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Interaction of apoHb and various Fe-porphyrins

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Abstract

Apohemoglobin (apoHb) was reconstituted with various iron porphyrins (FePs) such as hemin, TCPP-Fe, $TPPS_4$-Fe and TTMAPP-Fe. The binding constants and binding ratios of apoHb and FePs were determined by fluorescence quenching method. TCPP-Fe, $TPPS_4$-Fe and hemin (the nature prosthetic group in Hb) are metalloporphyrins (MPs) with negatively charged porphyrin ring. All of them bind to apoHb with a binding ratio of 3. However, the positively charged TTMAPP-Fe binds to apoHb with a binding ratio of 2. The binding constants of hemin, TCPP-Fe, $TPPS_4$-Fe and TTMAPP-Fe were 2.72×10^6, 1.17×10^6, 5.17×10^5 and 1.6×10^5, respectively. The Soret band of the reconstituted hemoglobin showed evident changes in the presence of H_2O_2. The peroxidase activities of these complexes were determined. The results showed that though apoHb could be reconstituted with all FePs, it showed different functions. Binding of apoHb with hemin can enhance the heterolytic cleavage of O—O bond of H_2O_2, but inhibiting the cleavage when TCPP-Fe and $TPPS_4$-Fe were used instead. The binding of apoHb with TTAMPP-Fe showed negligible influence on the cleavage of O—O bond in H_2O_2. These different results were discussed.
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Keywords: Apohemoglobin; Reconstitution; Metalloporphyrins; Peroxidase

1. Introduction

Hemoproteins perform a wide variety of functions, ranging from dioxygen transport and storage, electron transfer, oxygen activation, hydrogen peroxide destruction and several kinds of oxidation reactions such as alkane hydroxylation and olefin epoxidation [1]. These functions are governed by the nature of the prosthetic groups, the ligands to the iron atom, the catalytic residues provided by the apohemoproteins, and the general topological and physicochemical properties of the active site [2]. In order to investigate the nature of hemin-protein interaction, a heme-substitution technique has been used for various hemoproteins. It has been shown that the oxygen binding properties of hemoglobin and myoglobin are strongly affected by the modification at 2- and 4-positions of the porphyrin ring of the prosthetic group [3,4]. On the other hand, the modification at the 6- and 7-carboxyl groups does not affect the oxygen-binding properties of these hemoproteins [4,5]. An opposite relation was observed in horseradish peroxidase [6,7] and cytochrome c peroxidase [8]. The enzymatic activities are not affected by the substitution at 2- and 4-vinyl groups, whereas they lose their enzyme activities by modification of carboxyl groups.

As a series of important metalloporphyrins (MPs), the metal complexes of tetraphenyl porphyrin and its derivatives were commonly used as the substitution of hemin to mimic the properties of the hemoproteins [9,10]. Formerly, hemoglobin (Hb) was de-prosthetized, which was then reconstructed with its prosthetic groups with different central metal ions and the spectral properties along with the catalase and peroxidase activities of the reconstructed Hbs were studied [11]. In this report apohemoglobin (apoHb) was reconstructed with different phenyl-substituted iron-porphyrins (FePs), namely TCPP-Fe, $TPPS_4$-Fe, TTMAPP-Fe and hemin. Various kinds of properties of the reconstructed Hbs were studied.

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2. Experimental

2.1. Instruments

UV–visible spectra were recorded on a Varian Cary 1E Spectrophotometer (USA). All fluorescence data were obtained from a Perkin-Elmer LS-50B luminescence spectrometer (USA). The kinetic curves were recorded on an Ocean Optics spectrometer with a CCD detector (USA).

