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Heterotropic Interactions in Monomeric β^{SH} Chains from Human Hemoglobin

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The O_2 affinity of β^{SH} chains is lowered by H^+, inositol hexaphosphate (IHP), and CO_2. As the oxygen affinity of β^{SH} monomers (β_1^{SH}) is lower than that of β^{SH} tetramers (β_4^{SH}), it is possible that IHP and CO_2 exert their influence on the O_2 affinity of β^{SH} chains by increasing the dissociation constant of β_4^{SH} rather than by a direct effect on the molecule. In order to test for this hypothesis we have measured the O_2 affinity of β^{SH} chains as a function of protein concentration at various concentrations of IHP and inorganic phosphates in the absence and presence of CO_2. From these data association constants for the binding of IHP to β_1^{SH} and β_4^{SH} as well as for the equilibrium $4\beta_1^{SH} \rightleftharpoons \beta_4^{SH}$ were calculated. We found that IHP and CO_2 influence the oxygen affinity of β_1^{SH}. It was furthermore established that inorganic phosphate enhances the stability of β_4^{SH} while IHP favors its dissociation in monomers.

Recently it has become evident that heterotropic interactions between oxygen and protons, inositol hexaphosphate (IHP),[1] and CO_2 are not restricted to the normal hemoglobin tetramer, $\alpha_2\beta_2$, but also exist in homotetramers consisting of β^{SH} chains, β_4^{SH} (1-3). Furthermore, it has been shown that the oxygen affinity of β^{SH} monomers (β_1^{SH}) and β_4^{SH} is different (4, 5) and that it is pH dependent both in β_4^{SH} and β_1^{SH} (5). Moreover, it was established that the dissociation of β_4^{SH} in 4 β_1^{SH} is dependent upon pH (5). In view of these results we were interested to see if IHP and CO_2 also influence the oxygen affinity of β_1^{SH} and furthermore wanted to establish if the monomer-tetramer association constant of β^{SH} chains is affected by the polyanion IHP and by inorganic phosphates (P_i).

MATERIALS AND METHODS

α^{SH} and β^{SH} chains from human hemoglobin were prepared as described previously (2, 3). Sperm whale myoglobin was obtained from commercial sources (Serva, Heidelberg) and the heme iron converted from the trivalent to the divalent form as previously described (6). The proportion of oxidized heme groups was 3% in this material. The chains were stored in liquid nitrogen until use. Oxygen binding curves were measured at 20°C with heme concentrations ranging between 2 μM and 2 mM. Experiments at low heme concentration (<0.5 mM) were performed according to Benesch *et al.* (7) using tonometers with a 10- or 2-mm cuvette fused to it. For the experiments in the presence of CO_2 a tonometer with a rubber sealed sidearm was used through which the desired volume of CO_2 was injected (8). Oxygen binding curves at heme concentration > 0.5 mM were measured with a diffusion chamber (Eschweiler, Kiel) as described by Jelkmann and Bauer (9). Buffers were 0.05 M *N,N'*-methylenebisacrylamide-Tris in absence and $NaHCO_3$ in presence of CO_2. The pH was kept constant at 7.3 within 0.02 pH unit in all experiments. Total $[A^-]$ = 0.15 M and temperature 20°C. All buffers contained 0.1 mM EDTA. In the experiments at low-protein concentrations the oxygen binding curves were obtained from experiments in which deoxygenation was followed by only one single addition of air in order to avoid excessive heme oxidation during the experiment (7). Heme oxidation was found to be enhanced at low heme concentrations. The maximal percentage at the lowest heme concentration studied was 8% at the end of the experiment. For the following analysis it is assumed that the measured P_{50} is equivalent to the median ligand affinity. This assumption is corroborated by the fact that the n value was not significantly different from unity in our oxygen binding curves.

Mathematical procedure. In order to evaluate the concentration dependence of log P_{50} the following model was used:

[1] Abbreviation used: IHP, inositol hexaphosphate.

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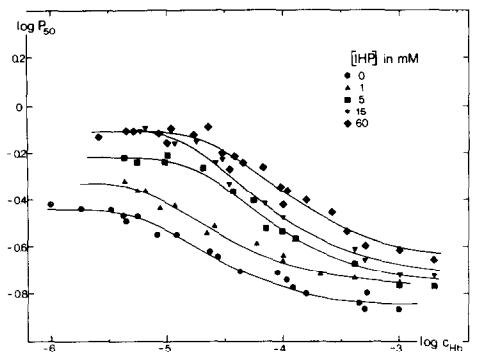

FIG. 1. Plot of log P_{50} (the partial pressure of O_2 at half saturation of the pigment) against log c, the concentration of β^{SH} chains expressed in heme equivalents at various concentrations of IHP. Temperature was 20°C, pH 7.3, and $Cl^- = 0.15$ M. The lines connecting experimental points were calculated as described in the text.

