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Conformational Aspects of the Interaction of Polyanions with Liganded β Chains of Human Hemoglobin†

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ABSTRACT: The interaction of carbon monoxide β chains with two allosteric effectors, namely inositol hexaphosphate and benzenehexacarboxylate, was studied. The sedimentation coefficient ($s_{20,w}$) of the liganded β chains was measured to be the same both in the presence and absence of the two effectors suggesting that the protein exists as a tetramer under the conditions of our titration and optical studies. The binding of benzenehexacarboxylate to the liganded β chains was investigated by potentiometric titration in the pH range 6.7–8.0. The results at pH 7.4 showed a binding of 2 mol of benzenehexacarboxylate per tetramer, with an association constant of 1.26×10^4 l. mol^{-1} at 20 °C. The Hill coefficient for the binding was determined to be 0.73. Similar experiments on the interaction of inositol hexaphosphate with the β chains showed a binding of 2 mol of the effector per tetramer with identical Hill coefficient (0.737) and comparable association constants (0.88×10^4 l. mol^{-1}). The value below unity of the Hill coefficient, found for the binding of the two effectors to the protein, probably reflected an anticooperativity produced by the different net electric charges of the free protein and the protein–effector complex. The difference in protons bound per mole of heme by the β subunits in the presence and absence of benzenehexacarboxylate appeared consistent with the proposal that two groups per chain changed their pK from 6.6 to 7.4 upon the interaction. In the presence of benzenehexacarboxylate, the protonation of these groups appeared to be cooperative, suggesting a conformational change of the protein upon the binding. The absorption spectra of carbon monoxide β chains in the Soret region was markedly altered by benzenehexacarboxylate and inositol hexaphosphate. The features in the difference spectra of the protein obtained with the two effectors were qualitatively identical and indicated changes in the heme environment produced by the interaction of the effectors with the β chains. Concomitant changes in circular dichroism and optical rotatory dispersion of the liganded β chains caused by the addition of the two effectors provided supporting evidence for the conformational change in the protein produced by the binding of the effectors.

The oxygen affinity of human hemoglobin is regulated in vivo through the action of the metabolic intermediate, 2,3-diphosphoglycerate, which lowers the oxygen affinity of hemoglobin by preferentially binding to its deoxy form (Benesch and Benesch, 1967; Chanutin and Curnish, 1967). Arnone (1972), on the basis of x-ray diffraction data, concluded that the effector binding to deoxyhemoglobin involves electrostatic interactions between anionic sites on DPG[1] and the cationic sites offered by Val-1, His-2, and His-143 of the two β chains and by Lys-82 of one of the β chains in hemoglobin. Inositol hexaphosphate is even more effective than DPG in lowering the oxygen affinity of hemoglobin (Benesch and Benesch, 1969) and the same binding sites on the chains of deoxyhemoglobin participate in its binding (Arnone and Perutz, 1974). These organic phosphates bind not only to the unliganded hemoglobin but to the fully liganded protein as well (Chanutin and Hermann, 1969; Luque et al., 1969; Garby et al., 1969; Gray and Gibson, 1971; Hedlund et al., 1972; Berger et al., 1973; de-Bruin et al., 1973). Other organic anions which have been shown to interact with deoxyhemoglobin through electrostatic interactions and thereby lower its oxygen affinity include benzenepentacarboxylate (Shimizu and Bucci, 1974; Bucci, 1974) and benzenehexacarboxylate (Ellis and Bucci, 1975; Desbois and Banerjee, 1975).

Inositol hexaphosphate and DPG both perturb the absorption spectra in the visible and Soret region of liganded hemoglobin (Adams and Schuster, 1974; Giardina et al., 1975; Knowles et al., 1975). Furthermore Lindstrom and Ho (1973) studied the effect of anions including IHP and DPG on the conformation of human adult carbon monoxyhemoglobin and

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[1] Abbreviations used are HbCO, carboxyhemoglobin A; COβ-SH, carboxy derivative of isolated β chains; DPG, 2,3-diphosphoglycerate; BHC, benzenehexacarboxylate; IHP, inositol hexaphosphate; Bistris, N,N^1-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; CD, circular dichroism; ORD, optical rotatory dispersion; R and T conformations according to Perutz's notation refer to the conformations of liganded and unliganded hemoglobin, respectively.

oxyhemoglobin by nuclear magnetic resonance. These authors concluded that DPG or IHP binding alters the tertiary structure of liganded hemoglobin with the greatest effect on the heme pocket and that these anions affect the tertiary structure of the chains without producing a T conformation. There is evidence to suggest that benzenehexacarboxylate also alters the conformation of carbon monoxy human hemoglobin (Ellis and Bucci, 1975).

As described above, organic anions seem to preferentially interact with the β chains in human adult hemoglobin. It would, therefore, be interesting to study the interaction of these anions with the isolated β chains. This paper reports results on the binding of benzenehexacarboxylate to carbon monoxy chains as measured by differential acid–base titration and on the effect of such binding on the conformation of the protein.

