SI Text

Data Collection and Analysis

Data Collection

Oxygen equilibrium curves depicting the saturation level of oxygen as a function of the partial pressure of oxygen were collected by an extensive literature survey. A list of the different data sources (1-15) and the extracted parameter values are given in SI Tables 1 and 2. In some cases, the saturation curve was given as the saturation Y as a function of the partial pressure pO_2 (linear space), and in other cases as $\log(Y/1\text{-}Y)$ as a function of $\log(pO_2)$ (Hill space). We used GraphClick (Arizona Software) to extract the numerical values from the figures. Repeated data extraction on different days and by different users resulted in a negligible difference in terms of estimation of the parameters L_4, K_T. Whenever possible we tried to use data that were collected by groups with extensive experience in collecting oxygen saturation curves. In most cases, the measurements are done at 37^oC using either whole blood or hemolysates (for different organisms) or purified hemoglobin reconstituted with physiological effectors (for different physiological conditions). Fig. 1*d* shows several exemplary measurements of human hemoglobin under different pH levels and of different organisms that reflect the extreme levels found in terms on p_{50} and n.

Varying experimental methods and protocols were used by different groups to measure the OEC. Further, the treatment of whole-blood solutions, and the physiological conditions of the organisms analyzed were not identical. This is an inherent limitation in using the knowledge base accumulated in several decades of research. On the other hand, any variation in physiological conditions should result in changes in p_{50} and L_{T0R4} more than in n and L_4; this only serves to strengthen the finding reported here. Still, it cannot be ruled out that systematic differences in the experimental techniques used give rise to appreciable effects, most notably in n and L_4. A control to estimate the error associated with changing experimental methodology, is to use cases where the same organism has been measured by different groups with different techniques. We could find only few cases where this was done and adequately reported (showing the raw measured data): humans, elephants, and cows. These cases indicate that the variability caused by experimental technique and different groups is well below the variability between organisms. Yet one should notice that these are mostly located at the center of the cooperativity range and thus do not cover the large span of values for n and L_4 seen for other organisms.

Criteria for Including Hemoglobin Data Sets in This Study

We included in this study only data sets of hemoglobin saturation measurements where the raw measured data points were shown [with one exception (8) from which the data for the shrew, hedgehog, and mole were extracted]. In this case, many papers from the same research group show a systematic approach to the choice of the saturation levels measured). In cases where only the fitted curve is reported one cannot estimate the level

of interpolation or extrapolation performed by the authors. We required at least four data points to be measured in the saturation curve. Similar results in terms of the patterns of parameters ranges were observed when limiting only to data sets with eight data points or more in the saturation curve. In a control test where data sets with many data points were pruned to include only four data points around 20%, 40%, 60%, and 80% saturation the extracted values of n and p_{50} were similar to the original, full data sets values. In cases where there were several instances of measuring the same organism (human, elephant, cow) only few of them were shown to limit overcrowding of the figures.

Averaging of Saturation Curves from Several Specimens Together

In some cases [for example, ref. 11 from which data for human, dog, horse, and cow were used] the reported values for the partial pressure of oxygen at a specific saturation level is the average over measurements of several specimens (in ref. 11, 68 humans, 23 dogs, 10 horses, 20 cows). This averaging process can affect the resulting cooperativity. Because the separate data points were not reported we did not control for this effect.

Numerical Curve Fitting and Parameter Extraction

The data from the saturation curves were used to extract the values of the parameters in the MWC model. The saturation level of hemoglobin in the MWC model as a function of the parameters K_R, K_T, and L_0 is given by:

$$Y = \frac{\frac{p}{K_R}\cdot(1+\frac{p}{K_R})^3 + L_0\cdot\frac{p}{K_T}\cdot(1+\frac{p}{K_T})^3}{(1+\frac{p}{K_R})^4 + L_0\cdot(1+\frac{p}{K_T})^4},$$

where p is the partial pressure of oxygen. Using the transformed parameters K_R, L_4, and L_{T0R4} the saturation is given by:

$$Y = \frac{\frac{p}{K_R}\cdot(1+\frac{p}{K_R})^3 + \frac{L_{T0R4}}{K_R^4}\cdot\frac{p}{(L_{T0R4}/L_4)^{1/4}}\cdot(1+\frac{p}{(L_{T0R4}/L_4)^{1/4}})^3}{(1+\frac{p}{K_R})^4 + \frac{L_{T0R4}}{K_R^4}\cdot(1+\frac{p}{(L_{T0R4}/L_4)^{1/4}})^4}.$$