2.2. Materials

Hemin (ferriprotoporphyrin IX chloride), pyrrole and Hb (lyophilized powder from bovine blood) were obtained from Sigma (St. Louis, MO, USA), and 4-carboxybenzaldehyde obtained from Acros (Pittsburgh, USA). Benzaldehyde, 4-*N*,*N*′-dimethylaminobenzaldehyde, hydrogen peroxide and guaiacol were supplied by Beijing Chemical Plant (Beijing, China). *Meso*-tetrakis (*p*-sulfonatophenyl)–porphyrin ($TPPS_4$), *meso*-tetrakis(4-carboxyphenyl)–porphyrin (TCPP), and *meso*-tetrakis[4-(*N*-trimethyl)aminophenyl]–porphyrin (TTMAPP) were synthesized according to the literature procedures [12]. The stock solutions of MPs (4.0×10^{-4} M) were prepared in deionized water. A working solution of H_2O_2 was freshly prepared by appropriate dilution of a stock solution, which was standardized by potassium permanganate method. The buffer solutions of Na_2HPO_4–NaH_2PO_4 (PBS) and $NaHCO_3$–Na_2CO_3 of specified pH were used to control the acidities of reaction medium.

2.3. Procedures

2.3.1. Preparation of TCPP-Fe, $TPPS_4$-Fe, TTMAPP-Fe and apoHb

TCPP-Fe, $TPPS_4$-Fe and TTMAPP-Fe were prepared with $FeCl_3$ or iron powder with corresponding porphyrins by the method of Adler [13]. ApoHb was prepared from hemoglobin by the HCl–butanone method of Teal [14].

2.3.2. Reconstitution of apoHb with FePs

ApoHb solution (5.0×10^{-6} M) of 0, 500, 1000, 1500 and 2000 μl were added in a 10 ml colorimetric cylinder, respectively, then diluted to 4.5 ml with $NaHCO_3$–Na_2CO_3 (10 mM, pH = 9.5). Under stirring, 500 μl MPs solution (8.0 $\times 10^{-5}$ M) was added slowly. The mixture solution was stayed for 30 min.

2.3.3. Measurement of binding constants (K_b) and binding ratios

Binding kinetics method is more precise for the determination of binding constants. Binding constants of apoHb with hemin, TCPP-Fe, $TPPS_4$-Fe and TTMAPP-Fe were determined, respectively, from the slop of the Scatchard plot based on the quenching of apoHb fluorescence [15]. The excitation and emission wavelengths were set at 295 and 345 nm, respectively. The binding ratios were determined by the break point of fluorescence quenching curves.

2.3.4. UV–vis spectra before and after H_2O_2 addition

The mixture solution prepared in 2.3.2 (1.0 ml) and 0.1 M NaH_2PO_4–Na_2HPO_4 buffer solution (1.0 ml, pH = 5.2) was added to 1 cm quartz cell and UV–vis spectra was determined. Then, 100 μl of H_2O_2 (4×10^{-2} M) was added under stirring, and the change of absorption of Soret band was recorded. The UV–vis spectra were also taken at 250 s after addition of H_2O_2.

2.3.5. Determination of peroxidase activity

Under room temperature, into a 2 ml quartz cell, 1.4 ml of 0.1 M NaH_2PO_4–Na_2HPO_4 buffer solution was added. Then, 400 μl of above mixture solution (2.3.2), 100 μl of guaiacol (0.1 M) and H_2O_2 (0.04 M) were added, in turn, under stirring. The absorption change at 470 nm was recorded.

3. Results and discussion

3.1. K_b and binding ratios of FePs and apoHb

K_b and binding ratios of apoHb and FePs were measured by fluorescence quenching method as explained in Section 2.3.3. The curves of fluorescence intensity vs. molar ratio of hemin and apoHb were displayed in Fig. 1. Binding ratios were determined by the break points of the curves and the K_b values were calculated from the slope of the Scatchard plot with assumption that all binding sites of apoHb are independent and the complexes of apoHb and FePs have no fluorescence. Comparison of the absorption ratio at 278 (the absorption of protein) and 405 nm (the Soret band of hemin in Hb) showed that only 90% of hemin was removed during the preparation of apoHb. Therefore, the binding ratios

