Henry *et al.*

Supplementary Material

June 30, 2021

Effect on ODC of HbF and rapid binding and dissociation of drug to the R conformation of Hb. Figure S1 compares the ODC for an individual, who is a compound heterozygote for β^0 thalassemia and pancellular persistence of fetal hemoglobin, and the range of ODC's for normal individuals. The Hb composition in this individual is 22.6% HbF, 75.4% HbA, and 2% HbA2. For a binomial distribution, the tetramer species are 56.9% $\alpha_2\beta^A{}_2$, 34.1% $\alpha_2\gamma\beta^A$, 5.1% $\alpha_2\gamma_2$, 3% $\alpha_2\delta\beta^A$, 1% $\alpha_2\gamma\delta$ (1, 2). Consequently, there is only 5% of a high affinity tetramer, so the it is not surprising that the ODC of this individual is within the normal range, albeit at the low end of normal. Although it is only a single ODC, it supports the expectation that the %HbF in the therapeutic range of 20-30% will have only a small offset from an increase in oxygen affinity of the $\alpha_2\gamma_2$ homotetramer to that reduce oxygen delivery,

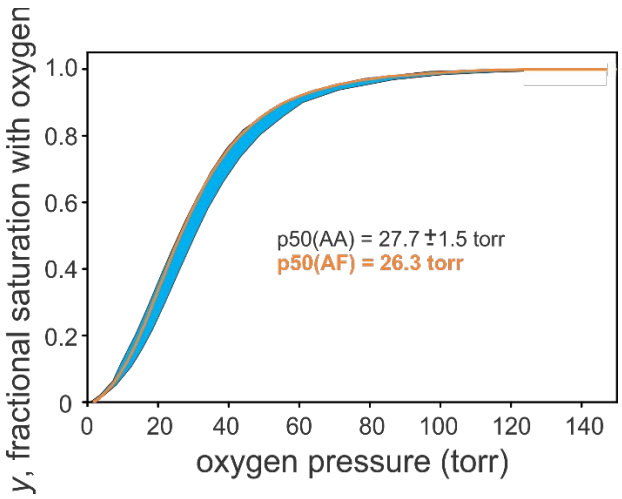

Figure S1. ODC of normal blood (blue shaded area) and ODC for compound heterozygote for $\delta\beta^0$ thalassemia and pancellular persistence of fetal hemoglobin (orange curve). The p50 for this individual is 26.3 torr. The normal range measured for 13 samples is 25.7 torr to 30.5 torr, with a mean of 27.7 torr and a standard deviation of 1.5 torr. The saturation is so close to zero at the lowest measured oxygen pressure that no correction to the saturation reported by the instrument was made.(3)

When a drug binds and dissociates rapidly to the non-polymerizing R conformation, the curve is no longer biphasic as it is with voxelotor, which remains bound to R and T during oxygen dissociation. This is shown in Figure S2 from Henry et al.(3), where the true equilibrium curve was calculated for the degree of modification of HbA of 40%

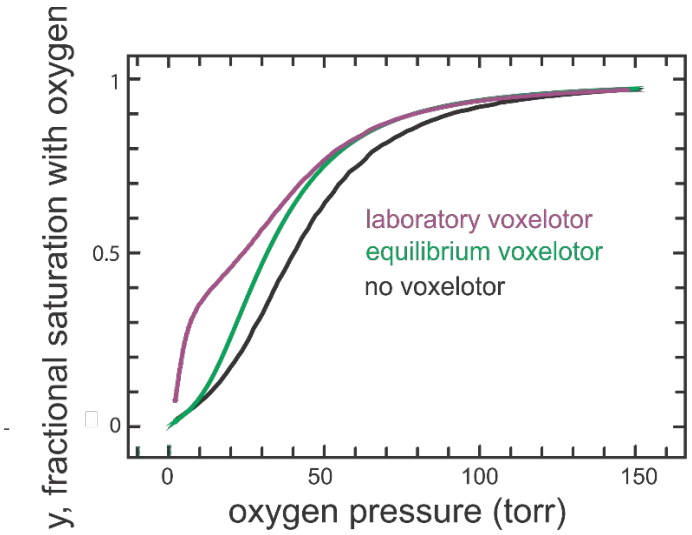

Figure S2. ODC measured at 37^0C for normal red cells and red cells treated with voxelotor from Henry et al.(3) Red cells were diluted 100-fold into pH 7.4 phosphate-buffered saline containing 12 μM voxelotor (purple curve), which produces 40% modification of HbA, or no voxleotor (black curve). The green curve is what would be measured if voxelotor bound and dissociated rapidly, as does 2,3-DPG.(4)

