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The Reduction Potential of Lactoperoxidase

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The reduction potential of Fe(III)/Fe(II) lactoperoxidase has been determined. Optical determinations of equilibria with 2-methyl-3-hydroxy-1,4-naphthoquinone as indicator and prereduced 9,10-anthraquinone-2-sulfonate as reducing agent gave $E_{m,7.0}$= −191±2 mV. Potentiometric determinations with 9,10-anthraquinone-2-sulfonate as mediator and, in the reduced form as reducing agent, gave $E_{m,7.0}$= −188±1 mV.

Addition of 0.5 % *N*-cetyl-*N,N,N*-trimethylammonium bromide, and using dithionite as reducing agent, gave $E_{m,7.0}$= −183 mV and −179 mV with 9,10-anthraquinone-2-sulfonate and 9,10-anthraquinone-2,6-disulfonate, respectively, as mediators.

Lactoperoxidase* consists of a single peptide chain,[1,2] carrying 10 % carbohydrates and one heme group[1] to give M_r= 78,500. The tertiary structure is maintained by eight disulfide bridges.[1,2] Acidic butanone will split LP into protoheme and protein only after digestion of the enzyme with pronase[3,4] or reduction of -S–S- bridges with dithiotreitol and rupture of H-bonds with 5 M guanidine (unpublished results). The spectrum of LP Fe(III) shows four absorption maxima within the range 450–650 nm, and its Soret maximum, at 412 nm, is shifted 10 nm bathochromically from that of HRP. If protoheme in HRP C2 is replaced by 2,4-diacetyldeuteroheme the spectrum becomes similar to that of LP and the redox potential at pH 7 increases from −246 mV to −109 mV.[5,6] A mixed type of spectrum is also found in turnip peroxidase P_7 with $E_{m,7.0}$−120 mV.[7] Thus in two other peroxidases a mixed type spectrum, such as that found in LP, is associated with a redox potential, rather positive for a peroxidase. The stability of the heme-protein bonds in LP and its peculiar spectrum have been attributed to an unusually narrow heme-accomodating cleft.[8] This would reduce the exposure of the heme to the solvent and increase the redox potential.[9] The properties of the heme surroundings as well as the spectrum focus interest on the redox potential of LP, hitherto unknown.

MATERIALS AND METHODS

LP was prepared from cow's milk.[10] The first main fraction to emerge from DEAE-Sephadex® (Pharmacia, Uppsala) in 10 mM TRIS-HCl buffer (pH 9.0 at 25 °C) at 5 °C was used. It showed A_{Soret}/A_{280}=0.96. On the basis of dry weight determinations after dialysis against 5 mM acetic acid, ε_{412}=112.3±0.9 (n=5) $mM^{-1} \cdot cm^{-1}$ was obtained, in agreement with ε_{mM}=112.2 for Carlström's corresponding fraction, B1.[3]

A detailed description of the redox cell used in this study is given elsewhere.[11] The bright Pt electrode surfaces were purified by cyclic voltammetry and submersed into an 0.1 KI solution for 3 min. The resulting absorbed layer of iodine prevented uncontrolled adsorption of dyes, enzyme, detergents and grease, but gave an active surface for potentiometry.[12] The high tendency of LP to precipitate could finally be overcome by using He for deaeration and a magnetic bar in KOH-treated glass for intermittent stirring. The turbidity caused by stirring, and its dependence

* Abbreviations: Lactoperoxidase, LP; horseradish peroxidase, HRP (E.C.1.11.1.7); 2-methyl-3-hydroxy-1,4-naphthoquionone, phthiocol; 9,10-anthraquinone-2-sulfonate, AQS; 9,10-anthraquinone-2,6-disulfonate, AQS_2; sodium dodecylsulfate, SDS; *N*-cetyl-*N,N,N*-trimethylammonium bromide, CETAB.

