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Aquoferric Derivatives of Natural and Artificial Leghemoglobins

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Leghemoglobins from soybean, kidney bean and pea root nodules were separated from endogenous nicotinic acid and their absorption spectra were measured at pH 6.0, where water occupies the sixth coordination site of Fe(III). The spectra of soybean leghemoglobin and kidney bean leghemoglobin represented spin equilibria, the former being slightly more of the high spin type than the latter. The spectrum of pea leghemoglobin had a predominantly low spin character. In this respect the leghemoglobins are different from myoglobins, where water is a high spin ligand. The pK_a-values for dissociation of proton from the heme-bound water molecule were determined spectrophotometrically for pea leghemoglobin and artificial leghemoglobins reconstituted from soybean apoleghemoglobin and meso-, deutero- and diacetyldeuteroheme. The pK_a-values for leghemoglobins with different hemes increased in the order of decreasing electron-withdrawing capacity of the 2,4-side chains of heme. This is the same order as was observed for the increasing high spin character for the same derivatives. The pK_a-value for pea leghemoglobin was found to be slightly lower than those reported earlier for soybean leghemoglobin and kidney bean leghemoglobin. Thus, for both natural and artificial leghemoglobins, a lower spin state of the ferric iron was related to facilitated proton dissociation from the ligand water molecule. It is suggested that the specific interaction between the heme side chains and the adjacent amino acid residues in leghemoglobin contributes to the spin state of iron, which influences the pK_a-value.

Leghemoglobin is a monomeric oxygen-binding hemoprotein occurring in legume root nodules.[1,2] X-Ray diffraction studies on leghemoglobin from yellow lupine have revealed the close resemblance of its tertiary structure to that of animal globins.[3] Leghemoglobin facilitates oxygen diffusion into the symbiotic nitrogen-fixing bacteria in root nodules. Most significant in the oxygen binding of leghemoglobin is the extremely fast combination reaction,[4] which makes oxygen transport possible in the environment of very low oxygen pressure required within the bacteroids.[5]

Leghemoglobin binds ligands more strongly than myoglobin does.[6] This may be in part due to the direct iron to ligand interactions which are different in these hemoproteins. On the basis of thermodynamic calculations on a number of hemoproteins, including leghemoglobin, a proposal was made by Banerjee *et al.*[7] that the ligand reactivity of a hemoprotein is primarily modulated by the electronic configuration of the heme interacting with globin, while only a minor role, if any, is attributed to the steric effects. Studies on artificial hemoproteins, in which unnatural hemes have been substituted for protoheme, have shown a relationship between reactivities of hemoproteins and the electron-withdrawal of the 2,4-substituents of heme.[8] Moreover, kinetic studies on artificial myoglobins have revealed that reduced electron density of the iron atom at the center of the porphyrin ring favors the combination and dissociation reactions with oxygen.[9] Recently, interesting observations have been made in resonance Raman studies on leghemoglobin and myoglobin, where the spectra have been interpreted to indicate distorted heme structure and relatively strong ligand field at iron in soybean leghemoglobin derivatives.[10] This could help to explain the fast combination reaction of oxygen, as well as the marked tendency of Fe(III) in leghemoglobin derivatives towards the low spin structure, even in the presence of a ligand which is expected to give a weak ligand field.[11]

In this study, nicotinic acid contamination was removed from leghemoglobin preparations before spectral measurements. This is essential because nicotinic acid binds tightly to the heme iron,[12] and

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affects the absorption spectra by a low spin contribution. In the spectra of pure aquoferric derivatives of soybean, kidney bean and pea leghemoglobin, the positions of the spin state equilibria were compared on the basis of the characteristic absorption bands. Different spin states are discussed in view of the specific protein structures adjoining heme. The p*K*-values for the dissociation of proton from the water ligand in natural and artificial leghemoglobins were measured in order to evaluate the magnitude of the effect of apoprotein and heme alteration on the properties of the sixth ligand of iron.

