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THE PROTOPORPHYRIN-APOPEROXIDASE COMPLEX AS A HORSERADISH PEROXIDASE ANALOG

A FLUORIMETRIC STUDY OF THE HEME POCKET

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Similarity of the protein tertiary structures of the native horseradish peroxidase (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) and protoporphyrin-apoperoxidase complex has been shown on the basis of identity of the tryptophan fluorescence parameter at pH 2.0–8.0 and of the circular dichroism spectra of the two proteins. Absorption and fluorescence spectra have been obtained for protoporphyrin in the complex in the pH range 7.0–1.6. A shift in the apparent p*K* by 4 units has been observed for protonation of the protoporphyrin pyrrolic ring in the complex. From this shift, the dielectric constant has been evaluated for the heme pocket of the peroxidase (approx. 20). Fluorescence quantum yield of protoporphyrin in the complex increased with pH decreasing from 5.0 to 3.5, whereas the spectrum pattern and fluorescence lifetime did not change. The ions, I^- and $[Fe(CN)_6]^{-4}$, peroxidase substrates, did not quench the protoporphyrin fluorescence in the complex at about neutral pH, whereas the quenching markedly enhanced with lowering pH. The bimolecular constant for the I^--quenching of the porphyrin fluorescence in the complex showed a pH-dependence similar to that of the bimolecular rate constant for the reaction of peroxidase compound I with I^-. A mechanism for I^- oxidation at an acid pH in the presence of peroxidase has been proposed.

Introduction

The structure of the active site in horseradish peroxidase still remains unclear [1]. The fluorescent spectroscopy, in many cases, helps elucidate a mechanism of physico-chemical interactions in the enzyme active site, if the latter contains an appropriate chromophore. Protoheme IX does not fluoresce. The iron-free analog of protoheme IX, protoporphyrin IX, shows an intensive red fluorescence [2] and forms a fairly stable complex with apoperoxidase [3]. This allowed us to study not the native enzyme, but its iron-free analog, a specific protoporphyrin-apoperoxidase complex. Comparison of ultraviolet fluorescence, pH fluorescence dependence and circular dichroism spectra for the native peroxidase, apoperoxidase and protoporphyrin-apoperoxidase complex proved, that the tertiary structure of the peroxidase iron-free analog is similar to that of the native enzyme. The investigation into the acid-base properties of protoporphyrin IX in the complex has made it possible to evaluate the effective dielectric constant in the heme pocket of the peroxidase. To elucidate the mechanism of interactions between inorganic ions, peroxidase substrates and the enzyme we analyzed the effect of the peroxidase substrates, I^- and $[Fe(CN)_6]^{-4}$, as fluorescence quenchers, on the pH fluorescence dependence of the protoporphyrin-apoperoxidase complex.

Experimental

Materials

Horseradish peroxidase (isozyme C according to

the Shannon classification [4]) with $RZ = A_{403}/A_{273} = 3.1$ was isolated from a commercial preparation of Reanal ($RZ = 0.6$), as previously described [4,5]. In some experiments peroxidase from Sigma or Calbiochem, type VI, was used. All the peroxidase preparations were indistinguishable under experimental conditions. Hemin and protoporphyrin IX were purchased from Koch-Light and Calbiochem, respectively. Other reagents were of analytical grade. The water was glass-distilled three times.

Apoperoxidase. Apoperoxidase was prepared according to the technique described previously [6], which we partially modified. The enzyme (5–10 mg) was dissolved in 1 ml 5 mM sodium phosphate (pH 7.0), cooled to 0°C and adjusted to pH 1.8 by 6 M HCl. Then hemin was extracted twice using 1 ml cooled 2-butanone. After the organic solvent had been separated, the aqueous solution was neutralized and extensively dialyzed against 0.01 M Tris-HCl, pH 8.0 at 4°C. The native apoperoxidase was quantified by spectrophotometric titration with hemin.

Protoporphyrin-apoperoxidase complex. The complex was prepared at 4°C. Free porphyrin was dissolved in a minimal volume of 3 M HCl and desiccated over KOH [7]. The dication salt thus obtained was dissolved in a minimal volume of 0.1 M NaOH and diluted with 0.01 M Tris-HCl, pH 8.0. Apoperoxidase was dissolved in 0.01 M Tris-HCl, pH 8.0 to a concentration of 0.5 mM. To 3 ml of 0.5 mM apoperoxidase a 1.5-fold excess of protoporphyrin was added with the following incubation in the dark for 3 h. The mixture was then passed through the composite column (17 × 2-Sephadex G-25, fine; 17 × 2-Sephadex A-25), equilibrated with 5 mM sodium phosphate buffer, pH 7.0. The complex was eluted with 5 mM sodium phosphate buffer, whereas the protein-unbound protoporphyrin was tightly absorbed at the top of the column in the form of a dark-brown ring. Apoperoxidase was eluted with ionic strength growing to 0.1.

Equipment

Absorption spectra were recorded by a B-25 Beckman spectrophotometer. The pH was measured by a Radiometer pH-meter 64. Fluorescence spectra were recorded by a Hitachi MPF-4 spectrofluorimeter. Circular dichroism spectra were recorded by a Dichrograph Mark II JOBIN-IVON. All measurements were made at 20°C.

