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Lactoperoxidase

Some Spectral Properties of a Haemoprotein with a Prosthetic Group of Unknown Structure

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The visible spectra of the major subfractions of LPO were compared. The spectra of homogeneous LPO B-1 and some of its derivatives were recorded. Significant spectral instabilities were observed for the reduced enzyme and its carbon monoxide compound. "Twin haemochromogen" spectra were observed by the addition of cyanide to the reduced enzyme and for the pyridine haemochromogen. The nature of the prosthetic group is discussed in connection to the spectral properties of LPO. By proteolytic digestion a haem was released which formed a pyridine haemochromogen with α- and β-bands at 556 and 526 mμ. It is suggested that the prosthetic group of LPO is protohaem.

Lactoperoxidase (LPO*) has been shown to be extensively heterogeneous [1–3] and to consist of ten electrophoretically different subfractions.[3] The major subfractions, separated by chromatography, are homogeneous in disc electrophoresis.[3] Conversion within these subfractions occurs [3,4] and a comparison of the physical properties and the composition of the predominant molecular forms was recently reported.[5] In the present study the spectral properties of the major subfractions are compared and the spectra of homogeneous LPO B-1 and its derivatives formed by interaction of the haem group with various ligands are recorded. These investigations were undertaken because the spectral characteristics of haemoproteins have provided understanding of the nature of the haem group and because earlier published spectra of LPO were obtained with preparations of enzyme that were only 49 % pure.[6] During the course of the present study deviations from the general patterns of the spectra of some derivatives of other protohaemoproteins were also observed. These observations are discussed in relation to the nature of the haem group in LPO.

* Abbreviations: LPO = lactoperoxidase; HRP = horse-radish peroxidase; Cat = liver catalase; Mb = horse myoglobin; Hb = horse haemoglobin.

MATERIALS AND METHODS

LPO. LPO B-1, LPO B-2_I, LPO B-2_{II}, LPO B-3, and LPO A were samples from the preparations used in a previous investigation.[5] All subfractions were homogeneous in disc electrophoresis [3] and their ratio of absorptivity, A_{412}/A_{280}, were 0.98, 0.94, 0.95, 0.92, 0.92, respectively. Equilibration with different buffers was obtained by passing the enzyme through a column of Sephadex G-25, fine, extensively washed with the appropriate buffer. Sephadex was purchased from AB Pharmacia, Uppsala, Sweden. Protein concentrations of the subfractions of the LPO B-group were determined spectrophotometrically using $E_{1\ \%}^{1\ cm} = 14.9$ at 280 mμ.[5] In the calculations of molar extinction coefficients, 78 500 was assumed to be the molecular weight of LPO B-1.[5]

Chemicals. All chemicals used were of analytical grade.

Spectrophotometry was performed in a Cary 14 spectrophotometer. Absorption spectra in the Soret region and in the visible part of the spectrum were recorded by means of the "0.0−1.0−2.0" and the "0.0−0.1−0.2" slidewires, respectively. For difference spectrophotometry the "0.0−0.1−0.2" slidewire was used. Unless otherwise noted, the absorption maxima were determined from spectra recorded by scanning at a rate of 3 mμ/min. Quartz cells with 1 cm light path were used. Measurements at single wavelengths were performed in a Beckman DU spectrophotometer.

For the measurements of the spectra of reduced LPO, a cell was used which was fitted by means of a ground glass joint to an extension tube similar to that from a Thunberg tube. Approximately 5 mg of sodium dithionite was placed in the side arm. By a stopcock fused to the extension tube, the cell was evacuated by means of a vacuum pump and then flushed with argon, which had been previously deoxygenated by passage through three wash-bottles containing vanadous sulfate.[7] The evacuation and flushing procedure were repeated six times and then the sodium dithionite was added to the peroxidase solution.

The compositions of the solutions used for the recordings of the spectra of various derivatives of LPO B-1 are shown in Table 1.

Table 1. Solutions used for the spectrophotometric measurements presented in Figs. 1 and 2.

