Interaction of Zinc Protoporphyrin with Intact Oxyhemoglobin†

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ABSTRACT: In erythropoietic protoporphyria and lead poisoning, free protoporphyrin (PPIX) and zinc protoporphyrin (ZPP), respectively, accumulate in erythrocytes. That PPIX and ZPP bind to human hemoglobin A (Hb_4) is established, but the site of binding is still a matter of controversy. We investigated the interaction of ZPP with intact, tetrameric oxy Hb_4, using batch microcalorimetry, front-face fluorometry, absorption difference spectroscopy, oxygen equilibrium studies, and isoelectric focusing (IEF). In the presence of oxy Hb_4 (pH 7.35, 0.05 M phosphate), the fluorescence emission maximum (excitation at 420 nm) of ZPP immediately shifts from 587 nm (ZPP alone) to 594 nm, as expected when binding to protein. The fluorescence intensity increases with time and is correlated with the ZPP:Hb_4 mole ratio. A slow, time-dependent reaction is also observed with microcalorimetry: the rate of heat of reaction exhibits both a fast and a slow component. The heats of reaction range from −2.1 to −14.8 mcal depending upon the ZPP:Hb_4 ratio of 4:1 (0.4 mM:0.1 mM) to 38:1 (3.8 mM:0.1 mM), respectively, and are typical of weak, noncovalent protein–ligand interactions. The optical difference spectra are a function of the ZPP:Hb_4 molar ratio and also exhibit a slow increase in intensity over time. No time-dependent optical difference spectra are observed with ZPP or with Hb_4 alone. The oxygen affinity of Hb_4 in the presence of ZPP decreases with increasing mole ratio. During IEF, all ZPP separates from Hb_4, consistent with a weak, noncovalent interaction at a non-heme pocket site. We conclude that ZPP binds to intact, tetrameric hemoglobin at non-heme pocket sites in a nonspecific, weak, noncovalent interaction.

The interaction of porphyrins with blood serum proteins is important in terms of porphyrin clearance from the circulation. Serum protein carriers such as albumin and hemopexin function to transport porphyrins to the liver (Lamola, 1982; Morgan et al., 1980; Eberhard & Vincent, 1985).

In the porphyrias, porphyrins may accumulate in certain organs to such an extent that organ damage occurs. In erythropoietic protoporphyria, protoporphyrin IX accumulates in red blood cells. The fact that free protoporphyrin (PPIX)[1] is bound to hemoglobin is well accepted, but the site of binding is still a matter of controversy, particularly in the case of erythropoietic protoporphyria (Lamola, 1982; Lamola et al., 1975; van Steveninck et al., 1977; De Goeij et al., 1975; Brun & Sandberg, 1985). As long as liver function is normal, there is a constant diffusion of protoporphyrin from red cells into the plasma; in a manner not understood, this porphyrin concentrates in the skin, and in the presence of light, photochemical reactions result in disfigurement and severe organ damage (Lamola, 1982). Fatal liver damage is reported with increased frequency (Bloomer & Straka, 1988).

Another porphyrin known to accumulate in erythrocytes is zinc protoporphyrin. ZPP accumulates in erythrocytes of victims of lead poisoning. Lamola et al. (1975), using low concentrations of ZPP, concluded that ZPP was bound to hemoglobin at the heme pocket, in contrast to their controversial conclusion that PPIX (in erythropoietic protoporphyria) is bound to non-heme pocket sites [re van Steveninck et al. (1977)].

ZPP also accumulates in erythrocytes of sickle cell patients. Hirsch et al. (1985a) showed that significantly higher levels of ZPP are found in sickle cell patients with low fetal hemoglobin levels as compared to sickle cell patients with high fetal hemoglobin. Again, the accumulation of excess porphyrins in erythrocytes raises the question whether porphyrins significantly interact with intact hemoglobin and bind to sites other than the heme pocket. Morgan et al. (1980) have shown that nonspecific binding of porphyrins to proteins may occur with high concentrations of porphyrins.

We investigated whether ZPP would interact with hemoglobin by first examining an excess of ZPP in the presence of intact tetrameric oxyhemoglobin. We present evidence, using microcalorimetry, front-face fluorescence spectroscopy, absorption difference spectroscopy, oxygen equilibrium studies, and isoelectric focusing, which demonstrates a significant noncovalent interaction of ZPP with intact hemoglobin, at sites other than the heme pocket. The interaction of ZPP with hemoglobin exhibits both fast and slow kinetic components.

