BISTABILITY IN ENZYMATIC OXIDATION OF HETEROAUXIN WITH INHIBITION BY CAFFEIC ACID

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Enzymatic oxidation of the phytohormone heteroauxin (indolyl-3-acetic acid, IAA) plays an important role in its metabolism [1]. Regulation of enzymatic oxidation of IAA in vivo occurs by action of natural inhibitants of this reaction. These include in particular some phenol compounds. The mechanism for the inhibiting effect of substituted phenols has been investigated in detail in a number of papers [2, 8]. As a result, the idea has persisted that substituted phenols are competing inhibitors for this reaction [2-6, 8]. This hypothesis has a number of objective disadvantages, in particular the Lineweaver–Burk plots certainly show a nonlinear dependence [9]. Accordingly, the mechanism of the effect of phenol inhibitors on enzymatic oxidation of IAA requires further experimental investigation.

This paper is devoted to the study of the mechanism of action on aerobic oxidation of IAA catalyzed by peroxidase (without addition of (H_2O_2)) for a natural inhibitor of this reaction: caffeic acid (CA) [10]. We have used the chemiluminescent method for the first time to study the effect of inhibitors on oxidation of IAA. This method is based on the high sensitivity of the kinetics and the chemiluminescence spectra for chemiluminescence arising during the course of this reaction to the reaction conditions [11]. Along with the chemiluminescent method, we used the spectrophotometric method. Formation of the oxidation products of IAA was observed from the difference in absorption at 242 and 296 nm (the wavelengths with equal absorption for IAA).

The differential optical density of the reaction mixture was determined on the Hitachi 557 spectrophotometer (Japan), using the two-wavelength mode and quartz cuvettes with optical path length 1 cm. The chemiluminescence kinetics were measured on a specially designed chemiluminometer [11]. We introduced the following addition to the design of the chemiluminometer compared with that used in [11]: six cuvettes with reaction mixtures were placed in a rotating drum, set into motion by a stepper motor. We used the OVS-1 illuminator for irradiation of the reaction mixture.

In this work, we used the following reagents: horseradish peroxidase (HRP) RZ 3.0, indolyl-3-acetic acid, salt components of the phosphate buffer, caffeic acid, Bengal Rose (Sigma, USA). Anfen, an original water-soluble free-radical trap, was synthesized and kindly provided by A. A. Volod'kin (Institute of Chemical Physics, Russian Academy of Sciences, Moscow). All the solutions were prepared using triply distilled deionized water. The standard reaction mixture, unless otherwise stipulated, contained 1 mM IAA, 0.1 μM HRP in 0.067 M phosphate buffer (pH 7.4) and had a volume of 3 ml.

In studying the effect of CA on the IAA/HRP/O_2 system, we observed that this enzymatic system is bistable. There are two stable states: "reaction occurs" and "no reaction." In the absence of CA, the system IAA/HRP/O_2 is always found in the "reaction occurs" state. The transition from the "reaction occurs" state to the "no reaction" state occurs when the concentration of the added CA exceeds some threshold value. In this case, the chemiluminescence stops and does not begin again over the entire priod of continuous monitoring (Fig. 1). Spectrophotometric measurements confirm that in the "no reaction" state, oxidation of IAA does not occur (Fig. 2). Transition from the "no reaction" to the "reaction occurs" state is achieved by introducing an additional amount of IAA solution into the system or by briefly illuminating the reaction miture with visible light in the presence of a photosensitizer, Bengal Rose. We must emphasize that introduction of an additional amount of HRP does not cause the transition "no reaction" → "reaction occurs."

