Synthesis of Fluorescent Organic Phosphates and Their Equilibrium Binding to Bovine Oxyhemoglobin†

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ABSTRACT: Fluorescent organic phosphates, β-naphthyl diphosphate, β-naphthyl triphosphate, and β-naphthyl tetraphosphate, were synthesized from β-naphthyl monophosphate using P_i and *N,N'*-dicyclohexylcarbodiimide. These organic phosphates were interacted with bovine oxyhemoglobin, all in no buffer, 0.1 M NaCl, at 25° and in the pH range 5.5–7.0. Equilibrium binding parameters were determined by measuring the fluorescence quenching upon their interactions. It is indicated that bovine oxyhemoglobin has more than one binding site, one of which is very strong. The strength of binding to the stronger site is in the order β-naphthyl tetraphosphate > β-naphthyl triphosphate > β-naphthyl diphosphate. The logarithms of association constants of these phosphates depend linearly on the net charges of these phosphates at any pH. The results were accounted for by electrostatic effects using a simple charge model. In that model, the average positive net charges in oxyhemoglobin involved in the binding of β-naphthyl phosphate are shown as a function of pH. It is shown that the binding of these fluorescent organic phosphates is prevented reversibly by the excess addition of nonfluorescent organic and inorganic phosphates, inositol hexaphosphate, tripolyphosphate, and pyrophosphate. Assuming competitive binding in a single strong site, the association constants of these nonfluorescent phosphates were also determined by measuring the recovery of the fluorescence intensity upon the release of fluorescent phosphates. At pH 6.18, the association constants of inositol hexaphosphate and tripolyphosphate are comparable to those of β-naphthyl triphosphate and β-naphthyl diphosphate, respectively. The association constant of pyrophosphate is lower than that of tripolyphosphate by one order.

This study is concerned with equilibrium binding of fluorescent phosphates to bovine oxyhemoglobin (HbO_2).[1] Recent reports by a number of authors have shown, under various solution conditions and by means of various experimental methods, that many organic and inorganic phosphates bind not only to deoxyhemoglobin, but also to HbO_2. As an example, by means of equilibrium dialysis, some investigators (Chanutin and Hermann, 1969; Hedlund *et al.*, 1972) determined the association constants of such organic phosphates as adenosine 5'-triphosphate (ATP) and 2,3-diphosphoglycerate (P_2glycerate) to human and horse HbO_2. But the methods, such as equilibrium dialysis or calorimetry (Hedlund *et al.*, 1972), take a long time or are accompanied by complicated operations. We have employed a simpler method. We made use of a fluorescent substance as an organic phosphate and determined the binding of that by quenching of its fluorescence upon interaction with HbO_2. MacQuarrie and Gibson (1971, 1972) made use of 8-hydroxy-1,3,6-pyrenetrisulfonate ($HOPyn(SO_3^-)_3$) as a fluorescent analog of P_2glycerate to study human hemoglobin conformational change. This $HOPyn(SO_3^-)_3$ has sulfonic groups instead of phosphoric groups. The binding of this analog to carboxyhemoglobin was much weaker than those of P_2glycerate and inositol hexaphosphate (P_6Ino) and was difficult to measure accurately. As the fluorescent organic phosphate, we chose β-naphthyl diphosphate ($NapP_2$), β-naphthyl triphosphate ($NapP_3$) and β-naphthyl tetraphosphate ($NapP_4$), which were synthesized from β-naphthyl monophosphate ($NapP_1$) using P_i and *N,N'*-dicyclohexylcarbodiimide (DCC). Chanutin and Hermann (1969) and Hedlund *et al.* (1972) performed experiments of equilibrium binding in the pH range 6.5–7.1, using cacodylate buffer which is less competitive for HbO_2 ion binding sites than other commonly used buffer systems. We used no buffer and performed experiments at the wider pH range 5.5–7.0 by adjusting with NaOH and HCl. Most studies on the interaction of hemoglobin with organic phosphates have been done with human and horse hemoglobin. We made use of bovine hemoglobin, which is slightly different from the former two species in titration behavior of histidines (Janssen *et al.*, 1972). The electrostatic nature between the binding site in bovine HbO_2 and organic fluorescent phosphates is analyzed and discussed in this paper.

The addition of a nonfluorescent phosphate to a mixed solution of HbO_2 and a fluorescent phosphate induces the recovery of the fluorescence intensity. From those data, the association constant of the nonfluorescent phosphate is also estimated.