Materials and Methods

Preparation of COβ-SH Chains. Human hemoglobin was obtained from fresh blood by the method of Drabkin (1946) using chloroform instead of toluene and was deionized by recycling for 1 h in cold through a mixed-bed, ion-exchange column. β chains with sulfhydryl groups blocked by *p*-hydroxymercuribenzoate were prepared from HbCO according to Bucci and Fronticelli (1965). These sulfhydryl groups were then regenerated by the method of Waks et al. (1973). The number of free sulfhydryl groups in COβ-SH chain, thus prepared, was determined by titration with *p*-hydroxymercuribenzoate as described by Boyer (1954) and was found to be close to 2 (1.85–1.95) per mol of heme. The protein concentration was measured spectrophotometrically using ϵ 14 000 cm^{-1} M^{-1} per heme at 540 nm or ϵ 196 000 cm^{-1} M^{-1} per heme at 420 nm. When required the stock solution of COβ-SH chains was diluted with Bistris buffer previously saturated with carbon monoxide.

BHC was obtained from Aldrich Chemical Co. and its purity was checked by measuring the melting point which was found to be 287 °C. The concentration of the BHC solution was determined both by pH titration and dry weight methods; the values determined by the two methods agreed within 2%. IHP (sodium salt) and *p*-hydroxymercuribenzoate were purchased from Sigma Chemical Co. The concentration of IHP solution was determined by the dry weight method assuming that the sodium salt of IHP contained 12 sodium ions per mol. Judging from the pH (~10) of the solution in water, the number of sodium ions might have been less than 12 but certainly more than eight; in fact IHP carries eight negative charges even at pH 7.3 (Arnone and Perutz, 1974). Assuming 9 sodium ions per mol of IHP, a maximum overestimation in the concentration as determined by the dry weight method would be about 7%.

Measurements of pH were made on a Radiometer pH meter M26, connected with a scale expander and Sargent recorder, using a GK 2301B combination electrode.

Differential Titration. The difference in the number of protons bound per mole of heme by COβ-SH chains in the presence and absence of BHC or IHP was measured in 0.05 M NaCl at 20 °C, as described in a previous paper for the interaction of benzenepentacarboxylate with deoxyhemoglobin (Bucci, 1974). The protein concentration in the titration experiments was between 1 and 5×10^{-4} M per heme.

Difference Absorption Spectra. Difference absorption spectra were measured with a Cary 14 recording spectrophotometer at 20 °C, maintained by circulating water from a thermostat. Two well-matched, double-sector cells, each sector having 1-cm path, were chosen. The two sectors of the *sample* cell contained respectively (i) COβ-SH chains and BHC in 0.05 M Bistris, pH 7.0, and (ii) 0.05 M Bistris, pH 7.0. Likewise the respective solutions in the two sectors of the *reference* cell were (i) COβ-SH chains and (ii) BHC, both in 0.05 M Bistris, pH 7.0. Exactly similar arrangements of solutions were made for measuring the difference spectra in the presence of IHP. The protein concentration in these experiments was such that the absorbance in 1-cm cells at 420 nm was less than 4. Extreme care was taken to ensure identical protein concentration both in the *sample* and in the *reference* cells. This was checked by measuring absorbances of the *sample* and the reference solutions at 540 nm in a cell of 2-cm path. No detectable influence of BHC or IHP on the absorbance of COβ-SH chain at 540 nm was found under the conditions of our experiments.

TABLE I: Sedimentation Velocity of COβ-SH Chains in the Presence and Absence of Effectors.

Protein Concn (mg/ml)	Effector	Effector Concn (mM)	Solvent	$s_{20,w}$
2.1	IHP	2.5	0.05 M NaCl	4.69
2.2	BHC	1.7	0.05 M NaCl	4.59
2.1			0.05 M NaCl	4.61
2.0	BHC	1.0	0.1 M Bistris	4.56
2.0			0.1 M Bistris	4.51

CD and ORD measurements of COβ-SH chains in 0.05 M Bistris, pH 7.0, were made at 20 °C in the presence and absence of BHC or IHP with a Jasco J-20 automatic recording spectropolarimeter. The temperature was kept constant by circulating water from a thermostat through a jacket covering the cell holder. CD and optical rotation were expressed as molar ellipticity, $[\theta]_\lambda$, in deg cm^2 (dmol of heme)$^{-1}$ and, as specific rotation, $[\alpha]_\lambda$, in deg cm^2 g^{-1}, respectively. The protein concentration and path lengths of cells in these experiments were such that the absorbance at 420 nm was always less than 2.0.

Preparation of Protein Solution. For difference absorption spectra, CD and ORD measurements, solutions of COβ-SH chains in the presence and absence of BHC or IHP were prepared in 0.05 M Bistris, pH 7.0, as follows. Two 10-ml portions of COβ-SH solution were measured in two 10-ml volumetric flasks. To one flask 0.1 ml of concentrated BHC or IHP solution was added and to the other 0.1 ml of 0.05 M Bistris buffer, pH 7.0, was added with the help of a micropipet. The optical measurements were made after 2 h.