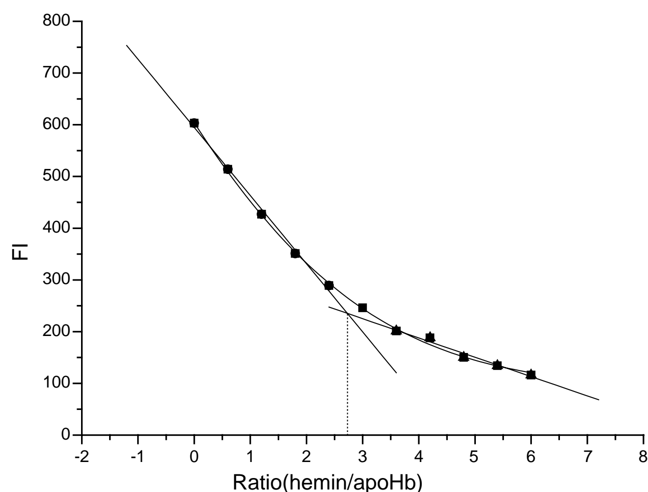

Fig. 1. Changes in apoHb fluorescence at 340 nm on gradual addition of hemin to fixed amount of apoHb (1.0×10^{-6} M).

Table 1
The binding constants and binding ratios of FePs and apoHb[a]

MPs	Binding constant	Binding ratio
Hemin	2.72×10^6	3.03
TCPP-Fe	1.17×10^6	3.34
$TPPS_4$-Fe	5.17×10^5	3.26
TTMAPP-Fe	1.6×10^5	2.05

[a] The concentration of apoHb was fixed at 1.0×10^{-6} M.

should be divided by 0.9. As shown in Table 1, the binding ratios of TCPP-Fe and $TPPS_4$-Fe are about three, similar to that of hemin, the natural prosthetic group in Hb. And that of TTMAPP-Fe was only two. It is well known that natural Hb contains four prosthetic groups, but in our experiment, only about three was determined. The reason may be due to incompleteness of the binding of hemin, TCPP-Fe or $TPPS_4$-Fe with apoHb under the current experimental conditions. According to the previous report on the binding of apoHRP and $TPPS_4$ described by Das [16], the binding site of TCPP-Fe and $TPPS_4$-Fe were suggested to be near the hemin binding site of apoHb and TTMAPP-Fe binds apoHb at other site. It seems that the natural binding site on apoHb only prefers negatively charged porphyrins, indicating the high possibility of the presence of positively charged residues nearby the binding site. Therefore, the charge interaction plays a very important role for the binding of water soluble porphyrins to apoHb. As the natural prosthetic group of Hb, hemin has the highest binding constant with apoHb. Though TCPP-Fe and $TPPS_4$-Fe have the negatively charged porphyrin ring similar to that of hemin, the structures of their ligands are different from that of hemin. So their binding constants with apoHb were relatively lower. The binding constant of Fe$TPPS_4$-Fe is even lower than that of TCPP-Fe, possibly due to the large size of Ph-SO_4^-. The lowest binding constant of TTMAPP-Fe maybe due to its nonspecific binding with apoHb, which lacks the shape and charge matching relationship between the negatively charged FePs and the natural binding site (cavity) of apoHb.

3.2. Spectral characteristics of the complexes of apoHb and FePs

The UV–vis spectra of the complexes of apoHb and FePs determined at various molar ratios were shown in Fig. 2.