Experimental Procedure

Materials

Preparation of $NapP_2$, $NapP_3$, and $NapP_4$. Polyphosphorylation from $NapP_1$ was performed by the similar method of Smith and Khorana (1958). The $NapP_1$ was obtained from Sigma Chemicals. The solid $NapP_1$ (2.5 mmol) was dissolved in dry pyridine (50 ml) containing tri-*n*-butylamine (50.0 mmol) and 85% orthophosphoric acid (18.0 mmol). Polyphosphorylation of $NapP_1$ was started by the

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[1] Abbreviations used are: HbO_2, oxyhemoglobin; NapP, β-naphthyl phosphate; $NapP_1$, β-naphthyl monophosphate; $NapP_2$, β-naphthyl diphosphate; $NapP_3$, β-naphthyl triphosphate; $NapP_4$, β-naphthyl tetraphosphate; ATP, adenosine 5'-triphosphate; P_2glycerate, 2,3-diphosphoglycerate; P_6Ino, inositol hexaphosphate; PP_i, inorganic pyrophosphate; PPP_i, tripolyphosphate; $HOPyn(SO_3^-)_3$, 8-hydroxy-1,3,6-pyrenetrisulfonate; DCC, *N,N'*-dicyclohexylcarbodiimide.

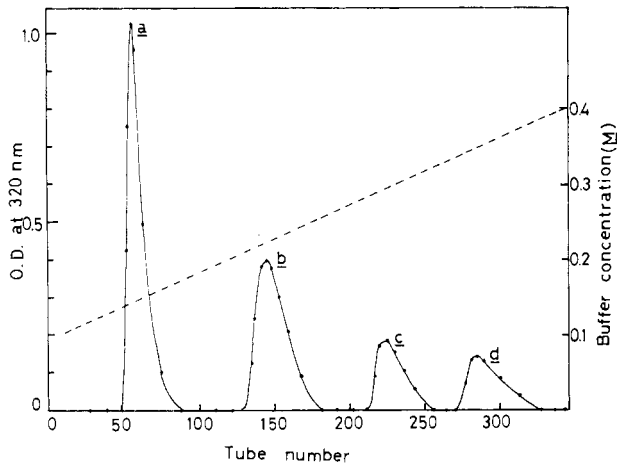

FIGURE 1: Column chromatographic elution profile of the reaction products for β-naphthyl polyphosphate synthesis.

addition of DCC (15.0 mmol) with stirring. After the reaction solution was kept at room temperature for 18 hr, the dicyclohexylurea precipitated was separated by filtration and then washed with water. The combined filtrate and washings (250 ml) were adjusted to pH 8.0 with 1 M sodium hydroxide, and extracted with 150 ml each of diethyl ether three times. Any organic solvent in the water layer was removed by evaporation. The product in the water layer was adsorbed on activated charcoal. Then the adsorbed was washed with water (2 l.) and eluted with 50% aqueous ethanol containing 2% ammonia (1.5 l.). The eluted solution was evaporated to dryness, and the residue was dissolved in 0.1 M triethylammonium bicarbonate buffer (pH 7.5). The above buffer solution containing the reaction product was applied to a column (2.5 cm × 40 cm) of DEAE-cellulose (carbonate form) and eluted with a linear gradient of triethylammonium bicarbonate (0.1–0.4 M, total 5 l., 15 ml/tube) at pH 7.5. As shown in Figure 1, this chromatography gave four well-separated peaks, a, b, c, and d. The fractions containing each peak were combined and evaporated with 25 ml each of methanol for three times to remove the solvent (triethylammonium bicarbonate). Dissolved in methanol (5 ml), each product corresponding to each peak (a, b, c, and d) was separately precipitated as a sodium salt by adding acetone (100 ml) containing NaI by the amounts of 8 times the number of moles of the product. Each precipitate was washed by centrifugation twice with 30 ml each of acetone–methanol (20:1, v/v) and diethyl ether (30 ml), and dried over P_2O_5 *in vacuo*. Each compound thus obtained showed one spot, under ultraviolet beam, on paper chromatography developed by isopropyl alcohol–1 M ammonium sulfate (65:35, v/v); R_F values of a, b, c, and d were 0.62, 0.39, 0.29, and 0.18, respectively. R_F values of a and $NapP_1$ before polyphosphorylation procedures were the same. The P_i content in each compound was analyzed by the method of Ames and Dubin (1960). The molar ratios of the naphthyl group to P_i in a, b, c, and d were approximately 1/1, 1/2, 1/3, and 1/4, respectively. We analyzed components c and d by cleaving the terminal phosphates with myosin ATPase which is well known for hydrolyzing triphosphate and tetraphosphate into diphosphate. By this method, it was shown that 1 mol of c and d released approximately 1 and 2 mol of phosphates, respectively, by hydrolysis. It was thus concluded that the compounds a, b, c, and d were $NapP_1$, $NapP_2$, $NapP_3$, and $NapP_4$, respectively.