$$\beta_4^{SH} \rightleftarrows 4\beta_1^{SH}$$

$$\beta_1^{SH} + O_2 \rightleftarrows \beta_1^{SH}(O_2)$$

$$\beta_4^{SH} + iO_2 \rightleftarrows \beta_4^{SH}(O_2)_i \quad i = 1, 2, 3, 4$$

Assuming that oxygen binds noncooperatively to β_4^{SH} the fractional saturation, Y, at a given partial oxygen pressure, p, is given by

$$Y = \frac{[\beta]\,K_M p + 4[\beta]^4 K_{1,4}^d \cdot K_T \cdot p(1 + K_T \cdot p)^3}{[\beta](1 + K_M p) + 4[\beta]^4 K_{1,4}^d (1 + K_T \cdot p)^4}$$

with K_M and K_T the microscopic association constants for oxygen binding to β_1^{SH} and β_4^{SH}, respectively; $K_{1,4}^d$ the association constant for deoxygenated β_1^{SH}; and $[\beta]$ the concentration of unliganded β_1^{SH} chains, i.e., β^{SH} monomers. $[\beta]$ is given as the root of the following equation:

$$c_{tot} = [\beta](1 + K_M \cdot p) + 4[\beta]^4 K_{1,4}^d (1 + K_T \cdot p)^4,$$

where c_{tot} is the total heme concentration. K_M, K_T, and $K_{1,4}^d$ were obtained from the relationship between the experimental log P_{50} and the protein concentration using an iterative least-squares procedure. Values for P_{50} of β_1^{SH} and β_4^{SH} were calculated from K_T and K_M, respectively. $K_{1,4}^{oxy}$ was computed from $K_{1,4}^d$, K_M, and K_T.

IHP binding constants for β_4^{SH} and β_1^{SH} were calculated from plots of log P_{50} against the IHP concentration for β_1^{SH} and β_4^{SH} according to the equations derived by Baldwin (10) and Szabo and Karplus (11), assuming four oxygen-linked IHP binding sites for β_4^{SH} and one for β_1^{SH}.

RESULTS

In Fig. 1 is shown a plot of log P_{50} against the β^{SH} concentration for a number of IHP concentrations. It can be seen that the oxygen affinity decreases with decreasing protein concentration at all IHP concentrations investigated. Experiments with α^{SH} chains showed that at pH 7.35 the oxygen affinity of α^{SH} is independent of protein concentration in a range between 10 and 120 μM heme and also independent of the presence of 30 mM IHP, log P_{50} being -0.42 ± 0.01 at 20°C. Likewise, P_{50} of sperm whale myoglobin was independent of both protein concentration and IHP concentration (log P_{50}: -0.35 ± 0.02).

A plot of log P_{50} against IHP concentration for β_1^{SH} and β_4^{SH} is shown in Fig. 2. Here it becomes evident that the oxygen affinity of both β_1^{SH} and β_4^{SH} depends upon the IHP concentration. Figure 3 shows a plot of log P_{50} against the concentration of β^{SH} chains in presence of CO_2 (pCO_2 = 100 Torr). It can be seen that over the whole range of protein concentrations investigated the oxygen affinity in the presence of CO_2 is lower than in the absence of CO_2. In the presence of 15 mM IHP, the addition of CO_2 does not result in a further decrease in oxygen affinity showing that the influence of IHP and CO_2 on the oxygen affinity of β^{SH} chains is not additional. At 10^{-4} M heme for example, the presence of CO_2 and IHP decreases P_{50} by a factor of 1.7 while the sum of the effects should decrease the ligand affinity by a factor of 3.3.

Table I shows log P_{50} values at various heme concentrations in the absence and presence of inorganic phosphate. It can be

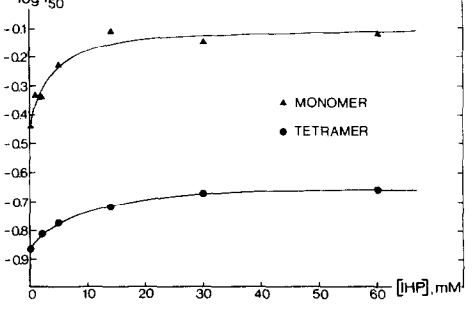

FIG. 2. Plot of log P_{50} against the concentration of IHP for β_1^{SH} (upper curve) and β_4^{SH} (lower curve). The lines were calculated as described in the text.