Sedimentation Velocity. The state of polymerization of COβ-SH chains in the presence and absence of BHC or IHP was checked by measuring the sedimentation velocity at 60 000 rpm, in 0.05 M Bistris buffer or in 0.05 M NaCl at pH 7.0, and 20 °C, with a Beckman Model E ultracentrifuge. The schlieren optics was used. Invariably single symmetrical peaks were detected.

Results

Sedimentation velocity results summarized in Table I show that COβ-SH chains exist as tetramers both in the presence and absence of BHC or IHP under the experimental conditions used in these studies. The values of $s_{20,w}$ are slightly higher than those generally found in this laboratory for tetrameric hemoglobin. This may be due to a minor difference in the partial specific volume and/or virial coefficient of isolated β chains and hemoglobin.

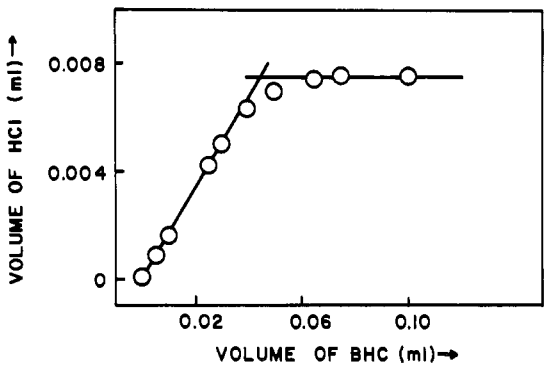

FIGURE 1: Titration of COβ-SH chain with BHC at pH 6.9 in 0.05 M NaCl and 20 °C. Protein concentration, 9.2×10^{-5} M per heme, 1×10^{-2} M BHC, 0.209 M HCl.

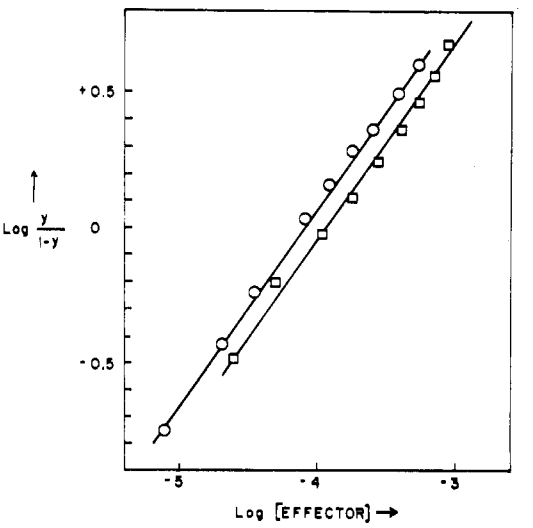

FIGURE 2: Hill plots for the titration of COβ-SH chains with effectors in 0.05 M NaCl, 20 °C (○) with 1×10^{-2} M BHC, pH 7.4 or (□) with 4×10^{-2} M IHP, pH 7.3. Protein concentration 2.67×10^{-4} M per heme, 0.209 M HCl.

Differential Titration. Titration of 0.01 M BHC with 0.1 M KOH indicated that (i) BHC possesses constant buffer capacity in the pH range 4–6, and that (ii) practically all its six protons were titrated near pH 7.0. The interaction of BHC with COβ-SH chains was investigated potentiometrically in the pH range 6.7–8.0.

Interaction of BHC with COβ-SH chains solution of identical pH resulted in an increase in pH which was back-titrated to the original pH by the addition of 0.2095 M HCl. The volume of HCl used was plotted against that of BHC. A typical such titration curve at pH 6.98 is shown in Figure 1. The stoichiometry of the reaction between BHC and COβ-SH chains was determined from the break-point of Figure 1. The respective concentrations of BHC and COβ-SH chains at the break-point were 4.5×10^{-8} and 2.3×10^{-8} mol which suggested a stoichiometry of 2 mol of BHC per tetramer of COβ-SH chains. Additional measurements conducted between pH 6.7 and 7.2 were all consistent with this stoichiometry. Similar titration of COβ-SH chains with 0.057 M IHP indicated a stoichiometry of 2.16 mol of IHP per tetramer of COβ-SH chains. As described above some (7%) overestimation of IHP concentration is possible. This uncertainty would reduce the stoichiometry from 2.16 to 2.0. This conclusion is apparently at variance with the view of Benesch et al. (1968b) who found by equilibrium dialysis that 1 mol of DPG was bound to both the liganded and unliganded β chains. However, the facts that their experimental conditions and experimental approach were different from those used in this study and that the interaction of hemoglobin with DPG is presumably weaker than with IHP (Benesch et al., 1968a) or with BHC (Ellis and Bucci, 1975) do not permit a meaningful comparison between the finding of this study and those reported by Benesch et al. (1968b).