Methods

Continuous recording of pH dependence of fluorescence intensity. The protein solution (3.3 ml) in 5 mM sodium phosphate was placed into a spectrofluorimetric cell. Radiometer microelectrode G-2222 and K-4112 were placed into the cell with the aid of a special holder. The solution was mixed with a magnetic stirrer. Radiometer DTS-633 digital titration system was used to add microquantities of 0.9 M H_3PO_4 to decrease the pH. The signal from the pH-meter was fed to the X axis, while that from a spectrofluorimeter amplifier to the Y axis of the X-Y recorder. The pH dependence of the fluorescence intensity was measured for no longer than 7 min in the pH 8.0–1.5 range and no longer than 2 min in the pH range 3.0–1.5. No appreciable irreversible denaturation of the peroxidase or its derivatives was observed for these time intervals [5].

Continuous recording of the pH dependence of the absorbance. This was performed by a Hitachi 356 double-wavelength double-beam spectrophotometer. The absorbance was recorded for the protoporphyrin-apoperoxidase complex at 408 nm and for the native peroxidase at 403 nm. A reference beam was at 700 nm. A signal from the log convertor was applied to the Y axis of the X-Y recorder. The pH was lowered using a Radiometer digital titration system DTS-633. The solution from the titration cell was transferred into the 0.08 ml flow cell of the spectrophotometer by a peristaltic pump. The solution in the cell was completely replaced by the fresh portion for 10 s. The total volume of the titrated mixture was 3 ml. The signal from the pH meter was applied to the X axis of the X-Y recorder.

Fluorescence lifetimes of chromophores. These were measured with the instrument described previously, which was laboratory-built from modules of Ortec [8]. For tryptophan, we used a pulsed lump filled with hydrogen under 5 atm as a source of light monochromated by a 4 cm chlorine filter. For protoporphyrin, an air-filled pulsed lamp was used with a 400 nm Perkin Elmer interference filter as monochromator. The emitted light was selected by glass filter. The flash configuration was eliminated by the Birks-Munro equation [9]:

$$\tau^2 = \tau_F^2 - \tau_E^2$$

where τ_F is the 'observed' time of the fluorescence decay and τ_E is the time of the flash decay. The fluorescence decay was exponential.

The pK for ionic groups. The pK values for ionic groups in the protoporphyrin-apoperoxidase complex were calculated from the spectrophoto- or spectrofluorimetric titration curves from the following equation:

$$\log\left(\frac{I_2 - I_1}{I - I_1} - 1\right) = -n \cdot \mathrm{pH} + \mathrm{p}K \qquad (1)$$

where I_1 and I_2 are fluorescence intensities or absorbances of either completely protonated, or non-protonated entities; I is fluorescence intensity or absorbance at an intermediate pH and n is the number of gained protons.

Results

Similarity of protein tertiary structures of the native peroxidase and protoporphyrin-apoperoxidase complex

The iron-free analog of the peroxidase can be used as a convenient model in the structure-function study of the enzyme if the tertiary structure of the complex in question does not strongly differ or is similar to that of the native enzyme. Tertiary structures of some iron-free hemoprotein analogs, such as cytochrome *C* and cytochrome *C* peroxidase do not appreciably differ from tertiary structure of appropriate native proteins [10,11]. No relevant data have yet been published for the iron-free analog of horseradish peroxidase. To compare the tertiary structures of the native horseradish peroxidase and the protoporphyrin-apoperoxidase complex, we have studied the tryptophan fluorescence properties, their pH dependence and the circular dichroism spectra for the apo- and native peroxidases as well as for the protoporphyrin-apoperoxidase complex.

Isozyme C of horseradish peroxidase contains one tryptophan residue, Trp-117, and five tyrosine residues [12]. Both tryptophan and tyrosine residues show fluorescence in the peroxidase [13,14]. The long-wavelength absorption band for the tyrosine residues is at 287 nm [15]. The quantum yield and spectrum patterns of the peroxidase ultraviolet fluorescence, obtained at an excitation wavelength of 290 nm (Table I) and 297 nm coincided. So, the peroxidase ultraviolet fluorescence characterized only

TABLE I

CHARACTERISTICS OF THE TRYPTOPHAN FLUORESCENCE IN THE NATIVE PEROXIDASE, APOPEROXIDASE AND PROTOPORPHYRIN-APOPEROXIDASE COMPLEX

Characteristics	Native peroxidase		Apoperoxidase		Protoporphyrin-apoperoxidase complex	
	pH 8.0	pH 2.0 [b]	pH 8.0	pH 2.0	pH 8.0	pH 2.0 [b]
Quantum yield vs. free tryptophan in solution, R_q [a]	0.014 ± 0.002	0.051 + 0.002	0.041 ± 0.002	0.058 ± 0.002	0.016 ± 0.002	0.048 ± 0.002
Fluorescence maximum, λ_{max} (nm)	328	345	335	345	328	345
Spectrum width at half its height $\Delta\lambda$ (nm)	56	65	65	65	56	65
Fluorescence lifetime, τ (ns)	3.3 ± 0.2	2.7 ± 0.2	2.6 ± 0.2	2.7 ± 0.2	3.1 ± 0.2	2.6 ± 0.2

[a] The relative quantum yield (R_q) of the tryptophan fluorescence in the peroxidase was measured vs. the quantum yield of the free tryptophan fluorescence under the same conditions according to Cowgill [32].