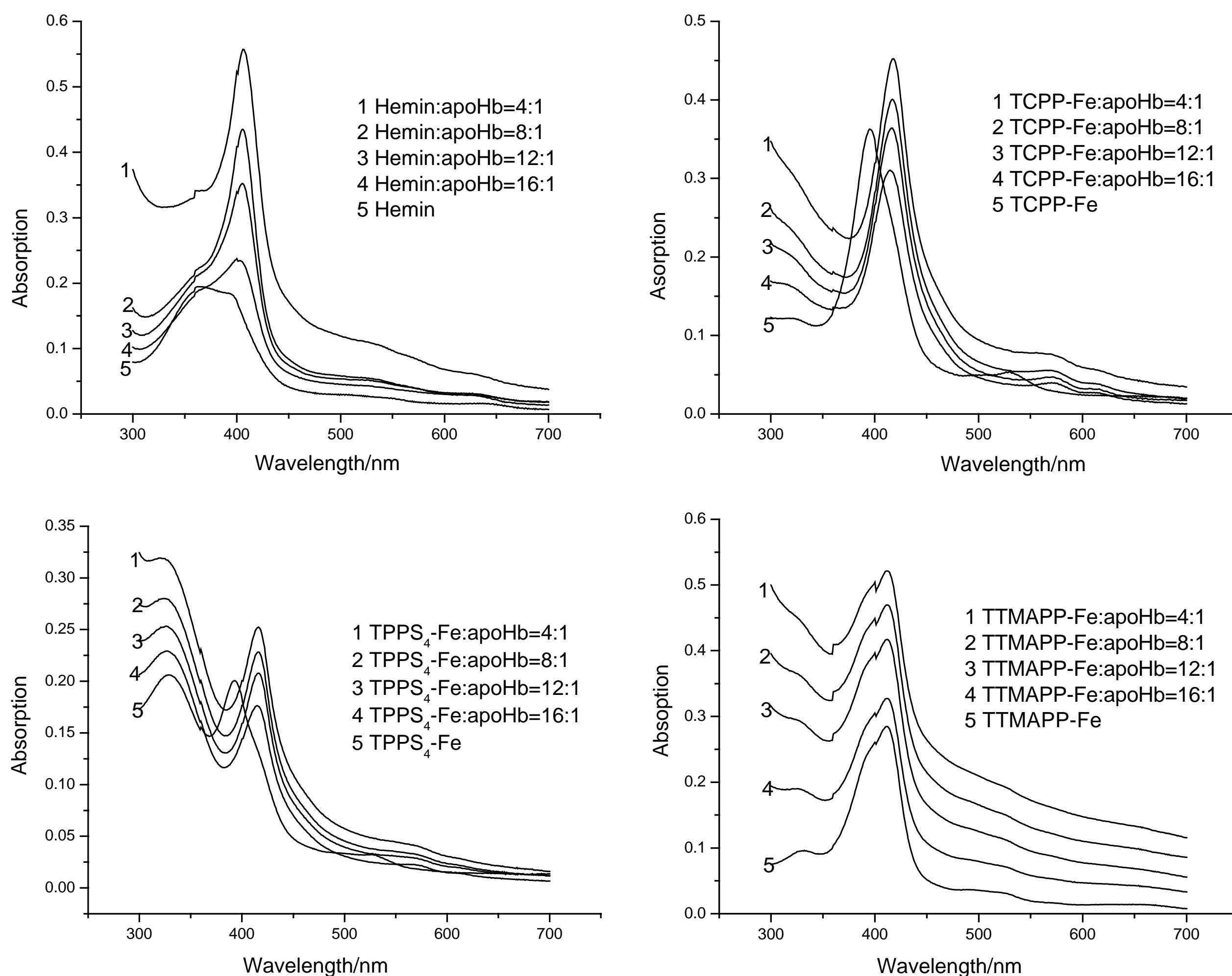

Fig. 2. The UV–Vis spectra of the complexes of apoHb and FePs at various molar ratios in 0.1 M phosphate solution buffer (pH = 5.2). C(FePs) = 4.0×10^{-6} M.

