The Hydrogen Ion Equilibria of Horseradish Peroxidase and Apoperoxidase

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1. The reversible proton dissociation equilibria of peroxidase, apoperoxidase and haem-recombined apoperoxidase have been explored in 150 mM-potassium chloride at 20°C at pH 3-11.5. 2. Complementary heat measurements have been made of the classes of titratable groups to determine their intrinsic ΔH dissociation. 3. These curves are interpreted as showing that there are two histidine residues capable of titration in peroxidase whereas there are three such in apoperoxidase. 4. Concomitant spectroscopic investigations indicate profound differences in the tyrosine ionizations in the two proteins. In peroxidase one group only of the five residues ionizes up to pH 11.5. In apoperoxidase four residues are titratable. 5. Spectroscopic titration in 6 M-guanidinium chloride and 150 mM-potassium chloride reveal one tyrosine residue fewer in peroxidase than in apoperoxidase. 6. These findings are discussed in terms of the 'side chain' groups responsible for binding the haem group in peroxidase. A proximal imidazole group seems probable as is also the involvement of a distally placed tyrosine. 7. The differences between apo- and holo-peroxidase are stressed, particularly in respect of abnormal carboxyl group titration in the former.

The object of the present investigation was to explore the reversible portions of the titration curves of peroxidase and apoperoxidase in the hope that any differences observed could be attributed to groups in the haem protein responsible for binding the haem group.

The classical study of Cannan, Kibrick & Palmer (1941) demonstrated that the dissociation of hydrogen ions from ovalbumin could be explained in terms of the intrinsic pK values of titrating groups on the protein when allowance was made for the electrostatic effect of charges present on the protein molecule. Since then this technique has been extended to cover similar studies on other proteins (Tanford, 1962).

The present paper reports an experimental study of the proton dissociation from a purified isoenzyme of horseradish peroxidase, namely isoenzyme B, and also of its apoenzyme and the haem-reconstituted peroxidase. The analysis of the results gives an estimate of the number of groups within each category of dissociating groups present in the molecule, together with information on their intrinsic pK values and the electrostatic interaction factor. This latter parameter adjusts the group dissociation coefficients to the continuously varying

coulombic forces as the charge on the protein varies with pH.

Many of the inherent ambiguities in the assignment of the number of groups being titrated can be overcome by comparison of simultaneous potentiometric and calorimetric titrations, by using the data on the heats of ionizations of the various groups derived from the latter to calibrate the type of group being titrated potentiometrically. This was found to be of special value in the present experiments.

The results of this work suggest that there is one fewer histidine residue available for titration in peroxidase than in the apoprotein, and complementary spectroscopic studies suggest that a tyrosyl residue may also be haem-linked in peroxidase.

Previous studies of this kind on horseradish peroxidase have probably been made with a mixed isoenzyme fraction (Theorell, 1943) and the study of Morita & Kameda (1958) was on the Japanese radish peroxidase.

METHODS AND MATERIALS

Horseradish peroxidase. Isoenzyme B (Shannon, Kay & Lew, 1966) was purified by methods described by Phelps & Antonini (1969) and apoperoxidase was prepared

from this by HCl-acetone treatment as described in the same reference. Reconstituted peroxidase was generated by stoichiometrically combining haematin (E. Merck, Darmstadt, Germany) in dilute NaOH with an aqueous solution of apoperoxidase in buffer.

Reagents. These were AnalaR and water was double-distilled. Guanidinium chloride was prepared as described by Nozaki & Tanford (1967) by neutralizing guanidinium carbonate with HCl and crystallizing the resulting hydrochloride from benzene, methanol and, finally, water. The extinction of the 6M solution at 280nm was less than 0.05.

Amino acid analyses. A Technicon amino acid analyser (Technicon Ltd., Chertsey, Surrey, U.K.) was used, operated under standard conditions. Samples for analysis were hydrolysed in constant-boiling HCl which had been redistilled over N_2 . The ampoules contained 1mg of protein/1ml of constant-boiling HCl and were evacuated and flushed with N_2 before hydrolysis. Duplicate analyses were performed at each of three times of hydrolysis (16, 27 and 35h hydrolysis), and the values for serine and threonine corrected for destruction by extrapolating to zero time of hydrolysis; the valine, leucine and isoleucine values were extrapolated for infinite time of hydrolysis.