EXPERIMENTAL

Leghemoglobins. Methods for isolation and purification of leghemoglobins from soybean *(Glycine max)*, kidney bean *(Phaseolus vulgaris)* and pea *(Pisum sativum)* root nodules were those described previously.[13-15] In the present work, component a of soybean leghemoglobin, component a of kidney bean leghemoglobin and component I of pea leghemoglobin were solely used. Experimental procedure for the preparation of artificial leghemoglobins reconstituted from soybean apoleghemoglobin and meso-, deutero- and diacetyldeuteroheme has been described previously.[16] The concentrations of leghemoglobin stock solutions were calculated from the heme content obtained by the pyridine hemochrome method.[17] The millimolar absorptivity of 33.2 for mesoheme,[18] 24.0 for deuteroheme,[18] and 14 for diacetyldeuteroheme[19] was used.

Absorption spectra. For the spectral study of aquoferric leghemoglobins, traces of endogenous nicotinic acid left in the purified preparations were removed by filtration at alkaline pH, where the affinity of leghemoglobin for nicotinic acid is expected to be negligible.[12,20] Leghemoglobin solutions (pH adjusted to near 9) were filtered through a G-15 Sephadex column and concentrated over a UM 10 Diaflo membrane. The absence of nicotinic acid was ascertained by recording the ratio of absorbance maxima around 560 nm and 620 nm, which on removal of nicotinic acid reached 1.7 for soybean leghemoglobin,[20] 2.4 for kidney bean leghemoglobin, and 3.7 for pea leghemoglobin, at pH 6. The non-interacting 2-(*N*-morpholino)ethanesulfonic acid buffer was used. The pH was adjusted to 6.0, where the water molecule bound to the sixth coordination site of the ferric iron is completely in the undissociated form. More acidic pH was avoided because, due to heme dissociation (pK near 4), leghemoglobins become unstable. All light absorption measurements were performed with a Cary 15 recording spectrophotometer at 22 °C.

Binding of nicotinic acid. The equilibrium association constants for the formation of the complex between Fe(III) leghemoglobin and nicotinic acid (pyridine-3-carboxylic acid) were obtained by measuring difference spectra upon successive additions of nicotinic acid to a 1 μM leghemoglobin, in 50 mM [2-(*N*-morpholino)ethanesulfonic acid]–NaOH buffer (pH 5.3).[12] The peaks in the difference spectra in the Soret wavelength region (around 400 nm) were used as a measure of fractional formation of the leghemoglobin–nicotinate complex.

Acid-alkaline transition. The apparent p*K* values for the acid–alkaline transition of leghemoglobins were determined by recording the absorption spectra for different pH-values in the wavelength region 350–700 nm. The buffers used were sodium phosphate (pH 6.6 and 7.0), Tris-HCl (pH 7.6 and 8.2) and sodium borate (pH 8.8–10.0). The ionic strength of the buffer solutions was 0.1. The pK_a-values had standard errors of ± 0.02 or less.

RESULTS

Binding of nicotinic acid. The Hill plots for the binding of nicotinic acid to Fe(III) leghemoglobins of soybean, kidney bean and pea gave equilibrium constants of 1.88, 0.95 and 0.76×10^6 M^{-1}, respectively (Fig. 1). The leghemoglobin–nicotinic acid complex has a low spin hemochrome spectrum.[12] The high affinity observed for each of the three leghemoglobins for nicotinic acid necessitates a careful removal of endogenous nicotinic acid before spectral studies.

Absorption spectra of aquoferric leghemoglobins. The spectra of pure aquoferric derivatives of soybean, kidney bean and pea leghemoglobin, shown in Fig. 2, reveal a different spin state of Fe(III) in each leghemoglobin, though they are all of mixed spin type. The charge transfer band at the wavelength 620–624 nm, which originates from electronic transitions between iron and ligand,[21] is characteristic of a high spin type of spectrum. This band is most intense in the spectrum of soybean leghemoglobin and lowest in the spectrum of pea leghemoglobin. The Q-bands (α and β) between 560–520 nm, arising from the porphyrin $\pi-\pi^*$-transitions,[21] are prominent in low spin spectra. The Q-bands are most intense in the spectrum of pea leghemoglobin, and least intense in that of soybean leghemoglobin, which has the greatest high spin character.

