AN ESR STUDY OF THE PEROXIDASE REACTION CATALYZED BY HUMAN METHAEMOGLOBIN AND METHAEMOGLOBIN--HAPTOGLOBIN COMPLEX

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The ESR spectra of peroxidase systems of methaemoglobin–ascorbic acid–hydrogen peroxide and methaemoglobin-haptoglobin complex–ascorbic acid–hydrogen peroxide have been measured in the acetate buffer of pH 4·5. For the system with methaemoglobin an asymmetrical signal with $g_{\perp} \sim 2$ has been observed which is interpreted as the perpendicular region of anisotropic spectrum of superoxide radical. On the other hand, for the system with methaemoglobin-haptoglobin complex the observed signal with $g \sim 2$ is symmetrical and is interpreted as a signal of delocalized electron. After realization of three repeatedly induced peroxidase processes the ESR signal of the perpendicular part of anisotropic spectrum of superoxide radical is distinctly diminished, whereas the signal of delocalized electron remains practically unchanged. An amino acid analysis of methaemoglobin along with results of the ESR measurements make it possible to derive a hypothesis about the role of haptoglobin in increasing of the peroxidase activity of methaemoglobin.

The study of peroxidase properties of haemoglobin (Hb)* by Polonovski and Jayle[1] in 1938 led to the discovery of the then unknown blood protein – haptoglobin (Hp). The said authors found that Hb can combine with Hp to form a very firm complex and that the bond mentioned increases the peroxidase properties of Hb very distinctly. This phenomenon has been continuously used[2] for determination of Hp. Investigations of this method showed that only the incorporation of Hb into the complex transforms Hb into a peroxidase "proper"[3], i.e., e.g., only after this incorporation there starts a linear dependence between the peroxidase activity (PA) and Hb concentration. A number of investigators[3] have focused attention on the reasons of the PA increase of Hb after its combination with Hp since the beginning of studies of Hp. These investigators found that the structure of globin section is very important in this combination since the complexes of Hb obtained from different

* Abbreviations: Hb haemoglobin, Hb^+ methaemoglobin, Hb-Hp haemoglobin-haptoglobin complex, Hb^+-Hp methaemoglobin-haptoglobin complex, HRP horse radish peroxidase, CcP cytochrome c peroxidase, AA ascorbic acid, DPPH diphenylpicrylhydrazyl, PA peroxidase activity.

animal species differ in their PA (ref.[4]). In addition, Bajic[5] presumed that the formation of this complex increases the stability of Hb and hence contributes to its higher catalytic activity. Makinen et al.[6] made this presumption more precise by interpreting the increase of PA as a consequence of stabilization of functional integrity of the heme centre of Hb against the denaturation effect of acid medium in which the peroxidase reaction takes place.

In the period of the last forty years a number of papers were published dealing with applications of the peroxidase reaction of Hb-Hp complex to determination of Hp for clinical purposes, genetic studies, as evidence of formation of the complex etc.[3]. The molecular mechanism itself of the increase in the peroxidase activity of Hb after its combination with Hp, however, remained unexplained. As early as at the beginning of studies of Hb it was found that the PA of its complex with Hp is higher regardless of whether oxyhaemoglobin, methaemoglobin (Hb^+), carbonylheamoglobin, or cyanomethaemoglobin was used for the reaction[4]. The study of the Hb^+-Hp complex following after the discovery of Hp showed that the complex fulfils the requirements connected with a true peroxidase better than Hb^+ itself does. It was important to find that Hb^+ itself loses its PA after several catalytic cycles induced by repeated additions of substrate and H_2O_2. However, if Hb^+ is bound with Hp in a complex, its PA will remain unchanged even after several catalytic processes as it is the case with true peroxidases.

Further studies[7,8] showed that the catalytic mechanisms of the individual peroxidases merely are various variants of the generally valid peroxidase mechanism[7,8] whose central feature consists in formation of the oxidized form of enzyme in the first step of the peroxidase reaction. In this oxidation of the heme enzyme with hydrogen peroxide one electron is supplied by the central iron atom which thereby becomes formally tetravalent. The second electron is then supplied either by the porphyrine system with formation of a π-cation-radical[7] (in the case of HRP) or by the amino acid residue with formation of the corresponding amino acid radical[9] (in the case of CcP). Thus it is highly probable that radicals will play role also in the study of catalytic peroxidase mechanism of Hb and its complex with Hp, and that the method of electron spin resonance (ESR) will represent an important research tool in this area. The ESR spectroscopy was applied earlier to studies[10] of coordination of amino acids in heme complexes of iron.