Spectrum obtained	Final concentration of LPO B-1 (μM) Soret reg.	visible reg.	Medium in which enzyme was dissolved
Peroxidase	6.05	6.85	0.2 M phosphate buffer, pH 6.0.
F^--peroxidase	8.98	8.98	0.5 M NaF in 0.2 M phosphate buffer, pH 6.0.
CN^--peroxidase	4.68	3.86	77 mM KCN in 0.2 M phosphate buffer, pH 6.0.
N_3^--peroxidase	5.52	5.52	0.5 M NaN_3 in 0.2 M phosphate buffer, pH 6.0.
Reduced peroxidase		5.16	0.2 M phosphate buffer, pH 6.0, $+\sim$5 mg $Na_2S_2O_4$.
CO-reduced peroxidase	5.83	5.40	0.2 M phosphate buffer, pH 6.0, saturated with CO $+\sim$5 mg $Na_2S_2O_4$.[a]
CN^--reduced peroxidase	4.68	3.86	77 mM KCN in 0.2 M phosphate buffer, pH 6.0, $+\sim$5 mg $Na_2S_2O_4$.

[a] Covered with parafilm during measurement.

RESULTS AND DISCUSSION

Difference spectrophotometry of the major subfractions of LPO. Subfractions of the LPO B-group have previously been reported to exhibit differences in the ratios A_{412}/A_{280}.[5] It was found that these differences were due to variations in absorptivity at 412 mμ, while that at 280 mμ was unchanged.[5] These results motivated a comparative investigation of the visible spectra of the subfractions of LPO. By adjusting the concentrations of the subfractions to about 0.6 mg/ml in pairs to give equal absorptions at the Soret band maximum at 412 mμ, difference spectra, LPO B-1 *minus* LPO B-2_I, LPO B-2_{II}, LPO B-3, or LPO A, revealed no significant differences in the visible part of the spectrum. In a previous study it was found that the A_{412}/A_{280} ratio varied in the eluate during chromatographic separation of the subfractions of LPO.[3] No foreign proteins were observed in disc electrophoresis that could account for this variation. The ratio was noted to correlate roughly to the concentration of the enzyme eluted from the column.[3] A concentration dependence of the extinction coefficient at the Soret band has been observed for pineapple peroxidase B[8] while that at 275 mμ was constant. However, when this relationship was examined by diluting successively from 0.5 mg/ml to 0.05 mg/ml in 0.01 M Tris-HCl buffer, pH 8.0 and 9.0, no variation in A_{414}/A_{280} was observed. Thus, the spectrophotometric ratio of LPO, in contrast to that of pineapple peroxidase, is not dependent on the concentration of the enzyme but it cannot be excluded that the variations in A_{412}/A_{280} are caused by dilution together with chromatographic separation. These events in combination conceivably can induce small structural changes in, *e.g.*, the local environment of the haem thus altering the absorptivity of the Soret band, which could also be the basis for the concentration dependence of the Soret band extinction seen for pineapple peroxidase B. Furthermore, pronounced spectral instability has been observed for some derivatives of LPO (*vide infra*). Theorell and Pedersen [9] found a A_{412}/A_{280} ratio of 0.79 for a recrystallized preparation of LPO that was homogeneous by moving boundary electrophoresis at pH 5.9 and with an iron content very similar to those found by the present author.[5] As this ratio is considerably lower than those found for any of the subfractions of LPO [3] it is very difficult to explain the value found by Theorell and Pedersen by a predominance of a subfraction of low spectrophotometric ratio, but may be related to differences in preparative procedures.

Spectral properties of LPO B-1 and some of its derivatives. The spectra of free lactoperoxidase, the reduced enzyme and certain derivatives with ligands are demonstrated in Figs. 1 and 2. The absorption maxima and extinction coefficients of the free enzyme, its cyanide, fluoride, and azide compounds and the carbon monoxide complex of the reduced enzyme (Table 2) are not very differerent from those observed for HRP,[10,11] Cat,[10,11] Mb,[10–12] and Hb.[10,13] In relation to the charge transfer bands at 501 mμ and 631 mμ, the absorption bands at 589 mμ and 541 mμ are more intense in the spectrum of free LPO than the corresponding bands of HRP, Cat, Mb, and Hb. Assuming protohaem as the prosthetic group of LPO (*vide infra*) this fact might indicate a higher proportion of a low-spin form in ferric LPO than in the "acidic forms" of HRP, Cat, metMb, and metHb. The cyanide compound of oxidized LPO

