**Heterotropic Interactions in Monomeric $\beta^{SH}$ Chains from Human Hemoglobin**

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The $O_2$ affinity of $\beta^{SH}$ chains is lowered by $H^+$, inositol hexaphosphate (IHP), and $CO_2$. As the oxygen affinity of $\beta^{SH}$ monomers ($\beta_1^{SH}$) is lower than that of $\beta^{SH}$ tetramers ($\beta_4^{SH}$), it is possible that IHP and $CO_2$ exert their influence on the $O_2$ affinity of $\beta^{SH}$ chains by increasing the dissociation constant of $\beta_4^{SH}$ rather than by a direct effect on the molecule. In order to test for this hypothesis we have measured the $O_2$ affinity of $\beta^{SH}$ 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 $\beta_1^{SH}$ and $\beta_4^{SH}$ as well as for the equilibrium $4\beta_1^{SH} = \beta_4^{SH}$ were calculated. We found that IHP and $CO_2$ influence the oxygen affinity of $\beta_2^{SH}$. It was furthermore established that inorganic phosphate enhances the stability of $\beta_4^{SH}$ while IHP favors its dissociation in monomers.

Recently it has become evident that heterotropic interactions between oxygen and protons, inositol hexaphosphate (IHP), and $CO_2$ are not restricted to the normal hemoglobin tetramer, $\alpha_2\beta_2$, but also exist in homotetramers consisting of $\beta^{SH}$ chains, $\beta_4^{SH}$ (1-3). Furthermore, it has been shown that the oxygen affinity of $\beta^{SH}$ monomers ($\beta_2^{SH}$) and $\beta_4^{SH}$ is different (4, 5) and that it is pH dependent both in $\beta_4^{SH}$ and $\beta_1^{SH}$ (5). Moreover, it was established that the dissociation of $\beta_4^{SH}$ in $4\beta_1^{SH}$ 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 $\beta_1^{SH}$ and furthermore wanted to establish if the monomer-tetramer association constant of $\beta^{SH}$ chains is affected by the polyanion IHP and by inorganic phosphates ($P_1$).

**MATERIALS AND METHODS**

$\alpha^{SH}$ and $\beta^{SH}$ 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 $\mu$M 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 (Eshchweiler, 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:
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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 $\beta^4_{SH}$ 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.

$$\beta^4_{SH} = 4\beta^1_{SH}$$

$$\beta^1_{SH} + O_2 \rightleftharpoons \beta^1_{SH}(O_2)$$

$$\beta^4_{SH} + iO_2 \rightleftharpoons \beta^4_{SH}(O_2), \ i = 1, 2, 3, 4$$

Assuming that oxygen binds noncooperatively to $\beta^4_{SH}$ the fractional saturation, $Y$, at a given partial oxygen pressure, $p$, is given by

$$Y = \frac{[\beta]K_M(p + 4[\beta]K^{14}_{1,4}K_Tp(1 + K_Tp)^3)}{[\beta](1 + K_Mp) + 4[\beta]K^{14}_{1,4}(1 + K_Tp)^3}$$

with $K_M$ and $K_T$ the microscopic association constants for oxygen binding to $\beta^1_{SH}$ and $\beta^4_{SH}$, respectively; $K^{14}_{1,4}$ the association constant for deoxygenated $\beta^1_{SH}$; and $[\beta]$ the concentration of unliganded $\beta^4_{SH}$ chains, i.e., $\beta^4_{SH}$ monomers. $[\beta]$ is given as the root of the following equation:

$$c_{tot} = [\beta](1 + K_Mp) + 4[\beta]K^{14}_{1,4}(1 + K_Tp)^4,$$

where $c_{tot}$ is the total heme concentration. $K_M$, $K_T$, and $K^{14}_{1,4}$, 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 $\beta^1_{SH}$ and $\beta^4_{SH}$ were calculated from $K_T$ and $K_M$, respectively. $K^{14}_{1,4}$ was computed from $K^{14}_{1,4}$, $K_M$, and $K_T$.