Sugar analyses. These were performed by g.l.c. of the trimethylsilyl ethers as described by Clamp, Dawson & Hough (1967).

Isoelectric point. These determinations were performed on an L.K.B. isoelectric-focusing electrophoretic apparatus by using wide-range ampholine carriers (pH3–10). Power (1W) was dissipated at 4°C for 36h and the resultant column was eluted by use of an L.K.B. Uvicord flow cell and Ultrarac fraction-collector; 1ml fractions were collected. Peroxidase, apoperoxidase and haem-recombined peroxidase were run at least twice on identical columns. The freshly collected effluent was immediately measured for pH and protein content.

Sedimentation. This was performed in a Beckman Analytical Ultracentrifuge operating under standard conditions. Sedimentation coefficients were obtained from the movement of the maximum ordinate of the schlieren pattern and were corrected to water as solvent at 20°C.

Potentiometric titrations. For these titrations the apparatus shown in Fig. 1 was used. Two procedures were used in calibration. Standard buffers at pH4, 7 and 10 were used for initial scale adjustment. Standardization was achieved as follows. A 4ml sample of 0.15M-KCl was added to the titration vessel and equilibrated at 20°C. The pH was lowered by adding a few microlitres of 1M-HCl from the acid syringe; this removed dissolved CO_2 . The pH was then adjusted to approx. 6–7 with 1M-KOH in the alkali syringe. The pH was then lowered by the addition of 52 μ l of 1M-HCl. Since the activity coefficient of HCl in 150mm-KCl at 20°C is 0.781, this corresponds to pH2.00. Volume additions were accurate to $\pm 0.2\mu$ l and the pH was measured on a Radiometer model 23pH-meter. Such standardization was used after each reversible titration of the protein sample. Titration of the peroxidase was most often undertaken from pH7 to 3, pH3 to 11.5, from pH11.5 to 3 and from pH7 to 11.5, 11.5 to 3 and 3 to 11.5. Protein concentrations were measured spectroscopically at pH7 on samples withdrawn from the titration vessel by using the following parameters: peroxidase E_{mM} (λ 404 nm) = 97; apoperoxidase E_{mM} (λ 280 nm) = 13.

Fig. 1. Diagram of potentiometric titration apparatus. A, B, μ l syringes containing standardized HCl and KOH respectively; C, thermostat-controlled chamber with lid; D, protein solution; E, glass electrode, F, calomel electrode; G, stirrer; H, inlet supply for CO_2 -free, humidified N_2 .

By a refinement of this technique with two thermostat-controlled water baths at different temperatures it was possible to perform rapid transitions of temperature after a fixed addition of alkali by switching the water circulating around the titration vessel from one temperature to the other, and to measure the Δ pH arising from this change in temperature. These measurements were confirmed by performing complete reversible titrations on the protein samples at each temperature. From the resultant data the heats of ionization of the various groups being titrated could be assigned by using the relationship: $\Delta H_{app} = 2.303R[\delta pH/\delta(1/T)]$, the pH difference being measured at a constant value of the net charge on the protein molecule.

Additional measurements were carried out by using a modified Aminco Titratheormograph (American Instrument Co., Silver Springs, Md., U.S.A.), where titrant, either 0.1M-KOH or 0.1M-HCl, was delivered from an accurately thermostat-controlled reservoir by an electrically driven syringe to the protein sample at the same temperature, and the heat liberated by the titrated groups on the protein was measured by a rapidly responding thermistor (response time about 100 ms), whose output was amplified through appropriate operational amplifier circuits and traced out on recorder chart paper. This system was calibrated by titrating 5 ml of 10 mM acid against 0.5 M alkali or 5 ml of 10 mM alkali against 0.5 M acid.

Other determinations. Tyrosine perturbation spectra were recorded on a Cary 14 spectrophotometer by using thermostat-controlled cuvette-holders. The pH-dependence of the ionization of the tyrosyl residues was followed at 293 nm for apoperoxidase by using a value of 2.3 for the millimolar absorption change (Hermans, 1962). The Soret peak of the haem proteins interfered considerably with similar measurements at the same wavelength and the method of Hermans (1962) was used, with E_{mM} 10 at 245 nm.

Tryptophan was determined by the method of Edelhoch (1967). Cysteine and cystine were measured by the method of Cavallini, Graziani & Dupré (1966).

