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Formation of adrenochrome from epinephrine by myeloperoxidase via a free radical : Its biological significance

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Abstract : Adrenochrome, 2, 3-dihydro-3-hydroxy-1-methyl-1H-indole-5, 6-dione, is an unstable red compound formed by the oxidation of epinephrine, (R)-4-[1-hydroxy-2-(methylamino) ethyl]-1, 2-benzenediol. A red pigment was generated during the reaction between epinephrine and hydrogen peroxide by myeloperoxidase via a short-lived intermediate having a free radical detected by electron paramagnetic (spin) resonance spectroscopy, possibly *o*-semiquinone free radical of the substrate (epinephrine), in a moderately alkaline pH buffer solution. The light absorption peaks of the pigment at 220, 302 and 485 nm were identical with those of adrenochrome, and the R_f-value of the pigment on thin layer chromatography was consistent with that of standard adrenochrome. From these findings, the pigment was concluded to be adrenochrome. The biological significance of the formation of adrenochrome by the myeloperoxidase system *in vivo* might relate to a possible role in the body's self-defense system : myeloperoxidase released from neutrophils may catalyze the conversion of epinephrine to adrenochrome at a site of trauma or phlogosis, and this adrenochrome would promote wound healing through the formation of a blood clot and/or scab by radical polymerization of body-fluid components around the affected part, resulting in acceleration of hemostasis.

抄録：ミエロペルオキシダーゼの触媒作用によって、弱アルカリ性の溶液中で、エピネフリン、(R)-4-[1-hydroxy-2-(methylamino) ethyl]-1, 2-benzenediol からアドレノクローム、2, 3-dihydro-3-hydroxy-1-methyl-1H-indole-5, 6-dione, が生成されることを吸収スペクトルの測定と薄層クロマトグラフィーを用いて証明した。このアドレノクロームの生成反応はエピネフリンの *o*-セミキノンと考えられるフリーラジカルをもつ短寿命の中間体を経由して進行していることが電子スピン共鳴 (ESR) スペクトルの観測から証明された。このミエロペルオキシダーゼによって生成されるアドレノクロームの生体内の役割として、創傷、炎症の場において、ラジカル重合によって止血し、痂皮形成を促し、病巣を速やかに治癒させることによって、生体を防御している可能性が考えられた。

Introduction

Adrenochrome is a well-known antihemorrhagic substance that is used clinically. It is formed from epinephrine^{1,2)}, a sympathomimetic hormone produced by the adrenal medulla as L-form in animals and man, as reviewed by Loewe³⁾, in a reaction at pH 5.0 catalyzed by catechol oxidase (EC 1. 10. 3. 1) from the

mushroom *Agaricus campestris*. It has been reported that at high pH, adrenochrome was formed from the oxidation of epinephrine by ceric, permanganate or ferricyanide⁴⁾ or during the autooxidation of epinephrine⁵⁾. We previously reported that myeloperoxidase catalyzes the reaction between epinephrine and hydrogen peroxide in neutral pH buffer solution to form a pigment having a light absorption peak at 485 nm, which was tentatively identified as

adrenochrome⁶). Myeloperoxidase (EC 1. 11. 1. 7) is a unique peroxidase, which has a heme similar to heme α as the prosthetic group^{7,8}) and catalyzes peroxidation of phenolic hydrogen donors such as *o*-methoxyphenol in neutral or alkaline solution⁷). It also catalyzes peroxidation of halides⁹), chlorination of prostaglandin E₁¹⁰), destruction of chemical compounds such as fuchsin basic and acid red No. 106¹¹), and cytotoxicity of B-16 melanoma tumor cells¹²) in the presence of a halide ion in moderately acidic conditions. It has been reported that free radicals were formed during the oxidation of epinephrine by inorganic redox agents⁴). It has also been reported that a semiquinone free radical was observed when hydroquinone as hydrogen donor was peroxidized by horseradish peroxidase, by using a flow method coupled with electron paramagnetic resonance spectroscopy¹³).