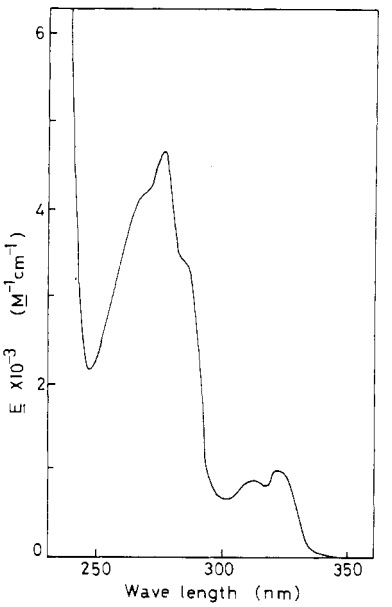

FIGURE 2: Absorption spectrum of $NapP_1$. The $NapP_1$ (1.5×10^{-4} M) was dissolved in 0.1 M NaCl at pH 7.0. E is the molar extinction coefficient.

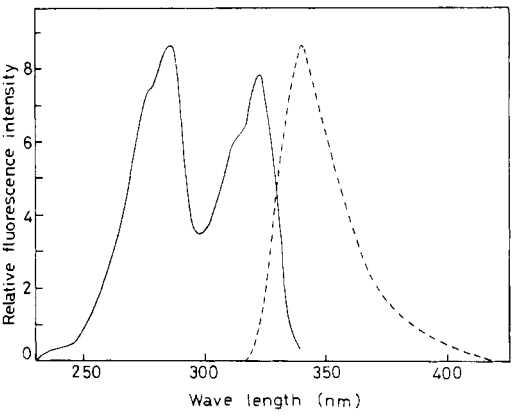

FIGURE 3: Excitation and emission spectra of $NapP_1$. The excitation spectrum (—) was observed at the emission wavelength of 343 nm. The emission spectrum (- - -) was observed at the excitation wavelength of 285 nm. All solution conditions were the same in Figure 2.

Absorption and Fluorescence Spectra. Figure 2 shows the absorption spectrum of $NapP_1$ at pH 7.0. The absorption maxima were obtained at 274, 312, and 321 nm. The absorption spectra of $NapP_1$ and its polyphosphorylated derivatives were slightly different from one another in the range 290–340 nm. Since the absorption spectra in the range 260–290 nm, on the other hand, were almost identical, their concentrations were estimated on the basis of an extinction coefficient $E_{1\,cm}^{1M} = 4600$ at 274 nm. Figure 3 shows the fluorescence excitation spectrum at the emission wavelength of 343 nm and the emission spectrum at the excitation wavelength of 285 nm for $NapP_1$. At the excitation wavelength of 285 nm, the fluorescence emission spectra of $NapP_1$, $NapP_2$, $NapP_3$, and $NapP_4$ were not remarkably different from one another.

Preparation of Hemoglobin. Fresh bovine blood was collected in acid–citrate–dextrose (ACD solution) and washed several times with ice-cold 0.9% NaCl. The washed red blood cells were hemolyzed by shaking vigorously with 1 vol of cold water and 1 vol of toluene, followed by centrifugation at 28,000 rpm using a Spinco No. 30 rotor for 40 min. The clear solution of hemoglobin (concentration about

17%), obtained by siphoning and filtering, was applied to a Sephadex G-25 column (2.5 cm × 45 cm) equilibrated with 0.1 M NaCl in order to eliminate the contaminations of P_2glycerate and of other inorganic and organic phosphates. Elution was carried out with 0.1 M NaCl at a flow rate of 15 ml/hr. The stripped hemoglobin solution thus prepared (concentration about 3%) was adjusted to pH 7.0 with a diluted NaOH solution, stored at 0°, and used for experiments within 11 days. Total phosphate contents in hemoglobin solutions before and after passage through Sephadex G-25 in 0.1 M NaCl were examined by the method of Ames and Dubin (1960). The result guaranteed that the treated hemoglobin is "stripped." The concentration of hemoglobin was accurately determined using an extinction coefficient $E_{1\,cm}^{1M} = 56{,}800$ at 542 nm for HbO_2. The molecular weight of HbO_2 tetramer was considered to be 64,460 (Hedlund *et al.*, 1972).

Methods

Absorption spectra were measured with a Hitachi 356 spectrophotometer. Fluorescence measurements were carried out with a Shimazu RF-501 recording spectrofluorophotometer, and the temperature of the cell was regulated at 25 ± 0.5° with a Taiyo Coolnit CL-15 circulating water system. The fluorescence spectra were not corrected for spectral responses of the photomultiplier, the monochrometer, and the Xenon lamp. Bandwidths of excitation and emission light, in the case of measuring fluorescence of NapP solution alone, were both 3 nm. In other cases containing HbO_2 in NapP solutions, they were 15 and 6 nm, respectively. Fluorescence intensity measurements were performed at the excitation wavelength of 285 nm and the emission wavelength of 340 nm. At the excitation wavelength of 285 nm, the fluorescence intensity of NapP is nearly maximum. At 340 nm, the emission fluorescence intensity is nearly maximum and the absorptions of HbO_2 and deoxyhemoglobin are identical.