RESULTS

Amino acid analysis. For work on the hydrogen ion equilibria of proteins a knowledge of the amino acid composition of the protein is necessary, particularly in respect of those residues which contribute to the titration curve. Table 1 shows the results of the amino acid analysis on a purified sample of isoenzyme B, of which a portion migrated as a single band in the isoelectric focusing apparatus. For comparison the values calculated from the data of Shannon *et al.* (1966) and the values of Weinryb (1968) are also given. Analyses on a sample of apoperoxidase gave values within ± 1 residue. Tryptophan, as measured in duplicated determinations by the method of Edelhoch (1967), was present as one residue per mol in both apoperoxidase and peroxidase. No cysteine residues were apparent on direct reaction with 5,5'-dithiobis-(2-nitrobenzoic acid). After 1 h of reduction with sodium borohydride, eight thiol groups (equivalent to four disulphide bridges in the parent protein) were measured by the method of Cavallini *et al.* (1966).

Isoelectric point. The isoelectric points of the peroxidase isoenzyme, of the apoprotein derived from it, and of the haem-recombined peroxidase were determined on an isoelectric focusing-column, as described in the Methods and Materials section, which created, when at equilibrium, a linear pH gradient between pH 3.3 and 9.7. The pH of these proteins was measured immediately after elution. The mean values for the isoelectric point obtained on duplicate runs were: peroxidase, pH 8.7; apoperoxidase, pH 6.8; haem-recombined peroxidase, pH 8.7. The maximum charge on the completely protonated apoprotein is made up of 1 (amino terminal group) + 5 (lysyl ϵ -NH₃⁺) + 21 (arginyl NH₃⁺) + 3 (histidyl), i.e. a total of 30 protons. The isoelectric point would thus occur when 30 carboxyl groups have ionized, forming corresponding negative charges. Table 1 shows that the maximum number of carboxyl and amide groups is 48 + 21, i.e. 69. Attempts were made to measure amide nitrogen, but the great variability of the results precluded any definitive assessment of the exact number of side-chain carboxyl groups present. Values of approx. 30 amide groups were obtained. This still leaves a deficit of approx. 10 carboxyl groups unaccounted for in the above scheme if the observed isoelectric point of 6.8 is accepted.

Sugar analysis. Peroxidase is a glycoprotein containing 16% sugar (Shannon *et al.* 1966). Since certain of these sugar moieties might be covalently linked to aspartic acid residues, analysis for the composition of these sugar prosthetic groups was undertaken. The results are shown at the foot of Table 1, where it is apparent that the glycoprotein

Table 1. *Amino acid and sugar analysis of horseradish peroxidase isoenzyme B*

Results are expressed in residues per 40 000 g of protein n.d., Not determined.

Amino acid residue	Present work	Shannon <i>et al.</i> (1966)	Weinryb (1968)
Lysine	6	6	6
Histidine	3	3	3
Arginine	20	22	18
Aspartic acid	47	54	48
Threonine	23	26	24
Serine	20	23	24
Glutamic acid	21	20	21
Proline	19	17	18
Glycine	18	18	18
Alanine	22	25	24
$\frac{1}{2}$ -Cystine	8	4	6-8
Valine	19	18	16
Methionine	3	3	3
Isoleucine	12	13	12
Leucine	34	39	31
Tyrosine	5	4	5
Phenylalanine	19	24	18
Tryptophan	1	1*	n.d.
Sugar analysis			
Mannose	24	Present	n.d.
Xylose	8	Present	n.d.
N-Acetylglucosamine	8	†	n.d.
Fucose	8	Present	n.d.

* Strickland, Kay, Shannon & Horowitz (1968).

† Sugars were identified by paper chromatography, and the amounts present were not quantified.

contains eight *N*-acetylglucosamine moieties in addition to xylose, fucose and mannose. It is probable that these amino sugars are coupled by aspartamido linkages to the protein chain, which would account for the apparent excess of carboxyl-function groups in the protein. Further attention will be drawn to anomalies of the aspartic acid and glutamic acid residues in discussion of the differences between the titration curves of apoperoxidase and peroxidase.