Determination of the Affintiy Constant of COβ-SH Chains for BHC. The difference in the number of protons bound per mole of protein in the presence and absence of the effectors would be given by the relations (Bucci, 1974)

$$\bar{h} - \bar{m} = (\bar{n} - \bar{m})[C]/([B] + [C]) \quad (1)$$

$$\Delta\bar{h} = YH_{max} \quad (2)$$

where $\bar{h}$ is the average number of protons bound per mole of COβ-SH chains in the presence of effectors; $\bar{m}$ and $\bar{n}$ are the average numbers of protons bound per mole of the free protein and the protein effector complex, respectively; [B] and [C] are the concentrations of the free protein and the complex, respectively; and Y is the fractional saturation of COβ-SH chains with the effector. At each addition of the effector, the amount of HCl used in the back-titration to achieve the original pH gave $\bar{h}$. The total amount of HCl used at the end point in the back-titration yielded H_{max}. Knowing $\bar{h}$ and H_{max}, the values of Y were calculated from eq 2 at each addition of the effector. The equilibrium concentration of the free effector was computed from the difference between the total amount of the effector added and that bound to the protein.

Figure 2 shows the Hill plots for the interactions of BHC and IHP with COβ-SH chains. The interaction of the protein with BHC was studied at pH 7.4 whereas that with IHP was investigated at pH 7.3. The Hill coefficient and association constant were 0.73 and 1.26×10^4 l. mol^{-1}. Essentially identical Hill coefficient (0.737) and comparable association constant (0.88×10^4 l. mol^{-1}) were measured for the binding of IHP to COβ-SH chains. At pH's lower than pH 7.3, the binding of the effectors to COβ-SH chains was essentially stoichiometric leaving too little free effector to permit a reliable determination of the association constants.

Difference in Protons Bound by CO-βSH Chains in the Presence and Absence of BHC. The correlation between H_{max} and the hydrogen ion concentration, (H^+), is described by

$$H_{max} = \sum_i \left(\frac{K_i'}{K_i' + (H^+)} - \frac{K_i}{K_i + (H^+)} \right) \quad (3)$$

where K_i and K_i' represent the ionization constants of the groups in the absence and presence of the effector, respectively.

In simulating the data, it was assumed that all of the protons absorbed by the interaction of BHC and COβ-SH chains was the result of the pK shift of the positively charged groups in the protein, forming salt bridges with the negative groups of BHC. This implied that all of the groups of BHC were completely titrated in the pH range investigated. This was not strictly true in the pH range 6.8–7.0. However, any correction of the experimental data based on the number of protons introduced into the system by the addition of BHC was too small to be meaningful. Since COβ-SH chains bind 2 mol of BHC per tetramer and one BHC molecule offers 6 negative charges upon ionization, the number of interacting groups per heme cannot be greater than 3. It should be pointed out that the assumption that all of the groups had identical acid–base characteristics kept their number to a minimum. Equation 3 could not re-

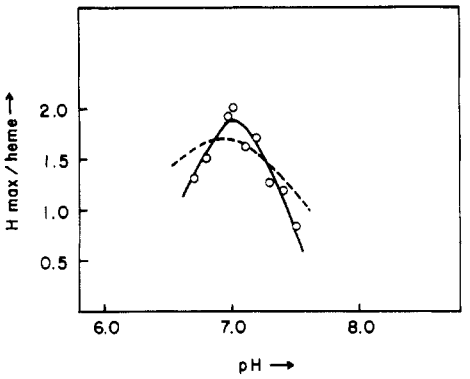

FIGURE 3: Difference in the number of protons bound per mole of heme by COβ-SH chains in the presence and absence of BHC in 0.05 M NaCl at 20 °C. The solid and dotted lines were drawn as described in the *text* and the experimental points are represented by circles.

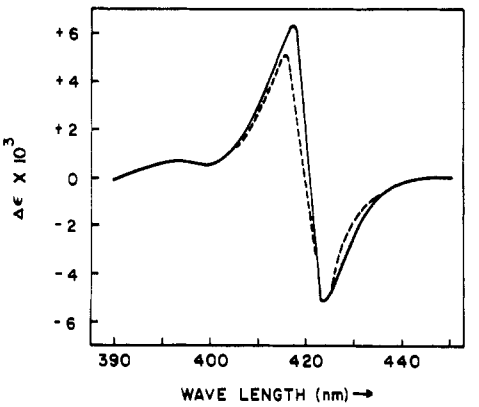

FIGURE 4: Difference spectra of COβ-SH chains at a concentration of 1.76 $\times 10^{-5}$ M per heme in the presence and absence of effectors in 0.05 M Bistris, pH 7.0, 20 °C (—) with 4 mM BHC or (- - -) with 5 mM IHP. The sample compartment contained the mixture of COβ-SH chains and the effector. For details, see text.

produce the data within the limit of 3 groups per heme. The dotted line of Figure 3 was obtained with 5.25 groups shifting their pK from 6.6 to 7.2 upon interaction with BHC. On the assumption that the protonation of the protein groups involved in the interaction with BHC was cooperative, a "Hill type" (Wyman, 1964) of exponent (q) was introduced in eq 3 which became

$$H_{max} = \sum_i \left(\frac{(K_i')^q}{(K_i')^q + (H^+)^q} - \frac{K_i{}^q}{K_i{}^q + (H^+)^q} \right) \quad (4)$$

The solid line shown in Figure 3 was obtained from eq 4, assuming that 2 groups per chain interacted with BHC changing their pK from 6.6 in the absence to 7.4 in the presence of the effector, with q = 3.2. A very similar curve was obtained assuming the pK shift of 3 groups from 6.7 to 7.3, with q = 2.15.