Materials and Methods

Hemolysates of red blood cells of human donors were prepared according to the method of Drabkin (1946) with small modifications. Hb A was separated into purified components

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[1] Abbreviations: Hb_4, human adult oxygenated hemoglobin (oxy Hb A) that is tetrameric and intact (all four heme pockets are occupied by hemes); ZPP, zinc protoporphyrin; IEF, isoelectric focusing; PPIX, free protoporphyrin.

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by chromatography on columns of DE-52 (Whatman) developed with 0.05 M Tris-HCl buffer, pH 8.1, eluted by addition of 0.5 M NaCl, and further purified and equilibrated on Sephadex G-200 columns, pH 7.35, in 0.05 M potassium phosphate. All samples were concentrated, dialyzed, and equilibrated against potassium phosphate buffer and stored in liquid nitrogen. Hemoglobin purity was determined by starch gel and cellulose acetate electrophoresis.

ZPP was purchased from Porphyrin Products, Logan, UT, and was always handled in the dark or dim light. ZPP was first dissolved in 0.1 N NaOH (0.1 of total volume) followed by the addition of 0.05 M potassium phosphate buffer, pH 7.35 (0.9 volume).

Front-face fluorometry was performed on a Perkin-Elmer 650-10S spectrofluorometer modified for front-face optics as previously described (Hirsch et al., 1985b). Front-face fluorometry eliminates inner filter effects caused by molecules, such as hemoglobin and ZPP, with high extinction coefficients of absorption, enabling the detection of fluorescence of such molecules at high concentrations which otherwise would not be observed with right-angle optics used in standard fluorescence measurements. The advantages of front-face fluorometry are discussed in great detail by Eisinger and Flores (1979) and Lamola (1982). The excitation wavelength was 420 nm, and both excitation and emission slit widths were set at 10 nm. The front-face accessory is adapted with a temperature-regulated jacket. A Neslab RTE-5 circulating water bath maintained the temperature at 30 °C, the temperature at which calorimetric measurements were made (see below). The concentration of Hb_4 was 0.20 mM. The reaction as monitored by front-face fluorescence is considered quasi-complete when the fluorescence intensity reaches a plateau. Since, in principle, the reaction is infinite, the term "quasi-completion" time refers to the "apparent" completion time as assayed by the methods described.

Oxygen equilibrium measurements (30 °C) were made by using an Imai cell (Imai et al., 1973) in conjunction with a recording Cary 17 absorption spectrophotometer. Multiple absorption spectra (500–700 nm) were recorded during the deoxygenation of hemoglobin or hemoglobin solutions containing ZPP. The concentration of Hb_4 was 0.02 mM, and various aliquots of ZPP were used to obtain the desired mole ratio. The values of the fractional saturation, Y, were determined by using the absorbances at 575 and 540 nm. Equilibrium data were fitted to the Hill equation (Hill, 1910).

Microcalorimetry was performed on a LKB Model 2107-111 batch calorimeter in conjunction with the LKB 2107-310 control unit, as previously described by Podgorski and Elbaum (1985). The working temperature of the calorimetric head was fixed at 30 °C. The two pairs of gold reaction vessels consisted of a chamber with an interior dividing wall which extends to two-thirds the height of the chamber. The reference vessels were filled with buffer, pH 7.35, in 0.05 M potassium phosphate. The sample compartment was filled with 0.2 mM hemoglobin tetramer extensively dialyzed against the reference buffer. The volume of the reactants placed in the sample compartments was always 1 mL each. ZPP was also dissolved in this same buffer and transferred into the vessel (sample compartment) in dim light. (Note that this reference buffer was also used for other assays described in this paper.) The reaction was initiated by rotating the entire instrument, effectively mixing the reactants. The sample compartments were rinsed once with 6 M HCl to remove any residual ZPP followed by five rinses with distilled water and five rinses with buffer. The area under the curve of thermal flow rate as a function of time represented the total amount of heat. The reaction is determined to be quasi-complete when the recording returns to the base line (i.e., the heat of reaction is no longer detectable) (Figure 4). The data were analyzed and interpreted according to accepted methodology (Atha & Ackers, 1974; Shiao & Sturtevant, 1969; Stoesser & Gill, 1967). The corrected heats of interactions (Q_c) were calculated from the expression:

$$Q_c = (Q_R - Q_{FR}) - [(Q_{D1} - Q_{FD1}) + (Q_{D2} - Q_{FD2})]$$

where Q_R is the heat of the reaction, Q_{D1} and Q_{D2} are the heats of reagent dilution, and Q_{FR}, Q_{FD1}, and Q_{FD2} are the heats of friction, measured by a separate rotation of the calorimeter unit after the reaction or dilution runs.