Molecular weight of apoperoxidase. Since the physical parameters of apoperoxidase have not received much attention, the sedimentation behaviour of the apoperoxidase was compared with that of peroxidase to confirm that no significant change in molecular weight had occurred as a result of treating peroxidase with acid-acetone at low temperatures. Fig. 2 shows the plot of the concentration-dependence of the sedimentation coefficient. For a similar series of experiments at low speeds the diffusion coefficient was calculated from the schlieren patterns obtained in a synthetic-boundary cell, at a series of protein concentrations. The molecular weight calculated from $s_{20,w}$ with a

Fig. 2. Sedimentation coefficients of apoperoxidase (●) and peroxidase (○) as a function of concentration. Analytical runs were performed at 20°C in 100 mM-NaCl-10 mM-sodium phosphate buffer, pH 7.0, at 59 780 rev./min and were corrected for density of solvent.

value of $\bar{v}$ (= 0.70) calculated from the amino acid and sugar composition was 39700 ± 2300 , and indicated no significant change in the molecular weight of the apoperoxidase from that of peroxidase.

Potentiometric titration curves. Figs. 3 and 4 show the results of the potentiometric titration of aqueous solutions of peroxidase and apoperoxidase in 150 mM-potassium chloride at 20°C. Each curve is the average of six titrations on separate samples of the protein and each has been shown to be reversible within the pH range 3–11. The average error in reproducibility within one experiment and between experiments was equivalent to ± 0.5 group dissociated/mol of protein.

The equation governing the dissociation of hydrogen ions from any set of identical groups is:

$$\text{pH} = \log \frac{x_1}{1-x_1} + \text{p}K_1 - 0.868 w Z$$

where x_1 is the average degree of dissociation of such a group at the particular value of pH, $\text{p}K_1$ is the intrinsic dissociation coefficient of this group and w is an electrostatic factor, which may or may not be a constant independent of Z , the net charge per molecule at the particular pH.

The theoretical curves so obtained are also shown in Figs. 3 and 4. The value of w derived from appropriate graphical procedures (Tanford, 1962) for peroxidase is in reasonable agreement with the value predicted from the theoretical treatment, where:

$$w = \frac{\epsilon^2}{DRkT} \left(1 - \frac{\kappa R}{1 + \kappa a} \right)$$

in which ϵ is the unit of electron charge, D is the dielectric constant of the medium, k is the Boltzmann constant, T is the temperature, κ is the Debye-Hückel parameter, R is the radius of the protein molecule and a is the radial distance of closest approach of the average salt ion ($a = R + 2.5 \text{ \AA}$). For peroxidase with a molecular weight of 40 000, a partial specific volume $\bar{v} = 0.70$ and, assuming 20% hydration, $R = 24.2 \text{ \AA}$, w would be 0.045 in 150 mM-potassium chloride at 20°C. The experimental value that gives the best fit is 0.058. It is noteworthy that as peroxidase is a glycoprotein containing uncharged sugars, which do not contribute to the proton equilibrium, the value of w may be re-calculated for the protein moiety alone by assuming its value of $\bar{v}$ as 0.73 but allowing an increase in a to $6.5 \text{ \AA}$ to accommodate these surface sugars. This gives $w = 0.053$ at 20°C in 150 mM-potassium chloride, a value in closer agreement with the experimental findings.

However, for apoperoxidase, though a large proportion of the titration curve can be fitted to the same experimental value of w as was used for peroxidase, anomalies are seen in the titration of the carboxyl region. Here a value of $w = 0.029$ seems best in agreement with the experimental data, though this value is unrealistic when compared with the radius of the protein particle that would possess such a value ($R = 31 \text{ \AA}$).

Apparent heat of dissociation. The measurement of heats of dissociation as an adjunct to the interpretation of the hydrogen ion equilibria in proteins is of fundamental importance, since it enables the transition from the titration of one group type to the titration of another to be defined sharply, as for instance from carboxyl to imidazole group or from α -amino to phenolic group.

Figs. 5, 6 and 7 show the apparent heats of dissociation of the various groups titrated in peroxidase and apoperoxidase respectively. These values were determined in two ways: in the first, two titrations were performed at different temperatures (8°C and 20°C). A second way directly measured the heats of dissociation by measuring the heat-evolution during the course of titration by means of a thermistor, and this method produced data for peroxidase in excellent agreement with the ΔH values derived in the first method (Fig. 5).

For apoperoxidase there was considerable disagreement in the values for the heats of dissociation. The heats obtained directly (second method; Fig. 7) as measured by titrating an alkaline solution of protein with acid did not agree with those obtained by titrating an acid solution of protein with alkali, and neither of them agreed with the values determined in the first method, which are shown in Fig. 6. The possible explanations for these discrepancies are discussed below.

