THE INFLUENCE OF 2,3 DIPHOSPHOGLYCERATE ON THE BOHR EFFECT OF SULFHEMOGLOBIN

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Summary. Using a pH titration method we have observed the ligation Bohr effect in sulfhemoglobin and find that it is increased upon the addition of 2,3-diphosphoglycerate. These results indicate that sulfhemoglobin undergoes a quaternary structure change upon ligation.

Introduction. Sulfhemoglobin is a nonfunctional hemoglobin derivative formed by the toxic action of certain nitro- and amino-aromatic drugs (1). It has recently been shown that sulf-Hb[1] can reversibly bind molecular oxygen, but with a substantially reduced affinity compared to Hb and a Hill's constant near unity. Nevertheless, sulf-Hb does exhibit a large alkaline Bohr effect (2). Since the presence of both a Bohr effect and an influence of DPG upon ligand affinity are characteristic of a quaternary structure change upon ligand binding, independent of Hill's constant (3), we have now investigated the influence of DPG on sulf-Hb ligation by observing the change in the Bohr effect by addition of DPG.

Materials and Methods. The Bohr effect of sulf-Hb was studied by measuring the change in proton binding upon CO ligation. Carbon monoxide (Matheson research grade) was passed through aminated silica-gel in dry

(1) Abbreviations: Hb, ferrous hemoglobin A; sulf-Hb, ferrous sulfhemoglobin; DPG, 2,3-diphosphoglycerate.

ice to remove acidic impurities. Titrants (0.01N HCl and NaOH) were thoroughly purged of oxygen by the freeze-pump-thaw method and then standardized with the primary standard potassium hydrogen phthalate (Fisher certified). DPG (Sigma) was converted to the acid form by treatment with Dowex 50W-X8 resin and subsequent neutralization with NaOH. Final concentrations of DPG were determined by the method of Ames and Dubin (4). Sulf-Hb, prepared from stripped (Human) Hb, is relatively unstable in solution and is routinely stored with catalase in phosphate buffer (pH = 8) in dry ice. Sulf-Hb has an absorption maximum at 619.5 nm (ε = 21.5 nM). The percentage of Hb impurity in sulf-Hb can be determined by using electron paramagnetic resonance to calculate the concentrations of the hydroxy forms of Hb and sulf-Hb in an oxidized sample at elevated pH (5). The ratio of optical absorptions, $A_{619.5}/A_{561}$, is however a satisfactory operational measure of sulf-Hb purity (2). In all cases this ratio was greater than 2, indicating at least 80% purity or better.

For a titration, approximately 5 ml of thawed sulf-Hb solution ($\sim$ 50 μM in tetramer) was passed through a G-25 Sephadex column (35 cm x 2.5 cm) which had previously been equilibrated with deoxygenated 0.05 M NaCl solution (4°). The protein was eluted with deoxygenated 0.05 M NaCl solution and collected in an N_2 purged Schlenk tube. A known volume of sulf-Hb solution was then syringed into a jacketed titration cell kept at 10°, pre-purged with N_2. The exact sulf-Hb concentration (usually $\sim$ 25 μM tetramer) was determined by transferring an aliquot to a pre-purged 0.1 cm path length quartz cell sealed with a rubber septum. Our conditions of N_2 purging were such that at the beginning of a titration Hb was in the oxy form, whereas the low-affinity sulf-Hb was deoxygenated. Thus any protons released upon addition of CO could be associated with ligation of sulf-Hb.

Each experiment involved the measurement of a portion of the titration curve of sulf-Hb, the addition of CO and measurement of proton release at a given pH, and then measurement of a portion of the titration curve of

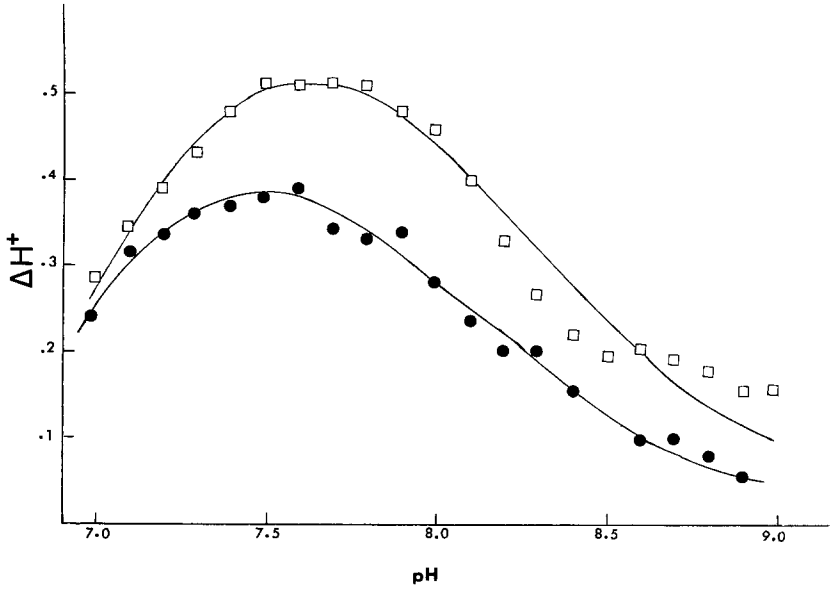

Figure 1. Change in protons bound per sulfheme (ΔH^+) upon CO binding to sulf-Hb (10°) with (△) and without (●) added DPG. For conditions see text. Solid curves are the results of least-squares fitting the data to equation 7.56 of reference 6.

the ligated protein. The two sets of data in fig. 1 are each composite results of four or more individual experiments with slightly differing sulf-Hb concentration.

Results. The Bohr effect in hemoglobin is usually observed as a change of ligand affinity with pH. In the presence of DPG, the magnitude of the Bohr effect is increased. By Wyman's equation, $-d \log p_{1/2}/d\ pH = -\Delta H^+$, the measurement of proton release upon ligand binding per sulfheme (ΔH^+) is equivalent to measurement of the change in ligand affinity with pH (see refs. 5, 6).

Figure 1a shows the Bohr effect for sulf-Hb at 10° in 0.05 M NaCl, as determined by measurements of ΔH^+ upon CO binding. Figure 1b shows that the Bohr effect for sulf-Hb (~ 25 μM tetramer) is increased by the addition of DPG (~ 64 μM). The largest observed increase in ΔH^+ produced by DPG is ~ 0.16 protons at a pH of approximately 7.7. However, if the sulf-Hb binding constant for DPG is the same as that of Hb ($K_{assoc} \sim 10^5\ M^{-1}$ (7))

the conditions of the sulf-Hb titration were such that no more than ~ 80% of the unligated tetramers bound DPG. Thus, utilization of saturating concentrations of DPG in these experiments might be expected to have produced a maximal increase in ΔH^+ of roughly 0.20 protons. A recent study of the DPG effect of Hb observed a maximum value of $\Delta H^+ \sim 0.24$ at pH ~ 7.4 (25°) with saturating amounts of DPG (8).

Because of uncertainties in the present measurement it is not possible to decide whether the DPG enhancements of the Bohr effect in Hb and in sulf-Hb are identical. However, the magnitudes of the increases in ΔH^+ upon DPG addition are clearly similar.

Discussion. This work confirms the previous observation that sulf-Hb exhibits the alkaline Bohr effect. We have further shown that the Bohr effect is increased by the addition of DPG. Therefore, following Perutz's discussion (3), we conclude that despite its reduced oxygen affinity and Hill's constant, sulf-Hb undergoes a quaternary structure change upon ligation.

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