GENERATION OF ELECTRONICALLY EXCITED STATES BY MEANS OF METHEMOGLOBIN AND METHEMOGLOBIN–HAPTOGLOBIN COMPLEX

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It has been found that both human methemoglobin and its complex with human haptoglobin in their enzymatic action can generate products in electronically excited states. The time dependence has been measured of the chemiluminescence generated by both the haem proteins in their peroxidase as well as oxidase actions. The methemoglobin–haptoglobin complex exhibited, in its peroxidase action, a higher chemiluminescence as compared with methemoglobin alone. The chemiluminescence observed in the oxidase reaction was the same with both the haem proteins. The Stern–Volmer plots for quenching of chemiluminescence with indole and acrylamide have been constructed for both enzymatic reactions.

Hemoglobin* (Hb) is a protein whose main physiological function consists in oxygen transport. Beside this important ability, Hb also exhibits enzymatic activities, viz. both a peroxidase one and an oxidase one. In this respect it is similar to the structurally cognant enzymes from the large group of haem oxidoreductases. Both peroxidases and Hb can catalyze a peroxidase reaction, i.e. oxidation of a suitable substrate with hydrogen peroxide. With Hb this ability is distinctly increased when it is bound to haptoglobin[1]. This phenomenon has been made use of for analytical determination of Hp since it was discovered[1].

The horse-radish peroxidase[2] (HRP) – an enzyme isolated from horse-radish – is a representative of plant peroxidases among haem proteins. In seventieths it was discovered that it is able to generate electronically excited states during both its peroxidase[3] and oxidase actions[4]. The electronically excited products generated in this way are considered to be able to induce photochemical reactions in plants without any participation of excitation radiation. The first oxidase reaction catalyzed with HRP and connected with emission of light was described for the system of horse-radish peroxidase–2-methylpropanal–oxygen[4,5]. In this case, 2-methylpropanal

* The abbreviations used: Hb hemoglobin, Hb^+ methemoglobin, Hp haptoglobin, Hb^+–Hp methemoglobin–haptoglobin complex, HRP horse-radish peroxidase, MPAL 2-methylpropanal, EDTA ethylenediaminetetraacetic acid, Hb^+–P methemoglobin–peroxide, Hb^+–II oxidation compound of methemoglobin analogous to Compound *II* of true peroxidases.

is oxidized with the oxygen present in the solution under catalytic action of HRP as a dioxygenase. A compound of dioxetane type is presumed as the oxidation intermediate which is decomposed into excited triplet acetone and formic acid. The triplet acetone can serve as a source of electron excitation energy for inducing a number of photochemical reactions "without light"[5]. Chemiluminescence was also observed in a system containing eosine, H_2O_2, and HRP in which a peroxidase reaction takes place[3]. In this case eosine is transformed into its electronically excited state exhibiting chemiluminescence.

In the present paper attention is focused on the human Hb^+ and Hb^+–Hp complex as proteins of animal origin possessing enzymatic effects of oxidoreductases. The aim of our work was to find whether or not the proteins mentioned are able to generate excited states in their enzymatic action. Attention was also directed to elucidation of the effect of high-molecular ligand of Hp.

EXPERIMENTAL

The human Hp was isolated from the fresh blood supplied by Institute of Hematology and Blood Transfusion, Prague[6]. The human Hb^+ was prepared by oxidation of Hb with a solution of $K_3Fe(CN)_6$ and was separated from the reaction mixture by means of gel filtration on Sephadex G-25. It was kept in lyophilized state.

The human Hp-II was isolated from the Cohn fraction *IV* by a method developed in our laboratory[7]. The Hb^+–Hp complex was prepared by addition of Hp solution to Hb^+ solution in such way that the molar ratio of the components was 1 : 1.

2-Methylpropanal was prepared by oxidation of 2-methyl-1-propanol with dichromate–sulfuric acid mixture[8] and was purified by distillation.

The chemiluminiscence was followed by means of a detection equipment of a scintillation counter ISOCAP 300 (Liquid Scintillation System, Nuclear Chicago). The peroxidase reaction in the measuring vessel was started by addition of a calculated amount of H_2O_2 to the system protein–fluoresceine–H_2O_2, the oxidase reaction was initiated by addition of MPAL to the system haem protein–MPAL–O_2. The solution of Sörensen phosphate buffer (pH 7·0), in which the measurements took place, always contained ethanol as a solubilization agent for fluoresceine and MPAL. The addition of EDTA to the reaction mixture was necessary in order to chelate the traces of heavy metal ions which otherwise would cause quenching of chemiluminescence.