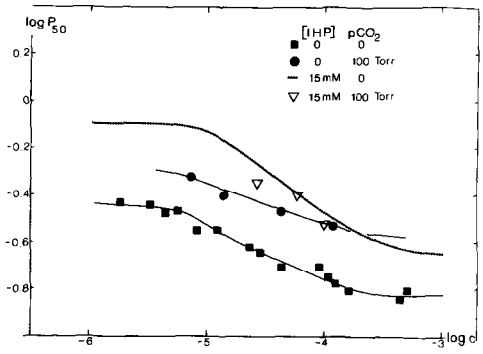

FIG. 3. Plot of log P_{50} against the concentration of β^{SH} chains in the presence and absence of either CO_2 or IHP or both. The line connecting the experimental points at pCO_2 = 100 Torr and in the absence of IHP was drawn by inspection. The dotted curve was taken from Fig. 1. The line connecting points obtained in the absence of CO_2 and IHP was calculated as described in the text.

seen that at a heme concentration higher than 40 μM the oxygen affinity of β^{SH} chains becomes essentially independent of protein concentration in the presence of 0.15 M P_i. From Table II it can be seen that the apparent association constant of the equilibrium $4\beta_1^{SH} \rightleftharpoons \beta_4^{SH}$ in the presence of IHP decreases with increasing IHP concentration approaching a constant value at high IHP concentrations.

DISCUSSION

The results obtained in this study clearly show that IHP and CO_2 reduce the oxygen affinity of β_1^{SH}. This, in turn, implies that oxygenation of the β_1^{SH} causes changes in tertiary structure of the molecule that are large enough to have a distinct influence on the binding sites of IHP and CO_2. The

TABLE I

DEPENDENCY OF LOG P_{50} ON [P_i] AND [Cl^-] AT VARIOUS CONCENTRATIONS OF β^{SH} CHAINS

	Log P_{50}		
		0.05 M Cl^-	
Log c	0.15 M Cl^-	0.1 M P_i	0.15 M P_i
−3.5	−0.82	−0.80	−0.75
−4.0	−0.75	−0.80	−0.75
−4.4	−0.68	−0.80	−0.75
−5.0	−0.55	−0.73	−0.73
−5.1	−0.53	−0.71	−0.72

effect of IHP on P_{50} is significantly stronger in β_1^{SH} than in β_4^{SH}. This result is of particular interest as both in isolated α chains and in sperm whale myoglobin, the P_{50} was completely independent of protein concentration (10–120 μM heme) and also of IHP.

From Table II it is seen that $K_{1,4}$ reaches a constant value at high IHP concentrations. Since $-\delta\log K_{1,4}/\delta\log$ [IHP] represents the number of IHP molecules released upon tetramer formation, this result means that at saturating IHP concentrations the $\beta_1^{SH} \rightarrow \beta_4^{SH}$ reaction is not associated with a change in the number of moles IHP bound. So if one assumes one binding site for IHP in β_1^{SH}, it follows that four IHP molecules are bound per β_4^{SH}.

Using the data given in Fig. 2 we calculated association constants for IHP to deoxygenated (K_D) and oxygenated (K_O) β_1^{SH} and β_4^{SH}, assuming one oxygen-linked binding site for IHP per β_1^{SH} and four equivalent oxygen-linked binding sites for β_4^{SH}. The following figures were obtained:

$$K_D = 476\ (M^{-1}), \quad K_O = 211\ (M^{-1}), \quad \text{for } \beta_1^{SH}$$

$$K_D = 140\ (M^{-1}), \quad K_O = 80\ (M^{-1}), \quad \text{for } \beta_4^{SH},$$

where K_D and K_O for β_4^{SH} represent the microscopic binding constants.

The value of K_O for β_4^{SH} is by two orders of magnitude smaller than the figure reported by Salahuddin and Bucci (12), who found only two binding sites for IHP per β_4^{SH} tetramer. The reason for this discrepancy is not clear. It is possible that the higher binding constant calculated by Salahuddin and Bucci is due to the lower salt concentration (0.05 M Cl^-) used by these authors in their experiments in comparison with the present experimental conditions. Experiments in which the absorption of protons by β_4^{SH} upon IHP binding was measured at pH 7.35 and 0.15 M Cl^- are in support of the low association

TABLE II

CALCULATED VALUES OF K_{app},[a] THE APPARENT MONOMER-TETRAMER ASSOCIATION CONSTANT FOR DEOXYGENATED AND OXYGENATED β^{SH} CHAINS AT VARIOUS CONCENTRATIONS OF *IHP*

[IHP] (mM)	K_{app}^{deoxy} $(1/M)^3$	K_{app}^{oxy} $(1/M)^3$
0	$1.6 \pm 0.3 \times 10^{12}$	$7.4 \pm 1.2 \times 10^{14}$
1	$1.2 \pm 0.4 \times 10^{13}$	$8.3 \pm 2.8 \times 10^{14}$
14	$4.1 \pm 0.9 \times 10^{11}$	$6.6 \pm 1.5 \times 10^{13}$
30	$1.1 \pm 0.3 \times 10^{11}$	$1.5 \pm 0.5 \times 10^{13}$
60	$1.1 \pm 0.1 \times 10^{11}$	$1.5 \pm 0.2 \times 10^{13}$