Difference Absorption Spectra. In the Soret region of the spectrum, COβ-SH chains in 0.05 M Bistris, pH 7.0, absorb maximally near 420 nm. Addition of BHC or IHP produced a slight blue shift (1.0 nm) in the spectra of COβ-SH chains. This can be seen in the difference spectra shown in Figure 4. The main features of the difference spectra of COβ-SH chains with BHC and IHP are summarized in Table II. It is clear that the difference spectra obtained with IHP was similar but not identical with that obtained with BHC.

Circular Dichroism Spectra. Circular dichroism spectrum of COβ-SH chains with and without BHC, in 0.05 M Bistris buffer, pH 7.0, and at 20 °C is shown in Figure 5. Below 431 nm, $[\theta]_\lambda$ was lower in presence of the effector down to 405 nm (see Figure 5). The value of $[\theta]_\lambda$ decreased from +96 000 at 424 nm in absence of BHC to +86 700 at 424.5 nm in presence of 1 mM BHC. Similar decrease in $[\theta]_\lambda$ from +96 000 to +86 000 was observed in presence of 1 mM IHP (see Figure 6). However, unlike BHC, the ellipticity in the range 410–419 nm was higher in the presence of IHP than in its absence. The ultraviolet circular dichroic spectra of COβ-SH chains in the range 250–300 nm were indistinguishable in the presence and absence of 3.7 mM BHC or 3.7 mM IHP (see Figure 7).

Optical Rotatory Dispersion. ORD of COβ-SH chains in the presence and absence of BHC and IHP is shown in Figure 8. The two effectors showed distinct effect on the heme Cotton effect which was slightly decreased and red shifted. The levorotation of COβ-SH chains in the range 300–422 nm was significantly decreased by BHC and IHP, the former being more effective than the latter.

TABLE II: Difference Absorption Spectra of COβ-SH Chains with BHC and IHP.

	BHC (1 mM)		IHP (1 mM)	
	Wavelength (nm)	$\Delta\epsilon^a \times 10^{-2}$ (cm^{-1} M^{-1})	Wavelength (nm)	$\Delta\epsilon^a \times 10^{-2}$ (cm^{-1} M^{-1})
Trough	423.5	−51.8	423.5	−51.8
Crossover	420.5		420.0	
Peak	417	+63.9	415.5	+50.0

[a] The difference in molar extinction is given on a per heme basis.

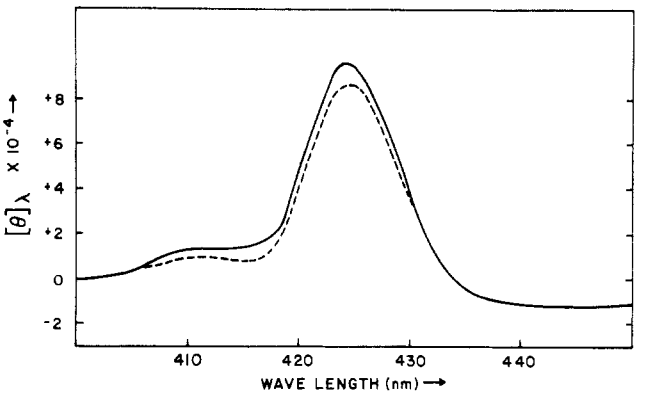

FIGURE 5: Circular dichroism spectra in the Soret region of COβ-SH chains at a concentration of 2.5 $\times 10^{-5}$ M per heme in the presence and absence of BHC in 0.05 M Bistris pH 7.0 at 20 °C (—) without BHC and (- - -) in the presence of 2 mM BHC.

Discussion

Differential Titration of COβ-SH Chains in the Presence and Absence of BHC. The restricted pH range in which meaningful titration data could be obtained and the way the simulation had to be conducted made it possible to analyze only certain details of the acid–base characteristics of the groups in the protein that interact with BHC. Nevertheless substantial information was obtained. The parameters calculated with the simulations can be considered average values that inform us of the pH range in which the ionization of those groups occurs, and of the magnitude of the pK shift upon the interaction. In regard to the number of groups involved in the formation of