We used the fminsearch algorithm in Matlab (The Mathworks, Framingham, MA) to perform the curve fitting. Examples of the fitted curves are given in Fig. 1*d*. The parameter extraction problem is solved by a nonlinear least-squares minimization algorithm that uses the simplex method. The results reported in this article used squared differences between the fits and the data in Hill space [i.e. log(Y/(1-Y)]. Instead of optimizing the least squares with respect to the parameter values themselves, we used the logarithms of the parameters as the free variables. These are physically significant (being related to the free energies of the various steps) and insure the enforcement of positivity on the equilibrium constants. We experimented with different methodologies for doing the fitting (e.g. using a different algorithm based on conjugate gradients (lsqrcurvefitting in Matlab), using a different cost function (square differences between the data and Y itself, i.e. in linear rather than Hill space), using random rather than constant initial

conditions, and using a different numerical solver (FindFit in Mathematica 6, Wolfram research). The different approaches give very similar results for the parameters L_4, K_T and L_{T0R4}. In contrast, the individual values of K_R and L_0 could not be reliably extracted and can be very sensitive to the method used.

The starting conditions for the numerical optimization was kept constant for the different data sets at values of K_R=10^0; L_4=10^{-3}; K_T=10^2. We find that varying the initial conditions over an order of magnitude has negligible affects on the resulting L_4 and L_{T0R4} values. We independently used optimization with the canonical set [K_R,L_0,c] or [K_R,L_4,K_T] and found that the results were similar. We chose the latter as we then have only one parameter with a large confidence interval as discussed above. As the equilibrium constants of the type L_4 and L_{T0R4} depend on the affinities K_R and K_T to the fourth order, when one is interested in seeing the effect of changes in parameters on the saturation curve it is sensible to use the same fold change done to K_R and K_T for $l_4 = L_4^{1/4}$ and $l_{T0R4} = L_{T0R4}^{1/4}$ as performed in Fig. 3*a*.

The values of the cooperativity n and p_{50} were extracted from the fitted MWC model by interpolating the resulting saturation curve to find the value of p_{50} and by calculating the slope at half-saturation in Hill space (also referred to as n_{50}).

Sensitivity Analysis

A major part of our analysis involved evaluating the error bars on our measurements. As emphasized above, the data sets we used were often very limited in the number of data points and saturation range measured, and there was a general lack of information about the accuracy of the measurements. To estimate the error bars, we thus extracted a model of the noise by analyzing the residuals of our fits. We aggregated all of the data sets, making the simplified assumption that they all have the same accuracy, and then computed the distribution of the residuals. We find that in Hill space [i.e. $\log(Y/(1-Y))$] the errors do not depend on the value of Y, in agreement with previous reports by Imai (16). We find that the errors have a SD of 0.05 in Hill space [Imai (16) using the automatic technique achieved a value of 0.035]. Using this model of the noise, we created an ensemble of synthetic data sets by adding simulated random measurement errors to each data set. We then fit each of the synthetic data sets, and thus extracted a distribution for the values of the extracted parameters. The SD of this distribution was taken as an estimate of our error bars. This error analysis does have limitations: when analyzing the residuals in the original measured data set we noticed that the errors between adjacent points tend to be correlated in sign rather than be randomly distributed. This indicates systematic errors in the measurements or limitations of the MWC framework that are not captured by our error model. Such correlations could lead to overestimates in the error bars on n (as uncorrelated noise would tend to increase the variability in values of the slope at p_{50}) and underestimates for the error bars on p_{50} (as uncorrelated noise would affect a smaller shift of p_{50} than correlated noise). Moreover, the accuracy of different data sets can vary significantly thus requiring separate error models that could not be constructed with the limited data available. The measurement of the partial pressure of oxygen, which serves as the "independent" variable in the experimental measurements may also contain a measurement error of comparable magnitude to that of the measurement error in the saturation level that is the "dependent" variable. We use an

error model only for the dependent variable, thus combining the respective errors to one "effective" error.