The aims of the present study were (a) to determine whether a semiquinone free radical of the substrate, epinephrine, is formed as an intermediate during the reaction between epinephrine and hydrogen peroxide catalyzed by myeloperoxidase, (b) to determine whether the light absorption peaks and R_f-value of the product of this reaction coincide in positions with those of standard adrenochrome, i. e., to confirm the identification of the pigment as adrenochrome, (c) to determine the pH-optimum of this reaction, and (d) to discuss the biological significance of the adrenochrome formed by this peroxidase system in terms of trauma and phlogosis.

Materials and Methods

Myeloperoxidase was prepared from polymorphonuclear leukocytes of normal pig blood⁹). The concentration of myeloperoxidase was calculated on the basis of a millimolar extinction coefficient of 95 mM⁻¹ cm⁻¹ at 430 nm. The myeloperoxidase preparation typically exhibited R. Z. value ($A_{430\text{nm}}/A_{280\text{nm}}$) of 0.85 and was previously confirmed to produce a single band on polyacrylamide gel electrophoresis. Adrenochrome and epinephrine (L-adrenalin) were purchased respectively from Sigma Co. (St. Louis) and Wako Chemical Industries (Osaka). Epinephrine and adrenochrome in the present study were dissolved in

0.1 M acetic acid and water respectively. The concentration of epinephrine was estimated from the molar extinction coefficient of 2,754 at 280 nm in 0.1 M HCl¹⁴). The concentration of adrenochrome was estimated from the molar extinction coefficient of 3,981 at 490 nm in water¹⁴). Spectrophotometry was carried out by Hitachi double beam spectrophotometer, type 200-20, or Hitachi double wavelength-double beam spectrophotometer, type 557. Thin layer chromatography was carried out using a Merck silica gel TLC plate (type RP-8 F_{254s}). Samples for the thin layer chromatography were prepared as follows. Hydrogen peroxide (0.4 mM) and myeloperoxidase (16 nM) were added to 7.5 mM epinephrine solution in 0.08 M acetic acid mixed with 0.1 M potassium phosphate (dibasic) solution (pH 6.0) and this reaction mixture was incubated for 20 min. Immediately, an aliquot of 10 μ l of the reaction mixture was applied. The colored spot was ascertained visually. Detection of the spots was also carried out under ultraviolet light at wavelength 254 nm. The electron paramagnetic (spin) resonance spectrometer used was a Varian V-4500 x-band instrument, utilizing a 100 kcycles field modulation attached flow apparatus, equipped with dual sample chambers¹⁵). One chamber was filled with 1 mM epinephrine and 0.2 mM hydrogen peroxide in 0.1 M phosphate buffer (pH 7.0), and the other with 0.7 μ M myeloperoxidase in the same buffer. The flow rate of the mixture was 8 ml per second. The control experiments were carried out using water instead of myeloperoxidase or hydrogen peroxide, or by stopping the flow.

The buffers used were acetate (acetic acid/sodium acetate) for pH 4.0-6.0, phosphate (potassium phosphate, monobasic/sodium phosphate, dibasic) for 6.0-8.0 and borate (boric acid/sodium carbonate) for pH 8.0-9.0.

All experiments were carried out at 25°C.

Results

A pigment with light absorption peaks at 220, 302 and 485 nm, clearly consistent with those of adrenochrome, was formed from the reaction between epinephrine, which does not absorb in the visible region,

and hydrogen peroxide catalyzed by myeloperoxidase (Fig. 1). As shown in the inset of Figure 1, the formation of this pigment was observed in the presence of myeloperoxidase, but no reaction was observed when myeloperoxidase was omitted. This reaction was accelerated by increasing the concentration of myeloperoxidase, hydrogen peroxide or epinephrine (Figs. 2, 3 and 4). The rate constant, k_4 , for the reaction between myeloperoxidase hydrogen peroxide complex (compound II) and epinephrine was calculated to be $8.8 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$ from the result of Figure 4 by Chance's equation¹⁶⁾ for overall kinetics. The effect of pH on the peroxidation of epinephrine mediated by myeloperoxidase was examined in the range of pH 4.0 to 9.0 (Fig. 5). The fastest rate was

Fig. 1 Light absorption spectra of pigment formed by the reaction between epinephrine and hydrogen peroxide catalyzed by myeloperoxidase and the standards, epinephrine and adrenochrome.