[b] At pH < 3.0 the dissociation constant of the peroxidase grows high enough for heme extraction from the enzyme into a water-organic system to become feasible. Even at pH 2.0 the heme (or protoporphyrin) remained, however, bound to the protein. This was evidenced by differences in fluorescence spectra of the protoporphyrin dication in solution and in the presence of apoperoxidase.

the tryptophan-117 fluorescence. In further experiments tryptophan was excited at 290 nm, because at this wavelength the protein shows a greater absorption.

The tryptophan fluorescence parameters in the apo- and native peroxidases (Table I) as well as the pH dependence of the fluorescence intensities (Fig. 1) markedly differed. The low quantum yield of the tryptophan fluorescence in hemoproteins is usually thought to be due to the heme quenching effect owing to energy migration [13,14,16]. As the energy migration process is dynamic quenching [9], removal of heme from the protein is expected to increase the lifetime, τ, and the quantum yield, R_q. We observed, on the contrary, a decrease in τ with R_q considerably risen for apoperoxidase (Table I). So, the apo- and native peroxidases differed considerably in their contributions of static and dynamic quenching of the tryptophan fluorescence. This can be accounted for only by difference in the protein conformation in proximity to the tryptophan residue for these two proteins. The pH fall from 4.0 to 2.0 gradually shifted the fluorescence maximum of apoperoxidase from 335 to 345 nm. Yet the rise in the fluorescence intensity at pH < 6.0 did not alter the fluorescence lifetime. This may be indicative of the change in the protein conformation supposed before [17]. For the native peroxidase, the fluorescence intensity did not alter at pH 8.0–3.0 (Fig. 1, curve 1). At pH < 3.0 the fluorescence intensity rapidly increased reaching its maximum at pH 1.6. Characteristics of the tryptophan fluorescence at pH 8.0 and 2.0 considerably differ (Table I). The Stern-Volmer constant of the tryptophan fluorescence quenching by I^- increased from 0.604 M^{-1} at pH 5.0 to 5.36 M^{-1} at pH 2.0, the change in the quenching by I^- taking place only at pH < 3.0. These results show that at pH < 3.0, conformation of the peroxidase protein molecule alters giving rise to the observed changes in tryptophan fluorescent properties and to a pronounced dissociation of the hemoprotein complex. So, the tryptophan fluorescent properties altered under the same conditions as those inducing the protein conformation changes: for apoperoxidase at pH 4.0–6.0, for native peroxidase at pH < 3.0. This shows that the tryptophan residue in the horseradish peroxidase is a fluorescent label sensitive to the overall conformation of the protein moiety.

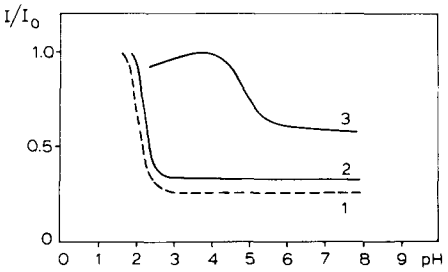

Fig. 1. pH Dependence of the tryptophan fluorescence in the native peroxidase (1), protoporphyrin-apoperoxidase complex (2) and apoperoxidase (3). Complex was purified (see Experimental). Protein concentration was 0.5 μM in 5 mM sodium phosphate. Excitation at 290 nm, fluorescence at 340 nm. 20°C.

The tryptophan fluorescent parameters in the protoporphyrin-apoperoxidase complex do not, in fact, differ from those in the native peroxidase (Table I). The pH dependence of the fluorescence intensity was also similar to that of the native enzyme (Fig. 1, curve 2). Thus, the tryptophan environment in the iron-free analog of the peroxidase was similar to that in the native enzyme and considerably differed from that in apoperoxidase. One can believe that similarity between the tryptophan fluorescent properties in the native enzyme and in the protoporphyrin-apoperoxidase complex is indicative of similarity between the protein tertiary structures in the native peroxidase and its iron-free analog. This was also supported by the following independent experiments. When heme is separated from the protein, the intensity of the negative band in the circular dichroism spectra of the peroxidase at 221 nm falls by 18% [18]. For the protoporphyrin-apoperoxidase complex, the intensity of the negative band increased by 18% at 221 nm compared to apoperoxidase. So, the circular dichroism spectra also indicated that the structures of the native peroxidase and its iron-free analog are similar.

Spectrophotometric and spectrofluorimetric titration of protoporphyrin-apoperoxidase complex

Protonation of the protoporphyrin pyrrolic ring can be described as follows:

$$H_2P \underset{K_3}{\overset{H^+}{\rightleftharpoons}} H_3P^{+1} \underset{K_3}{\overset{H^+}{\rightleftharpoons}} H_4P^{+2} \qquad (2)$$

Absorption and fluorescence spectra of the free base H_2P, monocation H_3P^{+1} and dication H_4P^{+2} markedly differ [2,19]. This facilitates a precise identification of various protonation entities of the protoporphyrin in the complex from the spectrophoto- and spectrofluorimetric titration curves and helps elucidate the microenvironment effect on acid-base properties of functional groups in the heme pocket.

