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THE OXYGEN AFFINITY OF HEMOGLOBIN β^{SH} CHAINS IS CONCENTRATION DEPENDENT

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SUMMARY: Oxygen binding to isolated hemoglobin β^{SH} chains exhibits heterotropic interactions with H^+, inositol hexaphosphate and CO_2 which implies different structures of the liganded and unliganded β chains. In order to find out if the dissociation behaviour of β_4^{SH} homotetramers is likewise linked to oxygenation, we have measured the oxygen affinity of the pigment as a function of the protein concentration at different pH values. We found that a decrease in protein concentration is associated with a decrease in oxygen affinity. This result accords with predictions reached from studies on the self-association of liganded and unliganded β chains. Furthermore,it was established that both at high and low protein concentrations the oxygen affinity of the β chains is pH dependent.

Tetramers consisting of four β^{SH} chains (β_4^{SH}) have long been considered as being devoid of any significant structural or functional property which is linked to oxygen binding (1). Whilst homotropic interactions have never been observed in β_4^{SH}, recent experiments have furnished proof that heterotropic interactions exist in this homotetramer with respect to protons (2, 3), inositol hexaphosphate (4) and carbon dioxide (5). In view of these results we were interested to see if another important property of β_4^{SH} namely its dissociation in monomers is likewise linked to oxygen binding. Such an oxygen-linked dependence of the aggregational state of β_4^{SH} should reflect itself in a change of the oxygen affinity with concentration. The oxygen affinity of isolated β chains of human hemoglobin was therefore measured at various concentrations and found to decrease with decreasing protein concentration, indicating that β^{SH} monomers have a lower oxygen affinity than β_4^{SH}. Whilst this study was in progress, a report by Valdes and Ackers appeared (6)

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in which it was shown that unliganded β^{SH} monomers form β_4^{SH} tetramers less readily than oxygenated β^{SH} monomers (6). This particular linkage of ligand binding and stability of β_4^{SH} implies a lower oxygen affinity of β^{SH} monomers compared to the homotetramer. Therefore, direct measurements on the self-association of β^{SH} chains (6) and inferences reached from oxygen binding experiments nicely complement each other.

MATERIALS and METHODS

Human hemoglobin α and β chains were prepared and their SH-groups regenerated as previously described (3). The proportion of oxidised heme groups was less than 5 % in this material. The chains were stored in liquid nitrogen until used in ligand binding experiments. Oxygen dissociation curves were done in tonometers with a 10 mm cuvette fused to it as described (8) using a Shimadzu (type UV-200) double beam spectrophotometer. The concentrations studied ranged from 2 μM heme to 250 μM heme. The experiments were performed at different wavelengths according to the heme concentration: 415 and 430 nm at [heme] $\leq$ 10 μM, 576, 555 and 540 nm at 15 μM < [heme] < 250 μM. Buffers were 0.05 M bis-Tris at pH $\leq$ 7.5 and 0.05 M Tris at pH > 7.5. Total [Cl^-] was 0.15 M and temperature 20 °C. All buffers contained 0.1 mM EDTA. P_{50} values (P_{50}: P_{O2} at which [Hb] = [HbO_2]) were constructed from experiments in which deoxygenation was followed by only one addition of air in order to avoid heme oxidation during stepwise oxygenation (8).

P_{50} values obtained at different heme concentrations were analyzed in the following way to obtain estimates for the association constant K_a ($4\ \beta^{SH} \rightleftharpoons \beta_4^{SH}$) both for the liganded and unliganded β chains: a log P_{50} value for the β^{SH}-monomer at a given pH was read off the curve relating log P_{50} and log [heme]. The association constant for the unliganded β chains, K_a, was then varied by a trial and error procedure and log P_{50} of the β^{SH}-monomer adjusted accordingly until the sum of the squares of differences between calculated and experimental points were at a minimum. In doing so it was assumed that P_{50} of the experiments done at 250 μM heme is equivalent to that of β_4^{SH}. The association constant for the liganded monomers, K_a^{O2} is then obtained from the log P_{50} values of β^{SH}, β_4^{SH} and K_a.