So far the ESR studies of haemoglobin and Hb-Hp complex have been mainly restricted to investigations of Hb or Hb-Hp alone, i.e. without the presence of hydrogen peroxide and an oxidizable substrate[6]. The ESR spectra of the enzyme–$-H_2O_2$ system were measured for myoglobin[11]. The existence of free radicals was proved in a study[12] of peroxidase reaction of HRP with compounds of AH_2 type using ESR. The aim of the present work was a study of differences between Hb^+ and Hb^+-Hp with respect to generation of radicals during the peroxidase reaction of ascorbic acid. For this study methaemoglobin was chosen because of the fact

that its central iron atom is in the same oxidation state as that in the best investigated true peroxidases (HRP and CcP).

MATERIALS AND METHODS

The human methaemoglobin (Hb^+) was prepared by oxidation of human oxyhaemoglobin[13] with potassium hexacyanoferrate(III). The hexacyanoferrate(II) formed was separated on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden). The Hb^+ solution was dialyzed overnight, and the pure Hb^+ was dried by lyophylization. The human haptoglobin of the II type (Hp II) was isolated in our laboratory[14] from the Cohn fraction IV.

The solution of methaemoglobin-haptoglobin complex (Hb^+-Hp) was prepared by mixing equal volumes of equimolar solutions of Hb^+ and Hp in acetate buffers of pH 4·5. Ascorbic acid (Lachema, Brno) was used as the substrate in the peroxidase reaction.[15]

The ESR spectra were measured by means of an ERS 200 spectrometer (Academy of Sciences G.D.R. Berlin) working in the X-region with the magnetic modulation of 100 kHz. The magnetic field was measured with an ^1H NMR magnetometer type MJ-110R (Radiopan, Poland), the microwave frequency was measured with a frequency counter type C 3—54 (U.S.S.R.). The given second-derivative spectra were obtained at the following conditions: modulation amplitude 1 mT, microwave frequency 9·3 GHz, microwave output 50 or 20 or 5 mW, attenuation within the limits of 30—18 dB, time constant within the limits of 0·1—5 s. Quartz cells of 4 mm outer diameter were used for the measurements, and diphenylpicrylhydrazyl was adopted as the standard (DPPH, $g = 2{\cdot}0037$). All the measurements were carried out at the temperature of liquid nitrogen (77 K). Ascorbic acid was mixed in the cell with Hb^+ or Hb^+-Hp, and the peroxidase reaction was started by addition of H_2O_2; after 32 s the cell content was frozen and the ESR spectrum was measured. If dissolved oxygen had to be removed, the solutions of Hb^+ and of ascorbic acid were bubbled through with N_2 gas before addition of H_2O_2, and also the acetate buffer for preparation of Hb^+ solution was bubbled through with N_2.

In the case of experiments with the Hb^+ which passed through several repeatedly induced catalytic processes the peroxidase reaction was let to proceed in the cell for 10 min. After this period, ascorbic acid followed by H_2O_2 were added to the reaction mixture, which was repeated after another 10 min. After 32 s the cell content was frozen and the ESR spectrum was measured. The spectral parameters were evaluated by usual procedures[16,17]. Irreversible changes of indole nucleus of tryptophan residues in Hb^+ due to the peroxidase reaction were inferred from changes in lifetime of their fluorescence[18]. The lifetimes were determined from the time-resolved fluorescence measured with a fluorimeter model 299 T (Edinburg Instruments). The wavelength of excitation radiation was 295 nm and the emission radiation was monitored at 340 nm. The fluorescence lifetime of tryptophan residues were calculated by means of the computer and programs which are supplied with the apparatus.

The analysis of the amino acid composition of methaemoglobin was carried out with a D-500 analyzer (Durrum Corp., U.S.A.). The protein samples studied were hydrolyzed in the standard way (6M HCl, 110°C, 20 h hydrolysis time) before the analysis proper.