Calculation of fraction sickled and fractional saturation versus oxygen pressure *in vivo*. Apart from the sinusoidal circulation in the spleen and bone marrow, red cells passing through the tissues from the arterial to the venous circulation experience a very rapid decrease in oxygen pressure on the seconds time scale. While oxygen dissociation from either the free or polymerized hemoglobin is a sub-second process and therefore very close to equilibrium, polymerization is far out of equilibrium because of the delay time prior to fiber formation, which can be much longer than the transit time through the tissues. The recently discovered universal relation between delay time and supersaturation(5) permits calculation of the time at which fibers form inside cells. The delay time (t_d) for fiber formation is continuously changing because the oxygen pressure and therefore the solubility and supersaturation are continuously changing. The time at which fibers form inside a cell, which we will call the sickling time (t_{sickle}), occurs when the following relation is satisfied (6)

$$\int_0^{t_{sickle}} \frac{d\tau}{t_d(\tau)} = 1 \qquad \text{(S1)}$$

Since we can accurately calculate the dependence of the solubility on fractional saturation with oxygen for the free Hb molecules in each cell for the average 88/12 mixture of HbS/Hb(F+A2) with and without voxelotor, as described below, and we know how the delay time depends on supersaturation (5), given a rate of pressure decrease, the integral can be evaluated at each time point for every intracellular hemoglobin concentration. Recognizing that polymerization proceeds

very rapidly to equilibrium at the sickling time,(7) the fraction polymerized can be calculated at the oxygen pressure corresponding to a given sickling time from

$$x_p = \frac{1-c_s/c_0}{1-c_s/c_p} \qquad \text{(S2)}$$

The average fraction polymerized $\langle x_p \rangle$ is the fraction polymerized for each group of cells having the same total Hb concentration c_0, averaged over the mean intracellular Hb concentration distribution for the 23 sickle cell patients. The whole blood saturation (y_t) is then calculated at each pressure from this using

$$y_t = \langle x_p \rangle y_p + \left(1 - \langle x_p \rangle\right) y_s \qquad \text{(S3)}$$

where y_p is the saturation of polymerized hemoglobin calculated from

$$y_p = \frac{K_P p}{1 + K_P p} \qquad \text{(S4)}$$

with K_p = 0.0059 torr^{-1} (7) (Fig. S1) and y_s in the absence of voxelotor is calculated from the MWC saturation function (Fig. S1) (8):

$$y_s = \frac{LK_T p\left(1+K_T p\right)^3 + K_R p\left(1+K_R p\right)^3}{L\left(1+K_T p\right)^4 + \left(1+K_R p\right)^4} \qquad \text{(S5)}$$

with L = 58,000 , K_T = 0.0076 torr^{-1} and K_R = 0.53 torr^{-1}, yielding a p50 of 31 torr(9) ; this is higher than normal because of the elevated 2,3-diphosphoglycerate in SS cells caused by the anemia(10). Both y_p and y_s are assumed to be the same for every cell, which is only approximately correct because the concentration of 2,3-diphosphoglycerate affects on y_s;(11) it is expected to affect y_p to a much lesser extent, because polymerized hemoglobin already has close to the lowest possible affinity for the T conformation. (6)

In the presence of voxelotor, the polymerized hemoglobin saturation, y_p, is assumed to be the same as for drug-free hemoglobin and is also calculated from equation S4 (Fig. S1). Gill et al. showed that the oxygen dissociation curves for HbS and HbA are identical (12). So, the free hemoglobin saturation, y_s, is based on our measurements of normal red cell suspensions for a known percent Hb modification with voxelotor that results in the biphasic green ODC shown in Fig. 5b of the main text, which is to a good approximation the sum of ODC's of free hemoglobin molecules for the 26% of molecules with drug bound and the 74% of drug-free Hb molecules. The overall p50 = 24 torr.