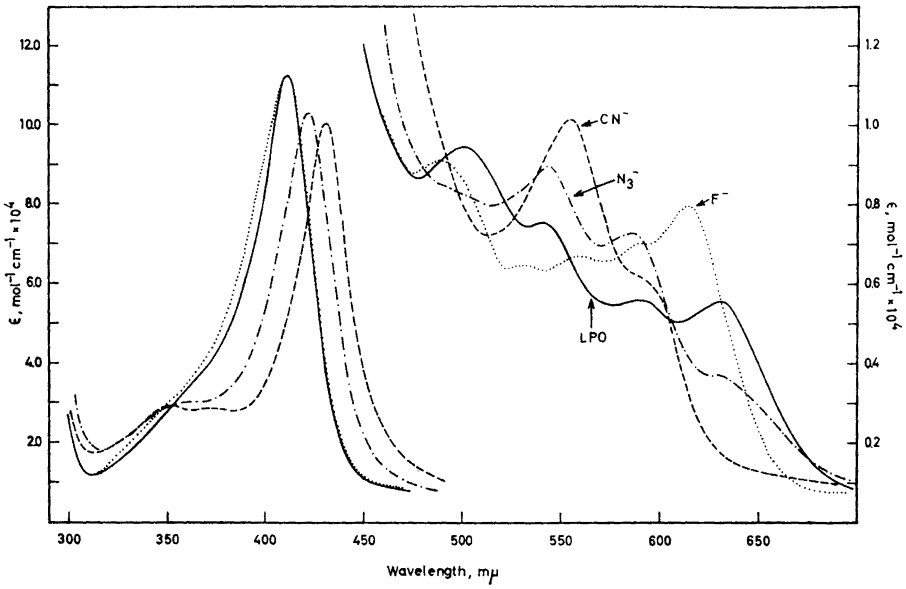

Fig. 1. Absorption spectra of LPO B-1 and its fluoride(F^-), cyanide(CN^-), and azide(N_3^-) compounds. For details of solutions used, see Table 1.

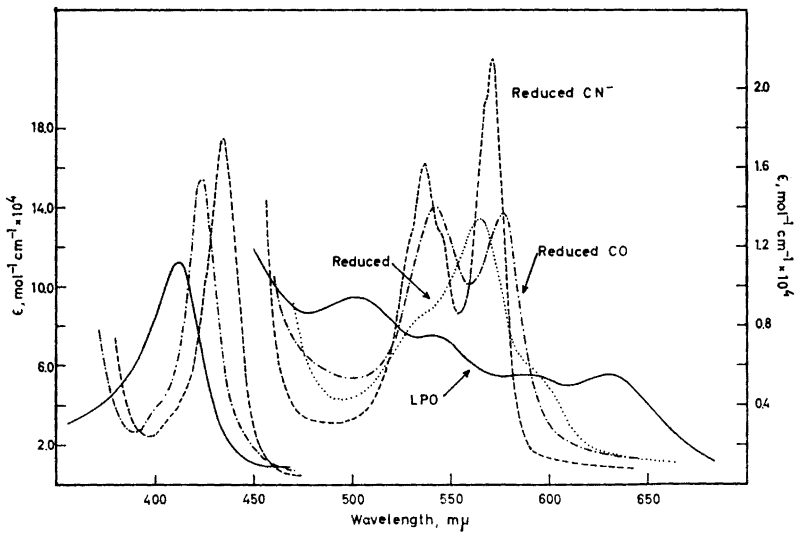

Fig. 2. Absorption spectra of LPO B-1, CO-reduced peroxidase, CN^--reduced peroxidase, and reduced peroxidase. The latter spectrum is that obtained 25 min after addition of sodium dithionite. For details of solutions used, see Table 1.

Table 2. Wavelength (mμ) of absorption maxima and the corresponding values of $\varepsilon \times 10^{-4}$ (in parantheses) for peroxidase and derivatives shown in Figs. 1 and 2.