The quantitative performance of the instrument was tested electrically. Calibration was shown to be linear in the range 0.5–15 mcal. The uncertainty in the values of heat evolved upon mixing (arising from slight irregularities in the base line) is 0.1 mcal.

Difference absorption spectra measurements were made at 30 °C, using tandem mixing cells (Hellma) on a Cary 17. The reference compartment contained separated ZPP and hemoglobin in a tandem mixing cell. The base line was obtained with the reactants separated in the sample compartment before mixing. The temperature was maintained with a circulating water bath at 30 °C.

Isoelectric focusing was performed in 4-mm glass tubes, at 0–4 °C as previously described by Park (1973). Optical spectra of bands on the gel were recorded by using a diode array spectrophotometer (Park et al., 1986).

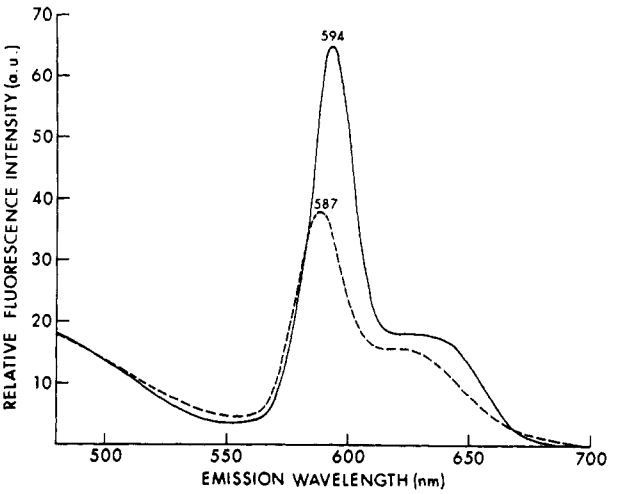

FIGURE 1: Front-face fluorescence emission spectra of ZPP in the presence (—) and absence (---) of intact tetrameric hemoglobin. The concentration of ZPP alone is 3.7 mM. In the combined solution, the concentration of hemoglobin is 0.1 mM tetramer and 3.7 mM ZPP (0.05 M potassium phosphate buffer, pH 7.35, 30 °C). Front-face optics are used (see Materials and Methods). Excitation wavelength is 420 nm with both excitation and emission slits widths at 10 nm.

Results

Front-Face Fluorescence. The front-face fluorescence emission spectra (excitation 420 nm) of a solution of ZPP in buffer (0.05 M potassium phosphate, pH 7.35) and ZPP in the presence of Hb_4 are shown in Figure 1. In the presence of hemoglobin, the fluorescence emission maximum of ZPP is shifted to longer wavelengths by 7 nm, from 587 to 594 nm. This emission maximum shift is observed with ZPP:Hb_4 ratios starting as low as 0.07:1 (not shown). The fluorescence intensity is seen to increase as a function of ZPP concentration.

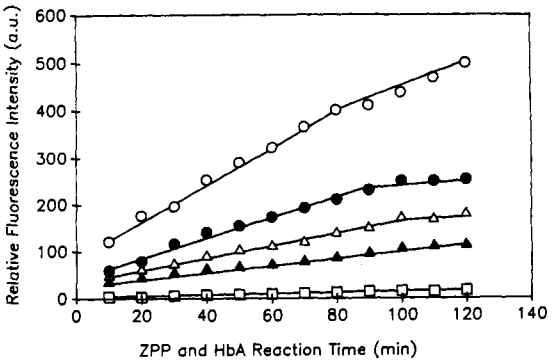

FIGURE 2: Relative front-face fluorescence intensity as a function of time for various mixtures of ZPP and intact tetrameric hemoglobin: (○) 38:1; (●) 28:1, (△) 20:1; (▲) 10:1; (□) 4:1. Excitation light was 420 nm, and the emission wavelength was fixed at the emission maximum of ZPP in the presence of hemoglobin, 594 nm. The temperature was 30 °C.