At pH 7.0, absorption spectra within 320–700 nm (Fig. 2) and red fluorescence spectra (not corrected for variation in detector response) (Fig. 3) of the protoporphyrin-apoperoxidase complex corresponded to the free base protoporphyrin in its monomeric form. At pH 3.0–8.0, the absorption spectrum pattern and extinction coefficients did not alter at different wavelengths. At pH < 3.0, the initial four-band spectrum in the visible region became diffused (Fig. 2; curve 2), and at pH 1.6 a two-band spectrum appeared (Fig. 2, curve 3) with maxima at 550 and 590 nm typical of the protoporphyrin dication. Absence of isobestic points suggested that a complicated equilibrium between more than two different spectrum forms was established. The spectrum recorded at pH 2.2 did not, however, correspond to the spectrum of the protoporphyrin monocation. The pH dependence of absorbance in the Soret band for the native peroxidase and the protoporphyrin-apoperoxidase complex were similar (Fig. 4a). The change in absorbance at pH < 3.0 was due to the protein denaturation at low pH. In the protoporphyrin-apoperoxidase complex the denaturation proceeded at a slightly higher pH, which was explained by the lack of the coordination bond between the heme iron and protein ligands in the

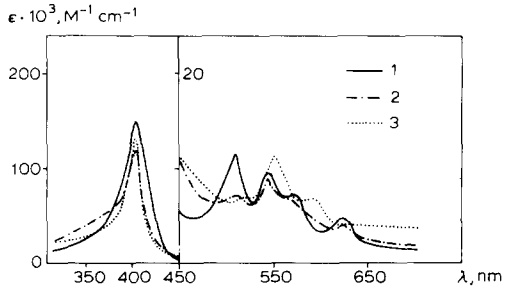

Fig. 2. Absorption spectra of the protoporphyrin-apoperoxidase complex at pH 7.0 (1), 2.2 (2) and 1.6 (3). 10 μM of the complex in 5 mM sodium phosphate, 20°C.

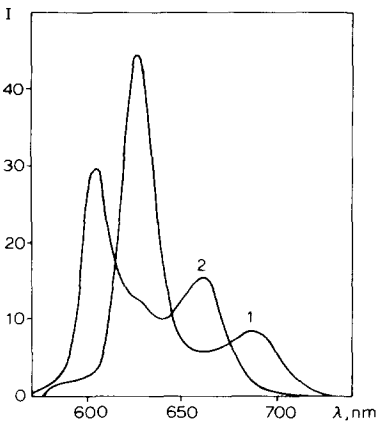

Fig. 3. Fluorescence spectra of the 0.05 μM protoporphyrin-apoperoxidase complex at pH 7.0 (1) and 1.6 (2). Excitation at 405 nm. 5 mM sodium phosphate, 20°C.

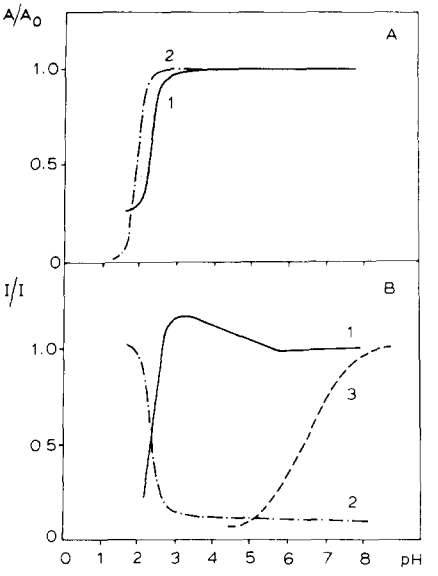

Fig. 4a. pH Dependence of absorbance in the Soret band for the protoporphyrin-apoperoxidase complex (1) (408 nm) and for the native peroxidase (2) (403 nm). Conditions are the same as in Fig. 3. b. pH Dependence of the fluorescence intensity of the protoporphyrin-apoperoxidase complex (1, 2) and of protoporphyrin in aqueous solution (3): 1 is the fluorescence of the porphyrin free base at 626 nm, excitation at 405 nm; 2 is the fluorescence of the protoporphyrin dication at 600 nm, excitation at 405 nm; 3 is the fluorescence of protoporphyrin in aqueous solution at 617 nm, excitation at 400 nm.

complex. This lowered stability of the complex at acid pH compared to the native peroxidase [5].

The pH dependence of the porphyrin free base fluorescence in the complex was roughly similar to the pH dependence of absorbance in the Soret band (Fig. 4). The fall in the fluorescence intensity of the protoporphyrin free base at pH < 3.0 was accompanied by growth in the fluorescence intensity of the protoporphyrin dication (Fig. 4). Besides, the pH dependence of fluorescence for the tryptophan residue in the protophorphyrin-apoperoxidase complex (Fig. 1, curve 2) largely coincided with the pH dependence of fluorescence for the protoporphyrin dication (Fig. 4b, curve 2). So, the protonation of the porphyrin molecule in the complex was a result of the acid denaturation of the protein molecule. The change in the medium in this case was abrupt, entailing an immediate conversion of porphyrin free base into dication. The apparent constant (K_{app}) of the protoporphyrin protonation was equal to approx. 2.5. On the other hand, the pH dependence of the porphyrin fluorescence in aqueous solution (Fig. 4b, curve 3) was characterized by K_{app} 6.5. Thus, a considerable shift in the pK_{app} (by 4 units) was observed for protonation of the porphyrin pyrrolic ring in the protoporphyrin-apoperoxidase complex compared to aqueous solutions.

