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Evidence for the high-spin heme iron in both stable and unstable reduced forms of lactoperoxidase: low-temperature magnetic circular dichroism data

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Abstract The unstable and stable ferrous lactoperoxidase at pH 6.0, 7.0 and 10.2 have been analysed using optical absorption and variable temperature MCD spectroscopy. The evidence is given that two high-spin forms of ferrous LPO are always observed when the enzyme is reduced in a buffer-glycerol mixture at low temperature (ca. −20°C) at which no spectral changes are seen for a long time after the reduction. Form 1 (the absorption band, 450 nm) dominates significantly over form 2 (the absorption band, 435 nm), but a relative content of form 2 increases on lowering the pH value. An annealing of the unstable LPO at high temperatures is followed by complete irreversible conversion of form 1 to form 2. In addition, at least one low-spin ferrous form exists in temperature-dependent equilibrium with the high-spin form(s) in both stable and unstable ferrous LPO. The reversible increase of its content is observed at least down to 140 K, suggesting that minor structural changes are sufficient for reaching the heme iron by a distal amino acid residue (presumably a histidine).

Key words: Lactoperoxidase; Hemoprotein; Magnetic circular dichroism; Spin state; Axial ligand

1. Introduction

Lactoperoxidase (LPO; EC 1.11.1.7) is a glycoprotein of molecular mass ~78500 found in milk, saliva and tears [1,2]. The enzyme catalyses an oxidation of thyocyanate to antimicrobal hypocyanate ion and functions as a component of the biological defense system [3,4]. Previous analysis using pronase digestion suggests that LPO has a non-covalently bound protoheme as a prosthetic group [5]. However, more recent investigation shows that the prosthetic group is bound to a protein moiety by a disulphide linkage which, when broken, gives a heme thiol [6]. A number of spectroscopic studies imply that LPO has a narrow heme pocket and a histidine imidazole is the fifth (proximal) ligand of the heme iron [7-10]. The covalent heme binding may be the cause of spectral and other differences observed between LPO and other peroxidases.

At least two spectroscopically distinct forms of LPO are observed when the enzyme is reduced either electrochemically with Pt-H2 and a mediator [11] or chemically by addition of sodium dithionite [1,7]. A primary unstable reduced form rearranges spontaneously into the stable product. The rate of conversion significantly increases at low pH values [12]. Although the unstable form is thought to have high-spin heme iron there are doubts concerning the spin state of the stable form. RR data in high-frequency region show noticeable difference in the position of a spin marker band for the two reduced forms [13]. An XAS analysis suggests a change in coordination number of the heme iron of ferrous LPO from 5 in the unstable form to 6 in the stable one as a result of shortening of one of the axial distances by ca. 0.2 A [14]. However, both methods do not unambiguously support the presence of two different spin states for ferrous LPO forms. Also, the visible absorption spectrum of the stable reduced LPO does not resemble that of a typical high- or low-spin heme [7].

This communication reports low-temperature MCD studies of the products produced by sodium dithionite reduction of LPO. Temperature-dependent MCD spectra were shown to be a very appropriate tool to monitor a spin state of hemoproteins [15]. Evidence is given that both unstable and stable reduced LPO have the high-spin heme iron. In addition, a small fraction of low-spin ferrous LPO is always observed at low temperatures.