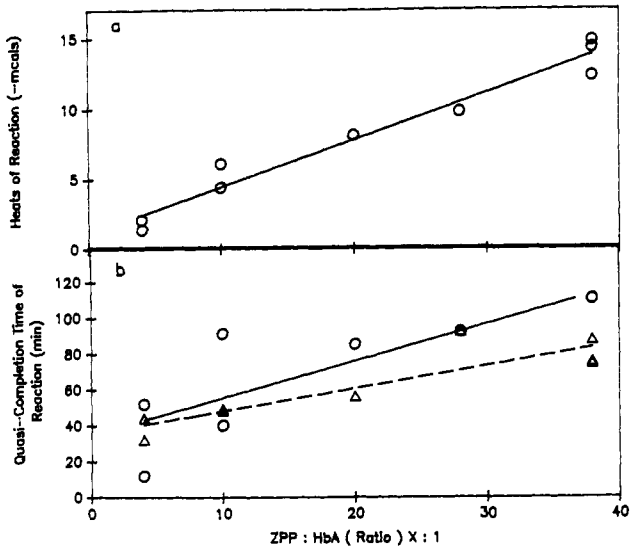

FIGURE 3: (a) Heats of reaction and (b) reaction quasi-completion times of various mixtures of ZPP and intact tetrameric hemoglobin determined respectively by microcalorimetry and front-face fluorescence. See Materials and Methods for conditions. Concentrations range from 0.4 mM:0.1 mM (ZPP:Hb_4, 4:1) to 3.8 mM:0.1 mM (ZPP:Hb_4, 38:1). (○) Reaction time assayed by microcalorimetry; (△) reaction time assayed by front-face fluorometry. The quasi-completion time for fluorescence was obtained from the original *continuous* recording over time (raw data not shown). Both assays exhibit similar times for quasi-completion of the reaction between ZPP and Hb A. All measurements were made at 30 °C in 0.05 M potassium phosphate buffer, pH 7.35.

Furthermore, the fluorescence intensity of ZPP in the presence of Hb_4 increases with time (Figure 2). This time-dependent increase in intensity reaches a maximum which appears to be correlated with the ZPP:Hb_4 mole ratio (from 4:1 to 38:1 ZPP:Hb_4) (Figure 2). We determined the visible spectra stability of the fluorometer with standard fluorescent blocks (Perkin-Elmer). Over 2.5 h, a 9% decrease (opposite to the direction of intensity change observed with ZPP in the presence of Hb_4) was observed. A concentrated solution of ZPP alone exhibited a 43% decrease in intensity after 2.5 h, an intensity change opposite to that observed for the interaction of ZPP with hemoglobin. Thus, this eliminates the possibility that the fluorescence changes are simply a result of ZPP aggregation or decomposition (see Discussion).

Microcalorimetry. The heats of reaction of the interaction of ZPP with Hb_4 are significant, ranging from −2.1 to −14.8 mcal, increasing as a function of the mole ratio of ZPP/Hb_4 4:1 (0.4 mM:0.1 mM) to ZPP/Hb_4 38:1 (3.8 mM:0.1 mM)

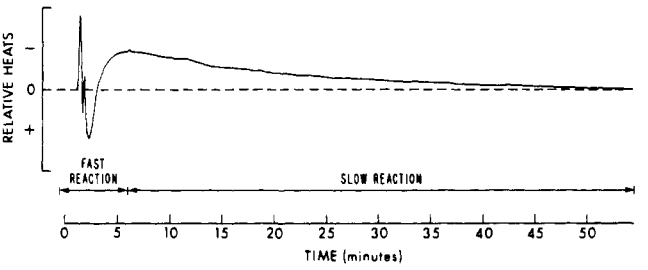

FIGURE 4: Fast and slow interaction of ZPP and intact tetrameric hemoglobin as exhibited by batch microcalorimetry. Raw data of heats of interaction mixing 1 mL each of ZPP (7.4 mM) and Hb A (0.2 mM), resulting in a final concentration of ZPP (3.8 mM) and Hb A (0.1 mM). Heats of friction and dilution are not shown. See Materials and Methods for conditions and calculations. Note that the kinetics of the "fast reaction" are faster than that shown in this figure. The limitation of the kinetics measurement is the time response of the instrument. Stopped-flow kinetic measurements would be required to assay the true kinetics of the fast reaction and will soon be studied.