At pH 3.0–6.0 the quantum yield of the porphyrin fluorescence in the complex increased by 18% (Fig. 4b, curve 1), while the fluorescence lifetime (16.5 ns) as well as the absorption and fluorescence spectra did not change. This increase in the quantum yield of the fluorescence was controlled by a group with pK 4.3.

Quenching the protoporphyrin-apoperoxidase complex fluorescence by inorganic anions

We studied the quenching by inorganic anions, such as I^-, Cl^-, Cs^+ and $[Fe(CN)_6]^{-4}$, of the protoporphyrin fluorescence in its complex with apoperoxidase at pH 3.0–8.0. At pH ⩾ 6.0, I^- did not markedly quench the fluorescence, though the enzyme molecule had a positive charge [17], and an electrostatic attraction between the protein and I^- should be conducive to the quenching. Cs^+ in aqueous solutions quenched the fluorescence of protoporphyrin almost as well as I^- did, but did not quench the protoporphyrin fluorescence in the complex. So, the effect of the local negative charge on the quenching was also excluded. At about neutral pH, the steric hindrances thus prevented the contact of the porphyrin ring with small molecules from an aqueous solution. At pH below 5.0, the I^- began to quench the protoporphyrin fluorescence in the complex, the quenching enhancing with falling pH (Fig. 5a). The fluorescence quenching of another chromophore, tryptophane residue, by I^- ions was observed only at pH < 3 (Fig. 5b), during an overall structural disordering of the peroxidase protein moiety. Therefore, at pH 5.0–3.0 a local loosening in the heme pocket occurred.

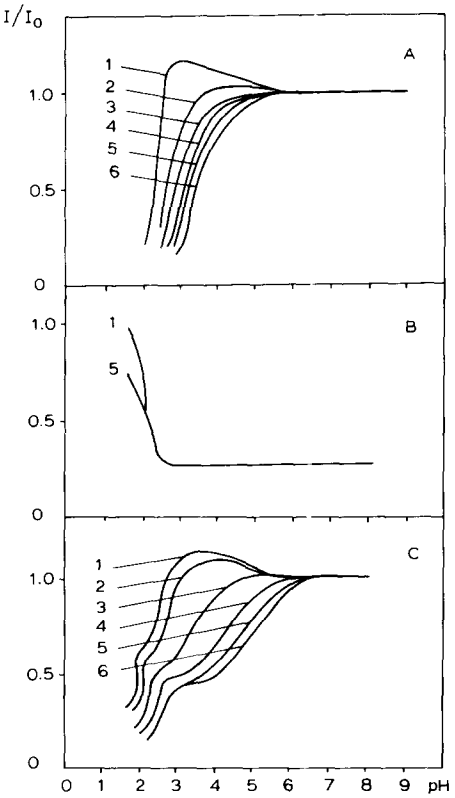

Fig. 5. pH Fluorescence dependence of porphyrin (A and C) and tryptophan residue (B) in the protoporphyrin-apoperoxidase complex at various concentrations of quenchers KI (A, B) and NaCl (C). KI concentrations: I, no KI; 2, 5.9 mM; 3, 11.8 mM; 4, 17.6 mM; 5, 23.3 mM; 6, 29 mM; NaCl concentrations: I, no NaCl; 2, 0.01 M; 3, 0.1 M; 4, 0.4 M; 5, 0.8 M; 6, 1 M. A, C are excitations at 400 nm, fluorescence at 628 nm; B is excitation at 290 nm, fluorescence at 340 nm; 0.1 μM solution of the protoporphyrin-apoperoxidase complex in 5 mM sodium phosphate.

The Cl^- weakly quenched the protoporphyrin fluorescence (K_{eff} 1.0 M^{-1}) and did not quench the tryptophan fluorescence in proteins [20]. For the protoporphyrin-apoperoxidase complex, increase in the Cl^- concentration up to 1 M had little effect on absorption spectrum, on the pH dependence of the absorbance in the Soret band and on the pH dependence of the tryptophan fluorescence at pH 3.0–6.0. So, high NaCl concentration did not affect the structure of the complex at pH 3.0–6.0. The porphyrin fluorescence in the complex was, however, quenched by Cl^- (Fig. 5c). The pH dependence of the fluorescence intensity at various NaCl concentrations had two distinct steps: the quenching at pH 3.0–6.0 related to the local loosening, whereas at pH < 3.0 to the overall loosening of the protein moiety.