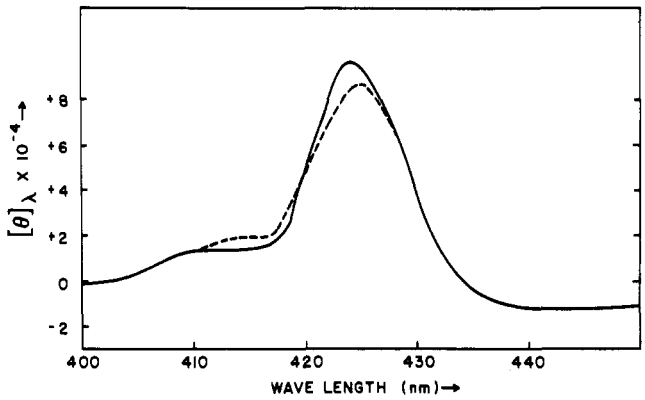

FIGURE 6: Circular dichroism spectra in the Soret region of COβ-SH chains at a concentration of 2.2×10^{-5} M per heme in the presence and absence of IHP in 0.05 M Bistris, pH 7.0 at 20 °C (—) without IHP or (- - -) in the presence of 5 mM IHP.

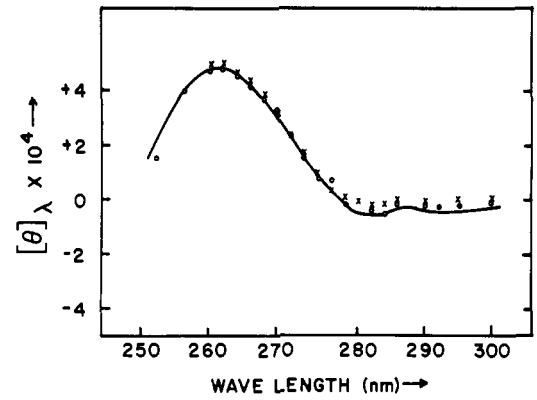

FIGURE 7: Ultraviolet circular dichroism spectra of COβ-SH chains near a concentration of 2.2×10^{-5} M per heme in the presence and absence of effectors in 0.05 M Bistris, pH 7.0, 20 °C (—) without effector, (×) in the presence of 3.7 mM BHC; (○) in the presence of 3.7 mM IHP.

salt bridges, it is proper to emphasize that the number of salt bridges cannot be less than 2 per heme, because this is the maximum number of protons absorbed by the interaction at pH near 7. Also they cannot be more than 3, because a total of six negative charges is present on the molecule of BHC. A possible involvement of lysyl residue (Arnone, 1972), which cannot be detected near pH 7, would keep this number close to 2 per heme.

The mere inspection of the titration data and the quick vanishing of the interaction above pH 7 (the proton absorption went down from nearly 2 per heme to zero in less than 1 unit of pH) suggest that the titration of the groups involved in the interaction is unusually sharp. The "visual" impression was supported by the numerical simulations that required "Hill exponents" higher than 1 in eq 4 in order to keep the number of interacting groups within the expected limits. These observations all point to a cooperative protonation of the groups involved in the interaction. This phenomenon can be explained on the basis of a conformational change of the protein produced by the binding of BHC to COβ-SH chains.

In the complex of COβ-SH chains with BHC, the cooperative protonation of the groups in the protein and the conformational change produced by the binding of BHC can be represented by the following scheme:

$$\begin{array}{ccccc} C_0 & \xrightarrow{P_1} & C_1 & \xrightarrow{P_2} & C_2 \\ \downarrow L_0 & & \downarrow L_1 & & \downarrow L_2 \\ C_0^* & \xrightarrow[S_1]{} & C_1^* & \xrightarrow[S_2]{} & C_2^* \end{array} \qquad (5)$$

where the C_i*'s and C_i's represent the respective concentrations of transformed and untransformed species of COβ-SH-BHC complex; the subscript i represents the number of moles of protons bound per heme in the groups that interacted with BHC; P_i's are the protonation constants for the C_i species; S_i's are the protonation constants for the C_i* species, and L_i's are the transformation constants. All P_i's and all S_i's are assumed equal. All L_i's are not necessarily equal since they are the expressions of a conformational change stabilized by the binding of BHC to COβ-SH chains, and the binding is in turn stabilized by the number of salt bridges formed, i.e., by the protonation of the groups involved in the interaction (Bucci, 1974). Inspection of the above scheme reveals that

$$L_i = L_0 R^i \qquad (6)$$

where R is the ratio, S_i/P_i. Also the following equation describes the overall apparent affintiy constant, $P_{i,\mathrm{app}}$, of each protonated species for the next proton

$$P_{i,\mathrm{app}} = P_i \frac{1 + L_{i+1}}{1 + L_i} \qquad (7)$$

Clearly the value of $P_{i,\mathrm{app}}$ increases with protonation of the groups producing an apparent cooperative protonation unless either (1) $L_i \ll 1$, so that $1 + L_i = 1$, and $P_{i,\mathrm{app}} = P_i$; or (2) $L_i \gg 1$, so that $1 + L_i = L_i$ and $P_{i,\mathrm{app}} = P_i R$. In other words cooperative protonation is produced only if the numerical values of L_i's are neither very different from 1.0 nor very near zero.