The stoichiometry of binding sites and the association constants of NapP for HbO_2 were determined by titrating a hemoglobin solution with NapP and measuring the extent of fluorescence quenching according to the same principle as that of MacQuarrie and Gibson (1971). On the basis of the large (42 Å) quenching radius of the heme group (Weber and Teale, 1959), it was assumed that bound NapP is completely quenched. Thus, the fluorescence intensity of NapP in a hemoglobin solution is a measure of the free NapP concentration. Sufficient addition of P_6Ino, which has no light absorption at the ultraviolet range of interest and which is nonfluorescent, causes the release of all NapP and the resulting fluorescence is a measure of the total NapP concentration. Since the total NapP concentration is known, the amount of bound NapP is calculated.

In addition to P_6Ino, pyrophosphate (PP_i) and tripolyphosphate (PPP_i) have no light absorption and are nonfluorescent. The exchange bindings of PP_i, PPP_i, and P_6Ino were also determined by titrating fluorescence of NapP in HbO_2 solutions with these reagents. In order to simplify treatment, it is assumed that these nonfluorescent phosphates compete with NapP for only one strong binding site, ignoring weak binding sites. The competitive equilibrium bindings of NapP and these nonfluorescent phosphates to bovine HbO_2 are summarized by eq 1 and 2, where F and L

$$HbO_2 + F \longrightarrow HbO_2{\cdot}F \qquad K_F = [HbO_2{\cdot}F]/[HbO_2][F] \tag{1}$$

$$HbO_2 + L \longrightarrow HbO_2{\cdot}L \qquad K_L = [HbO_2{\cdot}L]/[HbO_2][L] \tag{2}$$

$$[HbO_2] + [HbO_2{\cdot}F] + [HbO_2{\cdot}L] = [HbO_2]_T$$
$$[F] + [HbO_2{\cdot}F] = [F]_T$$
$$[L] + [HbO_2{\cdot}L] = [L]_T$$

denote the fluorescent and nonfluorescent phosphates, respectively, [F] and [L] denote the molar concentrations of nonbound F and L, respectively, $[HbO_2]$, $[HbO_2 \cdot F]$, and $[HbO_2 \cdot L]$ are the molar concentrations of HbO_2, $HbO_2 \cdot F$, and $HbO_2 \cdot L$, respectively, $[F]_T$, $[L]_T$, and $[HbO_2]_T$ are the molar concentrations of the total amount of F, L, and HbO_2, respectively, and K_F and K_L are the association constants of F and L to HbO_2, respectively. In the absence of L, the K_F value can be measured. Even in the presence of L, [F] and $[HbO_2 \cdot F]$ can be measured. $[HbO_2]_T$, $[F]_T$, and $[L]_T$ are known. Therefore, $[HbO_2 \cdot L]$, [L], and $[HbO_2]$ can easily be calculated. Changes in absorption by the addition of PP_i, PPP_i, and P_6Ino, at wavelengths of excitation and emission, were negligibly small. Control experiments by means of equilibrium dialysis showed no binding of NapP to HbO_2 in the presence of enough amounts of PP_i, PPP_i, and P_6Ino.

In all experiments, no buffer was used. Caldwell *et al.* (1971) showed that, at a low salt concentration of 0.01 M NaCl, the binding reaction of P_2glycerate to human deoxyhemoglobin is complex and probably involves nonspecific and multiple binding sites. We performed all experiments in 0.1 M NaCl, though we dealt with HbO_2 from bovine.

The pH values of solutions of hemoglobin, NapP, PP_i, PPP_i, and P_6Ino, before mixing, were adjusted to such appropriate values that the pH value of the solution after mixing may be finally the desired value. The pH values of hemoglobin solutions containing NapP and PP_i, PPP_i, or P_6Ino were also measured in the cell after the fluorescence measurements. A TOA HM-5A pH meter was used.

Results

Equilibrium Binding of NapP to Bovine HbO_2. Binding experiments were performed at several pH values, at 25°, and in 0.1 M NaCl by adding $NapP_2$, $NapP_3$, or $NapP_4$ to bovine HbO_2 solutions. The data were plotted according to Scatchard's method (Scatchard *et al.*, 1950): $\nu_{FT}/[F]$ *vs.* ν_{FT}. Scatchard plots of $NapP_3$ binding to bovine HbO_2 are illustrated in Figure 4. Similar Scatchard plots of $NapP_2$ and $NapP_4$ binding to bovine HbO_2 were obtained, but are not illustrated by figures. Abscissa intercepts of these data appear to be more than unity, which indicates at least two binding sites, in agreement with the result of Chanutin and Hermann (1969) for human HbO_2 by means of equilibrium dialysis. Generally, Scatchard's equation is described as