The hyperchromicity in Soret band was observed after all FePs bound to apoHb. Hemin, $TPPS_4$, TCPP and TTMAPP usually exit in aggregated forms in water, and upon binding with apoHb they will exist in the form of monomers. Therefore, addition of apoHb will break the aggeregates and causing hyperchromicity in the Soret band. The spectra of hemin showed bands at 364, 394, 507 and 638 nm. Upon binding with apoHb, the bands were red shifted to 406, 529 and 624 nm, characteristics of natural Hb of the high spin iron(III)-porphyrin with imidazole and water as axial ligands [17]. TCPP-Fe and $TPPS_4$-Fe showed similar Soret bands at about 396 and 532 nm, characteristics of a high-spin hexacoordination iron(III) species [18], probably resulting from the coordination of two water molecules to the iron atom. Obvious changes were induced by the insertion of them into the cavity of apoHb. About 20 nm red shift of the Soret band were observed together with the appearance of new band at 565 and 611 nm. It was obvious that the axial ligands to iron(III) ions were changed. As the absorption at the Soret band originates from π–π^* transitions, the red shift in this band suggests that the chromophore is in a more polar environment upon the binding. The phenomenon of red shift in Soret band of FePs with negatively charged porphyrin ring indicates these compounds bind to the interior part of apoHb or nearby the hemin binding site. No red shift in Soret band was observed when positively charged TTMAPP-Fe bind with apoHb, suggesting a different binding site, probably at the surface of apoHb.

To confirm the observation discussed above, the time dependence of the Soret band change of FePs and their complexes with apoHb after addition H_2O_2 was recorded and shown in Fig. 3. Their UV–vis spectra of the reaction systems after incubating with H_2O_2 for 250 s were depicted in Fig. 4. It was well known that in the present of H_2O_2, MPs could form compound I that is similar to natural peroxidase when reacting with H_2O_2 [9,10]. However, the compound I from MPs is easier to be destroyed through the intermolecular self-oxidation for the lacking of the protection microenvironment provided by protein. Figs. 3 and 4 clearly showed that the Soret bands from the three tetraphenyl-substituted MPs decreased quickly and the peak nearly disappeared upon adding of H_2O_2 while the Soret band from Hemin nearly remain unchanged, suggesting that hemin itself is not sensitive to the destruction of H_2O_2. The reason behind the big difference between hemin and other FePs is probably related to the electron effect of the substituents on the porphyrin ring of the other FePs. Fig. 4 also showed that, after incubating with H_2O_2, the Soret bands of TCPP-Fe, $TPPS_4$-Fe bathochrome move from 395, 393 to 411 and 418 nm while that of TTMPP-Fe hypsochrome moves from 419 to 400 nm. The bathochrome shifts refer to Fe–OO–H formation and the hypsochrome shift points to Fe=O porphyrin radical cation (Compound I)