RESULTS

The ESR spectra of the Hb^+–AA–H_2O_2 and Hb^+-Hp–AA–H_2O_2 systems measured exhibit the following features: the system with Hb^+ shows a signal of high-spin Fe^{3+} with $g_\perp \sim 6$ and an asymmetrical signal with $g_\perp \sim 2$ (Fig. 1, curve *1*); the

system with Hb^+-Hp complex shows a signal of high-spin Fe^{3+} with $g_\perp \sim 6$ and a signal with $g \sim 4{\cdot}3$ and, in contrast to the above system with Hb^+, a symmetrical signal with $g \sim 2$ (Fig. 1, curve 3). Also measured were the ESR spectra of both systems studied after three repeatedly induced peroxidase processes. Each system exhibited a signal of high-spin Fe^{3+} with $g_\perp \sim 6$ and a signal with $g \sim 4{\cdot}3$. In addition, the system with Hb^+ exhibited a signal with $g \sim 2$ which was also asymmetrical but substantially smaller than that in the system measured immediately after mixing the reactants (Fig. 1, curve 2). In contrast thereto, the system with Hb^+-Hp complex measured after three catalytic processes (Fig. 1, curve 4) exhibited the symmetrical signal with $g \sim 2$ of practically the same intensity like that with the fresh complex (Fig. 1, curve 3). The spectral parameters found for all the systems studied are presented in Table I.

The ESR spectrum of the Hb^+–H_2O_2 system (not containing the oxidizable substrate AA) exhibits an asymmetrical signal with $g_\perp \sim 2$ like that in Fig. 1, curve 4. The ESR spectrum of the Hb^+–AA–H_2O_2 system from which dissolved oxygen was removed by means of N_2 gas exhibited only an intensive signal of high-spin Fe^{3+} with $g_\perp \sim 6$, whereas the asymmetrical signal with $g_\perp \sim 2$ completely disappeared.

Table II presents the results of analyses of amino acid composition of native methaemoglobin and methaemoglobin after three repeatedly induced catalytic processes in the Hb^+–AA–H_2O_2 system. From the table it follows that after the three catalytic cycles the methaemoglobin shows the largest decrease in tyrosine, viz. to 40% as compared with the original value.

Table III presents the fluorescence lifetimes of native Hb^+ and the Hb^+ which had repeatedly catalyzed three reinduced peroxidase processes.

DISCUSSION

The reason of differences in peroxidase activity of Hb^+ and Hb^+-Hp complex has not been satisfactorily explained yet. Little attention is paid to this problem in literature and the conclusions hitherto made can be considered to be a partial explanation of the problem given. Makinen et al.[6] presume that Hb^+ in the Hb^+-Hp complex is more protected against effects of acid medium which is usually adopted in PA studies. Another conclusion was made on the basis of the finding that formation of the Hb^+--Hp complex is connected with conformational changes due to changed spin state of the central iron atom and changed quaternary structure of haemoglobin[19]. As a consequence of these changes it is possible to presume that the position of distal amino acid residues with respect to the bond of H_2O_2 in the heme cavity is more favourable in the Hb^+-Hp complex than in Hb^+.

Obviously, any explanation of PA differences between Hb^+ and Hb^+-Hp complex will necessitate the knowledge of catalytic mechanism of Hb^+ alone which is very poor at present. Nevertheless, it is very likely[7,8] that the catalytic mechanisms of

TABLE I

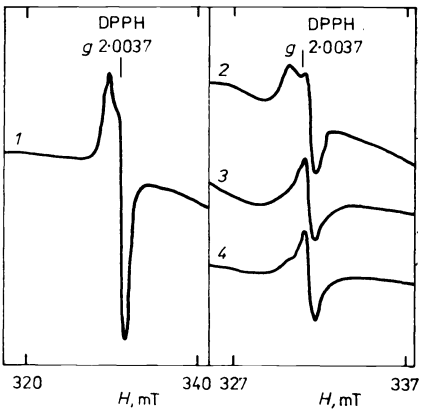
Spectral parameters of the peroxidase systems investigated