The acid–alkaline transition. The transition between the acid and the alkaline form of hemoglobin

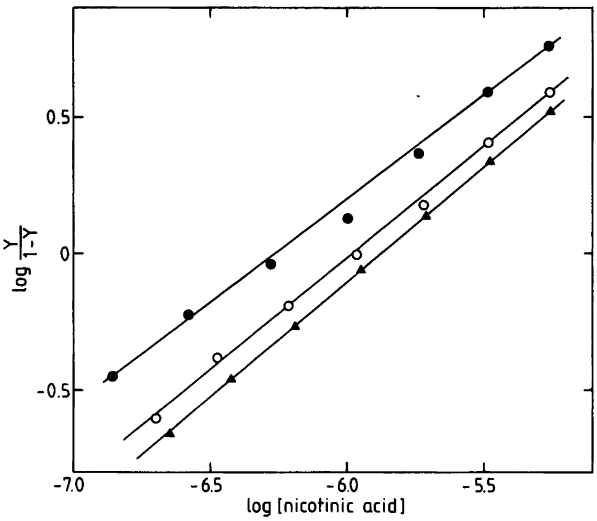

Fig. 1. Spectrophotometric titration of the extent of complex formation between Fe(III) leghemoglobin and nicotinic acid.

Y is the fractional saturation of leghemoglobin, the abscissa scale is log molarity. The difference spectra upon successive additions of nicotinic acid were measured with the 0–0.1 absorbance slidewire at the Soret wavelength region 350–450 nm. 1 μM leghemoglobin was in 50 mM [2-(*N*-morpholino)ethanesulfonic acid]–NaOH buffer (pH 5.3. Leghemoglobins were from soybean (●), kidney bean (○) and pea (▲).

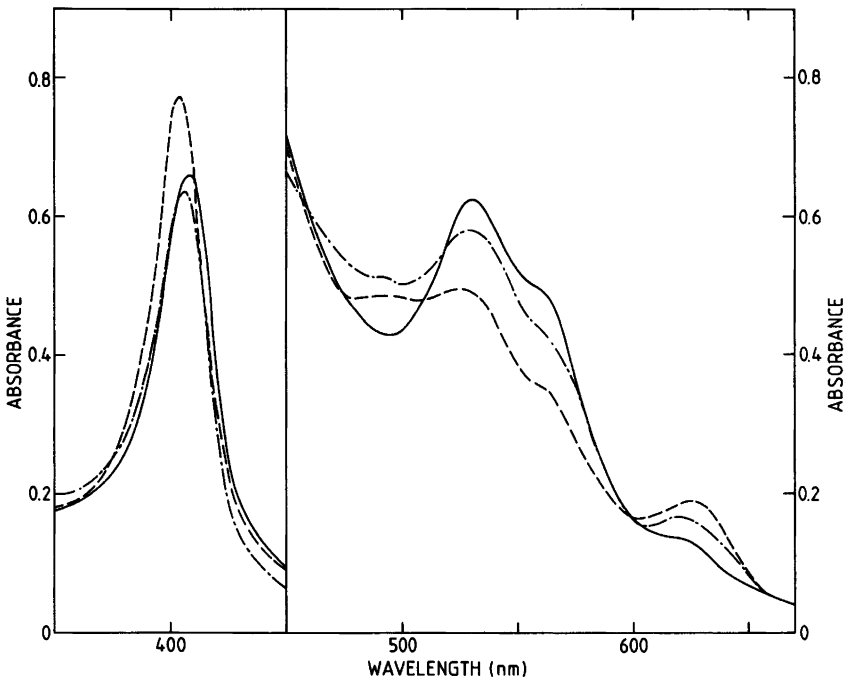

Fig. 2. Optical absorption spectra of the pure aquaferric derivatives of leghemoglobin a from soybean (– – –), leghemoglobin a from kidney bean (· · ·) and leghemoglobin I from pea (——). Leghemoglobins were freed from nicotinic acid as described in Experimental. Concentrations were 57 μM in the region 450–670 nm and 4.7 μM in the region 350–450 nm. The buffer was 50 mM [2-(*N*-morpholino)ethanesulfonic acid]–NaOH (pH 6.0).