Peroxidase	412 (11.22)	501 (0.94)	541 (0.78)		588–90 (0.55)	631 (0.56)
F^--peroxidase	411.5 (11.22)	488.5 (0.91)	533.5 (0.64)	560 (0.67)	590 (0.69)	615 (0.80)
CN^--peroxidase	430 (10.01)		555.5 (1.01)		595 (0.61)	
N_3^--peroxidase	422.5 (10.30)		549.5 (0.89)		587 (0.72)	630 (0.37)
CO-reduced peroxidase	424.5 (15.45)		543 (1.40)		577.5 (1.37)	
CN^--reduced peroxidase	435 (17.50)		537.5 (1.62)		571.5 (2.15)	

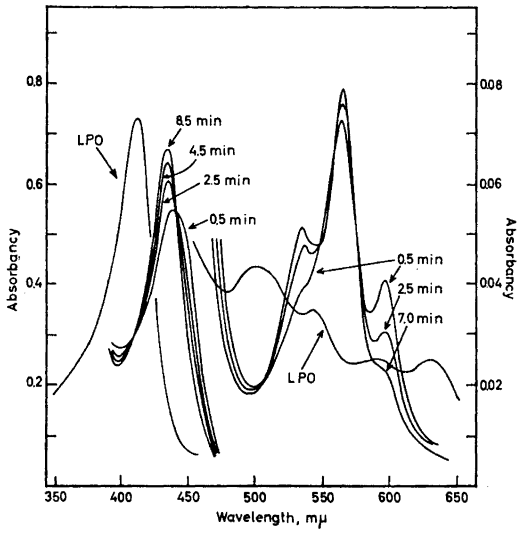

Fig. 3. Spectra recorded at different times after the addition of sodium dithionite to a solution of LPO B-1 in 0.2 M phosphate buffer, pH 6.0. The times indicated in the figure refer to that instance when the recorder was started at 650 mμ and 550 mμ, respectively. The wavelength scale was scanned at a rate of 150 mμ/min. The concentrations of enzyme used are different for the Soret band region, 6.2 μM, and for the visible part of the spectrum, 5.2 μM. The positions of the wavelength maxima given in the text are evaluated from these curves.

shows two minor bands in the region 340 to 380 mμ which are also found for other proteins containing protohaem.[14] Five absorption bands in the visible part of the spectrum are seen upon the addition of sodium fluoride to LPO; not more than four bands have been described for the fluoride compounds of any of the haemoproteins mentioned above. However, each of the absorption bands of the fluoride derivative of LPO corresponds to a band found in some of the other haemoproteins. Possibly the five-banded spectrum reflects a mixture of compounds of LPO in the presence of fluoride ions.

After reduction of LPO with sodium dithionite in 0.2 M phosphate buffer, pH 6.0, a spectrum (Fig. 3) with absorption maxima at 596 and 565 mμ was recorded. This spectrum changed within minutes to give a haemochromogen-like spectrum with α- and β-bands at 565 and 535 mμ, respectively, with a concomitant shift of the Soret band to shorter wavelength, 435 mμ. In the visible range, an inflection remained at the position of the former 596 mμ-band (Fig. 3). However, the spectral change continued and after approximately 20 min the Soret band had broadened, the extinction decreased and the peak at 535 mμ was also replaced by an inflection. This latter spectrum is very similar in shape to that of HRP when this enzyme is reduced with dithionite.[10] When LPO was reduced at pH 9.0 in 0.2 M Tris-HCl buffer, a visible spectrum was obtained that was almost identical to that first formed at pH 6.0 (Fig. 4). This spectrum remained relatively unchanged with time and no maximum at 535 mμ appeared. The Soret band was located at 446 mμ. By varying the pH