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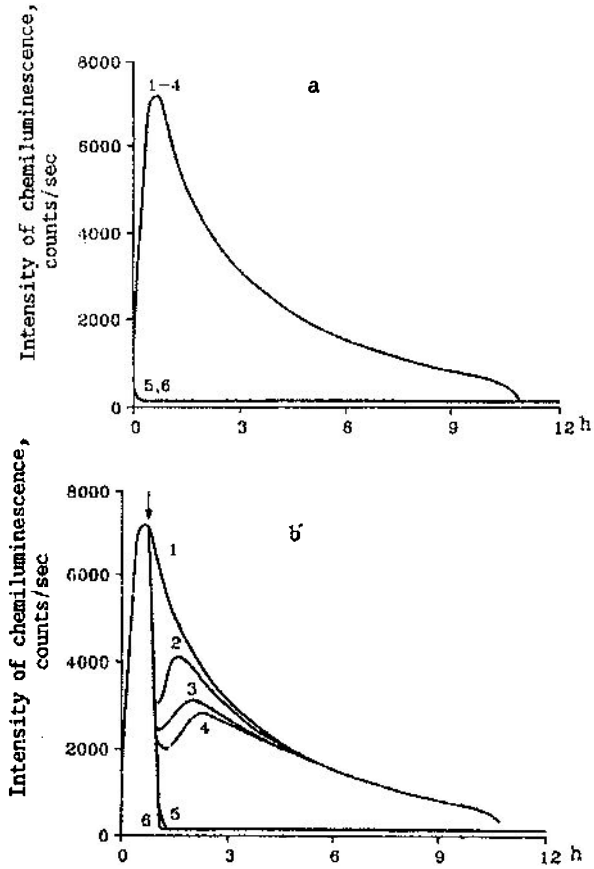

Fig. 1. Effect of CA on the chemiluminescence kinetics of the system IAA (1 mM)/HRP (0.1 μM)/O_2: a) HRP is added to the IAA–CA mixture: [CA], μM 0 (1), 0.08 (2), 0.16 (3), 0.24 (4), 0.33 (5), 0.41 (6); CA is added 45 min after preparation of the IAA–HRP mixture (the arrow indicates the moment at which the CA is added); [CA], μM: 0 (1), 1.3 (2), 2.3 (3), 3.2 (4), 4.0 (5), 4.9 (6).

If CA is added to the IAA–HRP mixture, then the threshold concentration of CA (at which transition occurs from the "reaction occurs" state to the "no reaction" state) increases with an increase in the HRP and IAA concentrations. If HRP is added to a IAA–CA mixture, then the threshold concentration of CA does not depend on the HRP concentration, but is directly proportional to the IAA concentration. The threshold concentration of CA depends on the moment it is added to the IAA/HRP/O_2 system. It reaches a maximum value when the CA is added 10 min after the beginning of the reaction. Upon further increase in this time interval, the thresold concentration of CA decreases. An effect analogous to that of CA is observed upon introduction into the IAA/HRP/O_2 system of a substance which is not specific for this reaction: anfen, which is known as a radical trap. The impossibility of initiating the transition from the "no reaction" state to the "reaction occurs" state by introduction of an additional amount of HRP clearly indicates that CA does not deactivate the enzyme. The lack of a dependence of the threshold concentration of CA on the HRP concentration (if the CA is added to IAA earlier than the HRP) also indicates a nonenzymatic pathway for the inhibition. Furthermore, the threshold concentration of CA is about three orders of magnitude lower than the initial IAA concentration in the reaction mixture. The latter refutes the possibility of removal of IAA from the reaction as a result of its binding with CA.

Let us assume that CA acts on some third hypothetical substance R. This substance obviously is found in trace amounts in the solution of the substrate, which is confirmed by the possibility of initiating the transition from the "no reaction" state to the "reaction occurs" state by introducing into the reaction mixture of an additional amount of IAA solution. Furthermore, the substance R is produced in the course of enzymatic oxidation of