[a] K_{app} ($K_{app} = \sum_{i=0}^{4} [\beta_4(IHP)_i]/\{[\beta_1] + [\beta_1(IHP)]\}^4$) for oxygenated β^{SH} chains were calculated as follows: $\log K_{app}^{oxy} = \log K_{app}^{deoxy} + 4\,(\log P_{50}^{monomer} - \log P_{50}^{tetramer})$.

constants reported in this paper (unpublished observation). The relatively weak effect of IHP on the monomer-tetramer association constant of β^{SH} chains as shown in Table II is in agreement with the results of Valdes and Ackers (4) who reported that at pH 7.4 IHP, up to a concentration of 1 mM, did not significantly change the monomer-tetramer association constant of deoxy β^{SH} chains.

From the fact that the influence of IHP and CO_2 on the oxygen affinity of β^{SH} chains is not additional (Fig. 3) it can be concluded that IHP and CO_2 compete for a common binding site. Previous results have shown that the effect of CO_2 on the oxygen affinity of β_4^{SH} requires free N-terminal α-amino groups (3). It is likely therefore that in β_4^{SH} the binding site for IHP involves the N-terminal α-amino groups which might also be true for β_1^{SH}.

Last, we wish to discuss the influence of IHP and P_i on the stability of β_4^{SH}. From the fact that IHP binds about three times stronger to β_1^{SH} than to β_4^{SH}, it can be concluded that the presence of IHP favors the dissociation of β_4^{SH} into 4 β_1^{SH}. Thus, IHP destabilizes the β_4^{SH} homotetramer. From the data given in Table I it can be seen that the oxygen affinity of β^{SH} chains in the presence of 0.05 M Cl^- and 0.1 M P_i is equal to that of the undissociated β_4^{SH} homotetramer down to a heme concentration of 4×10^{-5} M. At lower protein concentrations the oxygen affinity of the β^{SH} chains decreases. So it appears that the substitution of 0.1 M Cl^- by P_i causes a strong stabilization of β_4^{SH}. One explanation for this stabilizing action of P_i could be that P_i has a very small affinity for β_1^{SH} and is therefore bound essentially to β_4^{SH}. In this respect it would differ from IHP which was shown to interact effectively with the monomeric β_1^{SH} chains in an oxygen-linked fashion.

It is known that allosteric cofactors which reduce the oxygen affinity of human adult hemoglobin ($\alpha_2\beta_2$) can alter the tertiary structure of the protein (13). The experiments presented in this paper unequivocally demonstrate the occurrence of heterotropic interactions in isolated monomers of the β chains. This result is the more remarkable as neither isolated α chains nor sperm whale myoglobin exhibited such heterotropic interactions. It follows from these results that the tertiary structure of β^{SH} monomers changes upon oxygen binding to such an extent that the affinity for IHP and CO_2 significantly decreases. Therefore, the β chains retain some of their basic functional properties which they have in the intact heterotretramer ($\alpha_2\beta_2$) even in the monomeric form.

REFERENCES

1. BONAVENTURA, J., BONAVENTURA, C., AMICONI, G., TENTORI, L., BRUNORI, M., AND ANTONINI, E. (1975) *J. Biol. Chem.* **250,** 6278-6281.
2. ROLLEMA, H. S., DE BRUIN, S. H., AND VAN OS, G. A. J. (1976) *FEBS Lett.* **61,** 148-150.
3. BAUER, C., AND KURTZ, A. (1977) *J. Biol. Chem.* **252,** 2952-2955.
4. VALDES, R., AND ACKERS, G. K. (1978) *Proc. Nat. Acad. Sci. USA* **75,** 311-314.
5. KURTZ, A., AND BAUER, C. (1978) *Biochem. Biophys. Res. Commun.* **84,** 852-857.
6. BAUER, C., AND PACYNA, B. (1975) *Anal. Biochem.* **65,** 445-448.
7. BENESCH, R., MACDUFF, G., AND BENESCH, R. E. (1965) *Anal. Biochem.* **11,** 81-87.
8. BAUER, C., BAUMANN, R., ENGELS, U., AND PACYNA, B. (1975) *J. Biol. Chem.* **250,** 2173-2176.
9. JELKMANN, W., AND BAUER, C. (1978) *Pflügers Arch.* **377,** 75-80.
10. BALDWIN, J. (1975) *Progr. Biophys. Mol. Biol.* **29,** 225-320.
11. SZABO, A., AND KARPLUS, M. (1976) *Biochemistry* **15,** 2869-2877.
12. SALAHUDDIN, A., AND BUCCI, E. (1976) *Biochemistry* **15,** 3399-3405.
13. PERUTZ, M. F. (1976) *Brit. Med. Bull.* **32,** 195-208.