$$\frac{\nu_{FT}}{[F]} = \sum_i \frac{\nu_{Fi}}{[F]} = \sum_i K_{Fi}(n_i - \nu_{Fi}) \tag{3}$$

where K_{Fi} is the association constant for each of the n_i sites in class i, ν_{Fi} is the number of moles of F bound into the n_i sites in class i per mole of HbO_2, and $\nu_{FT} = \Sigma_i \nu_{Fi}$ is the experimentally observed value of moles of F bound per mole of HbO_2. In our experiments, we assumed that there are only two kind of sites and the number of sites in each class is $n_1 = n_2 = 1$. The solid lines in Figure 4 were obtained by calculating eq 3 using appropriate values of K_{F1} and K_{F2}, which were selected so that most of the calculated solid lines could fit to the experimental data. The pH dependency

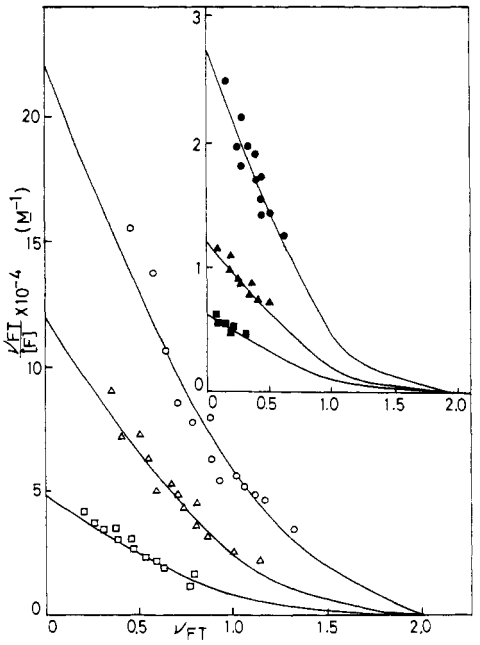

FIGURE 4: Scatchard plots of $NapP_3$ binding to bovine HbO_2 at various pH values in 0.1 M NaCl and 25°. Hemoglobin concentration was 2.2×10^{-5} M: (○) pH 5.50; (△) pH 6.00; (□) pH 6.40. The inserted graph is the similar Scatchard plots of $NapP_3$ binding at pH 6.59 (●), pH 6.80 (▲), and pH 6.96 (■). The solid lines represent the calculated curves at appropriate values of K_{F1} and K_{F2} at each pH value.

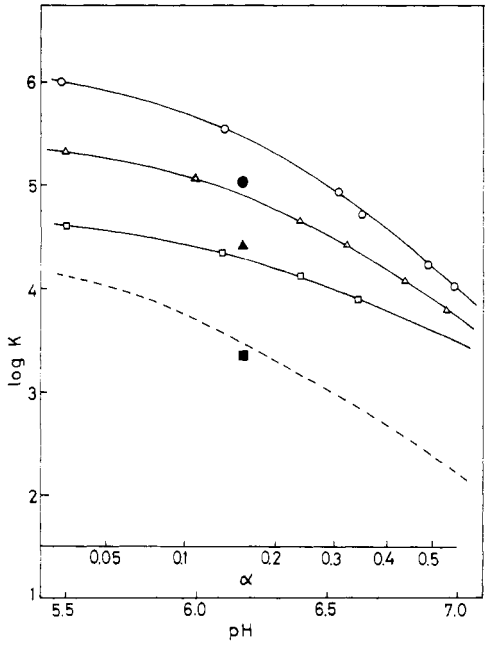

FIGURE 5: Logarithm of the association constants of the fluorescent and nonfluorescent phosphates as a function of pH: (○) log K_{F1} of $NapP_4$; (△) log K_{F1} of $NapP_3$; (□) log K_{F1} of $NapP_2$; (- - -) log K_{F2} of $NapP_4$, $NapP_3$, and $NapP_2$; (●) log K_L of P_6Ino; (▲) log K_L of PPP_i; (■) log K_L of PP_i. The unit of K is M^{-1}. Solution conditions are described in Figures 4 and 6. α denotes the degree of ionization of the terminal phosphate in NapP at various pH values.

of the logarithm of association constant K_{F1} was plotted in Figure 5. The exact estimation of the K_{F2} value was very difficult. The tentative feature of the logarithm of K_{F2} against pH is shown also in Figure 5. The discrepancy of the K_{F2} value for different NapP is unclear.