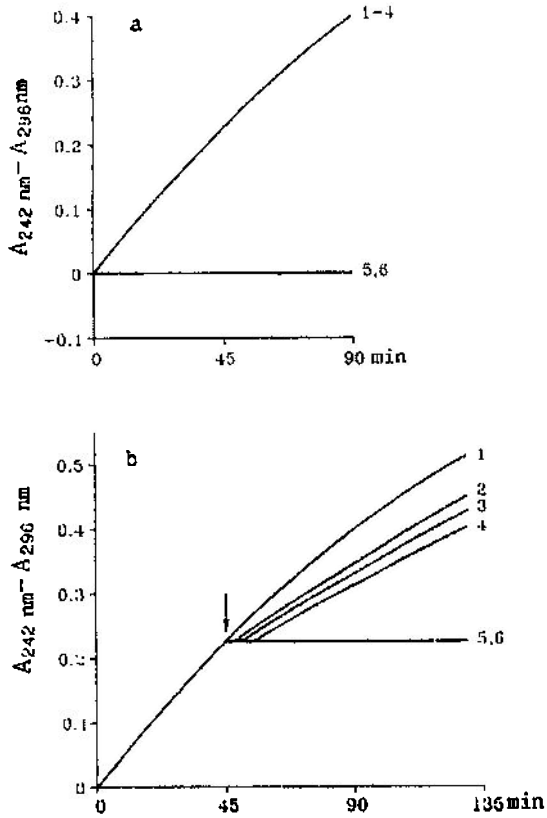

Fig. 2. Effect of CA on the kinetics of oxidation of IAA in the system IAA (1 mM)/HRP (0.1 μM)/C_2. Conditions were the same as in Fig. 1.

IAA. This is indicated by the increase in the threshold concentration of CA with an increase in the time interval before the beginning of the reaction and the moment of the threshold concentration of CA on the HRP and IAA concentration.

The nature of the substance R is unknown. However, the following indirect data allow us to hypothesize that R is a free radical. In fact, inhibition of the "no reaction" → "reaction occurs" transition may be induced by irradiation with light in the presence of a sensitizer, Bengal Rose, which upon irradiation by light gives a high yield of triplet-excited molecules [12]. The latter, being essentially biradicals, are capable of causing efficient formation of free radicals [13]. Furthermore, we know that peroxidase–oxidase reactions (as the system IAA/HRP/O_2 is classified) can occur according to a free-radical chain mechanism [14]. Formation of free radicals over the course of enzymatic oxidation of IAA was studied by Mottley and Mason [15]. They in particular identified the skatol-radical. If the hypothesis concerning the free-radical nature of R is correct, then CA should obviously play the role of a trap for the free radicals R· (considering the above hypothesis concerning the free-radical nature of the hypothetical substance R, for the following we will use R· instead of the symbol R). Confirmation of this hypothesis is the fact that the effects of CA and anfen (known as a radical trap) on the IAA/HRP/O_2 system are identical.

Thus in order to explain the bistability, let us hypothesize that enzymatic aerobic oxidation of IAA is a free-radical branched chain reaction, and that CA quenches the free radicals participating in the chain reaction by means of radical transfer. The following reaction scheme is minimal for describing the bistability:

$$\mathrm{IAA} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{R}^{\cdot}, \tag{1}$$

$$\mathrm{IAA} + \mathrm{R}^{\cdot} \xrightarrow[\mathrm{HRP},\ \mathrm{O_2}]{k_2} 2\mathrm{R}^{\cdot}, \tag{2}$$

$$\mathrm{R}^{\cdot} + \mathrm{R}^{\cdot} \xrightarrow{k_3}, \tag{3}$$

$$R^{\cdot} + CA \xrightarrow{k_4} P + CA^{\cdot}, \quad (4)$$

$$CA^{\cdot} \xrightarrow{k_5} CA, \quad (5)$$

$$CA^{\cdot} + CA^{\cdot} \xrightarrow{k_6}. \quad (6)$$

Reaction (1), which obviously proceeds at low rates with participation of the enzyme, is the source of trace amounts R· in the IAA solution. Reaction (2) is the reaction of enzymatic oxidation of IAA with positive feedback through R·, or in other words a free-radical branched chain reaction. Reaction (3) describes processes of quadratic chain termination. This reaction limits propagation of the chain for large R· concentrations. Reaction (4) is the transfer radical with R· to the CA molecule with formation of some product P and the radical CA·. We suggest that it is specifically this process which describes the inhibiting effect of CA·. Reaction (5) indicates the possibility of reduction of the CA· to the CA molecule, without specifying the electron donor. Reaction (6) is an obvious reaction of irreversible quadratic annihilation of CA·, for example as a result of dimer formation.