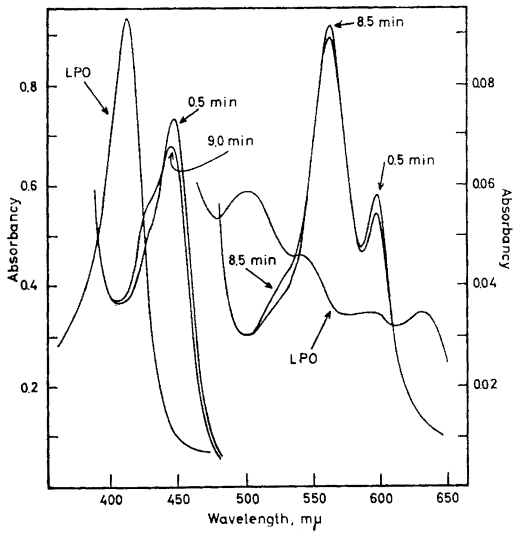

Fig. 4. Spectra recorded at different times after the addition of sodium dithionite to a solution of LPO B-1 in 0.2 M Tris-HCl buffer, pH 9.0. The times indicated in the figure refer to that instance when the recorder was started at 650 mμ and 550 mμ, respectively. The wavelength scale was scanned at a rate of 150 mμ/min. The enzyme concentrations used are 7.8 μM and 6.1 μM for the Soret band region and the visible region of the spectrum, respectively.

between 6.0 and 9.0 spectral conversions which were intermediate to those described above were found. These results motivated a detailed investigation of the pH-dependence of the spectral transitions. Due to the instability of the spectra and the presence of at least three forms of the enzyme during reduction at pH 6.0, only an estimation of the pH value at which the two first forms exist in approximately equal amounts was obtained, namely between 7 and 8. Moreover, the spectral changes accompanying reduction of paraperoxidase [10] are interesting and resemble that observed upon reduction of LPO at pH 6.0. The reduction of paraperoxidase gives a compound with absorption maxima at 565 and 535 mμ in the visible range of the spectrum. These bands are at exactly the same wavelengths as the maxima of the second form of reduced LPO. The spectrum of paraperoxidase is then slowly converted to the spectrum seen for ordinary ferro-HRP.[10] This is a conversion analogous to that observed with LPO.

For LPO the spectral shifts seen upon reduction at pH 6.0 might be interpreted as follows: the first spectrum formed is that of a high-spin ferroperoxidase with the fifth co-ordination site of the iron occupied by a protein ligand and the sixth co-ordination position free or bound to a ligand of low field strength. The reduction of the enzyme induced a slow conformation change that at one stage brings a protein side chain into such a position that it can form a low-spin compound by binding at the fifth co-ordinate. This state should correspond to the haemochromogen-like spectrum with absorption maxima at 565 and 535 mμ. However, the conformation change is not complete and the ligand at the sixth co-ordination position again dissociates from the iron and a compound is finally obtained that possibly is a mixture of low-spin and high-spin forms giving rise to the third spectrum.

A difference was found in the oxidizibility of the final product of the reduced enzyme which was obtained at pH 6.0 and the hig-spin form. The former was easily reverted to the ferric state merely by exposure of the reduced enzyme solution to air. Thus, 94 % of the Soret band absorption of the ferric form was regained from reduced peroxidase which was kept in the anaerobic cell for 90 min at pH 6.0. The corresponding treatment of LPO, reduced at pH 9.0, did not reoxidize the enzyme before bubbling with pure oxygen. This procedure, however, destroyed a considerable amount of enzyme as judged from the absorption at the Soret band.

Except for the ferro-peroxidase, the spectrum of the carbon monoxide complex of LPO was observed to vary with pH. By reducing a solution of LPO, saturated with carbon monoxide at pH 6.0, it was found that the spectrum first observed changed slowly to give the spectrum shown in Fig. 2. Rapid repeated scanning demonstrated that the first spectrum seen had absorption maxima slightly further to the red than that in Fig. 2, and the former and succeeding spectra formed isosbestic points with the final spectrum which was formed within a few minutes. The final spectrum was assumed to be developed when maximal extinction coefficients were obtained at the particular pH. The complex between ferroperoxidase and carbon monoxide at pH 9.0 showed a final spectrum with a Soret band at 427 mμ and α- and β-bands at 579 and 545 mμ, respectively, *i.e.* somewhat displaced to the red as compared to the spectrum at pH 6.0 (Table 2). Furthermore the extinction

coefficients at these maxima were lower and the β-band was higher relative to the α-band. The spectrum shown in Fig. 2 does not represent the fully developed spectrum of an "acidic form" of the carbon monoxide derivative, as by lowering the pH to 5.0 a Soret band still sharper and more intense was obtained.