Competitive Equilibrium Binding of NapP and Nonfluorescent Phosphate to Bovine HbO_2. Competitive equilibrium binding experiments were performed at pH 6.18. As shown in Figure 6, nonfluorescent phosphates are able to displace $NapP_2$. The curves in Figure 6 should yield the relative affinities between $NapP_2$ and nonfluorescent phosphates. If the affinities of these nonfluorescent reagents are much larger than that of $NapP_2$, these curves will consist of two closely linear limbs with sharp break points as reported by MacQuarrie and Gibson (1972). $NapP_2$ has the smallest affinity of three NapP's and P_6Ino has the largest affinity of three nonfluorescent phosphates. The curve in Figure 6 indicating the relative affinity between $NapP_2$ and P_6Ino has no break point. This fact shows that even the affinity of $NapP_2$ is not extremely different from that of P_6Ino. PP_i is shown to have a much smaller affinity than PPP_i and P_6Ino. To obtain the K_L value of PP_i, as an example, $[HbO_2]/[HbO_2 \cdot L]$ in eq 2 is plotted against $1/[L]$. The K_L value of PP_i is estimated as the inverse of slope in Figure 7a. The K_L value of PP_i should, in principle, be identical in all cases where F = $NapP_2$, $NapP_3$, and $NapP_4$. The K_L values of

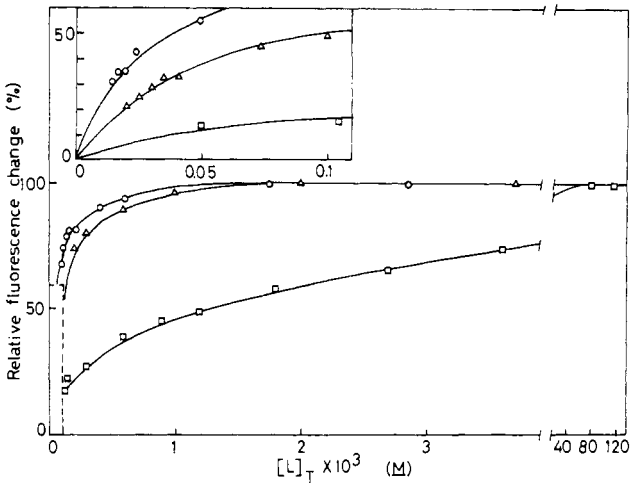

FIGURE 6: Displacement of $NapP_2$ from HbO_2 by the addition of nonfluorescent phosphates in 0.1 M NaCl at pH 6.18 and 25°. The top graph is an enlargement of the dotted-in area in the bottom graph. The $NapP_2$ concentration was 3.0×10^{-5} M. HbO_2 concentration was 2.2×10^{-5} M: (○) L = P_6Ino; (△) L = PPP_i; (□) L = PP_i.

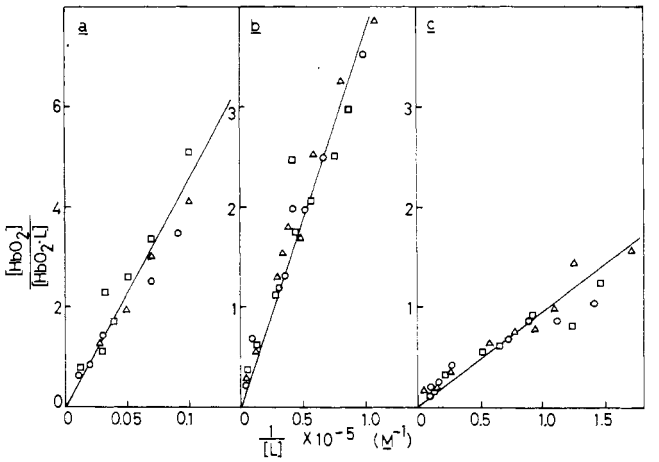

FIGURE 7: Plots of $[HbO_2]/[HbO_2 \cdot L]$ against $1/[L]$ in eq 2. The inverse of slope gives the association constant (K_L) of nonfluorescent phosphate: (a) L = PP_i; (b) L = PPP_i; (c) L = P_6Ino; (○) F = $NapP_2$; (△) F = $NapP_3$; (□) F = $NapP_4$. All other solution conditions were the same as in Figure 6.

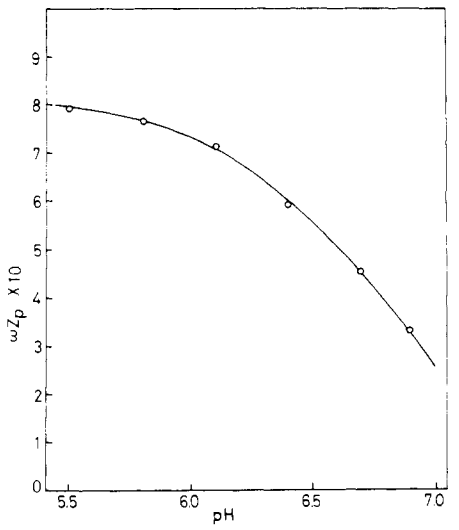

FIGURE 8: Calculated ωZ_P from Figure 5 using eq 6 as a function of pH.