Reaction system	*g*-factor	Signal width ΔHpp, mT	Spin number in sample N_b
Hb^+–AA–H_2O_2	$g_a = 6{\cdot}04$	17·0	$1{\cdot}5 \cdot 10^{15}$
	$g_x = 2{\cdot}0014$	0·8	$1{\cdot}4 \cdot 10^{14}$
	$g_x = 2{\cdot}0087$	4·4	
Hb^+-Hp–AA–H_2O_2	$g_a = 6{\cdot}04$	18·5	$1{\cdot}0 \cdot 10^{15}$
	$g_a = 4{\cdot}34$	7·5	$1{\cdot}4 \cdot 10^{14}$
	$g_c = 2{\cdot}002$	0·6	$2{\cdot}3 \cdot 10^{12}$
Hb^+–AA(3x)–H_2O_2(3x)	$g_a = 6{\cdot}02$	18·5	$1{\cdot}7 \cdot 10^{15}$
	$g_a = 4{\cdot}39$	8·0	$2{\cdot}6 \cdot 10^{14}$
	$g_x = 2{\cdot}0015$	0·6	$1{\cdot}4 \cdot 10^{13}$
	$g_x = 2{\cdot}0088$	2·0	
Hb^+-Hp–AA(3x)–H_2O_2(3x)	ó$_a$ = 5·93	13·0	$2{\cdot}9 \cdot 10^{14}$
	$g_a = 4{\cdot}33$	9·0	$2{\cdot}0 \cdot 10^{14}$
	$g_c = 2{\cdot}0017$	0·6	$2{\cdot}5 \cdot 10^{12}$

The actual concentrations of components in the systems measured correspond to the concentrations given in Fig. 1; a signal of heme iron, b $O_2^{\cdot-}$, c delocalized electron.

FIG. 1

Signals with $g \sim 2$ for systems 0·1 ml Hb^+–3 µl AA–3 µl H_2O_2 (curve *1*); 0·1 ml Hb^+–3 µl AA (3×)–3 µl H_2O_2 (3×) (curve *2*); 0·1 ml Hb^+-Hp–3 µl AA–3 µl H_2O_2 (curve *3*); 0·1 ml Hb^+-Hp–3 µl AA (3×)–3 µl H_2O_2 (3×) (curve *4*). Actual concentrations of components in the systems measured (mol . dm^{-3}): $c(Hb^+) = 2{\cdot}8 \cdot 10^{-4}$, $c(AA) = 0{\cdot}03$, $c(H_2O_2) = 0{\cdot}03$ (curve *1*); $c(Hb^+) = 2{\cdot}5 \cdot 10^{-4}$, $c(AA) = 0{\cdot}08$, $c(H_2O_2) = 0{\cdot}08$ (curve *2*); $c(Hb^+\text{-Hp}) = 2{\cdot}8 \cdot 10^{-4}$, $c(AA) = 0{\cdot}03$, $c(H_2O_2) = 0{\cdot}03$ (curve *3*); $c(Hb^+\text{-Hp}) = 2{\cdot}5 \cdot 10^{-4}$, $c(AA) = 0{\cdot}08$, $c(H_2O_2) = 0{\cdot}08$ (curve *4*); $T = 77$ K; pH 4·5



individual peroxidases merely are various variants of a generally valid peroxidase catalytic mechanism which can be described as follows:

$$Fe^{3+} \text{ . enzyme} - 2\,e \xrightarrow{H_2O_2} \text{Compound } I \qquad (1)$$

$$\text{Compound } I + e \rightarrow \text{Compound } II \qquad (2)$$

$$\text{Compound } II + e \rightarrow Fe^{3+} \text{ . enzyme} \qquad (3)$$

The central feature of this general catalytic mechanism consists in the formation of enzyme intermediate called Compound *I*. It was shown[7] that in the case of horse radish peroxidase (HRP) one oxidation equivalent in Compound *I* is stored as Fe^{4+} and the second one as π-cation-radical of porphyrine ring[8]. According to a recent paper[20] this cation-radical is not localized only in porphyrine but is markedly delocalized also to the residue of the proximal histidine. In the oxidized intermediate of cytochrome *c* peroxidase (CcP) one oxidation equivalent is also stored as Fe^{4+} (ref.[7]), the other one being present as a radical in the pair of methionine residues Met 230–Met 231 (ref.[9]). On the basis of analogy with the peroxidases mentioned (HRP, CcP) it can be presumed that the oxidized intermediate from Hb^+ will have radical character, too. Therefore, we decided to study the course of the peroxidase reaction catalyzed with Hb^+ or Hb^+-Hp with the help of ESR.