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upon the type of gas used during deaeration,[11] became more pronounced in the presence of non-ionic detergents like Triton® X-100 (0.1 %) and Tween 80 (0.5 %). 0.5 % SDS gave an instantaneous, complete precipitation of LP followed by a slow, but complete dissolution. 0.5 % CETAB, however, allowed stirring and flushing with Ar without any formation of turbidity, CETAB (0.5 %) in 100 mM sodium phosphate buffer pH 6.0 successfully eluted LP from an octyl-Sepharose® column whereas benzene, sodium octanoate, and several other additives did not.[10] Trials pointed at AQS, $E_{m,7.0}$= −225 mV, AQS_2, $E_{m,7.0}$= −184 mV[13,14], and phthiocol, $E_{m,7.0}$= −168 mV[11] as suitable mediators. Mediators and solutions were protected against light.

Commercially available He, Ar and H_2 (AGA, Stockholm) were purified by passage through an Oxy-Trap® system (Alltech. Ass., Ill.) to give O_2 <0.1 ppm. Fresh sodium dithionite (Merck, Darmstadt) was divided under argon into 2 ml tubes, which were sealed with paraffin wax and stored dark and dry at −20 °C. AQS and AQS_2 (BDH, England) were recrystallized from hot water and air-dried at room temperature. Other chemicals were of analytical grade. Water was bidistilled in glass vessels. All experiments were made in 100 mM sodium phosphate pH 7.0, 25 °C. Literature quotations refer to this pH and temperature. Potentials were registered with a battery powered potentiometer (Radiometer PHM 4, Copenhagen) and spectra with a Beckman ACTA®III or a Beckman DU 7® spectrophotometer. These were calibrated against a holmiumoxide filter and potassium dichromate solutions in 0.005 M sulphuric acid for wavelength accuracy and absorbance linearity, respectively.

Spectra were scanned over the range 450−700 nm, and the ratios $[mediator]_{ox}/[mediator]_{red}$ and $[LP]_{ox}/[LP]_{red}$ for the Nernst formula were determined from absorbance spectra at suitable wavelengths.

RESULTS AND DISCUSSION

The dithionite ion, $S_2O_4^{2-}$, rapidly reduces LP to give LP Fe(II)-1 that spontaneously shifts to a second stable form, LP Fe(II)-2 (Fig. 1, Table 1). Usually heme proteins are reduced by the dithionite monomer at a rate of 10^4 to 10^5 M^{-1} s^{-1}.[15–17] The first order transformation of LP Fe(II)-1 to LP Fe(II)-2 is pH-dependent[3] with $k_{obs}=3.0\times10^{-3}$ s^{-1} at pH 7.0 (Fig. 1). Since $S_2O_4^{2-}$ is catalytically decomposed by LP[18] an alternative reductant was desirable, and AQS, prereduced with H_2 and platinum black, was chosen. AQS must be kept at a concentration about three times that of LP to mediate reduction (Table 2). Spectrophotometric titration of LP with AQS, monotoring the change in absorptivity, showed a change in slope when 2.9 AQS/LP had been added. The mode of binding is not known but LP may bind AQS and other mediators not only in the vicinity of the heme but also at other areas of the molecule.[10] Unlike HRP, LP does not give a hyperbolic (1:1) spectral change when aromatic donors are added,[10] but from affinity chromatography we estimated that LP can bind aromatic donors with K_d about one fifth of that for HRP C, found as 0.5−10 mM.[10] Neither phthiocol nor AQS in their oxidized or reduced forms interfered with