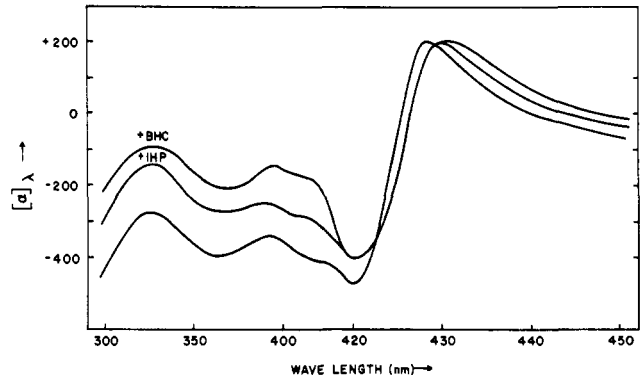

FIGURE 8: Optical rotatory dispersion curve for COβ-SH chains in the presence and absence of effectors in 0.05 M Bistris, pH 7.0 and at 20 °C. Above 430 nm, lower, middle, and upper curves represent specific rotations for COβ-SH alone, COβ-SH plus 4 mM IHP, and COβ-SH plus 4 mM BHC, respectively. The two curves obtained with BHC and IHP merge and appear as one in the range 419–430 nm. Protein concentration 2.1×10^{-5} M per heme.

We are not in any position of measuring the transformation constants. Nevertheless eq 7 and the distinct cooperative protonation present in our system suggest a low free energy for the conformational change. This implies that the two conformations of the protein can be simultaneously present in the system even after complete saturation of COβ-SH chains with the effectors.

Cooperative Protonation in Free COβ-SH Chains. Equation 4 assigned a Hill type of exponent also to the protonation of the groups that interact with BHC in "free" COβ-SH chains. This would entail a cooperative protonation of those groups even in the absence of the effector. With the data presently available, this is difficult to justify. It might be that the pH range investigated was too short to show redundancy of the exponent,

q, for the term that in eq 4 refers to free COβ-SH chains. However, cooperative protonation cannot be discarded a priori. At the relatively low ionic strength used in our experiments it is possible that the cooperative protonation is achieved through changes in the hydration of the pocket where the effector is to be bound.

Binding of the Effectors to Liganded β Chains. The Hill plots for the binding of BHC and IHP to COβ-SH chains are shown in Figure 2; both straight lines had a slope near 0.73. This value below unit can be explained as follows: (1) the β subunits exist in more than one conformations which possess slightly different affinities for the effector; (2) the binding is anticooperative because of the conformational change of the molecule upon binding of the first mole of the effector; and (3) the binding of the first effector, which increases the negative charges on the protein molecule, decreases the electrostatic interaction of the β subunits for the second molecule of the effector.

Difference Absorption Spectra. The difference spectra obtained indicated significant perturbation of the absorption spectra of COβ-SH chains in the Soret region produced by the binding of both BHC and IHP. The unequivocal assignment of the "fine" structures of the difference spectra (i.e., trough, peak, etc. of Figure 4) to changes in the spacial geometry of specific protein groups around heme is difficult. However, these results do indicate changes in the protein environment around the heme pocket. It seems unlikely that the effectors perturbed the heme spectra in COβ-SH chains merely by increasing the ionic strength of the solvent since an increase in NaCl concentration from 0.05 to 0.2 M did not influence the Soret spectra to any significant extent.

CD and ORD Measurements. The decrease in ellipticity at 420 nm and the shift in the circular dichroism spectra of COβ-SH chains in the presence of BHC and IHP provided additional evidence for the conformational change produced by the binding of the two effectors. It should be noted that heme itself is symmetric and optically inactive and acquires optical activity upon interaction with macromolecules (Stryer, 1961). Furthermore, CD (Geraci and Li, 1969; Sugita et al., 1971) and ORD (Li and Johnson, 1969) measurements showed that the optical activity of human hemoglobin in the Soret region was sensitive to protein conformation. In view of these considerations, the observed changes of the CD spectra induced by the binding of BHC and IHP would suggest alterations in the reciprocal orientation of the protein groups surrounding the heme and the heme. Results on ORD showing a marked shift in the extrinsic Cotton effects on Soret region and a decrease in levorotation, below 422 nm of COβ-SH chains upon its interaction with BHC and IHP also support this contention.

Significance of the Conformational Change. From the similarities of the difference absorption spectra and of the changes in CD and ORD of COβ-SH chains caused by the effectors, it appears that the effector induced conformational changes are qualitatively similar for both the anions. However, the extent of change of the optical properties produced by BHC is slightly but persistently higher than those found for IHP. It might be that the conformational change was similar in the two cases and that BHC was influencing the conformational equilibrium more than IHP.

A question may be posed whether the observed conformational change produced by the binding of the effectors to the liganded chains is a part of the R–T transition characteristic of the hemoglobin system. It should be noted that the three groups in deoxyhemoglobin whose pK shifted from near 7.2 near 8.5 upon interaction with benzenepentacarboxylate and BHC (Bucci, 1974; Ellis and Bucci, 1975) are not present in the interaction of BHC with liganded β chains. This suggests that the nature of BHC binding to COβ-SH chains is different from that found for BHC–deoxyhemoglobin interaction. This by implication would mean that COβ-SH chains probably do not exist in T conformation neither before nor after the interaction with BHC or IHP. This conclusion is at variance with the suggestion of Perutz and Mazzarella (1963) that a deoxy conformation prevails in hemoglobin H.