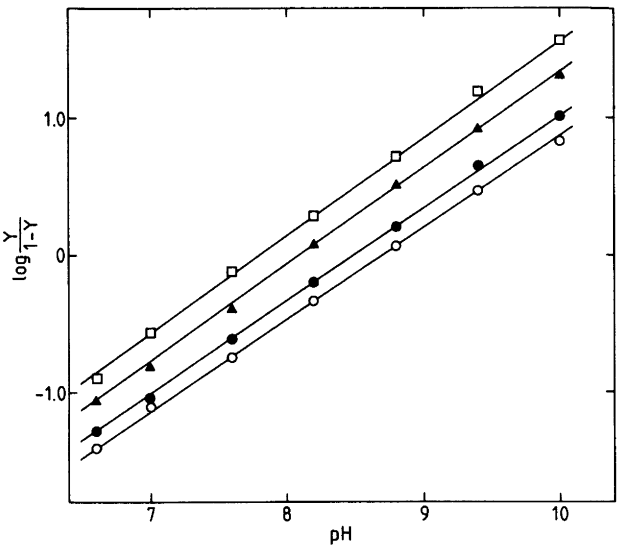

Fig. 3. Hill plots for determination of apparent pK for the acid–alkaline transition of leghemoglobin. Y is the fraction of alkaline leghemoglobin, evaluated at 520 nm for pea leghemoglobin (▲), at 596 nm for mesoleghemoglobin (○), at 600 nm for deuteroleghemoglobin (●), and at 530 nm for diacetyldeuteroleghemoglobin (□). The buffers were those described in Experimental.

is attributed to the ionization of the water molecule coordinated in the sixth position of the heme iron. Since the transition is accompanied by a characteristic change in the spectrum, it can be followed spectrophotometrically. The pK_a for the ionization of the heme-linked water was calculated from eqn. (1), in which n is the number of protons involved in

$$pK_a = n \times pH + \log \frac{Fe(OH)}{Fe^+(H_2O)} \quad (1)$$

the transition. The values for n obtained in the present experiments were about 0.7, which indicates dissociation of a single proton and shows also that the pH is electrostatically perturbed by a pH-dependent net charge of the protein.

The pK_a-value 8.10 was obtained for pea leghemoglobin (Fig. 3), while the pK_a's for kidney bean leghemoglobin and soybean leghemoglobin have been previously shown to be 8.25,[23] and 8.34,[24] respectively.

In Fig. 4, the pK_a-values of reconstituted leghemoglobins are plotted as a function of the pK_3-values of the respective free porphyrins. The pK_3-values are a measure of nitrogen basicity of the metal-free porphyrin and they correlate with the electron-withdrawing power of the 2,4-substituents, increasing in the order ethyl (meso), hydrogen (deutero), vinyl (proto), acetyl (diacetyldeutero).[22] This is also the order of a decrease in the pK_a-values for the acid–alkaline transition of leghemoglobin, as shown in Fig. 4. A similar observation has been made on sperm whale myoglobin, the values being superimposed in Fig. 4.[8] The shape of the line is quite similar for leghemoglobin and myoglobin in going from proto to deuetro and to mesoheme. However, the effect of substitution of acetyl side chains for vinyls is more pronounced in myoglobin than in leghemoglobin. Since the total charge of the protein molecule affects the pK_a's, the values for leghemoglobin, the more acidic protein of the two, are generally lower than those for myoglobin.

DISCUSSION

During active nitrogen fixation, large amounts of nicotinic acid occur in root nodules. Nicotinic acid has been proposed to play a role as a regulator in oxygen binding to leghemoglobin, because it could make the apparent oxygen affinity proton-dependent.[12] The observed high affinity in nicotinic acid binding suggests that this effector could have a