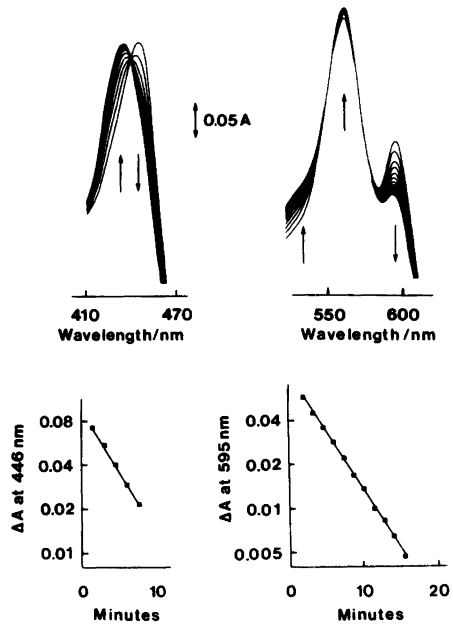

Fig. 1. Transformation of LP Fe(II)-1 to LP Fe(II)-2. In the Soret region (left) 5.3 and in the visible region 39.2 μM. 100 mM sodium phosphate, pH 7.0, 25 °C. First order plots of ΔA at 446 nm and 596 nm. 100 mM sodium phosphate pH 7.0, 25 °C.

Table 1. Absorption maxima LP Fe(III), LP Fe(II)-1 and LP Fe(II)-2. Isosbestic points for the two reduced forms and absorptivity in mM^{-1} cm^{-1} for some wavelengths are given (within parentheses). (s) Shoulder. 100 mM sodium phosphate pH 7.0, 25 °C.

Compound	Wavelength (nm)				
LP Fe(III)	412 (112.3)	501 (8.60)	542	585	630
LP Fe(II)-1	446 (77.8)		562 (13.6)	595	
Isosbestic points	408	440	502	552	573
LP Fe(II)-2	434 (94.6)		537(s)	565 (14.2)	595

the visible spectrum of oxidized or reduced LP. No precipitation occurred and the absorbances were additive.

Optical determinations of $E_{m,7.0}$. Phthiocol, with an absorptivity of the same magnitude as that of LP at about 500 nm, is only slowly reduced by H_2/Pt black. AQS was added to act as the mediator's mediator. AQS has too low absorptivity to be used alone. Typically, 29.9 μM LP was deaerated by bubbling He for eight hours without spectral changes. Deaerated solutions of phthiocol and AQS to give cuvette concentrations of 69.8 and 23.5 μM, respectively, were then added *via* the gas outlet from a gas-tight microsyringe. Then the gas was changed to H_2 and reduction *via* the Pt black electrode began. The spectrum of the system was scanned every 20 min during the first 2 h and then every 30 min. Data from this experiment are collected in Table 3. The ratios [ox]/[red] for phthiocol and LP were obtained from A_{630}, where only LP absorbs, and A_{565}, where both LP and phthiocol absorb. At both wavelengths the light absorption of AQS is negligible. The average of $E_{m,7.0}$ for three batches of LP was −191±2 mV (−191, −193, −189).

Potentiometric determination of E_m. The solution of LP (27.3 μM) was deaerated as above and deaerated solution of AQS in buffer to give a 4-fold excess of AQS over LP was added. Helium was then continuously passed over the solution, while AQS was being reduced by H_2 which had been dissolved in the platinum black prior to its submersion into the solution. Reduced AQS subsequently reduced LP. This procedure avoided the exposure of the bright Pt electrode to H_2 and permitted potentiometric measurements. The potential between the bright Pt electrode and the calomel electrode was registered at regular intervals, as was the spectrum (Table 4).

Table 2. Proportions of the mediator (AQS) needed to reduce LP and HRP C2 by means of H_2/platinum black.

[Mediator]/[Enzyme]	LP	HRP C2
0.12	no reduction	reduction
0.28	no reduction	reduction
1.2	no reduction	reduction
1.91	no reduction	reduction
2.32	no reduction	reduction
2.80	slow reduction	reduction
4.76	reduction	reduction

Table 3. Optical determination of $E_{m,7.0}$. For details see text. $E_{m,7}$ phthiocol= −168 mV. 100 mM sodium phosphate pH 7.0, 25 °C.