Curve a shows the light absorption spectrum of epinephrine ($73 \mu\text{M}$) in 0.05 M phosphate buffer ($\text{pH } 7.5$). Curve b shows the light absorption spectrum recorded 15 min after hydrogen peroxide ($40 \mu\text{M}$) and myeloperoxidase (16 nM) were added to epinephrine ($73 \mu\text{M}$) in 0.05 M phosphate buffer ($\text{pH } 7.5$). Curve c shows the light absorption spectrum of $68 \mu\text{M}$ adrenochrome in 0.05 M phosphate buffer ($\text{pH } 7.5$).

Inset shows the changes in absorbance at 485 nm with time for the reaction mixture in the presence (time course a) or absence (time course b) of myeloperoxidase. The conditions were the same as curve (b). The reaction was started by adding myeloperoxidase (time course a) or hydrogen peroxide (time course b) at the time shown with an arrow.

observed at $\text{pH } 8.0$ in phosphate buffer. To identify the pigment formed from epinephrine by the myeloperoxidase system, thin layer chromatography was performed. When the reaction mixture of epinephrine, hydrogen peroxide and myeloperoxidase was spotted to chromatoplate, air dried and developed, only one pink spot was observed (Fig. 6A). The R_f -value of the pink component was 0.74 , which was clearly consistent with that of the pink spot¹⁷⁾ of standard adrenochrome. Both were short-lived and darkened within approximately 20 min. Under ultraviolet light of 254 nm , two fluoresced spots were detected and their R_f -values, 0.92 and 0.74 were within the standards of epinephrine ($R_f, 0.92$) and adrenochrome ($R_f, 0.74$), respectively (Fig. 6B). When myeloperoxidase was omitted from the reaction mixture, no spot equal to adrenochrome was detected visibly or under UV. From these findings, it was concluded that the pigment having absorption peaks at $220, 302$ and 485 nm was adrenochrome. During the reaction, in which adrenochrome was formed from epinephrine catalyzed by myeloperoxidase, a short-lived intermediate with a free radical, possibly a semiquinone free radical of epinephrine, was detected by electron paramagnetic (spin) resonance spectroscopy (Fig. 7A). Small signals observed in the spectra

Fig. 2 Effect of myeloperoxidase concentration on the formation of adrenochrome.

The activity was assayed at 0.33 mM epinephrine, $40 \mu\text{M}$ hydrogen peroxide and 0.05 M phosphate buffer ($\text{pH } 7.5$) and at variable concentrations of myeloperoxidase.

Fig. 3 Effect of hydrogen peroxide concentration on the formation of adrenochrome.

The activity was assayed at 0.33 mM epinephrine and 16 nM myeloperoxidase and 0.05 M phosphate buffer (pH 7.5) and at variable concentrations of hydrogen peroxide.

Fig. 5 Effect of pH on the formation of adrenochrome.

Reaction mixture contained 0.33 mM epinephrine, 40 μ M hydrogen peroxide and 16 nM myeloperoxidase. The buffers used were acetate for pH 4.0-6.0 (—○—○—), phosphate for 6.0-8.0 (—●—●—) and borate for pH 8.0-9.0 (—△—△—) at concentrations of 0.05 M.

of control samples without myeloperoxidase or hydrogen peroxide were negligible in comparison with the intensity of signals in the complete system composed of epinephrine, hydrogen peroxide and myeloperoxidase (Fig. 7, curves C and E).

Discussion

The pigment formed by the peroxidation of epi-

Fig. 4 Effect of epinephrine concentration on the formation of adrenochrome.

The activity was assayed at 40 μ M hydrogen peroxide and 16 nM myeloperoxidase and 0.05 M phosphate buffer (pH 7.5) and at variable concentrations of epinephrine.

Fig. 6 Thin layer chromatograms of the product formed by the reaction of epinephrine, hydrogen peroxide and myeloperoxidase, and the standards, epinephrine and adrenochrome.