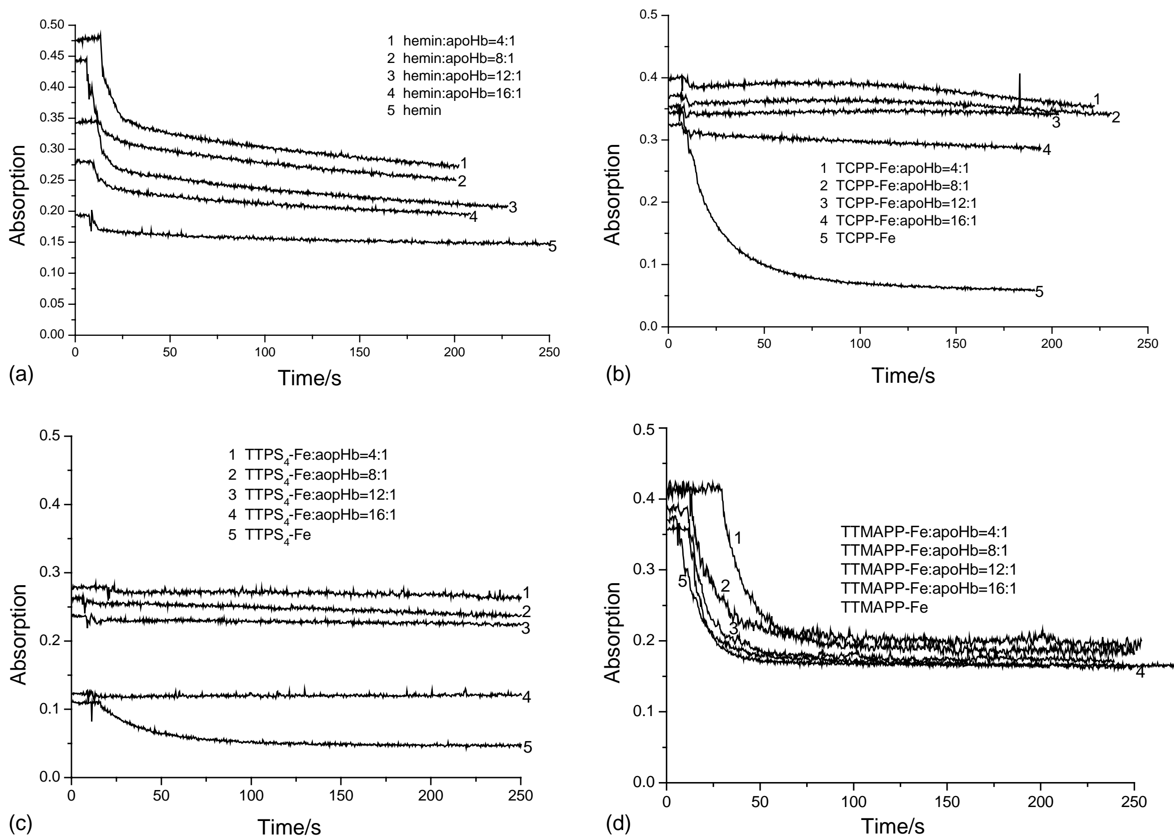

Fig. 3. Changes of Soret band of the complexes of apoHb and FePs at various molar ratios in 0.1 M phosphate solution buffer (pH = 5.2) after addition of H_2O_2. C(FePs) = 4.0×10^{-6} M and $C(H_2O_2) = 2.0 \times 10^{-3}$ M.