Quantifying and Contrasting Parameter Ranges

The main point of this study is to contrast physiological variation of parameters of oxygen saturation curves to evolutionary variation. To quantify the differences in the parameter ranges between the two data regimes we use the SD of the logged values of the parameters. Using the logged values of the parameters is sensible physically as that relates to the relevant free energies involved. It thus measures the range of fold change in the original parameter used. We use the notation σ_X to denote the SD of the log value of the parameter x. The use of the SD of the logged values of the parameters is advantageous also because this is independent of the units used. One can appreciate that from noting that any change of units that will results in some fold change in the value will give only an addition of a constant in the logged value and when calculating the SD will cancel out. Notice also that for small ranges of variation $\Delta(\log(x)) \approx \Delta x / x$, and thus it is equivalent to the coefficient of variation (CV) defined as the ratio of the SD to the mean that is widely used for quantifying variability in a unit less manner.

Cases Where the Optimization Diverges for Parameters Not Well Defined

There were cases where the least squares curve fitting optimal solution was achieved when $K_R \rightarrow 0$ and $L_0 \rightarrow$ infinity in such a way that their product $L_{T0R4}=K_R^4 * L_0$ is constant. This is understandable given the discussion of the energy diagram (see relevant section below), and the little effect of the actual location of the R_0 energy level. Because of numerical number representation issues the values are limited and we get values of $L_0 \sim 10^{60}$ and $K_R \sim 10^{-15}$. This happened when using the fitMil (i.e. the simplex method) for 12 out of 29 data sets, namely the cow, llama, yak, Asian elephant, black galago, brown galago, ringtailed lemur, shrew, hedgehog, echidna, gorilla male, and female. Even though these parameter values diverged, the values of L_4, L_{T0R4}, and K_T were well defined and repeatable using different initial conditions or fitting techniques (e.g. when using the reflective Newton technique or when using linear rather than log space to perform the search which tends to stop well before numeric divergence).

In two organisms (black lemur and kangaroo) the best fit to the experimental data resulted in a diverging value for L_4 ($L_4 \rightarrow 0$), and a finite value for L_0. This indicates a reversed asymmetry in the saturation curve either because of different properties of hemoglobin or as a result of the very limited experimental data for these organisms. These organisms thus do not appear in the figures for the relationship of cooperativity to L_4 and of L_{T0R4} versus L_4 as their L_4 values are not well defined.

The MWC Model and Its Parameterization

The Need to Reparameterize the MWC Model

The MWC model is traditionally analyzed in terms of the parameters L_0, K_R and K_T. This set of parameters has the advantage of having concrete, easy to visualize "mechanical" interpretation in terms of the protein. Though these may be natural parameters in terms of elemental chemical reactions, there is no reason to think that changes in structure (either physiological or evolutionary) will perturb these parameters independently or that they are the parameters most easily evaluated experimentally. The model can be reparameterized by any other combination of these parameters, say L_4, K_R and K_T. What are the differences between these approaches? For a given precision in the experimental data, the derived parameters will have different confidence intervals. Small error bars indicate that the parameter has a strong effect at the measured range and therefore the data constrain it. Large error bars on the other hand indicate that there could be very different values of the parameter that will give similar data, thus the data does not constrain its value. The traditional set is very sensitive in this respect. For extremely accurate measurements to very high saturation levels, the data reasonably constrains all parameters; this level of data is available almost exclusively in humans (Fig. 3*a* and SI Table 2). For other measurements of different mammals, L_0 and K_R could take a wide range of values that will fit the measured data, as long as their product $L_0*K_R^4$ is constant (17, 18). As a result there will be a large degree of uncertainty in the fitted values for two of the three parameters.