Immediately after mixing all the components needed for the chemiluminescence reaction the vessel was placed into the measuring apparatus which evaluated the number of scintillations (counts) per min, i.e. the frequency. This frequency, evaluated from a certain time interval of the reaction course, was identified with the chemiluminescence intensity at the given time point and was denoted as I. The measurement provided the time dependences of chemiluminescence which are characterized primarily by the height of the I_{max} maximum at the chemiluminescence curve. The quenching of chemiluminescence with acrylamide and indole was evaluated by the so-called Stern–Volmer plot[15] using the Stern–Volmer equation in the form:

$$I^0_{max}/I_{max} = 1 + K_{SV}[Q] ,$$

where I^0_{max} represents the maximum intensity of chemiluminescence at zero concentration of quenching agent, I_{max} gives the maximum intensity of chemiluminescence for the given concentra-

tion of quenching agent, K_{SV} is the quenching constant by Stern and Volmer, and [Q] means the concentration of quenching agent.

RESULTS

It was found that in the peroxidase reaction catalyzed with Hb^+ or Hb^+–Hp and using fluoresceine as the substrate, the electronically excited states were generated making themselves felt by chemiluminescnce. From the chemiluminescence–time dependence of the Hb^+ or (Hb^+–Hp)–fluoresceine–H_2O_2 system given in Fig. 1 it can be seen that the (Hb^+–Hp)–fluoresceine–H_2O_2 system exhibits a higher chemiluminescence intensity as compared with the system containing Hb^+. The chemiluminiscence of both the systems given was affected by addition of indole. The Stern–Volmer plot of chemiluminescence quenching by indole for the systems Hb^+ or (Hb^+-Hp)–fluoresceine–H_2O_2 is given in Fig. 2 wherefrom it can be seen that the quenching by indole is less in the system containing Hb^+ than in that with Hb^+–Hp. In the case of the reaction catalyzed with Hb^+ there even occurred an increase of the chemiluminescence at lower indole concentrations. The system containing the Hb^+–Hp complex exhibited a very intensive quenching by indole, and the curve of the Stern–Volmer plot is bent toward the I^0_{max}/I_{max} axis (Fig. 2).

It was found that also in the oxidase reaction taking place in the Hb^+ or (Hb^+–Hp)–MPAL–O_2 system there occurs chemiluminescence due to generation of electronically excited states. The chemiluminescence–time dependence of the Hb^+–MPAL–O_2 system alone and for that containing indole is given in Fig. 3, whereas Fig. 4 presents the respective time courses for the (Hb^+–Hp)–MPAL–O_2 systems without and with indole. From Figs 3 and 4 it can be seen that the chemiluminescence intensity in the absence of indole was the same for the system containing Hb^+ and Hb^+–Hp. The two systems exhibited differences in generation of electronically excited states only after addition of indole. As it is documented in Figs 3 and 4 addition of indole in a concentration of $1{\cdot}2 \,.\, 10^{-6}$ mol dm^{-3} decreased and increased the chemiluminescence intensity in the systems containing Hb^+–Hp and Hb^+, respectively. Acrylamide acted as a quenching agent in both the systems (Fig. 5). The Stern–Volmer dependences for both the systems studied are close to a straight line in the case of acrylamide. The quenching was stronger in the system with Hb^+ than in that with Hb^+–Hp.

DISCUSSION

So far the plant enzyme – horse-radish peroxidase (HRP) has been used almost exclusively in enzyme-catalyzed generation of electronically excited states of biological compounds without application of any excitation radiation. Recently increasing attention is given to the idea that the enzyme-generated electronic excita-

