tyr residues represents an irreversible process. This conclusion was drawn from the fact that after the dialysis of reaction mixture with TNM the values of PA was identical when compared with the values before the dialysis. So, the question arises, what is the reason of the destruction of Tyr residues in $Hb^+$ during its catalytic action, which has been observed in our preceding experiments[4]. The ESR study of $Hb^+$ showed that in the course of peroxidase action the superoxide anion radicals are generated. It is necessary to answer the question, what is the connection between Tyr destruction, oxygen dissociation in the solution and the superoxide anion radical generation.

With the aim to prove the role of Tyr residues and $O_2$ in solution for the $O_2^{\cdot}$ generation, the ESR spectra of $Hb^+$, modified by TNM, were measured during the $Hb^+$ peroxidase activity. Because the life time of superoxide anion radical in water solution is too short, the spin trapping method was used.

The time dependence of ESR signal intensity of superoxide anion radical with $g_\perp \sim 2$ in the course of chemical modification of $Hb^+$ with TNM is presented in Fig. 2. The

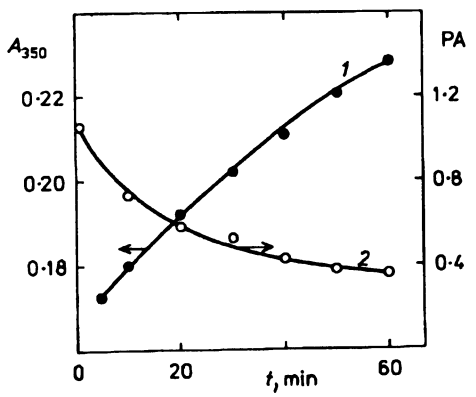


FIG. 1

The time dependence of $A_{350}$ absorbance (curve *1*) and the dependence of the relative peroxidase activity PA of methaemoglobin (curve *2*) in the course of the chemical modification with tetranitromethane (TNM). Actual concentrations in the system measured (mol $dm^{-3}$): $c(Hb^+) \approx 10^{-4}$, $c(TNM) \approx 10^{-3}$; pH 7.2

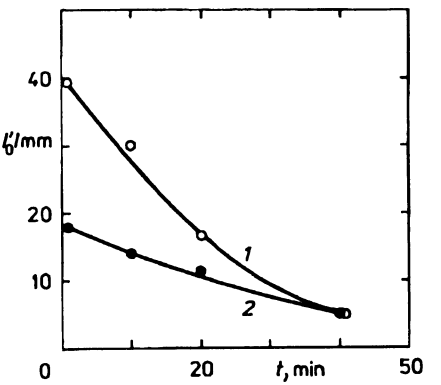


FIG. 2

The dependence of ESR signal intensity ($g_\perp \sim 2$) on the modification of $Hb^+$ with TNM in the solution containing dissolved oxygen (curve *1*) and without oxygen (curve *2*). Actual concentrations of compounds in the system measured (mol $dm^{-3}$): $c(Hb^{4-}) = 3 \cdot 10^{-4}$; $c$(phenol) = 1; $c$(TNM) = 3; $c(H_2O_2) = 1$; $T$ = 293 K; pH 7.2

ESR measurement was accomplished in the buffer solution containing dissolved $O_2$ (Fig. 2, curve *1*) and in the solution from which dissolved oxygen was removed by means of gaseous nitrogen (Fig. 2, curve *2*). It is obvious that oxygen in solution plays role in the peroxidase action of $Hb^+$. It was also confirmed that for the generation of superoxide anion radical the oxygen in solution and not $H_2O_2$ is necessary.

The fact that the generation of superoxide anion radical is connected with tyrosyl residues was confirmed in experiments with repeated catalytic cycles. After three peroxidase catalytic cycles, induced by repeat addition of phenol and $H_2O_2$, the ESR signal of superoxide anion radical rapidly decreased (Fig. 3, Table I). This corresponds to our hypothesis about irreversible destroying of Tyr residues during the catalytic process[4]. The fact that for the generation of superoxide anion radical the oxygen does not come from phenol, was confirmed in the experiment without phenol. In the system $Hb/H_2O_2$ the superoxide anion radical was found and in the system without $O_2$ the ESR signal was negligible.