Fig. 3. Titration curve for peroxidase at 20°C in 150 mM-KCl. The experimental points (●) are shown with curves theoretically determined (for the number of groups of intrinsic pK indicated) by using the electrostatic interaction factor (w) shown. Further details are described in the text.

Comparison of the titration curves of peroxidase and apoperoxidase. (a) The acid limb. Three features warrant attention: (i) there are six more carboxyl groups apparently titrating in apoperoxidase than in peroxidase; (ii) the value of w required to accommodate the titration curve, whilst in the normal range for peroxidase (0.058), is much less for apoperoxidase; (iii) the value of ΔH_{app} for the carboxyl region of the titration curve appears anomalous when the methods of deriving this quantity are compared.

Arguments are developed below which suggest both that apoperoxidase is an apparently substantially different protein from peroxidase, and also that in apoperoxidase, but not in peroxidase, a conformational change may occur below pH 7.

(b) The neutral region. The main difference observed here resides in the number of histidine residues that can be accommodated within the titration curve. In peroxidase, two histidine residues are apparent whereas in apoperoxidase three are compatible with the experimental data.

Fig. 4. Titration curve for apoperoxidase at 20°C in 150 mM-KCl. The experimental points and curves are as described for Fig. 3.

Fig. 5. Apparent heats of dissociation as a function of pH of peroxidase in 150 mM-KCl as calculated from titrations at 8°C and 20°C (○) and by direct calorimetry (●). The latter points correspond to both forward titrations of an acid protein solution with base and the titration of an alkaline protein solution with acid, where no differences were observed between the two experiments.

Fig. 7. Apparent heats of dissociation as a function of pH of apoperoxidase as determined by direct calorimetry. ○, Titration of an acid protein solution with alkali; ●, titration of an alkaline solution of protein with acid.

Fig. 6. Apparent heats of dissociation as a function of pH of apoperoxidase in 150 mM-KCl as calculated from titrations at 8°C and 20°C.

In both proteins the flat mid-region between pH 7 and 9 has been fitted to allow for the titration of one terminal amino group. In both proteins this group possesses a $pK_{int.}$ of 8.75, whereas the

histidine groups in apoperoxidase and peroxidase have pK values of 6.4 and 6.65 respectively.

The assignment of the histidine group that is not available for titration in peroxidase to a role in haem-binding is discussed below.

(c) The alkaline limb. Considerable experimental difficulty is encountered in this region. It is evident that at least one more group titrates in peroxidase than in apoperoxidase, and though it is to be expected that the ferrihaem group would titrate within this region in the former protein, experiments on the spectrophotometric titration of the tyrosine residues point to a considerable difference between the two proteins. It should also be remembered that, as these proteins contain a considerable proportion of arginine, with an expected $pK_{int.}$ about 12, the region of these titration curves between pH 11 and 11.5 will be contributed to by the dissociation of these groups, and a clear determination of the quantities of group titrating in each protein will be impossible.

Spectrophotometric titration of tyrosine residues. The potentiometric titration curve cannot resolve the individual contributions of lysine and tyrosine as the side-chain groups of these amino acids possess similar pK values. Measurements of the number of tyrosine groups ionizing across the relevant pH range would enable the separate contributions of lysine and tyrosine to be determined. Fig. 8 shows the results of experiments on the spectrophotometric titration. In peroxidase it is not feasible to measure the spectral perturbations at 295 nm because of substantial contributions from

Fig. 8. Spectrophotometric determination of tyrosine group ionizations/mol in peroxidase ($\circ$, $\square$) and apoperoxidase ($\bullet$, $\blacksquare$) as a function of pH, in 6M-guanidinium chloride + 150mM-KCl ($\circ$, $\bullet$) and in 150mM-KCl ($\square$, $\blacksquare$).

the Soret peak of the haem protein. The method of Hermans (1962) was therefore used and the ionization of tyrosine was followed at 246nm. In 150mM-potassium chloride at 20°C the surprising finding is that only one tyrosine can ionize up to pH 11.50. This is in contrast with the behaviour of apoperoxidase where four tyrosine residues ionize inside this pH region, with pK_{app} 10.9.