The unconstrained nature of either L_0 or K_R when data is limited can be significantly ameliorated by choosing a different parameterization. If instead of L_0 and K_R we use the combination $L_0*K_R^4$ which is equal to the equilibrium constant between the states T_0 and R_4 (L_{T0R4}) under standard conditions the data will now constrain its value. The other parameter, K_R for example, will still have a wide possible range of values indicating that given that L_{TOR4} has a given value, the value of K_R will only weakly affect the shape of the curve within the domain measured and will only be accurately estimated by measurements at very high saturation. Notice that here as in many other cases the effect of a change in a parameter while keeping the other parameters constant depends on what are the other parameters. When K_R is changed while keeping L_{T0R4} constant the effect on the OEC is only at the high saturation end but when keeping L_0 constant as in the traditional parameterization, the change affects most of the OEC including the value of p_{50}. We are thus led to parameter sets, such as K_T, L_{T0R4}, K_R, or L_4, L_{T0R4}, K_R, where we can reliably extract two parameters, and where the third parameter, K_R, does not constrain the OEC in most of the physiological range.

Parameters, Chosen for Precision in Estimation, Also Naturally Represent Phenotypic Changes in Hemoglobin

In fitting the OEC to the MWC model, we had two goals: first, as mentioned above to extract parameters that can be reliably estimated within a small confidence interval; second, to extract parameters that are phenotypically meaningful, namely that they have a

direct and quantifiable relationship to p_{50} and n. We have discovered that L_4 and L_{T0R4} share these characteristics:

(*i*) The parameter combination $L_4=L_0*c^4$, where ($c=K_R/K_T$) can be extracted robustly, with small confidence intervals, and in addition it strongly correlates (Fig. 3*b*) with the cooperativity, n. The connection between n and L_4 is a basic property of the MWC model in the parameters domain occupied by hemoglobin. This can be understood from an analysis of the energy level diagram and an analytic derivation (see below) where we demonstrate that

$$n \cong \frac{\left(4 + l_4 \cdot (1+l_4)^{3/4} \cdot (1-l_4)^{1/4}\right) \cdot (1+l_4)^3 \cdot (1-l_4)}{\left(1 + l_4 \cdot (1+l_4)^{3/4} \cdot (1-l_4)^{1/4}\right)^4} + l_4 \cdot (1+l_4)^{3/4} \cdot (1-l_4)^{1/4} = f(l_4).$$

where for simplicity of notation we use $l_4 = L_4^{1/4}$. Microscopically, L_4 is the equilibrium constant between the T_4 and R_4 states.

(*ii*) As mentioned above the MWC parameter that is known to be robustly estimated from curve fitting an OEC is the affinity of the T state, K_T. However K_T does not independently dictate a single phenotypic parameter. On the other hand, we find that the combination $L_{T0R4}=L_4*K_T^4$ can be both robustly estimated, and is strongly correlated (Fig. 3*c*) with the phenotypic parameter p_{50}. In fact, our analysis (see derivation below) demonstrates that to first order

$$p_{50} \cong g(L_{T0R4}) = L_{T0R4}^{1/4}$$
$$(= L_4^{1/4} \cdot K_T = L_0^{1/4} \cdot K_R).$$

Microscopically, L_{T0R4} is the equilibrium constant between the T_0 (fully deoxygenated tense conformation) and R_4 (fully oxygenated relaxed conformation) states under standard conditions (Fig. 1*c*). This relationship is based on the result that the value of p_{50} is almost equal to the partial pressure of oxygen at which the fully oxygenated state R_4 will have the same free energy as the fully deoxygenated state T_0. The difference in free energy under standard conditions (760 mmHg O_2) between these two states is $L_{T0R4}>>1$ and a lower partial pressure of oxygen is required to make the free energies of these two states equal. This partial pressure is p50 and its intimate relationship to L_{T0R4} explains the correlation we find.

While the MWC model requires three independent parameters, we are focusing on two parameters that are both robustly estimatable and phenotypically interpretable (L_{T0R4} and L_4); the extra degree of freedom (e.g. K_R) has only a minor effect on the oec when the other two parameters are fixed (Fig. 3 *ai*), and hence has only minor phenotypic significance under any realistic physiological conditions. We thus established a clear relationship between the common phenotypic parameters and the underlying microscopic parameters in the model.