Calculation of solubility as a function of oxygen pressure. This is a rather complicated thermodynamic calculation because of the many factors that must be taken into account. We

describe them here briefly. A more detailed description can be found in the review by Eaton and Hofrichter.(7)

These factors include the fact that in the mixture of 88% HbS and 12% Hb(F) (HbF and HbA2 exhibit identical copolymerization with HbS, justifying our use of the sum of the two in our analysis(13, 14)), the dissociation of the Hb tetramers into dimers results in the formation of hybrid molecules with a b chain from Hb S and a g chain from HbF.(1) There are now three different tetramers with different propensities to polymerize, quantified by a co-polymerization probability relative to $\alpha_2\beta_2^S$. The relative probabilities we use here are $e_1 = 1$ for the Hb S homotetramer, $\alpha_2\beta_2^S$, $e_2 = 0.1$ for the HbS/HbF heterotetramer, $\alpha_2\beta^S\gamma$, and $e_3 = 0$ for the Hb F homotetramer, $\alpha_2\gamma_2$. There are also very large non-ideality effects because of excluded volume effects (molecular crowding) in the concentrated Hb solutions of red cells. Consequently, the measured molar concentration of the Hb tetramers is not the effective thermodynamic concentration. The effective thermodynamic concentration is obtained by multiplying the molar concentration by a factor called the activity coefficient, which can be over 100. Molecular crowding also affects the thermodynamic properties of the water solvent, which are also taken into account. The last factor to consider is the dependence of the polymerization probability on the number (i) of oxygen molecules bound relative to tetramers with no oxygen molecules bound, which is given by $(K_P/K_T)^i$, where this factor is assumed to be the same for the two polymerizing Hb tetramers, $\alpha_2\beta_2^S$ and $\alpha_2\beta^S\gamma$. For a mixture of HbS and HbF, calculation of the solubility (c_s) as a function of oxygen pressure is accomplished by solving the following two simultaneous mass conservation equations (derived in Henry et al. (6)) for the two unknowns $x_{\beta S}$ and c_s, the quantity we seek:

$$\begin{aligned} x_{\beta S}c_s\left(c_p - c_0\right) + \Gamma Z\frac{c_s}{c_s^0}\left(e_1 x_{\beta S}^2 + e_2 x_{\beta S}\left(1-x_{\beta S}\right)\right)c_p\left(c_0 - c_s\right) &= X_{\beta S}c_0\left(c_p - c_s\right) \\ \left(1-x_{\beta S}\right)c_s\left(c_p - c_0\right) + \Gamma Z\frac{c_s}{c_s^0}\left(e_2 x_{\beta S}\left(1-x_{\beta S}\right)\right)c_p\left(c_0 - c_s\right) &= \left(1-X_{\beta S}\right)c_0\left(c_p - c_s\right) \end{aligned} \qquad \text{(S6)}$$

with the definitions:

$$Z \equiv \frac{L\left(1+K_P p\right)^4}{L\left(1+K_T p\right)^4 + \left(1+K_R p\right)^4} \qquad \text{and} \qquad \Gamma \equiv \frac{\gamma_s}{\gamma_s^0}\left(\frac{a_{H_2O}}{a_{H_2O}^0}\right)^n \qquad \text{(S7)}$$

where $X_{\beta S}$ is the total fraction of β^S chains (= HbS fraction, = 0.88) in the sample, (1- $X_{\beta S}$) is the total fraction of γ chains (= HbF fraction, = 0.12) in the cell, $x_{\beta S}$ is the fraction of β^S chains of the free (i.e. unpolymerized) Hb molecules, $a^o_{H_2O}$ is the activity of the water for pure HbS, a_{H_2O} is the activity of the water at the solubility for the mixture, n (= 2500) is the number of moles of water per mole of hemoglobin in the polymer phase, c_p is the Hb concentration in the polymer (= 0.69 g/mL), and c_0 is the total hemoglobin concentration in the cell ([HbS] + [HbF]).

A different formalism is required to calculate the solubility in the presence of voxelotor, since the drug binds and dissociates from the Hb very slowly(3). It is therefore not possible to use

the equilibrium theory that we did for the no-drug case above. Instead, we take advantage of the so-called polyphasic linkage relation by Gill and Wyman,(15) which permits calculation of the solubility from the ODC in the presence of the drug.(3) The quantity Z in equation (S7) represents the pressure-dependent probability of incorporating T-state Hb molecules into the polymer. For the drug-bound case, the ODC at a specified total drug concentration in general does not have an exact analytic form as it does for the drug-free case; in this case, Z as a function of pressure is computed by introducing a numerical approximation of the ODC into the more general expression

$$-p\frac{1}{Z(p)}\frac{d}{dp}Z(p) = 4\left(y_s(p) - y_p(p)\right) \qquad \text{(S8)}$$

which may be solved numerically for $Z(p)$, which may then be inserted into the system (S6) to obtain the solubility (c_s) at each oxygen pressure.

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