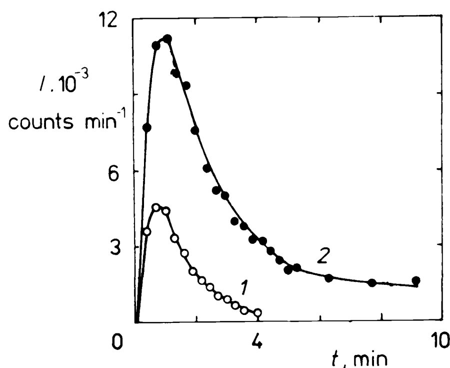

FIG. 1

Time course of chemiluminescence (I) of Hb^+–fluoresceine–H_2O_2 system (*1*) and (Hb^+–Hp)–fluoresceine–H_2O_2 system (**2**). Concentrations: Hb^+ and Hb^+–Hp 7·5 . 10^{-7} mol dm^{-3}; H_2O_2 9·7 . 10^{-4} mol . dm^{-3}; fluoresceine 4·9 . 10^{-5} mol dm^{-3}; EDTA 4 . 10^{-4} mol dm^{-3}; ethanol 1·7 . 10^{-1} mol dm^{-3}. Acetate buffer pH 4·5

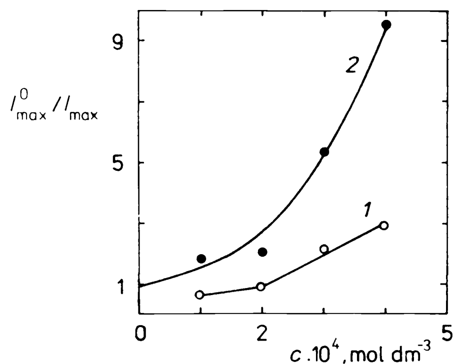

FIG. 2

The Stern–Volmer plot of quenching of chemiluminescence by indol of Hb^+–fluoresceine–H_2O_2 system (*1*) and (Hb^+–Hp)–fluoresceine–H_2O_2 system (**2**); c indole concentration. Acetate buffer pH 4·5

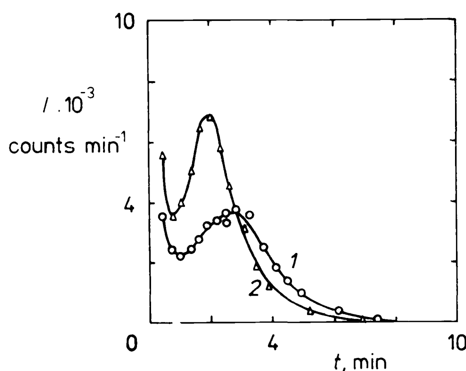

FIG. 3

Time course of chemiluminescence (I) of the Hb^+–MPAL–O_2 system alone (*1*) and in the presence of indole (**2**). Phosphate buffer pH 7·0

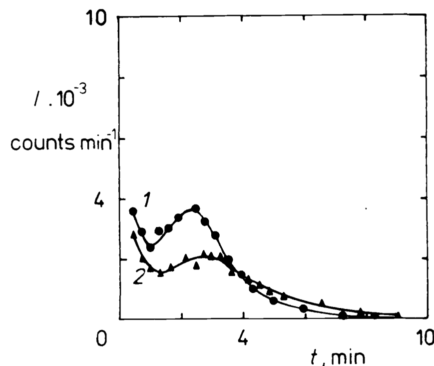

FIG. 4

Time course of chemiluminescence (I) of the (Hb^+–Hp)–MPAl–O_2 system alone (*1*) and in the presence of indole (**2**). Phosphate buffer pH 7·0

tion energy can induce a number of important biochemical reactions in living organisms. One example is the photochemical transformation of the plant growth hormone auxin (indolylacetic acid)[9]. This transformation is catalyzed by HRP and proceeds without excitation radiation. As the reactions of the given type can obviously take place also in animal organisms, we tried, in the context of this work, to find proteins of animal origin able to generate electronically excited states during their enzymatic action. The human Hb^+ and its complex with human haptoglobin – Hb^+–Hp were intensively studied from physical-chemical standpoint in our laboratory in past few years[10,11]. These haem proteins are known to exhibit (in analogy with HRP) both peroxidase and oxidase enzymatic activities. Therefore we tried to find whether or not the haem proteins studied (Hb^+ and Hb^+–Hp) can generate electronically excited reaction products in their enzymatic action. From Figs 1 and 3 it can be seen that the proteins mentioned can exhibit chemiluminescnce in their enzymatic action and, hence, can generate electronically excited states. The chemiluminescence of the Hb^+ or (Hb^+–Hp)–fluoresceine–H_2O_2 system accompanies the peroxidase reaction and is proportional to the peroxidase activity of the haem proteins used (Fig. 1). The higher chemiluminescence found in the system with Hb^+–Hp as the enzyme is in accordance with the earlier-found fact that Hp increases its peroxidase activity when bound to Hb^+ (ref.[1]). On the other hand, the chemiluminescence curves measured in the oxidase reactions in the systems Hb^+ or (Hb^+–Hp)–MPAL–O_2 in the absence of indol (Figs 3 and 4) indicate that the ability to generate electronically excited states in this case is the same for Hb^+ and Hb^+–Hp.