2. Materials and methods

Bovine LPO from Sigma (ratio $A_{413}/A_{280} = 0.91$) was used without further purification. The EPR spectra obtained for Sigma protein were shown to be identical with those of the fresh LPO [16]. The enzyme was dissolved either in 0.1 M sodium phosphate (pH 6.0 and 7.0) or in 0.1 glycine-phosphate (pH 10.2) buffer. The LPO concentration was determined using $\varepsilon = 112\ mM^{-1}\ cm^{-1}$ at 412 nm for native enzyme [11]. To obtain transparent glasses for the low-temperature measurements, the enzyme solutions were mixed with glycerol (Sigma) at a volume ratio 1:1.5. The MCD and absorption spectra were recorded using a hermetic contact type cell with an optical pathlength of 1 mm. The low-temperature reduction procedure was adopted to slow down conversion of the unstable reduced LPO to the stable one. The enzyme in a buffer-glycerol mixture was cooled in the optical cell to about −40°C and few grains of sodium dithionite were added to the mixture. After stirring the solution, the cell was sealed and inserted into a precooled spectrophotometer cryostat. The preparation had previously been purged of air with a stream of He gas at least for 15 min. Reduction of native enzyme and interconversion between the unstable and stable ferrous LPO were followed spectrophotometrically by exposure of the sample at appropriate temperature. After recording the absorption spectrum and its second derivative, the sample was frozen to 77 K and the cell was inserted into cryostat of an MCD instrument for measurements of MCD spectra at various temperatures down to 2.1 K in a magnetic field of 1.45 T. The application of the MCD method, experimental techniques and details of low-temperature measurements are described in [15]. Since MCD signals are linear in magnetic field up to 1.45 T at the lowest temperatures, the MCD spectra are normalized with respect to magnetic field and expressed in units $\Delta\varepsilon(M^{-1}\ cm^{-1}\ T^{-1})$, where

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Abbreviations: LPO, lactoperoxidase; MCD, magnetic circular dichroism; RR, resonance raman; XAS, extended X-ray absorption fine structure; EPR, electron paramagnetic resonance.

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$\Delta\varepsilon = \varepsilon_L - \varepsilon_R$, ε_L and ε_R are extinction coefficients for left and right circularly polarized light, respectively. Absorption spectra and its second derivatives were recorded on a Shimadzu UV-160 spectrophotometer equipped with a home-made cryostat.

3. Results and discussion

3.1. Components observed in ferrous LPO

The aim of this work is to characterize the spin state of the stable and unstable ferrous LPO by MCD spectroscopy. To slow down the interconversion between the two ferrous forms observed after dithionite addition, a low-temperature reduction procedure was adopted. It has been found that the initial absorption spectrum produced by reduction of LPO at about −15°C remains unchanged for a long time. Slow changes are observed only on rising the temperature up to ~0°C. Fig. 1 shows the absorption spectra and their second derivatives recorded at −15°C following reduction at this temperature of LPO at pH 7.0 and 10.2 and those obtained after annealing the pH 7.0 ferrous LPO for 15 min at +25°C. The absorption spectra the of initial ferrous LPO at pH 7.0 and 10.2 have maxima at 444 nm and 446 nm, respectively, and are commonly refered to as those of form 1 (unstable) of the ferrous LPO. The absorption spectrum of the annealed ferrous LPO with a maximum at 435 nm is typical for the ferrous LPO known as form 2 (stable) of the ferrous LPO. The more close examination shows, however, that none of the spectra displayed in Fig. 1 represents a pure individual LPO form. Therefore, by forms 1 and 2 we mean the main components of the unstable and stable ferrous LPO, respectively. The second derivatives show that, except form 1 with an absorption band at ca. 450 nm, in both spectra produced by low-temperature reduction there is an admixture of form 2 (with the absorption band at ca. 435 nm) and of the third ferrous component with the absorption band at 424–427 nm (form 3). In the annealed reduced LPO sample, form 1 completely disappears in favor of form 2 and partially of form 3. The content of form 3 increases on lowering the temperature as it is seen from the spectral changes in Fig. 1 for LPO sample at an alkaline pH value.