RESULTS and DISCUSSION

In Fig. 1 is shown a plot of log P_{50} against the concentration of isolated β chains at acid, neutral and alkaline pH. It can be seen that the oxygen affinity decreases with decreasing protein concentration at all pH values investigated. At pH 7.35 for example, log P_{50} of β_4^{SH} is -0.81 and log P_{50} of β^{SH} is -0.42 which amounts to a difference in standard free energy of -0.55 kcal/mol O_2 bound. In contrast to the β^{SH} chains, the oxygen affinity of α^{SH} chains

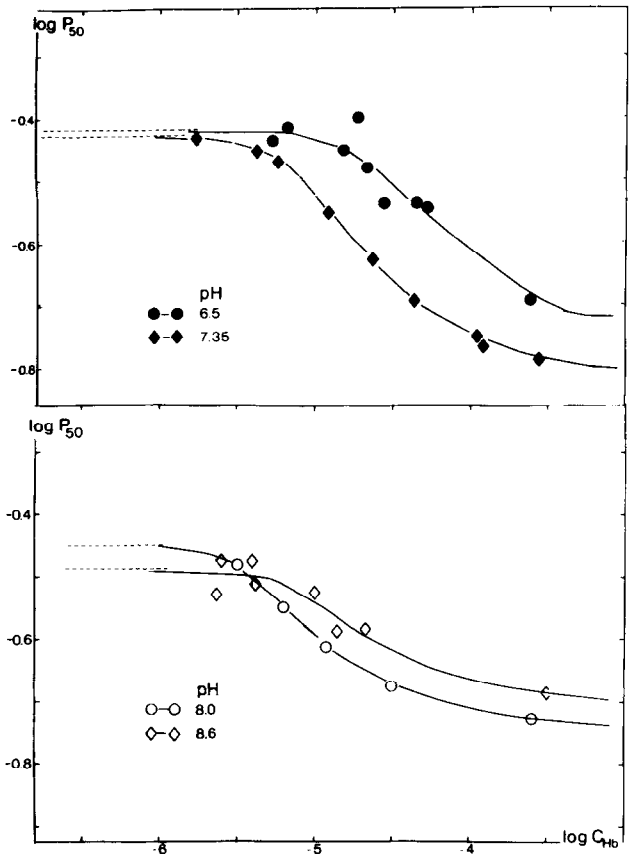
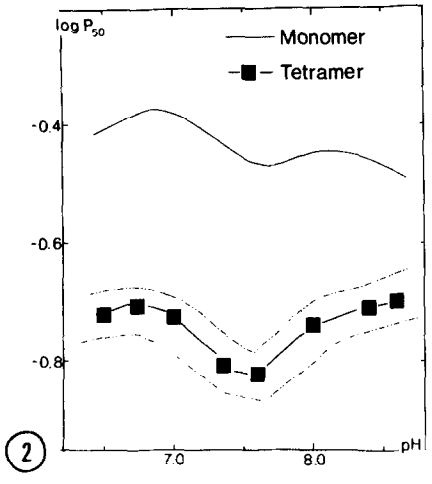


Fig. 1. Plot of log P_{50}, the partial pressure of O_2 at which $[Hb] = [HbO_2]$ against log c, the concentration of β^{SH} chains expressed in heme equivalents at pH 6.5 and 7.35 (upper panel) and pH 8.0 and 8.6 (lower panel). Temperature was 20 °C. The lines connecting experimental points were calculated using estimated values for P_{50} of β^{SH} monomers, the association constant for the unliganded β^{SH} monomers, P_{50} of β_4^{SH} tetramers and the protein concentration. Details are given in the text.

was independent of concentration between 12 and 120 µM heme, log P_{50} being -0.42 ± 0.02 (S.D.) at 20 ° and pH 7.35.

A plot of log P_{50} against pH for β^{SH} and β_4^{SH} is shown in Fig. 2. Here it becomes apparent that the oxygen affinity of β_4^{SH} and of β^{SH} depends upon the pH in the solution. From the titration data of Rollema et al. (3) it can be predicted that, starting at pH 9, P_{50} of β_4^{SH} should first decrease with increasing acidity, then increase, and at around pH 6.0 decrease again. The curve shown in Fig. 2 follows qualitatively the predicted course, except that the minimal and