The ESR spectrum of Hb^+–AA–H_2O_2 system exhibits beside the signal[21] of high-spin Fe^{3+} an asymmetrical signal with $g_\perp \sim 2$. On the basis of the asymmetry and the spectral parameters (Table I) the signal was interpreted as the perpendicular component of anisotropic spectrum of superoxide radical $O_2^{\cdot-}$ (Fig. 1, curve *1*). For comparison, Fig. 2 presents the usual well-resolved spectrum of $O_2^{\cdot-}$. It must be noted, however, that – depending on the way of and conditions during formation of $O_2^{\cdot-}$ in the reaction mixture – the components of its ESR spectrum exhibit various shifts and that especially the extent of *g*-factor is relatively large[22–24]. The ESR spectrum of Hb^+-Hp–AA–H_2O_2 again exhibits the signal of high-spin Fe^{3+}, the signal with $g \sim 4{\cdot}3$ and the signal with $g \sim 2$. The signal with $g \sim 2$ is symmetrical in this case, and on the basis of spectral parameters (Table I) it was interpreted as a signal of a delocalized electron[25] (Fig. 1, curve *3*). The decomposition of methaemoglobin (and of analogous porphyrine complexes of Fe^{3+}) produces an intermediate exhibiting an ESR signal with $g \sim 4{\cdot}3$ which corresponds to Fe^{3+} in tetrahedral environment. The rate of formation and the stationary concentration of this intermediate depend on the type and concentration of oxidizing agents, and hence the ESR spectrum of this intermediate varies in its intensity or is absent.

It was found earlier[26] that in the reduction of hydrogen peroxide with methaemoglobin one electron comes from iron which thereby is converted into tetravalent state. On the basis of analogy with HRP and CcP we suggest that the second electron

comes from the porphyrine ring, the short-lived π-cation-radical of porphyrine immediately accepting an electron, the charge being transferred to the proximal His

TABLE II

Analysis of amino acid composition of native methaemoglobin and modified methaemoglobin after three peroxidase catalytic cycles

Amino acid	Hb^+ (ref.[27])	Hb^+ (nat.)	Hb^+ (modif.)	$\frac{Hb^+ \text{ modif.})}{Hb^+ \text{ (nat.)}} \cdot 100$
Lys	22	21	18	85
His	19	17	16	94·1
Arg	6	6	5	83·3
Asp	25	25	26	104
Thr	16	14	15	107·1
Ser	16	16	15	93·75
Gen	16	17	17	100
Pro	14	15	15	100
Gly	20	20	21	105
Ala	36	38	40	105·2
Val	31	28	29	103·5
Met	3	2	2	100
Leu	36	36	35	97·2
Tyr	6	5	2	40
Phe	15	15	15	100

TABLE III

Lifetimes of fluorescence of tryptophane residues of native methaemoglobin and modified methaemoglobin after three peroxidase catalytic cycles

Protein	Lifetime, ns[a]	
	τ_1	τ_2
Hb^+ (nat.)	0·72	4·06
Hb^+ (modif.)	0·60	4·29
$\frac{Hb^+ \text{ (modif.)}}{Hb^+ \text{ (nat.)}} \cdot 100$	83	105

[a] The course of extinction of fluorescence was biexponential with the lifetimes τ_1 and τ_2 typically with the parameter $\chi^2 = 1{\cdot}2$.