The I^- quenched the porphyrin fluorescence at pH $\leqslant 5.8$, whereas $[Fe(CN)_6]^{-4}$ at pH $\leqslant 4.2$. To assess the size of $[Fe(CN)_6]^{-4}$ and I^- we used a parameter a of a closest approach of ions from the Debye-Hückel equation. For $[Fe(CN)_6]^{-4}$ ion, a is 5 Å and for I^- ion, 3 Å [21]. The fluorescence quenching of the complex by $[Fe(CN)_6]^{-4}$ showed the existence of steric hindrances for a bulky quencher to contact with the protoporphyrin at pH > 4.2. The quenching

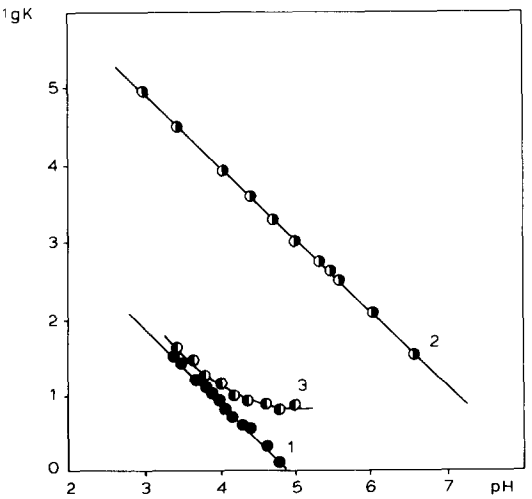

Fig. 6. pH Dependence of log K_{eff} and log K for fluorescence quenching of protoporphyrin in the protoporphyrin-apoperoxidase complex by I^-; line (I) is for log K_{eff}; line (3) is for log K. pH Dependence of log of the bimolecular rate constant for the reaction between I^- and compound I of the peroxidase according to data of Ref. 31, line 2.

of the porphyrin fluorescence in the complex by I^- was quantitatively characterized by the Stern-Volmer quenching constant, K_{eff}. At pH 3.0–5.0, the log K_{eff} vs. pH was a straight line with a slope equal to −1 (Fig. 6).

Discussion

Effective dielectric constant in the peroxidase heme pocket

The shift in protoporphyrin K_{app} by 6 units in micellar solutions was accounted for by a considerable decrease in the dielectric constant (up to approx. 10) in the micelle nucleus [22]. The low polarity in the peroxidase heme pocket was attested earlier by the data on binding the fluorescent label, 1-aminonaphthalene-8-sulfonic acid, with horseradish apoperoxidase [23–25]. The observed protoporphyrin pK shift (by 4 units) in the complex can be also accounted for by a low polarity in the heme pocket. At pH < 3.0, apoperoxidase bears about 29 positive charges [12,17], which can also entail a pK shift within 1 unit. Furthermore, the local positive charge in proximity to the heme pocket can turn out higher than the charge averaged along the entire protein surface. In any case a mere positive charge could not shift pK by 4 units. This could be induced only by a low polarity in the heme pocket. From this shift, the effective dielectric constant (approx. 20) in the heme pocket of the peroxidase was calculated by the method described elsewhere [22]. One can surmise from the above that the K_{app} of other functional groups in the peroxidase heme pocket thus markedly differed from those in aqueous solutions.

Conformation change in the protoporphyrin-apoperoxidase complex at pH 3.0–5.0

Increase in the quantum yield of the protoporphyrin-apoperoxidase complex fluorescence as the pH changed from 6.0 to 3.3 (whereas the absorption and fluorescence spectra and the fluorescence lifetime of the porphyrin remained unaltered) can be accounted for by conformational changes in the complex, which are controlled by the group with pK 4.3. This pK value is close to the pK earlier determined for the group of the peroxidase active site which affects the enzyme-H_2O_2 interaction [26] and the peroxidase complexing with CN^- and F^- [27,28]. A group with

pK 4.2 was also revealed for the native peroxidase from the NMR data [29]. This pK value was ascribed by some authors to one of the propionic acid residues of the heme [28,29]. The presence of the static component in the quenching of the porphyrin fluorescence in the complex allows us to interpret the nature of this conformational change in detail.

The equilibria between different conformers with changing pH can be schematically represented as follows:

$$\begin{array}{lcc} \text{no fluorescence} & EH(H_2P \cdot Q) \underset{H^+}{\overset{K'_H}{\rightleftharpoons}} E(H_2P \cdot Q) & (3) \\ & \downarrow\uparrow \; K'_c \qquad\qquad \downarrow\uparrow \; K_c & \\ \text{fluorescence} & EH(H_2P \ldots Q) \underset{H^+}{\overset{K_H}{\rightleftharpoons}} E(H_2P \ldots Q) & (4) \end{array}$$

(Scheme 1)

where E is the protein moiety; EH is its protonated entity; Q is an intraprotein quencher (sulfur of methyonine or disulfide bond, carboxylate ion, etc. [30]), contacting with the porphyrin ring in the 'closed' conformers $EH(H_2P \cdot Q)$ and $E(H_2P \cdot Q)$; $EH(H_2P...Q)$ and $E(H_2P...Q)$ are 'open' conformers in which the porphyrin ring and the quencher are spaced; K'_c and K_c are the equilibrium constants of the conformational change; K'_H and K_H are the protonation constants of appropriate conformers. The lifetime of each conformer should be considerably over the lifetime of the excited porphyrin molecule, i.e., it should be measured in microseconds or in greater units. The growth in the quantum yield with pH changing from 6.0 to 3.3 implies an increase in probability for emergence of the conformer $EH(H_2P...Q)$. So, the equilibrium constants of conformation changes, K'_c and K_c, should differ ($K'_c > K_c$).

pH Dependence of Stern-Volmer constants for quenching the protoporphyrin fluorescence in the complex

For the quenching controlled by a diffusion (such as quenching by I^- and $[Fe(CN)_6]^{-4}$), the Stern-Volmer constant K_{eff} is proportional to the number of collisions between the chromophore and a quencher with an accuracy to a factor. Thus, one can state that at pH 3.0–5.0, the log of the number of I^- collisions with the porphyrin ring was a linear function of pH, with a slope equal to −1. The log of the rate constant for the bimolecular reaction of the I^- with peroxidase compounds I is similarly pH dependent (Fig. 6). So, the oxidation of I^- by peroxidase compound I seems to require a direct contact of the I^- with the porphyrin ring.