Addition of potassium cyanide to ferro-peroxidase yields spectra with some interesting features. The spectrum shown in Fig. 2 is very similar in shape to the "twin haemochromogen" spectrum observed by J. Keilin [15] for ferro-myoglobin by the addition of certain nitrogeneous bases and the low-temperature spectra of cytochrome c reported by Estabrook.[16] The α- and β-bands are each split into two bands which will be called α_1, α_2, β_1, and β_2, with the α_1- and β_1-bands at longer wavelengths than the α_2 and β_2-bands. The α_1- and β_1-bands of the cyanide compound shown in Fig. 2 are further to the red, 571 mμ and approximately 545 mμ, than the α- and β-bands of the reduced cyanide compound described for HRP and Mb by Keilin and Hartree,[25] 565.5 and 535 mμ, respectively. The α_2- and β_2-bands of the LPO derivative lies at approximately 567 mμ and at 537.5 mμ, but by making allowances for the additive effect of the α_1- and β_1-bands, the α_2- and β_2-bands are very close to those of the cyanide compounds of HRP and Mb.[25] The Soret band is not split and has a maximum at 435 mμ. Addition of cyanide at high concentrations at pH 10.2 (Fig. 5) gives a spectrum with a β-band more intense than the α-band, which is characteristic for the bis-cyanoferroprotoporphyrin as shown by J. Keilin.[17] However, the maxima of the derivative of LPO are somewhat displaced toward long wavelengths namely at 576 and 544 mμ. Thus, the "twin haemochromogen" of LPO might be interpreted as a

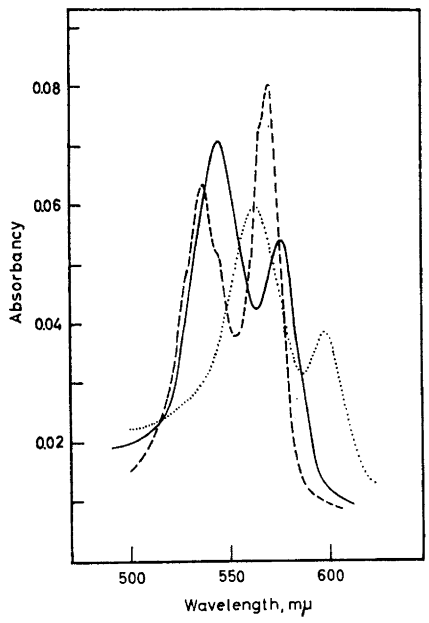

Fig. 5. Spectra obtained by the addition of KCN, 0.067 mM final concentration, to LPO B-1, reduced in 0.2 M Na_2HPO_4, pH 9.0 (- - - -), and in a final concentration of 460 mM in 0.2 M Na_2HPO_4, pH 10.2 (———). Reduced enzyme: (· · · ·) Enzyme concentration: 4.4 μM.

mixture of mono- and biscyanoderivatives. This explanation is very similar to that once put forward by J. Keilin [18] for the "twin-haemochromogen" formed from Mb in aqueous pyridine. This mechanism was refuted in a more recent publication,[15] where a stoichiometric ratio of 1:1 was postulated for ligand and Mb.