1. Reaction mixture. The reaction mixture contained 7.5 mM epinephrine, 0.4 mM hydrogen peroxide and 16 nM myeloperoxidase (pH 6.0) ; 2. standard adrenochrome ; 3. standard epinephrine. A : Self-indicating (a pink color) ; B : fluorescing when exposed to ultraviolet light of wavelength 254 nm. The developing solvent was a mixture of methanol and 0.1 M acetate buffer (pH 4.5) (9 : 1, v/v). Other conditions are detailed in Materials and Methods.

nephrine mediated by myeloperoxidase at neutral pH was identified as adrenochrome by measuring its absorption spectrum and R_f-value by thin layer chromatography and comparing it with those of standard adrenochrome (Figs. 1 and 6). The adrenochrome formation rate increased with the increased concentration of myeloperoxidase, hydrogen peroxide

Fig. 7 Electron paramagnetic (spin) resonance spectrum of semiquinone free radical observed during the reaction between epinephrine and hydrogen peroxide mediated by myeloperoxidase.

A : Semiquinone free radical. B : Control, traced by stopping the flow under the conditions of (A). C : Control, traced under flowing (water was used instead of myeloperoxidase). D : Control, traced by stopping the flow under the conditions of (C). E : Control, traced under flowing (water was used instead of hydrogen peroxide). F : Control, traced by stopping the flow under the conditions of (E). The reaction conditions are detailed in Materials and Methods.

or epinephrine (Figs. 2, 3 and 4). The value of a rate constant, k_4 , which is a parameter to show the magnitude of peroxidase activity of myeloperoxidase toward epinephrine, was comparable with that of the k_4 of this enzyme toward *o*-methoxyphenol⁷⁾. From this, it was concluded that the conversion of epinephrine to adrenochrome mediated by myeloperoxidase was due to the peroxidase activity of this enzyme. We also detected free radicals, possibly the *o*-semiquinone free radical of epinephrine, generated from epinephrine by myeloperoxidase. Adrenochrome may be generated by the following reactions :

This mechanism is basically the same as that of the peroxidation of hydroquinone catalyzed by horseradish peroxidase reported by Yamazaki *et al.*¹³⁾. However the myeloperoxidase also catalyzes the peroxidation of halides, chloride, bromide and iodide

at low acidity⁹⁾. Furthermore, as reported in previous papers¹⁰⁻¹²⁾, at moderately acidic pH, the optimum pH values being between 4.7 and 5.5, this peroxidase together with hydrogen peroxide and a halide constitutes a potent oxidation system, which destroys targets such as fuchsin basic and acid red No.106, halogenates prostaglandin E₁ and cytolyzes melanoma cells, resulting in the death of the cells. This range of pH change, from neutral or slightly acidic to moderately acidic (pH 4.7 to 5.2), occurs in the cytoplasm of leukocytes during phagocytosis as reported by Pavlov and Solov'ev¹⁸⁾, suggesting that the action of myeloperoxidase in neutrophils at acidic pH is dependent on a halide ion.

The present study suggests a further biological role of myeloperoxidase in the promotion of healing. At a site of trauma or phlogosis, where there is an accumulation of body fluids, which normally lie within the range from neutral to slightly or moderately alkaline, or where the environment becomes neutral to slightly alkaline, the myeloperoxidase released from neutrophils^{19,20)} functions as part of a system generating adrenochrome. This, in turn, may participate in wound healing by promoting the formation of a blood

clot and/or scab by radical polymerization, forming a melanin-like substance¹⁾, and resulting in the acceleration of hemostasis^{2,21,22)}.

In conclusion, myeloperoxidase may act in two ways in the body's self-defense system. One is in a halide-dependent reaction system scavenging xenobiotics directly in moderately acidic conditions in lysosomes acidified on stimulation by xenogenous substances. The other is in the adrenochrome-generating system in moderately alkaline conditions in resting lysosomes of neutrophils, outside of the lysosomes in neutrophils, or outside of the cells. Thus, it appears that the biological function of myeloperoxidase is regulated by the change of pH in the environment.

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