The pH dependence of K_{eff} is accounted for by a change in probability of various protein conformers, where the porphyrin is accessible or inaccessible for an external quencher. According to Scheme 1, the pH dependence of the porphyrin fluorescence intensity in the complex in the absence of an external quencher is given by the equation:

$$I_0 = \frac{(1 + 1/K_c) \cdot I_{min} + (1 + 1/K'_c) \cdot I_{max} \cdot [H^+]/K'_H}{1 + 1/K_c + (1 + 1/K'_c) \cdot [H^+]/K'_H} \quad (5)$$

where I_{min} and I_{max} are fluorescence intensities of conformers $EH(H_2P...Q)$ and $E(H_2P...Q)$, respectively. If to assume that quenching occurs only during an interaction between I^- and the conformer $EH(H_2P...Q)$, in the presence of the quencher, Eqn. 5 can be transformed as follows:

$$I = \{(1 + 1/K_c) \cdot I_{min} + (1 + 1/K'_c) \cdot I_{max}([H^+]/K_H) \cdot 1/(1 + K \cdot [I^-])\} \times \{1 + 1/K_c + (1 + 1/K'_c)\ [H^+]/K_H\}^{-1} \quad (6)$$

where K is a true Stern-Volmer constant for the conformer $EH(H_2P...Q)$. Transforming Eqns. 5 and 6 one can show that at low concentrations of quencher ($K \cdot [I^-] \ll 1$) K_{eff} is given by the following expression:

$$K_{eff} = \frac{K}{1 + K_H/[H^+]} \quad (7)$$

At $[H^+] \ll K_H$

$$\log K_{eff} = \log \frac{K}{K_H} - pH \quad (8)$$

i.e., at $pH \ll pK_H$, the log K_{eff} should be a linear function of the pH, with a slope equal to −1. However, at pH close to the pK_H, a marked deviation of

log K_{eff}–pH dependence from a straight line should be observed. In our case the above dependence was linear, which can be explained only if the K value was pH dependent. The K was calculated at different pH from Eqn. 9 obtained from transformations of Eqns. 5 and 6:

$$\frac{I}{I_0 - I} = \left(1 + \frac{K_H}{[H^+]}\right) \cdot \frac{1}{K} \cdot \frac{1}{[I^-]} \cdot \frac{K_H}{[H^+]} \qquad (9)$$

The true Stern-Volmer constant K grew with falling pH (Fig. 6). This was evidence for the existance of steric hindrances in quenching for the conformer $EH(H_2P...Q)$ at $pH \geqslant 5.0$. For instance, the Stern-Volmer constant K at pH 5.0 was 8 M^{-1} compared to 22 M^{-1} for porphyrin in aqueous solution. Elimination of the steric hindrances in quenching for the conformer $EH(H_2P...Q)$ at $pH \leqslant 4.4$ showed that its conformation was changeable and there should be at least yet another group with pK near 4.3, but it cannot be detected by the method used.

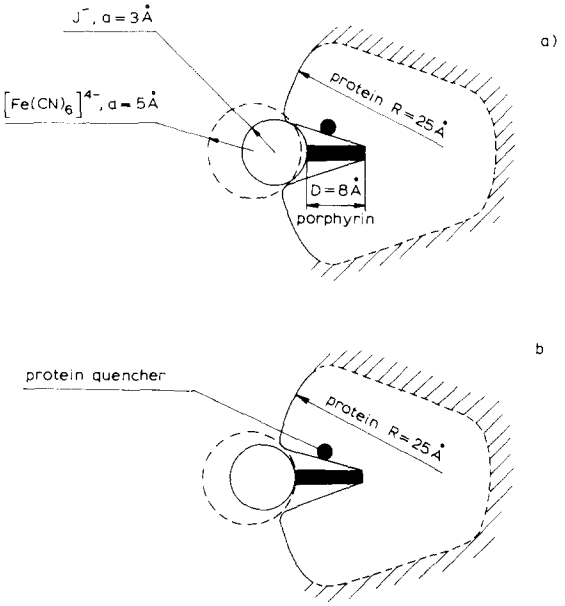

Fig. 7. Effect of the oscillation amplitude in the heme pocket of the peroxidase on interaction between ions from aqueous solution and the protoporphyrin ring; (a) is for a small amplitude, only small I^- can contact with the porphyrin, bulky $[Fe(CN)_6]^{-4}$ ions have steric hindrances for interaction; (b) is for a large amplitude, both types of ions can contact with the porphyrin.