and (by the tunnel effect) probably to some further amino acid residue. This amino acid residue, obviously, undergoes irreversible oxidation after the first catalytic cycles, it loses its ability to form the cation-radical whereby the PA of Hb^+ decreases. The role of this internal electron donor can obviously be played by some electron-rich amino acid residues, particularly Met, Trp, His, and Tyr. The Met residues in Hb^+ molecule are placed at the distal part of the heme group[27] and, hence, they cannot act as electron sources in the catalytic reaction for sterical reasons. In accordance therewith, the content of Met in Hb^+ is not changed in the course of peroxidase catalytic activity, which was confirmed by the analysis of amino acids (Table II). The same result was also obtained for the His residues. In the case of Trp residues the measurements of time-resolved fluorescence after repeated peroxidase reaction showed that the changes in lifetime are smaller than the observed decrease in enzyme activity of Hb^+ (Table III), hence an irreversible destruction of tryptophane residues is not indicated. On the other hand, the Tyr content in Hb^+ decreased as much as to 40% of the original value during the catalytic reaction. On the basis of this finding it can be concluded that the tyrosine residues act as the internal electron donor in the Hb^+ molecule. This idea does not contradict the finding by Tew and Ortiz de Montellano[28] who proved the important role of tyrosyl radical in the dimerization of myoglobine by action of hydrogen peroxide, the radical being generated by an intramolecular electron transfer to the ferryl heme group.

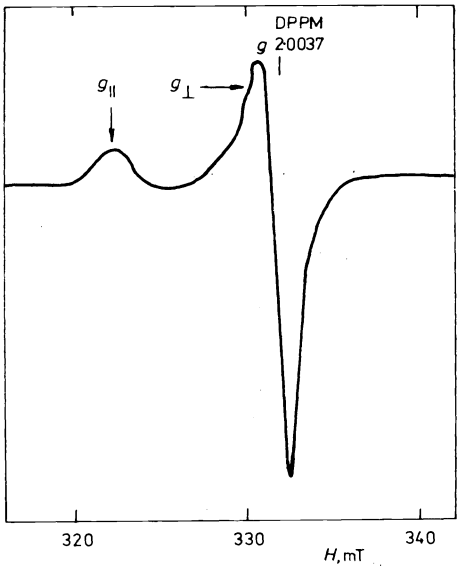


FIG. 2

ESR spectrum of $O_2^{\bullet-}$ generated by mixing NaOH and H_2O_2 solutions. Spectral parameters: $g_{\parallel} = 2{\cdot}0653$ (ΔHpp = 4·4 mT), $g_{\perp} = 2{\cdot}0068$ (ΔHpp = 1·6 mT). Concentrations (mol dm^{-3}): $c(NaOH) = 1$, $c(H_2O_2) = 1$; $T = 77$ K. Experimental conditions: modulation amplitude 1 mT, microwave frequency 9·3 GHz, microwave output 20 mW, attenuation 18 dB, time constant 2 s, scan rate of spectrum 3·7 mT/min

However, regardless of the nature of the amino-acid electron donor we presume the most important effect of Hp on Hb^+ in the complex to consist in the protection of the amino-acid electron donor against oxidation action during the peroxidase reaction. This is in accordance with the ESR spectra measured for the Hb^+–AA–H_2O_2 system (Fig. 1, curve *1*, Table I) and for the same system after three catalytic, repeatedly induced processes (Fig. 1, curve 2). In the case of Hb^+ after the three catalytic processes the measured perpendicular component of anisotropic spectrum of $O_2^{\cdot-}$ was substantially smaller than that of the initial system. In the case of the Hb^+-Hp complex the signal of delocalized electron measured for the initial system Hb^+-Hp–AA–H_2O_2 (Fig. 1, curve *3*) had the same intensity as that measured after three catalytic processes (Fig. 1, curve *4*).

The fact that in the peroxidase reactions with Hb^+ and Hb^+-Hp the ESR spectra exhibited the signals of $O_2^{\cdot-}$ and of delocalized electron, respectively, seems to indicate that the amino-acid electron donor in the Hb^+ bound with Hp in the complex is less accessible to oxygen in the solution. This agrees with the idea that the bond with Hp protects Hb^+ in the complex against effects of the solution and, hence, against oxidation during the catalytic reaction. The relation between $O_2^{\cdot-}$ radical and oxygen in solutions was proved by means of the ESR spectrum of the Hb^+–AA–H_2O_2 system from which dissolved oxygen was removed by means of gaseous nitrogen. In the system mentioned the signal of superoxide radical ($g_\perp \sim 2$) completely disappeared.

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