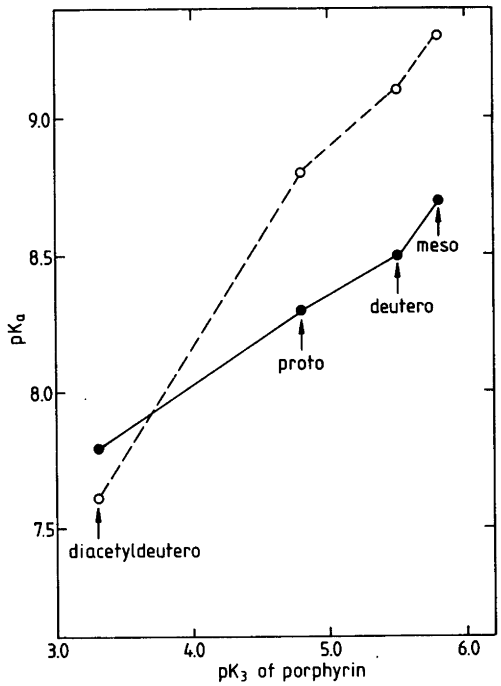

Fig. 4. Correlation between pK_3 for protonation of metal-free porphyrin and apparent pK for the acid–alkaline transition of artificial leghemoglobins (●). The values for pK_3 are those published by Falk[22] and refer to proton binding to the neutral porphyrin species. The superimposed data (○) on artificial sperm whale myoglobins are those determined by Makino and Yamazaki.[8] Artificial leghemoglobins were prepared according to Ref. 16. The acid–alkaline transition was determined spectrophotometrically as described in Experimental and the pK-values were evaluated graphically in Fig. 3. The 2,4-substituents of heme are acetyl in diacetyldeutero-, vinyl in proto-, hydrogen in deutero-, and ethyl in mesoleghemoglobin.

similar role in the oxygen binding of soybean, kidney bean and pea leghemoglobins. However, nicotinic acid is not a general regulator for leghemoglobins, since subclover leghemoglobin is unable to bind it.[25] Because the amino acid sequence of subclover leghemoglobin is not available, the structural basis for its unability to bind nicotinic acid cannot be evaluated.

The acid–alkaline transitions of leghemoglobins in the present study occur in a narrow pH range. However, the small differences between the observed pK_a's are quite indicative. Proton dissociation from water appears to be facilitated by increasing electron-withdrawal of the 2,4-substituents of heme, whereas the size of the substituents seems unimportant. A correlation exists for Fe(III) leghemoglobins in the present investigation between the pK_a-values of the acid–alkaline transition and the spin state of iron. A lower spin is related to facilitated proton dissociation from the water molecule. In soybean, kidney bean and pea leghemoglobins, the different pK_a's for the proton dissociation from the heme-bound water are due to the specific protein arrangement in the heme–protein contact region.

The spin equilibrium spectrum of soybean aquoferric leghemoglobin has been previously[26] attributed to the flexibility of the *E*-helix opposite to the heme, which would allow the mobile distal histidine imidazole to move on and off the iron, corresponding to the low spin form and the high spin form, respectively. Assuming the same structural basis for the iron spin in pea leghemoglobin, the predominantly low spin state would be due to the location of the distal histidine side chain on the heme iron, prevailing over the off location.

However, it has been shown before[16] that when the electron affinity of the 2,4-side chains of heme in reconstituted leghemoglobins increases, a change towards low spin occurs in absorption spectra. A similar observation can be made on reconstituted myoglobins,[16,27] but the change in the position of the spin state equilibrium is less remarkable in myoglobin than in leghemoglobin. Since only the heme side chains vary in artificial leghemoglobins and myoglobins, it is unlikely that the distal histidine imidazole would be involved in the relationship between the electron affinity of the 2,4-substituents of heme and the spin state of Fe(III).

The resonance Raman spectra obtained for soybean leghemoglobin have been interpreted to indicate an expanded porphyrin core, or greater ruffling or doming of the porphyrin ring, in deoxyleghemoglobin than in deoxymyoglobin, which property is retained in various low spin complexes.[10] This also implies that the displacement of iron from the plane of the pyrrole nitrogens in high spin derivatives of leghemoglobin could be shorter than in myoglobin, which would explain the tendency of the leghemoglobin iron towards low spin. The differences in the position of the spin state equilibrium in soybean, kidney bean and pea leghemoglobins are attributable to the few amino acid variations in the heme–apoprotein contact

region. The magnitude of the influence of these variations is unexpected, and could perhaps be due to the distortion of the heme group. In view of the results obtained with artificial leghemoglobins, it is suggested that in pea leghemoglobin the amino acid residues adjoining the vinyl side chains cause a small withdrawal of the π-electron density towards the periphery of the porphyrin ring. As a consequence, the displacement of the iron from the heme plane decreases and the low spin character of the absorption spectrum increases.

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