PPP_i and P_6Ino can be obtained in a similar fashion. The logarithms of K_L at pH 6.18 are indicated in Figure 5. At pH 6.18, the K_L values of PPP_i and P_6Ino are shown to be comparable to the K_{F1} values of $NapP_2$ and $NapP_3$, respectively. The K_L value of PP_i is also shown to be the smallest of all K_F and K_L values at pH 6.18.

Discussion

By measuring the fluorescence quenching of NapP upon interaction with bovine HbO_2, we could, easily and speedily, estimate the association constants of fluorescent phosphates. If we extrapolate the straight-line region in $\nu_{FT} < 1$ of our Scatchard plots back to the abscissa, we would have the intercepted point on the abscissa between 1 and 2. The result is similar to results of MacQuarrie and Gibson (1972) and Hedlund *et al.* (1972).

In the discussion about the pH dependency of K_F values, treatments of Riggs (1971) and Tanford (1961) are useful. Riggs assumed that P_2glycerate binds between the protonated α-NH_2 terminal groups of the two β chains in deoxy- and oxyhemoglobin, and discussed the relation between the P_2glycerate binding to their groups and the protonation reaction of their groups. Furthermore, Arnone (1972) revealed by X-ray analysis that the anionic groups of P_2glycerate form salt bridges with seven cationic groups including α-NH_2 groups of the β chains in human deoxyhemoglobin. Such features of binding may be extended to other species of hemoglobin, for example, bovine hemoglobin. However, there are reports (Tomita and Riggs, 1971; De Bruin *et al.*, 1973, 1974) that the P_2glycerate binding site of HbO_2 may exist not only in β chains, but also in α chains. Benesch *et al.* (1972, 1973) studied the interaction of N termini of α and β chains in hemoglobin with various pyridoxal compounds instead of polyphosphate. In brief, we have no detailed information on the P_2glycerate binding site of HbO_2. Therefore, we employed Tanford's (1961) treatment, which is a rough but more general one. According to the treatment of Hedlund *et al.* (1972), based on Tanford's theory (1961), we assume that there exists general coulombic interaction between charged protein and charged ligand, with pH control of both these charges, Z_P and z_F, respectively. We consider the charged protein as a charged spherical macroion with the interaction parameter ω. Here, we restrict our discussion to K_{F1}. If the intrinsic association constant of NapP at $Z_P = 0$ is given by K_0, then

$$K_{F1} = K_0 e^{-2\omega Z_P z_F} \quad (4)$$

$$\log K_{F1} = \log K_0 - 0.868\omega Z_P z_F \quad (5)$$

We intend here to evaluate the Z_P or ωZ_P value in eq 5 as a function of pH from experimental data in Figure 5 by determining the three kinds of z_F and $\log K_{F1}$ for $NapP_2$, $NapP_3$, and $NapP_4$ at a given pH value, and by cancelling $\log K_0$. Usually, the z_F value is dependent on pH. The pK values for the reaction $H_2ATP^{2-} \rightarrow H^+ + HATP^{3-}$ and $HATP^{3-} \rightarrow H^+ + ATP^{4-}$ are 4.06 and 6.95, respectively (Alberty, 1969). In the pH range (5.5–7.0) of interest here, the latter reaction is significant. Assuming tentatively that the pK values of ionization for $HNapP_2^{2-}$, $HNapP_3^{3-}$, and $HNapP_4^{4-}$ are the same value, 6.90, the degrees (α) of their ionizations are the same at the same pH value (see Figure 5). Therefore, at a given pH, the net charges z_F of $NapP_2$, $NapP_3$, and $NapP_4$ differ from one another by one charge. As an example, at pH 6.90, we can consider that the z_F values of $NapP_2$, $NapP_3$, and $NapP_4$ are 2.5, 3.5, and 4.5, respectively. Practically, the pK values of each NapP may be slightly different from one another. Since errors caused by the slightly different pK scarcely influence our discussion, we can discuss the change of the K_{F1} value as a function of the z_F value for different F at a given pH. If we assume that, at a given pH, ω and Z_P are always constant for any F ($NapP_2$, $NapP_3$, and $NapP_4$) $\log K_{F1}$ should be linear against z_F at any pH. Indeed, such a linear relation is experimentally recognized in Figure 5. A similar linear relationship of interaction energy against the net charge of ligand has been observed by Lo and Schimmel (1969) in the case of human deoxyhemoglobin at pH 7.0. If we differentiate eq 5 with the net charge z_F of each NapP at a given pH, we obtain eq 6. From eq 6 and experimental data in