The reparameterization of the MWC model requires some explanation. The original set of parameters (K_R. K_T, and L_0) is not only familiar but natural in that they refer to simple

equilibriums. One can imagine measuring them independently, if one could measure the relative abundance of unliganded R and T states and if one could isolate these states to measure oxygen saturation. However, in the normal range of measurement they are not equally measurable. Furthermore, for most experiments it is not possible to measure accurately enough L_0 and K_R independently. Hence, though the MWC model is a three-parameter model, it is generally not possible to extract precisely enough free parameters from the published data. Reparameterization allowed us to extract accurately two parameters, reserving the third parameter, K_R, which has very little influence on the OEC. Yet these two parameters, L_4 and L_{T0R4}, are natural in a different sense; they are independently reflective of two physiological parameters, p_{50} and n, respectively. Finally, they turn out to be orthogonal for evolutionary and physiological change. Another kind of orthogonality would be if single-amino acid substitutions affected single model parameters. *A priori* there is no reason to believe that the canonical MWC parameters would be particularly natural by this criterion.

Description In Terms of Energy Levels

We gain insight into the relationship between the phenotypic properties and the microscopic parameters of hemoglobin by analyzing the energy levels of the different states in the MWC model. In the MWC model, hemoglobin is assumed to have two conformations (R and T), each with five possible levels of oxygenation (R_0-R_4, T_0-T_4), for a total of 10 possible states. The ratio between the occupancies of two states of different conformations (Ti and Rj), under standard conditions (1 Atm = 760 mmHg O_2), is given by the equilibrium constant L_{TiRj}. There are 25 such pairs of states. For example, L_{T0R4} between the fully deoxygenated T state (T_0) and fully oxygenated R state (R_4). By convention, equilibrium constants between states with the same level of oxygenation are denoted by only a single index, such that L_{T0R0} is denoted as L_0, and similarly, L_{T4R4} as L_4. The standard free energy difference is denoted by ΔG^0. In a transition between two states, for concreteness, say T_0 and R_0, the free energy difference is related to the equilibrium concentrations or occupancies of the substrates and the products via:

$$\Delta G^0_{TOR0} = -R * T * \log([T_0] /[R_0]) \equiv -\log([T_0]/[R_0]) = -\log([L_0]),$$

where the square brackets denote the equilibrium concentration or level of occupancy, and for simplicity in later notation we use free energy units such that R * T =1, where R and T in this equation are the gas constant and the temperature, respectively.
For a case where O_2 is involved, for concreteness, say T_0 and T_1 (T_0+O_2-->T_1)

$$\Delta G^0_{TOT1} = -\log(([T_0]* [O_2]/[T_1]).$$

The ratio between the substrates and products for binding of oxygen defines the affinity:

$$K_T=[T_0]*[O_2]/[T_1],$$

where $[O_2]$ is the concentration of oxygen (also denoted as the partial pressure of oxygen, pO_2). An analogous expression defines K_R.

Under standard conditions of oxygen ($[O_2]$ = 1 Atm) we find

$$\Delta G^0_{T0T1} = -\log([T_0]*[O_2]/[T_1]) = -\log([T_0]/[T_1]) = -\log(K_T).$$

The relationships between the 10 states can be visualized by an energy diagram where the relative free energy of state x at the given oxygen level, denoted $G^0_x(O_2)$ is on the y axis (Fig. 1*c*). This diagram enables the evaluation of any equilibrium constant based on the vertical distance between the two respective states. The occupancy of a state is proportional to $\exp(-\Delta G^0_x)$. The lowest lying configuration is the one that is most prevalent. One can easily appreciate relationships between different parameters, for example L_{T0R4} is equal to $L_4*K_T^4$ but also equivalently to $K_R^4*L_0$, we thus see that these two expressions are equal to each other.

Because it is important to notice that the depiction of the energy levels in Fig. 1*c* is under a specific (standard) level of oxygen, we are interested in the change in saturation when oxygen partial pressure is varied we need to understand how this diagram gets transformed under varying oxygen levels (SI Fig. 12).