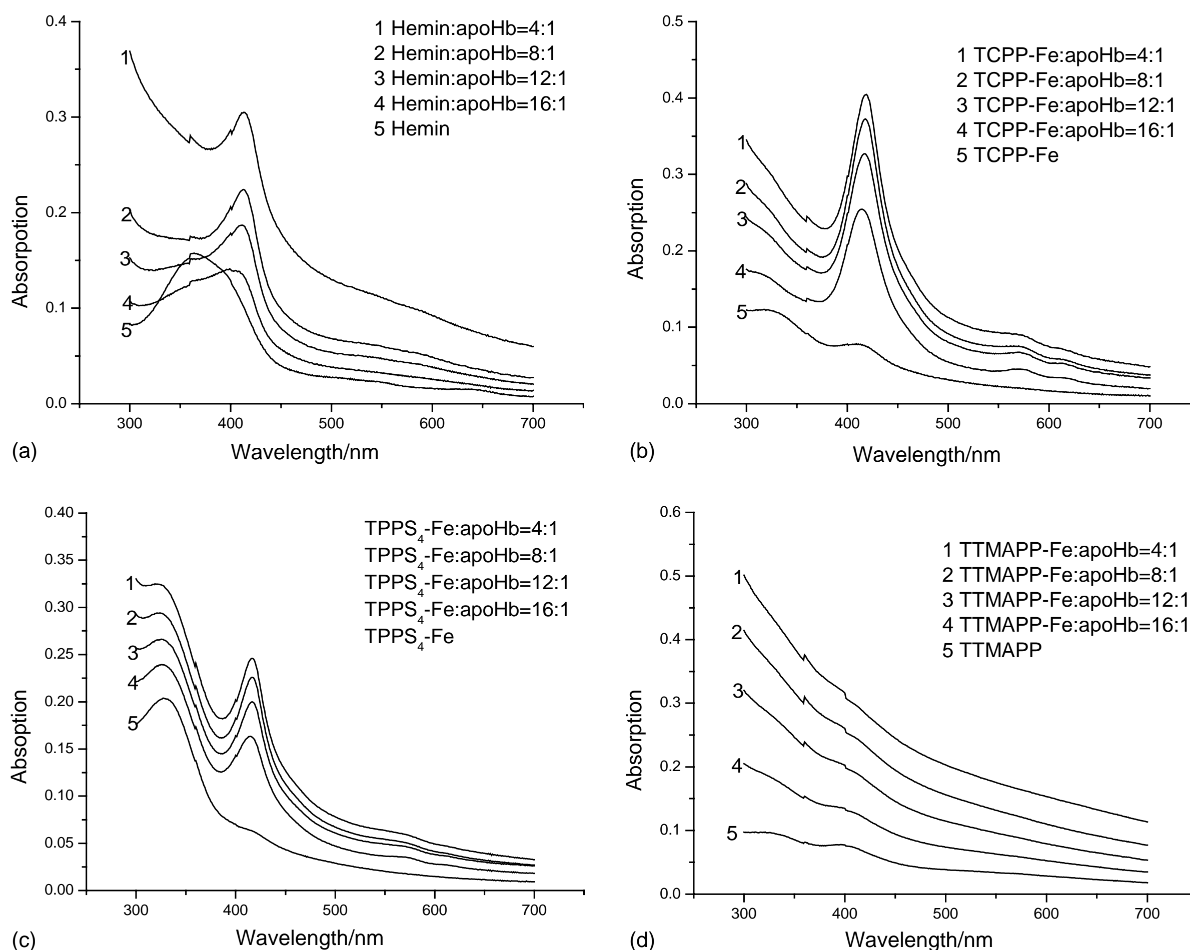

Fig. 4. The UV–vis spectra of the complexes of apoHb and FePs at various molar ratios in 0.1 M phosphate solution buffer (pH = 5.2) after incubating with H_2O_2 for 250 s. C(FePs) = 4.0×10^{-6} M and $C(H_2O_2) = 2.0 \times 10^{-3}$ M.

formation, and these is related to their catalytical activities (refer to the discussion below). Different phenomenon was observed when FePs bind to apoHb. The complex of hemin and apoHb were destroyed by H_2O_2 much faster than hemin itself. The Soret band was bathochrome shifted from 406 to 415 nm hinting enhanced formation of Fe–OO–H. The band of 415 nm was formerly described as the Soret band of peroxide compound that was regarded as the active intermediate in the catalytic reaction [19]. This confirmed that the microenvironment of apoHb was in favor of the heterolytic cleavage of O–O bond in H_2O_2 and hemin–apoHb complex. The rapid increasing of the active intermediate leaded to faster decrease at the Soret band. The destroying of TCPP-Fe and $TPPS_4$-Fe was almost fully restrained when they bound to apoHb and no obvious spectral change was observed after incubating with H_2O_2. Though TCPP-Fe and $TPPS_4$-Fe can bind the interior part of apoHb for having negatively charges, the different ligand structure made these FePs not locate at the binding sites of hemin or near the binding sites but incorrectly positioned and hence don't have an access channel for H_2O_2. Thus, the active intermediate formation was completely blocked. The binding of apoHb to TTMAPP-Fe showed little influence on the reaction between TTMAPP-Fe and H_2O_2. This can be explained reasonably for the weak or surface interaction between apoHb and TTMAPP-Fe, and this is quite different from that between apoHb and TCPP-Fe or $TPPS_4$-Fe.