The system of reactions (1)-(6) is minimal for description of the bistability in the reaction of enzymatic oxidation of IAA with inhibition by caffeic acid. According to the given equations, there exists some critical concentration of CA at which the multiplication coefficient of the R· radicals will be equal to unity. If the CA concentration is above the critical level, then the multiplication coefficient is less than unity; if the CA concentration is lower than the critical level, then the multiplication coefficient is greater than unity. In the first case, the reaction stops (the "no reaction" state). In the second case, the chain reation propagates in an avalanche fashion until its rate is limited by reaction (3) (the "reaction occurs" state). In this case, the CA concentration tends toward zero relatively rapidly as a result of reactions (4) and (6). The critical concentration of CA obviously corresponds to the experimentally determined threshold CA concentration.

Summing up, we must say that the bistability we observed in the reaction of enzymatic oxidation of IAA with inhibition by caffeic acid and the study of the characteristics of the bistability clearly show that the idea persisting in the literature [2-6, 8] concerning the competing enzymatic character of the inhibition of this reaction by phenol inhibitors is mistaken.

In conclusion, we thank V. V. Lazarev and F. I. Ataullakhanov for useful discussion of the results of our work.

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STUDY OF REACTIONS OF PHOSPHOLIPASE A_2 WITH POLYMERIC LIPOSOMES

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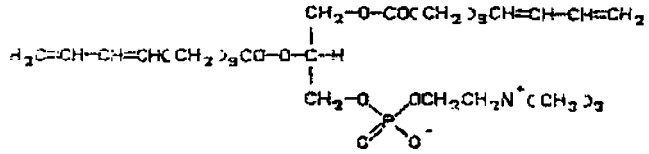
Over the last decade, there has been intense research in the area of polymeric lipid membranes [1, 2]. The high stability of polymeric membranes allows us to use them to model some properties of biological membranes, and also to solve a number of practical problems. Thus it was shown in [3, 4] that deposition of polymeric bilayers of diacetylene phosphatidylcholines on the surface of some synthetic materials significantly increases the heme compatibility of the latter. This effect is due to the fact that lipid bilayers are located on the surface which are structurally analogous to the lipid matrix of biological membranes.

In order to solve such problems, the lipid bilayers must have high stability and be stored in biological media. Furthermore, the polymeric matrix within the bilayer should not distort the surface of the latter or disrupt the mechanisms of interaction with various biomolecules.

In this paper, we present the results of a study of the interaction between polymeric liposomes and the lipolytic enzyme phospholipase A_2, for which a natural substrate is phospholipid molecules with bilayer membrane. Interaction of the phospholipase A_2 with lipid molecules is a sensitive test for the native character of the properties of the surface of a lipid membrane.

Earlier we suggested 1,2-di(11,13-tetradecadienoyl)-sn-glycero-3-phosphocholine (1) for obtaining polymeric phosphatidylcholine membranes. The polymerizable diene groups in this lipid are found at the ends of the hydrocarbon chains, i.e., in the bilayer they are removed as far as possible from the surface. Polymerization of this phosphatidylcholine within the liposome imparts high stability to the latter. Polymeric liposomes of lipid (1) are not destroyed in the presence of detergents, in a medium of organic solvents, or under the action of ultrasound [5].



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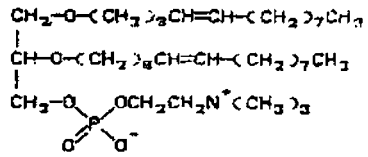

(2)

As a model for biological membranes, we used multilamellar liposomes. In order to obtain the comparative characteristics, we studied the reaction of the phospholipase characteristics, we studied the reaction of the phospholipase A_2 with monomeric and polymeric

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