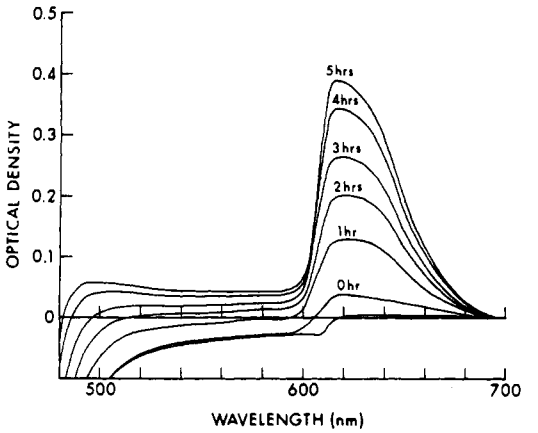

FIGURE 5: Absorption difference spectroscopy—change of optical density with time for ZPP and intact tetrameric hemoglobin (28:1). A spectrum was recorded before mixing. Up to 5 h after mixing, spectra were recorded at 1-h intervals, up to 5 h after mixing. The temperature was 30 °C. See Materials and Methods.

(Figure 3a). The rate exhibited both a fast and a slow component, similar to that observed with the fluorescence measurements (Figure 4).

It is important to point out that on 3 out of 10 occasions the total reaction time to the point where it can be resolved by batch microcalorimetry was exactly the same or very close to the "apparent" reaction time measured by fluorescence. A *t*-test comparing the quasi-completion time of the reaction as assayed by fluorescence vs microcalorimetry shows that there is no significance difference between the results obtained from both methods ($p = 0.26$) (Figure 3b). Furthermore, the reaction completion times, as assayed by microcalorimetry and fluorescence, are correlated with increasing mole ratio of ZPP (Figure 3b).

Optical Difference Spectroscopy. Upon mixing ZPP with Hb_4 (28:1), a difference spectrum is observed with a maximum at 598 nm (Figure 5). The changes in the absorption difference spectra increased with ZPP:Hb_4 molar ratio. Also observed is a change in intensity over time; however, the total time of the reaction appeared significantly longer (i.e., 5 h) than that observed with fluorescence and microcalorimetry. When we removed the separated Hb_4 and ZPP solution from the reference tandem cuvette and measured their respective absorption spectrum, there was no difference compared to the spectra measured 5 h earlier. Hence, the new spectra could only result from a reaction between ZPP and Hb_4. The high optical density resulting from the overlapping absorption

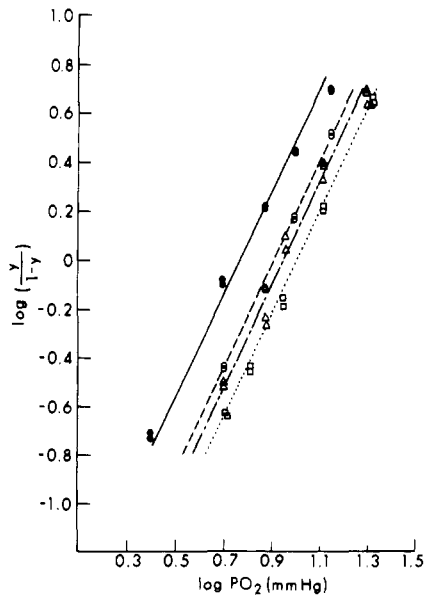

FIGURE 6: Effect of ZPP on the oxygen equilibrium of hemoglobin: Hill plots of oxygen binding by hemoglobin A in the absence and presence of ZPP. (●) Hb_4 alone; (○) ZPP:Hb_4 4:1; (△) ZPP:Hb_4 8:1; (□) ZPP:Hb_4 10:1. See Materials and Methods.

spectra of ZPP and Hb_4 limited the maximum mole ratio of Hb_4 to ZPP which could be studied.

In order to further rule out dimerization and higher aggregation of ZPP in our stock solution, the optical density of ZPP as a function of concentration was measured. In compliance with Beer's law, complete linearity was observed with concentrations of ZPP ranging from 0.1 to 2.8 mM (corresponding to the 1:1 through 1:28 ratios with Hb_4).