% reduced LP 630 nm	$\frac{RT}{F} \cdot \ln\frac{[ox]}{[red]}$ LP at 630 nm mV	$\frac{RT}{2F} \cdot \ln\frac{[ox]}{[red]}$ phthiocol from 565 nm mV	$E_{m,7.0}$ mV
13.9	46.8	24.9	−189.9
23.2	30.7	8.5	−190.2
28.6	23.5	1.9	−189.6
37.7	12.9	− 9.8	−190.6
48.6	1.5	−23.6	−193.1
59.2	− 9.6	−32.4	−190.8
		Mv	−190.7±1.3 mV

Table 4. Potentiometric determination of $E_{m,7.0}$. For details see text. $E_{m,7}$ calomel electrode 249.9 mV. 100 mM sodium phosphate pH 7.0, 25 °C.

% reduced LP 630 nm	$\frac{RT}{F} \cdot \ln\frac{[ox]}{[red]}$ Mv	E_h mV	$E_{m,7.0}$ mV
0			
47.2	+ 2.9	−182.7	−185.6
60.2	−10.7	−198.1	−187.4
65.8	−16.8	−204.8	−188.0
73.0	−25.6	−214.7	−189.1
78.2	−30.3	−218.9	−188.6
100			
		Mv	−187.7±1.4 mV

A maximum of 80 % reduction of LP could be achieved with the amount of H_2 dissolved in the Pt black. At the end of the experiment an addition of a slight excess of $S_2O_4^{2-}$ was used to achieve 100 % reduction. The extent of reduction was measured at A_{630}, where the absorbance of AQS is negligible. The average of E_m from four experiments on different batches of LP was −188±1 mV (−188, −188, −187, −190).

The reduction rate of LP and mediator under the above described conditions is slow in relation to the transformation of LP Fe(II)-1 to the stable LP Fe(II)-2. The reduction potential in concern is therefore that of the equilibrium LP Fe(III)/LP Fe(II)-2. Plots of ln ($[LP]_{ox}/[LP]_{red}$) *versus* E_h from the two experiments detailed above gave straight lines shifted in parallel by 3 mV. The optical and potentiometric analyses gave n=1.05 and 1.11, respectively, the presumed value being n=1.

Effects of CETAB. Potentiometric titration in buffer with 0.5 % CETAB was carried out with sodium dithionite as reducing agent, presuming a higher rate of reduction than that of catalyzed disproportionation of the dithionite ion. Spectrophotometric titration of LP with AQS in the presence of 0.5 % CETAB reduced the ratio of AQS to LP from 2.9 to 2.4 in the optical test (*cf.* above). Proper potentiometric titration curves could be obtained with a 3.5-fold excess of AQS or AQS_2. The potentials obtained in single experiments were −182.9±1.9 and −179.2±0.8 mV, respectively. Nernst's plots gave straight lines, n=0.99 and 1.07. To permit comparison with the non-detergent experiments, LP was allowed to transform to LP Fe(II)-2 before potentiometric readings were made. The extent of reduction was calculated from A_{630}. The reason for the low increase, 10 mV, of the reduction potential cannot with certainty be identified. It may be attributed to detergent effects on the heme, or possibly on the equilibrium enzyme–mediator–electrode.

In summary, the Fe(III)/Fe(II)-2 reduction potential of LP has been determined as −189±2 mV in 100 mM sodium phosphate pH 7.0, 25 °C. $E_{m,7.0}$ for HRP A, HRP C, turnip peroxidase P_1 and chloroperoxidase is −212 mV, −263 mV, −218 mV[19] and −144 mV,[19] respectively. Thus, in spite of the similarities in spectrum between LP, 2,4-diacetyldeuteroheme HRP C, and turnip peroxidase P_7, and the alleged narrowness of the heme cleft in LP, $E_{m,7.0}$ of LP falls within a range common to several peroxidases also carrying protoheme as prosthetic group. There may be several reasons for the discrepancy between expected and observed E_m-values. Environmental and structural alterations to the heme may cause similar optical effects without influencing E_m to equal extents.

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