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THE ROLE OF SUPEROXIDE RADICALS IN LACTOPEROXIDASE-CATALYSED H_2O_2 - METABOLISM AND IN IRREVERSIBLE ENZYME INACTIVATION

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Irreversible inactivation of lactoperoxidase in the presence of excess H_2O_2 has been investigated. Serial overlay absorption spectra of the Soret region show that the rate and total amount of enzyme inactivation depend on the proton concentration. Perhydroxyl or superoxide radicals ($HO_2^{\cdot}$ or O_2^{-}) cannot be established as the inactivating species in this mechanism, but they influence the rate of reconversion of the intermediate lactoperoxidase-compound III back to the resting ferric form of the enzyme.

Lactoperoxidase (LPO, EC 1.11.1.7) displays different UV/VIS absorption spectra in the Soret region in the course of the reaction with hydrogen peroxide (H_2O_2) depending on the excess of H_2O_2 [1-3]. The first intermediate, referred to as compound I (cpd I), which is formed by addition of H_2O_2 in an equimolar ratio with LPO, can only be observed using special techniques such as stopped-flow [2,3]. However, cpd II (λ_{max} = 430 nm), the reaction product of cpd I with a 1-e^- donor molecule [3], is easily produced and recorded by addition of H_2O_2 (up to about 50 μM at physiological pH) to the resting state of LPO (3 μM, λ_{max} = 411 nm). Reconversion to native LPO occurs by way of an additional 1-e^- transfer reaction. Addition of H_2O_2 in excess of 50 μM leads, via cpd II, to formation of cpd III (λ_{max} = 423 nm) [1]. This state is reconverted to the resting ferric enzyme via ferrous LPO (λ_{max} = 435 nm) [2]. The λ_{max}

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of these intermediates are indicated in Figs 1 and 2. Cpd III has been suggested to be an intermediate in the reaction leading to irreversible inactivation of the enzyme by cleavage of the heme moiety and liberation of iron [1,2].

The aim of this communication is to shed light on the role of perhydroxyl or superoxide radicals ($HO_2^{\cdot}$, O_2^{-}, pK_a 4.8 [4]), which are very likely to be formed in a system lacking in an additional e^- donor [1,2] and/or containing free ferric iron and H_2O_2 [5], in the irreversible inactivation of LPO.

MATERIALS AND METHODS

All chemicals were of analytical grade and obtained from Merck (Darmstadt, FRG) or Fluka (Buchs, Switzerland) except for hydrogen peroxide (H_2O_2, obtained from Siegfried, Zofingen, Switzerland), lactoperoxidase (LPO, EC 1.11.1.7, 70 Units/mg solid, ratio A_{412}/A_{280} = 0.85, obtained from Sigma, St. Louis, MO), and superoxide dismutase (SOD, EC 1.15.1.1, used as Peroxinorm®, Grünenthal, FRG, containing orgotein (=SOD) and saccharose in a ratio of 1:2).

Compound conversion of LPO was measured by UV/VIS absorption spectroscopy and serial overlay plotting of the spectra. Fast conversions of LPO to cpd II via cpd I or to cpd III via cpds I and II were monitored on a Hewlett-Packard 8451 A diode array spectrophotometer (0.2 or 0.5 second cycles) and the slower reconversion from cpd III back to the resting ferric enzyme via the ferrous state on a UVIKON model 810 spectrophotometer combined with a UVIKON 21 recorder (0.5 or 1.0 minutes intervals for the Soret region (350-450 nm)). Recovery of LPO was calculated from the remaining absorbance at 411 nm compared to the initial absorbance before the reaction. All reactions were performed in 0.05 M phosphate buffer of pH 5.8, 7.33, or 8.0.

RESULTS AND DISCUSSION

Addition of excess H_2O_2 (e.g. 2 mM) to LPO (3 µM) leads to formation of cpd III (Fig. 1A-C). In parallel to the longer lifetime of cpd III at acidic pH conditions, destruction of the enzyme increases from pH 8.0 (79 % recovery, Fig. 1C) to pH 7.33 (59 % recovery,