Oxygen Equilibrium Studies. If ZPP binds to a site on the hemoglobin molecule, a change in the functional properties of hemoglobin might be expected. When a modified Imai cell apparatus is used to study the oxygen equilibrium of Hb A in the presence of ZPP, the oxygen affinity of hemoglobin is observed to decrease in the presence of ZPP (Figure 6). Under the conditions employed (see Materials and Methods), the P_{50} of hemoglobin alone (i.e., in the absence of ZPP) was 5.9 mmHg, whereas hemoglobin in the presence of ZPP was 8.1, 8.9, and 10.1 mmHg, respectively, for mole ratios of ZPP to Hb_4 of 4:1, 8:1, and 10:1. The n value, which reflects cooperativity, did not differ in the presence or absence of ZPP. The high optical density resulting from the two overlapping visible absorption spectra limited the use of hemoglobin concentrations to no more than 0.02 mM tetramer and limited the study of mole ratios to no higher than 10:1 ZPP:Hb_4. At these concentrations, the optical difference spectrum is too small to be observed. Hence, there is no interference with the oxygen equilibrium measurements by the fast reaction. The dilute concentrations employed here were actually an advantage since the slow, time-dependent reaction is greatly reduced at these concentrations.

Isoelectric Focusing. Aerobic isoelectric focusing of an Hb_4 solution incubated 2 h with ZPP (38:1 ZPP:Hb_4) resulted in a pattern consisting of one band focused at the isoelectric point of Hb_4 and another diffuse brown-red band of partially precipitated material at the acid end of the gel. The optical spectrum of the red band, taken on the gel, as previously described (Park et al., 1986), was identical with that of Hb_4. Further, pure Hb_4 added to the gel focused coincident with this red band from the Hb_4/ZPP mixture. When ZPP solutions alone are run on the gels, the lower diffuse brown-red band at the acid end of the pH 6–9 gels is the only band that appears. We conclude that all the ZPP is removed from the Hb_4 during electrofocusing and migrates to the bottom of the gel.

Discussion

It is well-known that ZPP binds to many different proteins such as albumin, hemopexin, and ligandin (Lamola et al., 1975; Morgan et al., 1980; Muller-Eberhard & Vincent, 1985). When ZPP binds to a protein such as albumin, the ZPP fluorescence emission maximum shifts to the red (Leonard et al., 1974; Morgan, 1980; Lamola, 1982). The 7-nm shift in the ZPP fluorescence maximum observed in the presence of intact hemoglobin strongly suggests that binding has occurred, most likely in a hydrophobic area (Morgan et al., 1980). This shift is observed immediately after mixing. However, if one continues to follow the fluorescence spectrum over time, an increase in the fluorescence intensity is observed, indicating a secondary or slow interaction or process. Since this pattern is also observed with microcalorimetry and absorption difference spectroscopy, this supports the conclusion that a significant secondary or slow interaction is occurring.

The question arises as to the binding site. Porphyrins in the presence of apohemoglobin exhibit a high affinity for the heme pocket. When binding occurs, a shift in the fluorescence emission maximum is observed. Lamola et al. (1975) concluded that in lead poisoning, ZPP, present in low concentrations in the red cell, binds to an empty heme pocket of hemoglobin. However, the conditions of this present study differ from those of Lamola et al. (1975) in the following: (1) high concentrations of intact purified hemoglobin are employed to ensure its tetrameric state, and (2) ZPP is present in excess in order to probe any secondary binding sites. In fact, Morgan et al. (1980) have shown that sites of nonspecific binding of prophyrins to proteins may occur at higher concentrations of prophyrins. We propose that multiple non-heme pocket sites exist because the reactions or interactions of ZPP with hemoglobin are still observed at a ZPP:Hb_4 ratio of 38:1.

That ZPP binds to hemoglobin by weak, noncovalent bonding is strongly supported by the microcalorimetric data. The measured heats of reaction (Figure 3a) are typical of weak protein–ligand interactions of a noncovalent nature (Wadso, 1972). Since stoichiometry could not be observed with calorimetry (or with any of the other assays used in this work), this also implies and is consistent with binding of ZPP to non-heme pocket sites in a weak, noncovalent interaction. Alternatively, we may be looking at different classes of binding sites or, possibly, a single, very weak binding site. These alternatives cannot be resolved under the present conditions and require further study.