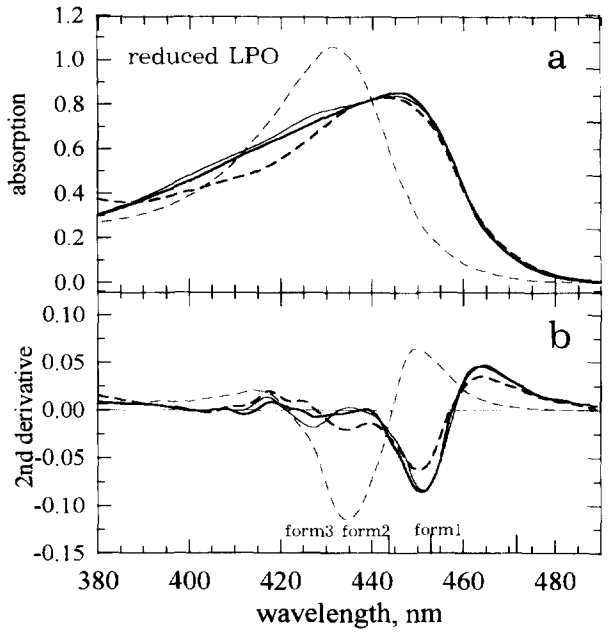

Fig. 1. Absorption spectra (a) and their second derivatives (b) of the unstable (thick dashed) and stable (thin dashed) ferrous LPO at pH 7.0 recorded at −15°C and +25°C, respectively, and of the unstable ferrous LPO at pH 10.2 recorded at −15°C (thick solid) and −40°C (thin solid). The unstable ferrous LPO at both pH values was prepared by adding of sodium dithionite into a cell at −15°C. The stable ferrous LPO was obtained by annealing of the unstable sample at +25°C.

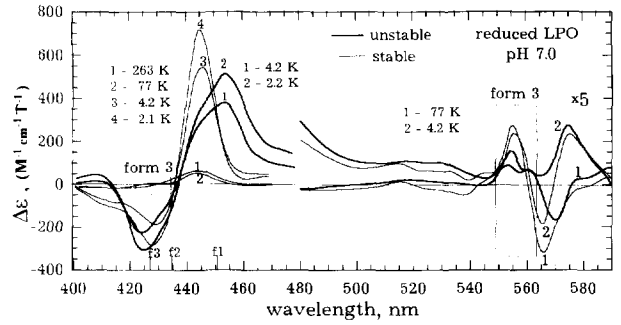

Fig. 2. MCD spectra of the unstable (thick solid) and stable (thin solid) ferrous LPO at pH 7.0 at several temperatures in a magnetic field of 1.45 T. The MCD signals from the low-spin ferrous form 3 are outlined by boxes.

Analysis of the absorption spectra and their second derivatives for different preparation of LPO at pH 6.0, 7.0 and 10.2 reduced at low temperatures confirms the presence of at least three forms in the non-annealed and partially annealed ferrous LPO. The relative content of the three forms depends on a pH value of the preparation, on the reduction temperature and on the time of annealing at a given temperature. Lowering the temperature is always followed by reversible increase of form 3 content, while rising the temperature and/or annealing of the sample is accompanied by irreversible conversion of form 1 mainly into form 2. The mean positions and standard deviations (in brackets) of absorption bands for forms 1, 2 and 3 of the ferrous LPO are 450.0 (1.3) nm, 436.8 (1.5) nm and 423.9 (1.7) nm, respectively. They have been obtained by decomposition of 14 absorption spectra and their second derivatives into spectral components using the least squares fitting procedure.

3.2. Identification of a spin state of the ferrous LPO forms by MCD spectroscopy

The MCD spectroscopy is a very appropriate tool to distinguish between paramagnetic and diamagnetic hemoproteins derivatives [15]. An MCD intensity of paramagnetic hemoproteins is strongly temperature-dependent down to the lowest temperatures due to the Boltzmann redistribution between sublevels of the ground manifold accessible for thermal population [17,18]. In contrast, the MCD spectra of diamagnetic hemoprotein derivatives are temperature-independent below 77 K, while at higher temperatures some changes in MCD may occur, caused by a conformation transition and/or by a band narrowing.

Fig. 2 shows the MCD spectra at several temperatures of the non-annealed and annealed ferrous LPO at pH 7.0 whose absorption spectra and second derivatives are displayed in Fig. 1. It is clearly seen that both MCD spectra in the Soret region are temperature-dependent even at temperatures below the liquid helium temperature. The presence of the two overlapping temperature-dependent MCD signals is evident for the non-an-