$$-\frac{\delta \log K_{F1}}{\delta z_F}\frac{1}{0.868} = \omega Z_P \quad (6)$$

Figure 5, we can obtain ωZ_P as a function of pH. Figure 8 shows that the ωZ_P obtained decreases with an increase in pH. If we give an appropriate constant value to ω, Figure 8 will directly show the pH dependency of Z_P. De Bruin *et al.* (1969) studied the proton titration behavior in bovine HbO_2 and showed that there are many titratable groups especially in the pH range 6.0–7.5. Some of these titratable groups may correspond to the net charge Z_P in our data shown in Figure 8. At the ionic strength of 0.1 M and 25°, the Debye–Hückel parameter κ is 0.10 Å^{-1}. As the radius of the hemoglobin molecule is about 30 Å and the Debye radius $1/\kappa$ is about 10 Å, not all charges in hemoglobin but the positive charges in the vicinity of binding sites can contribute to the phosphate binding. Therefore, the net charge Z_P in our data (Figure 8), practically, must signify the positive net charge involved in the binding in HbO_2, although the plausibility for quantitative estimation on the Z_P value is dependent on the estimate of the ω value and also the validity of the electrostatic model used. According to Perutz (1970), the positively charged groups involved in the phosphate binding in hemoglobin have pK values above approximately 7.0. At a pH lower than 5.5, the phosphate binding sites are fully protonated. Indeed, Z_P in Figure 8 (ω is a constant) is shown to be saturated to a value at the decreasing pH. It appears that we succeeded in accounting for our results on the association constants at various pH values by electrostatic effects.

Table I: Relation between R and Z_P; ωZ_P = 0.80 at Low pH, Ionic Strength = 0.1 M, 25°, $1/\kappa$ = 10 Å.

R (Å)	ω	Z_P
6	0.40	2.0
9	0.23	3.5
12	0.15	5.3
15	0.11	7.3
30	0.034	24

We tentatively calculated ω against R. From Figure 8, we employed the saturated value 0.80 at low pH as ωZ_P, and calculated Z_P using the ω value (Table I).

If we differentiate eq 5 with pH, we obtain eq 7. Hedlund

$$-\frac{\delta \log K_{F1}}{\delta \mathrm{pH}} \frac{1}{0.868} = z_F \frac{\delta(\omega Z_P)}{\delta \mathrm{pH}} + (\omega Z_P) \frac{\delta z_F}{\delta \mathrm{pH}} \quad (7)$$

et al. (1972) regarded $\delta z_F/\delta \mathrm{pH} = 0$ in the pH range 6.5–7.0. On the basis of our data (Figure 8), we examined the contribution of the second term in eq 7 in comparison with the first term. It was found that the per cent ratios, the absolute value of the second term to the absolute value of the first term at pH 5.5 and 7.0, are 24–47 and 5–9%, respectively. The second term in eq 7 is negligible in the neighborhood of pH 7.0. Therefore, the treatment of Hedlund *et al.* is approximately valid at this pH.

As shown in Figure 5, the K_{F1} value of $NapP_3$ is larger than the K_L value of PPP_i, although both substances have the same number of phosphoric groups. Similarly, the K_{F1} value of $NapP_2$ is larger than the K_L value of PP_i. This fact suggests that not only phosphoric groups but also hydrophobic groups contribute to the binding to HbO_2. It is impractical to compare our results with many literature references about organic phosphate binding to hemoglobins of other species under somewhat different conditions. Keeping in mind these differences, we will make a try to compare our values with the association constants of P_2glycerate, ATP, and so on measured by various investigators. Hedlund *et al.* (1972) gave $K = 4 \times 10^2\ M^{-1}$ for the P_2glycerate binding to horse HbO_2, and $K = 8 \times 10^2\ M^{-1}$ for the ATP binding, at pH 7.0 and 25° in 0.1 M NaCl. De Bruin *et al.* (1974) found $K = 1.2 \times 10^3\ M^{-1}$ for P_2glycerate binding to human HbO_2, at pH 6.8 and 25° in 0.1 M KCl. The association constants of $NapP_2$, $NapP_3$, and $NapP_4$ are larger than those of P_2glycerate and ATP.

The association constant of $NapP_3$ is comparable to that of P_6Ino, which is the strongest allosteric effector ever found (Benesch *et al.*, 1968; Gibson and Gray, 1970; Tyuma *et al.*, 1971). We found also that, as well as P_6Ino, $NapP_2$, $NapP_3$, and $NapP_4$ influence O_2 affinity of bovine hemoglobin. The natures of three fluorescent organic phosphates as strong allosteric effectors will be reported elsewhere.

It is worthwhile to mention here that the fluorescence of NapP has a very interesting feature. At the excitation wavelength in the range 290–340 nm, the fluorescence intensities of $NapP_2$, $NapP_3$, and $NapP_4$ are clearly different from one another. Therefore, we made use of $NapP_3$ as a fluorescent ATP analog, and succeeded in measuring ATPase activity directly by the fluorimetric method (Kagawa *et al.*, 1974).

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