A possible nature of conformational change in the peroxidase at pH 3.0–5.0

From the analysis of the results obtained we can propose the following interpretation for the nature of the conformational change in the peroxidase heme pocket at pH 3.0–5.0. Protein fragments oscilate against one another with certain frequencies and amplitudes. The positions corresponding to extremes of the amplitudes can be considered as two conformations. In the peroxidase at about neutral pH, the conformers, 'closed' $E(H_2P \cdot Q)$ and 'open' $E(H_2P...Q)$, correspond to two extremes. Protonation of the complex increases the portions of the 'open' conformer $EH(H_2P...Q)$. The lifetime of the non-protonated 'open' conformer $E(H_2P...Q)$ is short and I^- fails to diffuse inside the protein and to quench the protoporphyrin fluorescence. During the lifetime of the protonated 'open' conformer $EH(H_2P...Q)$, small I^- manage to diffuse inside the protein and quench the porphyrin fluorescence. Increase in the oscillation amplitude of protein fragments in the vicinity of porphyrin at $pH \leqslant 4.4$ decreases steric hindrances for quenching by I^- (K grows) and makes possible the contact of $[Fe(CN)_6]^{-4}$ with the porphyrin ring (Fig. 7).

References

1 Morrison, M. and Schonbaum, G.K. (1976) Annu. Rev. Biochem. 45, 861–888
2 Gurinovitch, G.P., Sevtchenko, A.N. and Soloviev, K.P. (1968) Spectroscopy of chlorophyll and related compounds, Izd. Nauka i Tekhnika, Minsk (Russ.)
3 Asakura, T., Kobayashi, T. and Chance, B. (1974) J. Biol. Chem. 249, 1799–1805
4 Shannon, L.M., Kay, E. and Lew, J.Y. (1966) J. Biol. Chem. 241, 2166–2172
5 Berezin, I.V., Ugarova, N.N., Kershengolts, B.M. and Brovko, L. Yu. (1975) Biochimia (Russ.) 40, 297–301
6 Yonetani, T. (1967) J. Biol. Chem. 242, 5008–5013
7 Falk, J.E. (1964) Porphyrins and Metalloporphyrins, Elsevier Amsterdam
8 Kuzmin, M.G. and Sadovsky, N.A. (1975) Chimia Vysokich Energy 9, 291–310 (Russ.)
9 Birks, Y.B. and Munro, J.H. (1967) in Progress in Reaction Kinetics, Vol. 4, pp. 239–303, Pergamon Press, Oxford
10 Fisher, W.R., Taniuchi, H. and Anfinsen, C.B. (1973) J. Biol. Chem. 248, 3188–3195
11 Larsson, L.O., Hagman, L.-O., Kierkegaard, P. and Yonetani, T. (1970) J. Biol. Chem. 245, 902–903
12 Welinder, K.G. (1976) FEBS Lett. 72, 19–23

13 Varkonyi, Z. and Kabok, K. (1975) Acta Biochem. Biophys. Acad. Sci. Hung. 10, 129–137
14 Varkonyi, Z. and Szalay, L. (1974) Acta Biochem. Biophys. Acad. Sci. Hung. 9, 255–264
15 Weber, G. and Teale, F.I.W. (1959) Disc. Faraday Soc. 27, 134–141
16 Strickland, E.H., Horwitz, J., Kay, E., Shannon, L.M., Wilchek, M. and Billups, C. (1971) Biochemistry 10, 2631–2638
17 Phelps, Ch., Forlani, L. and Antonini, E. (1971) Biochem. J. 124, 605–614
18 Strickland, E.H., Kay, E., Shannon, L.M. and Horwitz, K. (1968) J. Biol. Chem. 243, 3560–3665
19 Tsvirko, M.P., Solo'ev, K.N., Knyukshto, V.N. and Gradyushko, A.G. (1975) Zhurnal Prikladnoi Spektroskopii (Russ.) 23, 643–647
20 Altekar, W. (1975) Ind. J. Biochem. Biophys. 12, 397–399
21 Kieland, J.G. (1937) J. Am. Chem. Soc. 59, 1675–1678
22 Savitski, A.P., Ugarova, N.N. and Berezin, I.V. (1979) Bioorg. Chem. (Russ.) 5, 259–267
23 Rosen, C.G. and Nilsen, R. (1971) Biochim. Biophys. Acta 236, 1–7
24 Rosen, C.G. (1970) FEBS Lett. 6, 158–160
25 Tao, T. (1969) Biopolymers 8, 609–632
26 Dolman, D., Newell, G.A., Thurlow, M.D. and Dunford, H.B. (1975) Can. J. Biochem. 53, 495–501
27 Ellis, W.D. and Dunford, H.B. (1968) Biochemistry 7, 2054–2062
28 Roman, D. and Dunford, H.B. (1972) Biochemistry 11, 2076–2082
29 Morishima, J., Ogawa, S., Inubushi, T., Gonezawa, T. and Iizuka, D. (1977) Biochemistry 16, 5109–5117
30 Burshtein, E.A. (1977) Biophysica (Russ.), Vol. 7, pp. 120–160, VINITI, M
31 Roman, D., Dunford, H.B. and Evett, M. (1971) Can. J. Chem. 49, 3059–3063
32 Cowgill, R.W. (1968) Biochim. Biophys. Acta 168, 417–430