TABLE I
The ESR spectral parameters of the measured radicals in the studied systems obtained after three peroxidase catalytic cycles

| System | *g*-factor | Signal with $[H_{pp}]$, mT | *f*, GHz | Splitting constants | |
|---|---|---|---|---|---|
| | | | | $a(H_0)$, mT | $a(H_p)$, mT |
| $Hb^+$ + phenol + $H_2O_2$ + Tiron pH 7.2 | 2.0049 | 0.076 | 9.4705 | 0.19 | 0.37 |
| $Hb^+/N_2$ + phenol/$N_2$ + $H_2O_2$ + Tiron pH 7.2 | 2.0052 | 0.064 | 9.4703 | 0.18 | 0.36 |

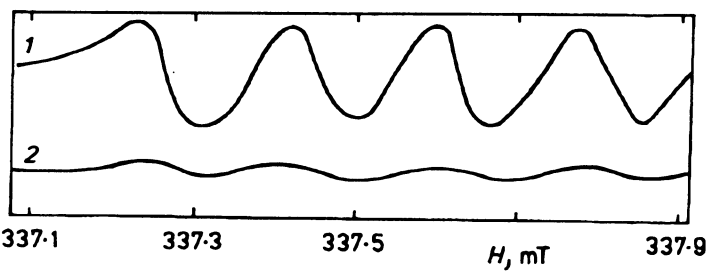


FIG. 3
The ESR spectrum of radicals generated in the system $Hb^+$–phenol–$H_2O_2$ and radical scavenger Tiron (curve *1*) and this one after the three catalytic peroxidase actions (curve *2*)

On the basis of our study it can be concluded that the tyrosyl residues act as the internal electrondonor in the course of $Hb^+$ catalysis. This idea does not contradicts the finding by Tew and Ortiz de Montellano[10] who proved the important role of tyrosyl radical in the dimerization of myoglobin by action of hydrogen peroxide. Tyrosine peroxyl radicals, formed by addition of oxygen to tyrosyl radicals were also observed as an initiating species in myoglobin-induced peroxidation[11]. Gutteridge[12] suggested that haptoglobin may play an important role in vivo by preventing tyrosyl stimulated formation of oxygen radicals. This result agrees with our finding that the bond with haptoglobin protects $Hb^+$ in the complex against effects of the solvent and hence against oxidation[4].

## REFERENCES

1. King N. K., Winfield M. E.: J. Biol. Chem. *238*, 1520 (1963).
2. Fox J. B., Nicholas R. A., Ackerman S. A., Swift C. E.: Biochemistry *13*, 5178 (1974).
3. Chance M., Powers L., Kirmar C., Chance B.: Biochemistry *25*, 1259 (1986).
4. Stejskalová J., Stopka P., Pavlíček Z.: Collect. Czech. Chem. Commun. *56*, 923 (1991).
5. Chance M., Powers L., Poulos T., Chance B.: Biochemistry *25*, 1266 (1986).
6. Edwards S. L., Xuong N., Hamlin R. C., Kraut J.: Biochemistry *26*, 1503 (1987).
7. Kramlová M., Přistoupil T. I., Ulrych S., Hrkal Z.: Haematologia *10*, 365 (1976).
8. Lundblad R. L., Noyes C. M. in: *Chemical Reagents for Protein Modification*, Vol. 2, p. 73. CRC Press, 1984.
9. Connel G. E., Smithies O.: Biochem. J. *72*, 115 (1959).
10. Tew D., Ortiz de Montellano R. R.: J. Biol. Chem. *263*, 17880 (1988).
11. Newman E. S. R., Rice-Evans C. A., Davies M. J.: Biochem. Biophys. Res. Commun. *179*, 1414 (1991).
12. Gutteridge J. M. C.: Biochim. Biophys. Acta *917*, 219 (1987).

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