In order to explain the experimental facts mentioned, which are different in the cases of peroxidase and oxidase reactions, one must consider the molecular mechanism of both the enzymatic reactions. In the case of the generation of electronically ex-

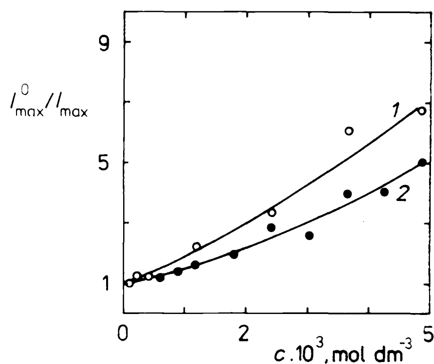

FIG. 5

The Stern–Volmer plot of quenching of chemiluminescence by acrylamide in Hb^+–MPAL–O_2 system (*1*) and in (Hb^+–Hp)–MPAL–O_2 system (*2*). Phosphate buffer pH 7·0; c acrylamide concentration

cited states in the peroxidase reaction catalyzed with HRP Brien et al.[3] suggest a formation of electronically excited product in a one-electron reduction of the compound HRP-II (i.e. the oxidized form of HRP known as the Compound *II*) to the original enzyme HRP. However, from the available knowledge about mechanisms of chemiluminescence reactions[10] it follows that an electronically excited state of compound is formed very often during reduction of its cation-radical or oxidation of its anion-radical. At any rate, however, this mechanism, which is denoted as CIEEL (Chemically Initiated Electron Exchange Luminescence) by Schuster[12], necessitates the presence of an ion-radical for the formation of electronically excited state. In the peroxidase reaction studied in the present work the transient oxidation compound is Hb^+ with H_2O_2 the so-called Hb^+-peroxide (Hb^+-P) which can accept two electrons from the substrate whereby the latter is oxidized. In this oxidation reaction one electron reduces the cationic radical of some of the globin amino acid residues, whereas the other reduces the ferryl iron to trivalent state. In contrast to Brien et al.[3] we suppose that the electronically excited state of the peroxidase reaction is already formed during the reduction of the cation-radical Hb^+-P to the compound Hb^+-II (similar to HRP-II) which is not a radical. The substrate oxidized in the reaction is fluoresceine in our case: it is the source proper of the chemiluminescence radiation, since obviously the excitation energy in the reduction of cation-radical is transferred from it. The number of electronically excited molecules and, hence, the intensity of chemiluminiscence are determined by the Hb–P concentration which is higher in the systems with higher peroxidase activity, i.e. with the Hb^+–Hp complex.

As far as the oxidase reaction is concerned, the chemiluminescence is the same for Hb^+ and Hb^+–Hp used as catalysts. A reaction scheme for the oxidase reaction of the HRP–MPAL–O_2 system was suggested by Baader et al.[13]. The mechanism suggested involves as a part also the conventional peroxidase cycle known from the peroxidase reaction. In the oxidase reaction with MPAL as the substrate the electronically excited states are formed (in contrast to the peroxidase reaction) by the so-called dioxetane mechanism, i.e. decomposition of 2-peroxymethylpropanal to triplet acetone and formic acid. In accordance with the work by Bohne et al.[14] we suppose that in the oxidase reaction catalyzed with Hb^+ or Hb^+–Hp the rate-limiting step also does not consist in the peroxidase cycle but consists in the rate of transformation of the methylpropanal keto-form into enol-form, the latter being the actual substrate for the haem enzyme. The final consequence of it, of course, is that the chemiluminescence intensity in the oxidase reaction of methylpropanal is the same for Hb^+ and Hb^+–Hp complex (Figs 3 and 4).

The electronic excitation energy generated enzymatically in biologically important molecules can be transferred to acceptor molecules, whereby the latter can be excited and undergo photophysical and photochemical transformations. The transfer of electronic excitation energy can be studied either with respect to the resulting

photochemical transformations or often with respect to quenching of electronically excited states of donor by molecules of a quenching agent.