3.3. *The peroxidase activities of FePs and their complexes with apoHb*

As the formation of Fe–OO–H or Fe=O porphyrin radical cation (Compound I) is very possibly related to their peroxidase activities the enzyme activities of Hb, apoHb, FePs as well as the complexes of apoHb and FePs at various molar ratios were determined. As shown in Fig. 5, TCPP-Fe, $TPPS_4$-Fe and TTMAPP-Fe had higher peroxidase activities than hemin. The reason is that the heterolytic cleavage of O–O bond in the complex of phenyl-substituted FePs is easier than that of hemin and it is coincident with the Soret band change shown in Fig. 3. The positively charged TTMAPP-Fe showed higher peroxidase activity than negatively charged TCPP-Fe and $TPPS_4$-Fe, in consistent with the finding that the Soret band from TTMAPP-Fe showed hypsochome shift while those from TCPP-Fe and $TPPS_4$-Fe showed bathochome shift. The hypsochome shift means the formation of Fe=O porphyrin radical cation and bathochome shift means

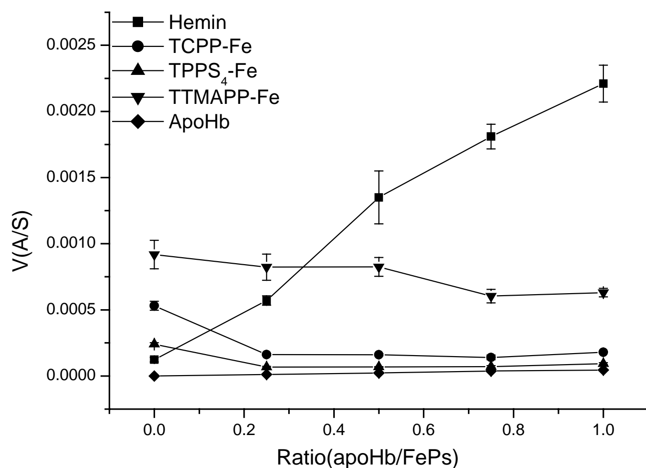

Fig. 5. Relative peroxidase activities of FePs and their complexes with apoHb at various apoHb/FePs ratios. C(FePs)=1.6 × 10^{-6} M, C(guaiacol) = 5.0 × 10^{-3} M, $C(H_2O_2)$ = 2.0 × 10^{-3} M. The reactions were carried out at room temperature and data were based on three replicate measurements. ApoHb was reconstituted with heme analogues (FePs). The binding of the FePs on apoHb as well as the peroxidase activities and other properties of the reconstituted Hb were studied.

the formation of Fe–OO–H, the former is the most important active intermediate of peroxidase. Opposite results were observed when they were reconstituted with apoHb. The activity of hemin was obviously increased with the increasing of the ratio of apoHb and hemin. The activities of TCPP-Fe and $TPPS_4$-Fe were almost quenched when they bound to apoHb. Less influence was found for the binding of apoHb to TTMAPP-Fe. All of these are in good agreement with that found for Soret band change induced by the binding of apoHb to FePs in the present of H_2O_2. Negatively charged TCPP-Fe and $TPPS_4$-Fe may bind with apoHb at interior part or the binding site of hemin but with incorrect positioning since no enhanced peroxidase activity was not observed. The positively charged TTMAPP-Fe is probably non-specifically binds on the surface of apoHb. It should be emphasized that the reconstituted hemin and apoHb complex (4:1 in molar ratio) showed about 63% peroxidase activity of that from natural Hb [11]. This is another hint for successful reconstitution between hemin and apoHb.

4. Conclusion

Hemin is very possibly binds to the natural binding site of apoHb, which is supported by the red shift of Soret band due to reconstitution and the enhanced peroxidase activity of the reconstituted hemin-apoHb complex as well as the comparable perroxidase activity of the natural Hb and and the reconstituted hemin-apoHb complex. Negatively charged TCPP-Fe and $TPPS_4$-Fe might bind at the interior part of apoHb or nearby the natural binding site but with incorrect positioning due to the substituents on the porphyrin ring. This is supported by the lack of an access channel for H_2O_2. The relatively high peroxidase activity of positively charged TTMAPP-Fe compared with negatively charged FePs along with the lower K_b and less change of peroxidase activity and Soret band are well in agreement with the proposed surficial binding of TTMAPP-Fe on apoHb.

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