IHP binding constants for $\beta^4_{SH}$ and $\beta^4_{SH}$ were calculated from plots of log $P_{50}$ against the IHP concentration for $\beta^1_{SH}$ and $\beta^4_{SH}$ according to the equations derived by Baldwin (10) and Szabo and Karplus (11), assuming four oxygen-linked IHP binding sites for $\beta^4_{SH}$ and one for $\beta^1_{SH}$.

RESULTS

In Fig. 1 is shown a plot of log $P_{50}$ against the $\beta^4_{SH}$ 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 $\alpha^4_{SH}$ chains showed that at pH 7.35 the oxygen affinity of $\alpha^4_{SH}$ is independent of protein concentration in a range between 10 and 120 $\mu$M heme and also independent of the presence of 30 mM IHP, log $P_{50}$ being $-0.42 \pm 0.01$ at 20°C. Likewise, $P_{50}$ of sperm whale myoglobin was independent of both protein concentration and IHP concentration (log $P_{50}$: $-0.35 \pm 0.02$).

A plot of log $P_{50}$ against IHP concentration for $\beta^1_{SH}$ and $\beta^4_{SH}$ is shown in Fig. 2. Here it becomes evident that the oxygen affinity of both $\beta^1_{SH}$ and $\beta^4_{SH}$ depends upon the IHP concentration. Figure 3 shows a plot of log $P_{50}$ against the concentration of $\beta^4_{SH}$ chains in presence of CO$_2$ ($p$CO$_2$ = 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 $\beta^4_{SH}$ 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 $\beta^4_{SH}$ (upper curve) and $\beta^4_{SH}$ (lower curve). The lines were calculated as described in the text.
seen that at a heme concentration higher than 40 μM the oxygen affinity of βSH chains becomes essentially independent of protein concentration in the presence of 0.15 M Pi. From Table II it can be seen that the apparent association constant of the equilibrium $4\beta_{1}^{\text{SH}} \rightleftharpoons \beta_{4}^{\text{SH}}$ 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 CO₂ reduce the oxygen affinity of β₁SH. This, in turn, implies that oxygenation of the β₁SH causes changes in tertiary structure of the molecule that are large enough to have a distinct influence on the binding sites of IHP and CO₂.

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

$$K_D = 140 \text{ (M}^{-1}\text{)}, \quad K_O = 80 \text{ (M}^{-1}\text{)}, \quad \text{for } \beta_{4}^{\text{SH}},$$

where $K_D$ and $K_O$ for β₄SH represent the microscopic binding constants.

The value of $K_O$ for β₄SH 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 β₁SH tetramer. The reason for this discrepancy is not clear. It is possible that the higher binding constant calculated by effect of IHP on $P_{50}$ is significantly stronger in β₁SH than in β₄SH. 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 $-\delta \log K_{1,4}/\delta \log [\text{IHP}]$ represents the number of IHP molecules released upon tetramer formation, this result means that at saturating IHP concentrations the $\beta_{1}^{\text{SH}} \rightarrow \beta_{4}^{\text{SH}}$ reaction is not associated with a change in the number of moles IHP bound. So if one assumes one binding site for IHP in β₁SH, it follows that four IHP molecules are bound per β₄SH.

Using the data given in Fig. 2 we calculated association constants for IHP to deoxygenated ($K_D$) and oxygenated ($K_O$) β₁SH and β₄SH, assuming one oxygen-linked binding site for IHP per β₁SH and four equivalent oxygen-linked binding sites for β₄SH. The following figures were obtained:

<table>
<thead>
<tr>
<th>Log c</th>
<th>0.15 M Cl⁻</th>
<th>0.1 M $P_i$</th>
<th>0.15 M $P_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>-3.5</td>
<td>-0.82</td>
<td>-0.80</td>
<td>-0.75</td>
</tr>
<tr>
<td>-4.0</td>
<td>-0.75</td>
<td>-0.80</td>
<td>-0.75</td>
</tr>
<tr>
<td>-4.4</td>
<td>-0.68</td>
<td>-0.80</td>
<td>-0.75</td>
</tr>
<tr>
<td>-5.0</td>
<td>-0.55</td>
<td>-0.73</td>
<td>-0.73</td>
</tr>
<tr>
<td>-5.1</td>
<td>-0.53</td>
<td>-0.71</td>
<td>-0.72</td>
</tr>
</tbody>
</table>