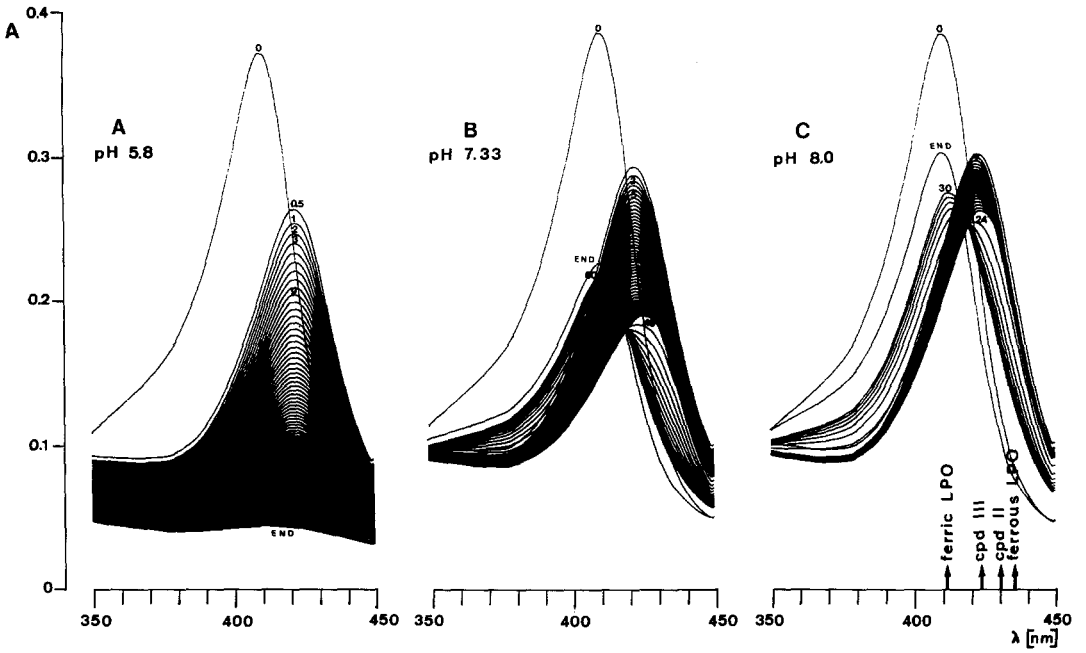

Fig. 1 LPO inactivation as a function of pH monitored by serial overlay plotting of spectra in the Soret region (350-450 nm). Numbers inserted indicate minutes after the start of the reaction. Addition of H_2O_2 (2 mM) to LPO (3 μM) results in the formation of cpd III (λ_{max} = 423 nm). Decreasing absorption without compensatory formation of another cpd indicates irreversible enzyme inactivation. When most of the excess H_2O_2 is used up, the peak of cpd III shifts towards higher wavelengths and back to the resting ferric enzyme (λ_{max} = 411 nm) forming an isosbestic point at 420 nm. Whereas at pH 5.8 the enzyme is fully destroyed, a part of LPO escapes from inactivation at pH 7.33 and 8.0, most probably due to the activation of an alternative pathway which is able to metabolize H_2O_2 faster than the cpd III way.

Fig. 1B) and pH 5.8 (all LPO destroyed after 90 minutes, Fig. 1A). Less H_2O_2 (e.g. 250 μM) destroys 21 % LPO at pH 5.8, 10.5 % at pH 7.33, and only 1.5 % at pH 8.0, where cpd III formation is nearly omitted apart from a slight blueshift (results not shown). Although the exact nature of this pH effect on enzyme inactivation is not known at present, different pathways of H_2O_2 metabolism could be activated depending on the proton concentration. At acidic pH a reaction dominates which favours cpd III formation and destroys the enzyme (Fig. 1A). Protonation of O_2^- leads to $HO_2^\cdot$ which is much more

reactive than is O_2^- [6] and may cause formation of further reactive oxygen species such as hydroxyl radicals [5,6]. Iron liberated in the course of LPO inactivation [1,2] could well catalyse this OH· formation [5,6]. The inactivation rate is slowed down at neutral (Fig. 1B) and alkaline pH (Fig. 1C), where H_2O_2 seems to be used by a faster non-inactivating competing reaction, which omits the rate-limiting "cpd III-way". As a consequence, H_2O_2 is withdrawn from maintaining the cpd III state.

Degradation products of H_2O_2 metabolism such as molecular oxygen in its triplet ($^3\Sigma_g^-$ O_2) or singlet state ($^1\Delta g$ O_2), superoxide radicals ($HO_2^\cdot$, O_2^-, pK_a 4.8 [4]), or hydroxyl radicals (OH·) would affect the enzyme differently (for reviews see [1,2,6-8]). The correlation of irreversible LPO inactivation with oxygen production and the partial escape from degradation after supply of alcoholic OH· scavengers are described elsewhere [1,2]. In the present report we try to point out the effects of superoxide radicals on compound formation and enzyme inactivation. For this purpose the experiment of Fig. 1B (LPO 3 μM, H_2O_2 2 mM) has been repeated including superoxide dismutase at various concentrations (no SOD in Fig. 2A, 3.7 μM in Fig. 2B, 7.4 μM (not shown), 12.3 μM SOD in Fig. 2C). Whereas inactivation rate and enzyme recovery are not significantly affected by SOD, reconversion of cpd III via ferroperoxidase back to the resting ferric enzyme [2] is considerably slowed down.