When the experiments were repeated with protein in 150mM-potassium chloride at 20°C with 6M-guanidinium chloride, the results suggested that there are four titratable tyrosine residues in peroxidase and five in the apoprotein.

With peroxidase in 6M-guanidinium chloride, one event is noteworthy. Across the neutral region, the protein changes from the characteristically red-brown colour on the acid side to a green solution on the alkaline side. The Soret band changes from a peak position at 394nm (acid) to a broad band with a maximum around 360nm (alkaline). The midpoint in this conversion occurs at pH 6.5, and would appear to indicate the change in environment of the haem group from one of low polarity to a more aqueous phase. The change is reversible.

DISCUSSION

The object of these experiments was to determine any significant difference between the protein and its apoprotein that might be involved in the process

of binding the haem prosthetic group. The following comments seem pertinent.

(1) The titration curves are fully reversible and the behaviour observed with apoperoxidase can be readily converted into that of peroxidase by stoichiometric titration with haem. The reversibility ensures that a proton dissociation equilibrium is being measured, and that this process is not linked to denaturation changes causing the hysteresis effects noted by Morita & Kameda (1958).

(2) The titration curves and spectrophotometric information suggests that in many respects the two proteins are dissimilar, e.g. in their carboxyl, tyrosine and lysine group titration behaviour (Table 2), and since the removal of the haem group is solely responsible for a change which is restored by its replacement, the conclusion seems inescapable that the haem group contributes essentially to the structure of the peroxidase protein; removal of it leads to a change in the protein far beyond what would be reasonably expected on the sole grounds of steric cover by the haem group.

In addition, the concentration-dependence of the sedimentation constant of apoperoxidase is significantly larger than that of peroxidase, as shown in Fig. 2, suggesting that the apoprotein has a higher frictional coefficient than its parent protein.

Further evidence of the difference between the two proteins is shown in a comparison of the far-violet circular dichroism (Strickland *et al.* 1968). This work has been corroborated by C. Phelps, P. Fasella & E. Antonini (unpublished work) who found that the optical rotatory dispersion of the two proteins follows similar characteristics to those shown in the former reference.

(3) The differences between the holo- and apo-protein are summarized in Table 2.

(a) Carboxyl groups: potentiometric titration reveals that there are six more groups titrating with ΔH_{diss} 0–2kcal/mol in apoperoxidase than in peroxidase. Since peroxidase contains a proto-haem group with two carboxyl groups, and these should titrate in this region, the net difference may be eight carboxyl groups. It is difficult to see a connexion between this large disparity of carboxyl groups and any functional significance of these groups in haem binding.

(b) Neutral region. Apoperoxidase apparently contains one more titratable histidine residue than peroxidase. The close similarity of the spectral data for liganded forms of ferric and ferrous forms of peroxidase to those of myoglobin and haemoglobin (Keilin & Hartree, 1951) suggests that there are close similarities in the ligand field around the iron atom of the haem group in these proteins and would favour the idea of a proximal histidine group co-ordinating to position 5 of the iron atom. The pK of the Soret spectral transition in 6M-guanidinium

Table 2. *Summary of group differences between peroxidase and apoperoxidase*

In peroxidase, the iron atom in the haem titrates with a pK of 10.70, as determined spectrophotometrically. Values in parentheses are assumed. n.d., Not determined.

	Peroxidase		Amino acid analysis	Apoperoxidase	
	no. of groups	pK		no. of groups	pK
α -Carboxyl	(1)	(3.75)	(1)	(1)	3.75
β , γ -Carboxyl	16	4.50	68 total (—8 <i>N</i> -acetylglucosamine, —39 amide) = 21 groups*	22	4.25
Imidazole	2	6.65	3	3	6.40
α -Amino	(1)	8.70	(1)	(1)	8.70
ϵ -Amino	6	10.55	6	3	10.70
Phenolic	1	10.55	5	4	10.70
Guanidyl		n.d.	20		n.d.

* In peroxidase, in addition to the number of β -, γ -carboxyl groups quoted, one would expect two additional carboxyl groups of the haem group with similar pK values to titrate.

chloride at pH 6.5 further implicates a histidine residue as being responsible for preserving the haem group in an environment of low polarity.