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 β₁SH upon IHP binding was measured at pH 7.35 and 0.15 M Cl⁻ are in support of the low association
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TABLE II
CALCULATED VALUES OF $K_{\text{app}}$* THE APPARENT MONOMER-TETRAMER ASSOCIATION CONSTANT FOR DEOXYGENATED AND OXYGENATED $\beta$SH CHAINS AT VARIOUS CONCENTRATIONS OF IHP

<table>
<thead>
<tr>
<th>[IHP] (mM)</th>
<th>$K_{\text{app}}^\text{oxy}$ (1/M)$^3$</th>
<th>$K_{\text{app}}^\text{deg}$ (1/M)$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.6 ± 0.3 x 10$^{-10}$</td>
<td>7.4 ± 1.2 x 10$^{-14}$</td>
</tr>
<tr>
<td>1</td>
<td>1.2 ± 0.4 x 10$^{-13}$</td>
<td>8.3 ± 2.8 x 10$^{-14}$</td>
</tr>
<tr>
<td>14</td>
<td>4.1 ± 0.9 x 10$^{-11}$</td>
<td>6.6 ± 1.5 x 10$^{-13}$</td>
</tr>
<tr>
<td>30</td>
<td>1.1 ± 0.3 x 10$^{-11}$</td>
<td>1.5 ± 0.5 x 10$^{-13}$</td>
</tr>
<tr>
<td>60</td>
<td>1.1 ± 0.1 x 10$^{-11}$</td>
<td>1.5 ± 0.2 x 10$^{-13}$</td>
</tr>
</tbody>
</table>

$^*$ $K_{\text{app}} = \sum_{i=0}^{4} [\beta_i(IHP)]/[\beta_1 + [\beta_i(IHP)]]^4$ for oxygenated $\beta$SH chains were calculated as follows: $\log K_{\text{app}}^\text{deg} = \log K_{\text{app}}^\text{oxy} + 4(\log P_{\text{monomer}} - \log P_{\text{tetramer}})$.

constants reported in this paper (unpublished observation). The relatively weak effect of IHP on the monomer–tetramer association constant of $\beta$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 $\beta$SH chains.

From the fact that the influence of IHP and CO$_2$ on the oxygen affinity of $\beta$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 $\beta$SH requires free N-terminal $\alpha$-amino groups (3). It is likely therefore that in $\beta_4$SH the binding site for IHP involves the N-terminal $\alpha$-amino groups which might also be true for $\beta_4$SH.

Last, we wish to discuss the influence of IHP and $P_i$ on the stability of $\beta_4$SH. From the fact that IHP binds about three times stronger to $\beta_4$SH than to $\beta_4$SH, it can be concluded that the presence of IHP favors the dissociation of $\beta_4$SH into $4\beta_1$SH. Thus, IHP destabilizes the $\beta_4$SH homotetramer. From the data given in Table I it can be seen that the oxygen affinity of $\beta$SH chains in the presence of 0.05 M Cl$^-$ and 0.1 M $P_i$ is equal to that of the undissociated $\beta_4$SH homotetramer down to a heme concentration of $4 \times 10^{-5}$ M. At lower protein concentrations the oxygen affinity of the $\beta$SH chains decreases. So it appears that the substitution of 0.1 M Cl$^-$ by $P_i$ causes a strong stabilization of $\beta_4$SH. One explanation for this stabilizing action of $P_i$ could be that $P_i$ has a very small affinity for $\beta_4$SH and is therefore bound essentially to $\beta_4$SH. In this respect it would differ from IHP which was shown to interact effectively with the monomeric $\beta_4$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 $\beta$ chains. This result is the more remarkable as neither isolated $\alpha$ chains nor sperm whale myoglobin exhibited such heterotropic interactions. It follows from these results that the tertiary structure of $\beta$SH monomers changes upon oxygen binding to such an extent that the affinity for IHP and CO$_2$ significantly decreases. Therefore, the $\beta$ chains retain some of their basic functional properties which they have in the intact heterotetramer ($\alpha_2\beta_2$) even in the monomeric form.

REFERENCES