The conclusion that ZPP is binding to sites other than the heme pocket is also supported by the isoelectric focusing studies. The focusing behavior of ZPP in the presence of hemoglobin results in only two bands which focus at sites of pure intact human adult hemoglobin and ZPP alone. If indeed ZPP were to replace heme at the heme pocket, we would expect that the interaction would be strong enough to allow the ZPP-globin to focus as a separate species. ZPP-globin does not bind oxygen and is known to be in the T conformation (Fiechtner et al., 1980; Hoffman, 1979), and on the basis of previous work of Park (1973), T-state hemoglobins should focus with a higher isoelectric point than oxy Hb A because of the presence of Bohr protons. The isoelectric focusing pattern observed in this investigation showed no evidence, either by optical spectrum or by isoelectric point of ZPP-globin with ZPP in the heme pocket. Thus, our isoelectric focusing studies also suggest that ZPP is forming a weak, noncovalent

interaction with intact tetrameric hemoglobin at a non-heme pocket site.

These results demonstrate the importance in hemoglobin reconstitution experiments (which substitute different porphyrins at the heme pocket) to run the final product through a separation technique (e.g., column chromatography) in order to remove any excess prophyrins bound to non-heme pocket sites.

The observed change in the oxygen affinity of hemoglobin in the presence of ZPP is further evidence for a significant interaction of ZPP with hemoglobin. The observation that the P_{50} changes with increasing mole ratios greater than 4 ZPP:1 Hb_4 indicates that binding is taking place at non-heme pocket sites. It also suggests that the binding occurs at site(s) affecting the quaternary structural changes which hemoglobin undergoes during oxygen binding. Since the buffer is 50 mM and the maximum ZPP concentration added is 0.74 mM for solutions used in the oxygen equilibrium studies, ionic strength differences are negligible and therefore are not a factor in the oxygen affinity changes observed for hemoglobin in the presence of ZPP.

The slow reaction of ZPP with hemoglobin, as exhibited by front-face fluorescence spectroscopy, absorption difference spectroscopy, and microcalorimetry studies, also suggests multiple, weak binding sites on the hemoglobin molecule. However, the slow reaction remains to be fully understood. It should be emphasized that it is unlikely that the fast or slow reaction is a photoreaction since batch microcalorimetric measurements are made in complete darkness. On the other hand, it is possible that the longer slow reaction observed by absorption spectroscopy may include a photochemical component. Further investigations are required to understand the process or processes involved in the slow reaction.

A possible objection to the data of this study is that we are working with ZPP solutions at relatively high concentrations that could promote the formation of porphyrin dimers or higher aggregates. The spectroscopic results are not consistent with dimer formation. Holden (1941) showed that ZPP in aqueous solution remains primarily monomeric. Furthermore, (1) the linearity of the titration curve of ZPP to Hb_4 and (2) the linearity of ZPP concentration with optical density (Beer's law) are also consistent with a monomeric solution of ZPP. Even if possible dimers or higher aggregates exist, the data presented here still cannot be explained without a binding interaction of ZPP with Hb_4.

The significance of our results is that it may give new insight into diverse investigations of porphyrins in red cells. First, in contrast to present dogma, this study implies that ZPP in lead poisoning may not be bound to the heme pocket of hemoglobin but rather to sites other than the heme pocket. Second, a closer study of the interaction of other porphyrins with hemoglobin needs to be reevaluated. For example, the controversy of the free protoporphyrin binding site of hemoglobin (Van Steppeninck, 1977; Lamola et al., 1975; Brun & Sandberg, 1985) could be resolved by using these methods. Third, this study is also relevant to recent evidence demonstrating the accumulation of heme in sickle cells (Shaklai et al., 1985; Hebbel et al., 1988). If heme and other porphyrins bind to non-heme pocket sites on hemoglobin, the resulting lowered oxygen affinity could increase the tendency for Hb S to polymerize. Further investigations are under way to determine the affects of ZPP and other species of porphyrins on hemoglobin function and its role in sickle cell disease.

In conclusion, (1) ZPP significantly interacts with intact, tetrameric hemoglobin, (2) ZPP binds to hemoglobin at sites other than the heme pocket, (3) this appears to be a weak binding of a noncovalent nature, and (4) the kinetics include a fast and slow reaction. Further investigation is required to understand the nature of the slow reaction and to determine if more than one class of weak binding sites exists.

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