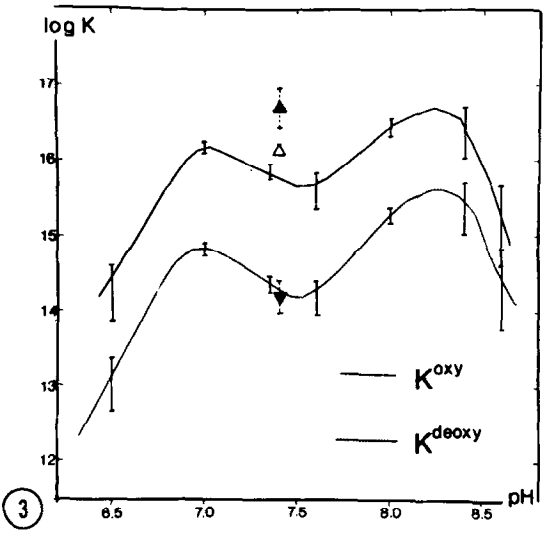


Fig. 2. Plot of log P_{50} against pH for β^{SH} monomers and β^{SH} tetramers. P_{50} values of β^{SH} monomers were estimated as described in the text. The dotted lines around the curves relating experimentally determined P_{50} values of the β^{SH} tetramer (■) indicate the total range of experimental scatter.

Fig. 3. Plot of log K, the association constant of the reaction 4 $\beta^{SH} \rightleftharpoons \beta_4^{SH}$ in the absence (lower line) and presence (upper line) of oxygen as a function of pH. Association constants were calculated as indicated in the text. Vertical bars represent one standard deviation. Symbols refer to direct estimates of K obtained by Valdes and Ackers in the presence (▲ ref. 6; △ ref. 7) and absence (▼ ref. 6) of heme ligands.

maximal values of P_{50} are shifted to the alkaline range. It is possible that the lower temperature and the higher $[Cl^-]$ in the present study accounts for this difference.

Fig. 3 shows the association constants (K_a) that were calculated using the procedure outlined above for the reaction 4 $\beta^{SH} \rightleftharpoons \beta_4^{SH}$ as a function of pH. It became evident that K_a for the deoxygenated chains was lower than K_a for the oxygenated derivative at all pH values investigated. At pH 7.35 for example K_a was estimated to be $2.3 \pm 0.5 \times 10^{14}$ (liter^3/mol^3) and $7.2 \pm 1.6 \times 10^{15}$ (liter^3/mol^3) for deoxygenated and oxygenated β^{SH} chains respectively. Confidence limits represent one standard deviation. We thus infer from oxygen equilibrium studies that the liganded β_4^{SH} tetramer is more stable

than the unliganded one. This conclusion is in accord with recent results obtained by Valdes and Ackers using an equilibrium gel permeation method (6) by which they measured directly K_a for the liganded and unliganded β^{SH} chains. Actually, these authors predicted from their measurement that the β^{SH} monomers must have a lower oxygen affinity than the β^{SH} tetramers which is exactly what we observed. If one compares their estimate for K_a (at pH 7.4, 21.5 ^{o} and 0.1 M NaCl) with our value obtained under similar conditions (pH 7.35, 20 ^{o}, 0.15 M Cl^-) it turns out that K_a for the deoxygenated derivative derived from gel-filtration experiments (K_a = 1.52 $\pm$ 0.75 x 10^{14} liter^3/mol^3) is practically identical to our estimate. Our K_a value for liganded β^{SH} chains is closer to the earlier estimate of Valdes and Ackers (7) than to their more recent number (Fig. 3). Nevertheless, in view of the difficulties involved in obtaining P_{50} values of high-affinity heme proteins at low concentrations, it is reassuring to see that the general conclusion of two independent experimental approaches is in harmony in that the stability of β_4^{SH} is linked to oxygenation.

The pH dependence of the association of β^{SH} to β_4^{SH} deserves some comment. As can be seen from Fig. 3, in the physiological pH range, K_a is at a local minimum around pH 7.5 and rises on both sides of this pH value, running through a maximum at pH 7.0 and 8.2. At the acid and alkaline end of the pH range investigated, K_a falls. The decrease of K_a at the acid end of the pH range investigated could be explained if α-amino groups or imidazol groups are situated opposite to each other at the contacts which hold the β_4^{SH} tetramer together. A rise in acidity would increase the protonation of these groups and lead to electrostatic repulsions. The fall of K_a at alkaline pH values is more difficult to explain on such a basis because there are no amino acid residues which normally titrate in this pH range. Note, however, that the experimental scatter of our data is large at the extreme ends of the pH range investigated so that it is difficulat to analyze quantitatively the change of P_{50} and K_a with pH. Nonetheless, an exact knowledge of the residues forming the contacts in the homotetramer β_4^{SH}, would be helpful not only for the interpretation of the pH dependence of K_a but also for evolutionary considerations since β_4^{SH} is regarded as an ancestor of the normal hemoglobins consisting of two pairs of unlike subunits (9

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