The investigation of quenching of chemiluminescence was used in the present work to follow the properties of enzymatically generated electronically excited states. The Stern–Volmer plot for the quenching in the systems Hb^+–fluoresceine–H_2O_2 and (Hb^+–Hp)–fluoresceine–H_2O_2 are given in Fig. 2. As it can be seen from the picture the system with Hb^+–Hp complex exhibits larger quenching by indole, and the dependence observed is not linear. We presume that in this case the Hp molecule contributes to the quenching, too. An interesting and unexpected increase of chemiluminescence in the system Hb^+–fluoresceine–H_2O_2 after addition of indole, in our opinion, must be ascribed to the transfer of electronic excitation energy to indole under the conditions when the luminescence of indole itself takes place. We could verify that indole does not affect the peroxidase reaction proper which is responsible for the generation of electronically excited states of fluoresceine.

An increase of chemiluminescence was also observed after the addition of indole to a system in which the oxidase reaction catalyzed with Hb^+ took place (Fig. 3). In this case, too, we presume that the transfer of electronic excitation energy from triplet acetone to indole caused excitation of the latter species, and the luminescence of indole contributed to the total luminescence of the system studied. On the other hand, the addition of indole to the same system containing the Hb^+–Hp complex caused quenching of chemiluminescence (Fig. 4).

Acrylamide acts as quenching agent of the triplet acetone generated in the oxidase reactions with both Hb^+ and Hb^+–Hp (Fig. 5). The observed fact that the system with the Hb^+–Hp complex shows stronger quenching than that with Hb^+ in the presence of acrylamide is explained by conformational changes of the haem cavity of Hb^+ during formation of the complex with Hp (ref.[10]). These conformational changes obviously hinder the access of quenching agent molecules into the haem cavity in the complex where the generation proper of triplet acetone takes place.

From Figs 2 and 5 it can be seen that the quenching of chemiluminescence always distinctly depended on whether the system contained Hb^+ or the Hb^+–Hp complex. It can be stated that the electronically excited state formed in the close vicinity of the haem group is affected by the conformation of the nearest surroundings of the haem goup and by changes of this conformation due to formation of the complex. The haem cavity itself protects the electronically excited product molecules from collision quenching by the oxygen molecules dissolved in water. Due to this protection it is possible that the chemiluminescence of fluoresceine and triplet acetone are detected.

REFERENCES

1. Nyman M.: Scand. J. Clin. Lab. Invest., Suppl. 39, Vol. *11* (1959).

2. Frew J. E., Jones P. in: *Advances in Inorganic and Bioinorganic Mechanisms*, Vol. 3, p. 175. Academic Press, London 1984.
3. O'Brien P. J., Bechara E. J. H., O'Brien C. R., Duran N., Cilento G.: Biochem. Biophys. Res. Commun. *81*, 75 (1978).
4. Faria Oliveira O. M. M., Haun M., Durán N., O'Brien P. J., O'Brien C. R., Bechara E. J. H., Cilento G.: J. Biol. Chem. *253*, 4707 (1978).
5. Cilento G.: Pure Appl. Chem. *56*, 1179 (1984).
6. Kramlová M., Přistoupil T. I., Ulrych S., Hrkal Z.: Hematologia *10*, 365 (1976).
7. Pavlíček Z., Kalous V.: Collect. Czech. Chem. Commun. *29*, 1851 (1964).
8. Lipp A.: Justus Liebigs Ann. Chem. *1*, 305 (1880).
9. Vidigal C. C. C., Faljoni-Alário A., Durán N., Zinner K., Shimizu Y., Cilento G.: Photochem. Photobiol. *30*, 195 (1979).
10. Pavlíček Z., Jaenicke R.: Eur. J. Biochem. *18*, 305 (1971).
11. Pavlíček Z., Kalous V.: Collect. Czech. Chem. Commun. *38*, 3675 (1973).
12. Schuster G. B., Horn K. A. in: *Chemical and Biological Generation of Excited States* (W. Adam and G. Cilento, Eds), p. 229. Academic Press, London 1982.
13. Baader W. J., Bohne C., Cilento G., Dunford H. B.: J. Biol. Chem. *260*, 10217 (1985).
14. Bohne C., MacDonald I. D., Dunford H. B.: J. Biol. Chem. *262*, 3572 (1987).
15. Barltrop J. A., Coyle J. D.: *Principles of Photochemistry*. Wiley, New York 1978.

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