(c) The alkaline region. The results of the potentiometric titration alone reveal that one additional group is titrated in peroxidase. This would be expected since the conversion of peroxidase into alkaline peroxidase involves an additional ionization of the Fe^{3+} in the haem protein, with a pK value approx. 11.0 (Theorell, 1943). However, comparison of the spectrophotometric titrations of tyrosyl residues shows that the explanation must be more complicated. The major finding is that only one tyrosine residue ionizes in the haem protein in 150mM-potassium chloride whereas four residues do so in the apoenzyme under the same conditions. Thus in the haem protein the eight groups, determined potentiometrically, which ionize with a collective pK of 10.55, must include the one tyrosine residue, the six lysine residues and the one ferric haem transformation. In the apoprotein the titration value of seven groups must include the four tyrosine residues detected spectrophotometrically and three lysyl side chains, leaving three lysine groups unaccounted for in the potentiometric titration. No explanation is apparent for these findings at present, but titration of peroxidase and apoperoxidase in which the ϵ -amino groups of lysine have been blocked with maleic anhydride (Butler, Harris, Hartley & Leberman, 1969) may resolve some of the apparent discrepancies, as this technique should reveal the presence of the correct number of tyrosine groups in both proteins.

In respect of the possible implication of tyrosine as an additional haem-binding group, the experiments in 6M-guanidinium chloride are important. Spectrophotometric titration reveals four of the five

tyrosine residues in peroxidase, whereas for apoperoxidase it gives exact agreement with the five tyrosine residues demanded by the amino acid analysis. Moreover, back-titration, with acid, of a solution of peroxidase in 6M-guanidinium chloride to pH 7 and subsequent chromatography on Sephadex G-50 produces a product virtually indistinguishable from the parent peroxidase before treatment with denaturing agent. It seems probable that an additional attachment to the haem may be mediated by one tyrosyl residue. In his classic paper, Theorell (1943) suggested that the iron atom is bound to a carboxyl group in the protein component and that one of the carboxyl groups of the hemin is bound to a group with a $pK = 10.2$ in the free protein compartment. From the present work it seems likely that tyrosine may be implicated. This suggestion receives support from the work of Blumberg, Peisach, Wittenberg & Wittenberg (1968), in which the interpretation of the electron paramagnetic resonance spectra of ferriperoxidase at low pH, or of the high-spin form, suggested a symmetry which could be no higher than orthorhombic, where the electronic environment of the d electron system of the iron atom is not equivalent in the three principal cases x , y and z . Similarities were seen in the abnormal ferrihaemoglobins Osaka and Iwate where there is a replacement by tyrosine of histidine as a z -plane ligand for iron.

(4) Conformational changes in peroxidase and apoperoxidase. The potentiometric titration of apoperoxidase demands for its interpretation a change in the electrostatic interaction factor, w , from a reasonable value of 0.058 to one of 0.029. This is not sufficient evidence to claim that the apoprotein undergoes a conformational change below pH 7.0, but the lack of reversibility of the heat

measurements and the disagreement between the direct and derivative methods of measuring ΔH_{diss} for apoperoxidase lend some support to this argument. A comment on these heat measurements is needed. One difference between the two methods of measuring the heats of dissociation is the time required for their recording. Customarily, a full potentiometric titration curve took 40–60 min to achieve whereas the calorimetric measurements required 30–40 s. There is thus a difference of almost two orders of magnitude in the time needed to collect the data, and whereas the Van't Hoff calculations probably truly refer to an equilibrium situation, those obtained by calorimetric means may have been influenced by non-equilibrium effects.

It is instructive, finally, to restate the categories of functions of haem proteins first delineated by Theorell (1943), in which the protohaem prosthetic group can play many roles including those of (1) reversible combinations of the ferro form with ligands such as O_2 and CO as evinced by myoglobin and haemoglobin, (2) reversible oxidoreduction as in cytochrome *c* and (3) decomposition reactions involving hydrogen peroxide, either to water and oxygen as in catalase or to water and oxidizing equivalents as in peroxidase. All these activities are inherent in free haem, but each specific reactivity is enormously enhanced when haem is coupled to a specific protein; this requires that the environment of the haem site is favourable to the specific reaction and that, particularly, the proximal or distal haem-binding groups contribute specifically to the reaction. It is hoped that experiments in which data from the electron-paramagnetic-resonance spectra with nitric oxide are compared with those of similar derivatives of single chains

derived from mutant haemoglobins, in which the distal histidine has been replaced by tyrosine, may add support to the present work.

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