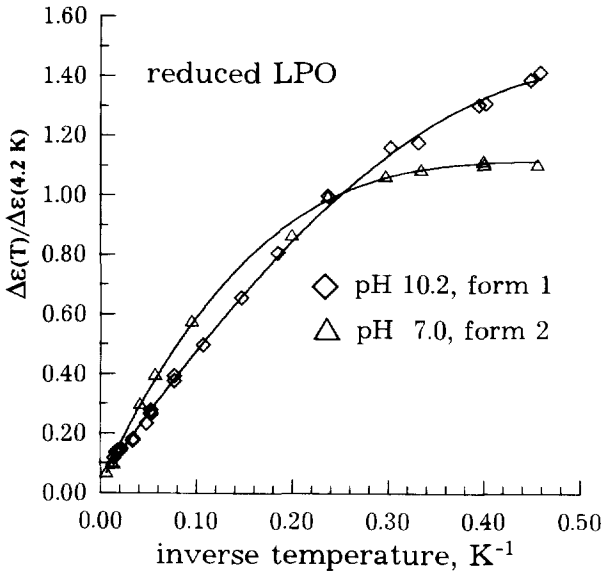

Fig. 3. Temperature dependencies of the MCD intensity at 453 nm for the unstable and at 444 nm for the stable ferrous LPO. The dependencies corresponds to the high-spin forms 1 and 2, respectively, and are normalized to the intensity at 4.2 K.

nealed sample in the form 1 and 2 absorption regions. Therefore, both forms are paramagnetic, the most likely they are high-spin ones as all other paramagnetic ferrous hemoproteins and model compounds studied. The annealing of the sample is accompanied by disappearance of the signal from form 1 and by increase of the derivative-shaped signal at 435 nm from form 2. Fig. 3 shows temperature the dependencies of the peak intensity in the Soret MCD spectra of the non-annealed and annealed samples which correspond mainly to forms 1 and 2, respectively. The temperature interval, where the plot is linear, for form 1 is much larger than for form 2. This implies that the energy gap between the components of the ground manifold of form 1 is smaller than that of form 2. The shape and sign pattern of the Soret MCD for form 2 are very similar to those typical for high-spin ferrous hemoproteins whose protein-derived ligand of the heme iron is an imidazole of histidine (deoxyhemoglobin, deoxymyoglobin and reduced HRP) [15]. This finding gives a further evidence that the heme iron in LPO is bound to a protein moiety through a histidine residue [7–10].

No other MCD signal is seen in the Soret region of the annealed sample at the lowest temperatures (4.2 and 2.1 K). However, the MCD signal in the absorption region of form 3 is clearly observed at 77 K. The observation indicates that form 3 is diamagnetic low-spin ferrous LPO, and its MCD, being temperature-independent, is obscured at the lower temperatures by a very strong temperature-dependent MCD signal from form 2 of the ferrous LPO. The apparent absence of the MCD signal from form 3 in the MCD spectrum recorded at 263 K may be connected with the decreased content of this form. The temperature-independent derivative-shaped MCD signal located at 558 nm is clearly seen in the visible region.

This signal is very similar to that observed for bis(imidazole)-heme in cytochrome b5 [19], denatured deoxyhemoglobin and model compound [20]. Therefore, the most likely candidate for the sixth ligand in the low-spin form 3 is an imidazole of a distal histidine residue.

In conclusion, two high-spin forms of the ferrous LPO are always observed when the enzyme is reduced at low temperatures at which the absorption spectra remain unchanged for a long time. Form 1 (with the absorption band at 450 nm) always dominates over form 2 (with the absorption band at 435 nm), but the relative content of form 2 increases on lowering the pH value. At high temperatures, form 1 is irreversibly converted to form 2. These observations suggest that: (1) the relative content of the two high-spin ferrous forms observed on low-temperature reduction of LPO reflects frozen pH-dependent equilibrium between two high-spin ferric forms pre-existing in native enzyme [16]; (2) only one high-spin ferrous form is stable at pH between 6.0 and 10.2; and (3) the large conformation rearrangements are required for conversion of the unstable high-spin ferrous LPO to the stable one. In addition, at least one low-spin ferrous form exists in temperature-dependent equilibrium with the high-spin ferrous form(s). Reversible increase of the content of the low-spin ferrous LPO is observed at least down to ca. 140 K, suggesting that minor structural changes are sufficient for the distal amino acid residue (presumably a histidine) can reach the heme iron. Appearance of one or two low-spin species on cooling was also observed for the oxidized LPO [8,13,16].

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