Theorell and Åkeson [19] found that the α- and β-bands of the pyridine haemochromogen of LPO were situated at 565 and 530 mμ, respectively. Later Morell [20] reported that these maxima were only initial and that, with time, the bands shifted to shorter wavelengths. After 10 min the position of the maximum of the α-band was at 556—558 mμ,[20] *i.e.* the maximum of the pyridine haemochromogen of protohaem. Morell concluded that the spectral shift was not only due to the incubation in sodium hydroxide but also influenced by the presence of pyridine. In our hands, the pyridine haemochromogen of LPO in 0.1 M sodium hydroxide and 25 % pyridine showed bands initially at 564 and 527 mμ. However, the spectrum had split α- and β-bands, the splitting being less pronounced than that obtained for the reduced cyanide compound. By using higher pyridine concentrations, 50 %, the bands shifted to 560 and 526 mμ, respectively, and the splitting was no longer noticeable. Thus there are good reasons to believe that these haemochromogen spectra reflect a mixture of mono- and bis-pyridinohaemochromogens in analogy with the interpretation of the spectra of the cyanide adducts. It is very likely that the prosthetic group still stays in a crevice of the protein at pH 13 and the fifth and/or sixth co-ordination positions are not completely accessible to pyridine. Therefore, LPO was digested with pronase (1:1) in 0.1 M ammonium bicarbonate at 37°C overnight. The solution was then acidified with hydrochloric acid to pH 2 and extracted with methylethylketone. Most of the colour was transferred to the organic phase, leaving some residual haem in the water phase. The pyridine haemochromogen obtained from the organic phase in 0.1 M sodium hydroxide and 25 % pyridine showed α- and β-band maxima at 556 and 526 mμ, which are also found for the pyridine haemochromogen of haem obtained by acidic extraction of myoglobin with methylethylketone.

The evidence put forward by Morell [6] that the prosthetic group of LPO is not a protohaem are: (1) the absorption bands of LPO and its derivatives are consistently found at longer wavelengths than those of the corresponding derivatives of HRP, Cat, and Hb, and (2) the pyridine hemochromogen of LPO does not show the typical wavelength maxima obtained from protohaem. By using the absorption maxima obtained in this study and, in addition to the haemoproteins used by Morell,[6] including Mb in the comparison with other haemoproteins, it is found that the first statement above is not justified and could not be used as an evidence against protohaem as the prosthetic group of LPO. Furthermore, the spectral properties of the reduced enzyme and its pyridine and cyanide compounds and the difficulty in releasing the prosthetic group from the protein moiety could easily be explained by steric arrangements in the environment of the haem group. The prosthetic group might be burried in a deep, hydrophobic crevice. Possibly this crevice is formed by the two similar subunits of LPO which were postulated from the peptide mapping experiments by Rombauts *et al.*[22] Disulfide bridges are abundant in LPO [5] and these might help to lock the crevice and prevent the prosthetic group

from being extracted until the protein is almost completely broken down, *e.g.* by proteolytic enzymes. All the methods which have been effective for the release of haem groups from LPO will also readily break peptide bonds.[20,22]

Thus, it is unnecessary to assume a covalent binding of the prosthetic group or a haem group different from protohaem to explain the properties of LPO. The results obtained by Morell[20] and Hultquist and Morrison[22] from investigations of porphyrin derivatives obtained from LPO are somewhat conflicting. Both authors, however, suggest that electrophilic groups, more electrophilic than the vinyl groups of protoporphyrin, are bound to the porphyrin nucleus of LPO. This would also explain the absorption bands of LPO and its derivatives which were consistently found to be at longer wavelengths by Morell.[6] From the properties of a porphyrin derivative obtained by treating LPO with sodium methoxide, Hultquist and Morrison[22] concluded that the prosthetic group of LPO is a derivative of mesohaem 9, in which two double bonds are in conjugation with the tetrapyrrole nucleus and with one or more hydroxyl groups attached to the side chains. On the other hand the nature of the porphyrins obtained from LPO by treatment with hydrogen iodide and hydrazine in acetic acid[22] favours the possibility that protohaem is the prosthetic group of LPO.

It is interesting to note the results of Foulkes *et al.*[23] who found that myeloperoxidase gave the bands of a protohaemochromogen when the enzyme was boiled in the presence of pyridine, sodium hydroxide, and sodium dithionite. This enzyme does not normally form a pyridine haemochromogen. In analogy with the instability of the absorption spectra of certain derivatives of LPO, Agner and Ehrenberg met considerable difficulties in getting stable spectra of some derivatives of myeloperoxidase.[24] These observations and other similarities demonstrated in a previous study[5] might indicate that there is some relation between lactoperoxidase and myeloperoxidase.

The results obtained in this investigation do not exclude, but rather give some indications in favour of protohaem being the prosthetic group of LPO.

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