The stoichiometry of the binding of BHC to COβ-SH chains is 2 per tetramer. It is conceivable that the binding sites in COβ-SH chains may be situated between the two adjacent β subunits. Comparing the acid–base characteristics of the binding of BHC to the liganded β chains and deoxyhemoglobin, the following explanation may be formulated regarding the nature of binding sites on the two proteins: (1) the binding sites on the two proteins are different; (2) the binding sites are identical but different protein conformations produce different pK's of the groups involved in the binding; (3) the binding sites are topologically the same on deoxyhemoglobin and COβ-SH chains but the different conformations of the proteins make different groups available for the interaction. The available data do not permit the choice of one view against the other two.

Bonaventura et al. (1975) found that the addition of IHP decreased the oxygen affinity of isolated β chains. The change was small and would be consistent with a small free energy of conformational change of the system. This is consistent with what we find for the interaction of liganded β chains with effectors. The high concentration of the effector (30 mM) used by Bonaventura et al. (1975) would suggest that the system was always saturated with the effector in their equilibrium studies.

In our experiments the absorption of protons produced by the interaction of COβ-SH chains with IHP appeared to have characteristics similar to those of the interaction with BHC. We did not pursue a detailed investigation of the phenomenon because its interpretation in terms of ionizable groups was complicated by the ionization of the phosphoric residues in the pH range amenable to the investigation.

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Anomalous Azide Binding to Metmanganomyoglobin†

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ABSTRACT: The reaction of metmanganomyoglobin ($Mn^{III}Mb$) with azide presents a novel pattern with direct evidence for kinetic complexity. Although the kinetic analysis may not be unique, it appears that the azide complex cannot be fully formed, as judged by spectrophotometric changes, even with an infinitely great $[N_3^-]$. This is interpreted as resulting from an equilibrium between the final spectroscopically observable azide complex and an intermediate species whose spectrum is not substantially different from that of $Mn^{III}Mb$ itself. The two forms of the azide complex appear to exhibit roughly equal proportions at 3 °C. We propose that this intermediate is a weak Mn^{3+}–azide complex in which the metal ion remains out-of-plane toward the imidazole of the proximal histidine, but that the metal lies toward the anion in the "final" complex.

Although it is usually possible to represent heme-protein, ligand reactions by one or more simple second-order forward (on) reactions and first-order reverse (off) reactions, this does not mean that the reactions themselves are simple, but more probably, that single steps are rate determining. There are, indeed, indications that this simple approach may not always be sufficient. For example, Gibson and Roughton (1955) found a discrepancy between the rate of oxygen dissociation from oxymyoglobin as measured by a CO replacement procedure, and as measured by mixing with dithionite. More recently Gibson and Kamen (1966) found multiple deviations from simple expectations in examining the reaction of CO with cytochrome *c*'s from *Rhodospirillum* and *Chromatium,* and their observations were confirmed and extended by Cusanovich and Gibson (1973). Their results could be explained by supposing that the cytochrome *c*'s are able to bind CO in two ways, only one of which is associated with the characteristic spectrophotometric change to the CO cytochrome. We report here an analogous situation in the binding of azide by the Mn^{3+} form of the manganese-substituted globins, metmanganoglobin ($Mn^{III}Hb$) and metmanganomyoglobin ($Mn^{III}Mb$).[1]

The parallels between the functional (Thiele et al., 1964; Yonetani and Asakura, 1969; Bull et al., 1974; Gibson et al., 1974; Hoffman et al., 1975) and structural (Moffat et al., 1974) properties of hemoglobin and myoglobin and the manganese-substituted proteins are particularly close. However, the ligation properties of Mn^{3+} and Fe^{3+} porphyrins are drastically affected by the difference of a single electron in the d-orbital populations. Thus, for example, cyanide, nitrite, and azide bind to met-Hb and met-Mb producing the low-spin, six-coordinate hemochrome (see Antonini and Brunori, 1971), whereas only azide binds to $Mn^{III}Mb$ and $Mn^{III}Hb$ (Thiele et al., 1964) and the resultant six-coordinate metal complex remains high spin ($S = 2$) (Boucher, 1972). Furthermore, water as the sixth ligand to met-Hb undergoes an ionization to OH^- with $pK \sim 8$, whereas $Mn^{III}Hb$ shows no such ionization below

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[1] Abbreviations used: $Mn^{III}Hb$; metmanganoglobin, the Mn^{3+} substituted hemoglobin; $Mn^{III}Mb$; metmanganomyoglobin, the Mn^{3+} substituted myoglobin; Tris, tris(hydroxymethyl)aminomethane; TPP, tetraphenylporphinato; IHP, inositol hexaphosphate.