The results suggest that superoxide radicals do not seem to be directly involved in the mechanism leading to irreversible inactivation of LPO, but take part in the reaction where cpd III is converted back to the resting ferric enzyme [2]. Due to the fact that SOD influences a reaction which displays an isosbestic point between two intermediate states (Figs 2A-C), a damaging effect of superoxide radicals in bulk solution on LPO can probably be excluded. It should be realized however that the radicals could be generated at or near

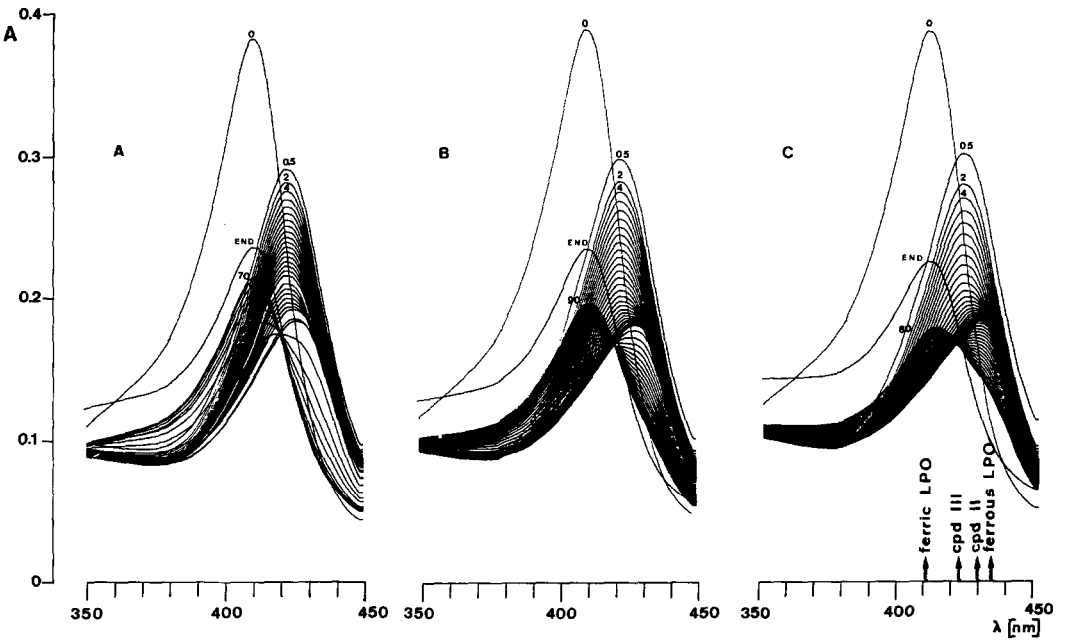

Fig. 2 Influence of SOD on the rate of conversion from the intermediate ferrous LPO back to the resting ferric enzyme. Numbers inserted indicate minutes after the start of the reaction.
Addition of SOD (3.7 μM in Fig. 2B, 12.3 μM in Fig. 2C) slows down the reconversion rate to native LPO compared to a sample without SOD (Fig. 2A). Concentrations of LPO 3 μM and of H_2O_2 2 mM. Due to the fact that SOD influences a reaction which displays an isosbestic point between two intermediate states, a damaging effect of superoxide radicals on LPO can probably be excluded.

the active center of the enzyme at a site where the bulky SOD molecule might not have free access. In this case, $HO_2^{\cdot}$ or O_2^- or consequently formed $OH^{\cdot}$ could still damage the enzyme. On the other hand, there is little doubt on the fact that superoxide radicals take part in the conversion of cpd III to ferric LPO. Control experiments with albumin and (goat-anti rabbit) γ-globulin, added in the same concentrations as SOD to exclude non-specific protein effects, did not significantly alter the conversion rate of cpd III to ferric LPO.

Although the molecular mechanism of this reaction is not yet known, it is conceivable that $HO_2^{\cdot}$ and/or O_2^- act as e^- acceptors in the redox reaction converting LPO[Fe-II] to the resting LPO[Fe-III]

[2]. SOD, which dismutates superoxide radicals to peroxide and molecular oxygen, competitively reacts with the e^- acceptor and may thus slow down the conversion rate. Other mechanisms involving superoxide radicals and cpd III conversion to ferric LPO are conceivable. We are investigating this aspect further using spin trapping experiments to attempt to elucidate the single steps and the components involved in this reaction.

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