Because we deal only with ΔG there is no absolute reference point, and we choose G^0_{T0} to serve as a constant reference point, or anchor (and equivalently G^0_{R0} because L_0 is a constant, independent of the oxygen pressure). When the oxygen level is changed the value of ΔG changes. For example, ΔG^0_{T0T1} that by definition is $-\log([T_0]*[O_2]/[T_1])$ can be written using the free energy difference when $[O_2]$=1, $\Delta G^0_{T0T1}([O_2]=1) \equiv \Delta G^0_{T0T1} = -\log([T_0]/[T_1])$ as:

$$\Delta G^0_{T0T1}(O_2) = \Delta G^0_{T0T1} + \log([O_2]).$$

Thus, we see that as $[O_2]$ changes the distance between T_0 and T_1 ($\Delta G^0_{T0T1}(O_2)$) changes. Starting with $\Delta G^0_{T0T1}(O_2) = \Delta G^0_{T0T1} > 0$ under standard oxygen pressure (where $(\log([O_2])=0)$, and decreasing $[O_2]$ (negative $\log([O_2])$) we find that at some point $\Delta G^0_{T0T1}(O_2)$ will change sign. At the point where the free energy is equal to zero, the concentration of both states is by definition equal, and from the definition of the affinity the concentration of oxygen at that condition is equal to the affinity (SI Fig. 12*b*). Depending on the number of oxygen molecules that can bind, the dependence on $[O_2]$ will have different coefficients, for example, for the process $T_0+4*O_2 \rightarrow T_4$:

$$\Delta G^0_{T0T4}(O_2) = \Delta G^0_{T0T4} + 4*\log([O_2]).$$

At each oxygenation level we can visualize the resulting energy diagram as shown in SI Fig. 12. This depicts the changing occupancies of different states of hemoglobin when the oxygen level changes by following the locations of the respective energy levels of each state. The different oxygen levels used in SI Fig. 12 are (a) 760 mmHg, the standard conditions of 1 atmosphere, (b) 100 mmHg, near K_T, (c) 27 mmHg, close to p_{50}, and (d) 5mmHg, near K_R.

Provided with this visualization aid we can relate the phenotypic properties *n* and p_{50} to the microscopic parameters. Some microscopic parameters play a dominant role

whereas others are negligible. We begin with the value of p_{50}. Under high levels of oxygen the lowest lying and thus most probable configuration is R_4 and we find hemoglobin to be fully oxygenated (SI Fig. 12*a*). As oxygen levels are lowered all energy levels shift upward in respect to the T_0 and R_0 anchor. At some point R_4 will be at the same level as T_0 (SI Fig. 12*c*). As $L_0>1$ and $L_4<1$ (for mammalian hemoglobin characteristic values are $L_0=10^5$ and $L_4=10^{-3}$), all the other levels will be higher and therefore less probable. Thus the most and equally probable configurations are at full (R_4) and no (T_0) oxygenation. Assuming that to first order the other configurations are negligible (i.e. $L_0>>1$ and $L_4<<1$) this is the point where hemoglobin is half saturated which is by definition p_{50}. When does this condition occur? It the two levels are at the same energy level then $\Delta G^0_{T0R4}(O_2)=0$. Then because:

$$\Delta G^0_{T0R4}(O_2) = \Delta G^0_{T0R4} + 4*\log([O_2]),$$

we get:

$$\log([O_2]) = -1/4*\Delta G^0_{T0R4} = 1/4*\log(L_{T0R4}) = \log(L_{T0R4}^{1/4}) \equiv \log(l_{T0R4}).$$

and this is the point where $\log([O_2]) = \log(p_{50})$. So we arrive at a relationship between a phenotypic property and a microscopic parameter:

$$p_{50} \approx L_{T0R4}^{1/4} = l_{T0R4}.$$

As seen from the energy diagram under standard conditions $L_{T0R4}=L_4*K_T^4=L_0*K_R^4= l_{T0R4}^4$. We can similarly analyze the cooperativity n in a similar manner. If there were only the states T_0 and R_4, the cooperativity would be 4 as implied by the coefficient in front of $\log([O_2])$. Lower cooperativities are the result of the fact that other states have non-negligible probabilities. Because we find that for hemoglobin under normal conditions in nature $abs(\log(L_0))>>abs(\log(L_4))$, T_1 is the next most probable state (SI Figs. 11 and 12*c*) and its effect should be included. The closer this state will be to the T_0 and R_4 energy levels at p_{50} the lower the cooperativity will be. The locations of T_1 and the other T states near p_{50} are governed by the value of L_4. When $\Delta G^0_{T0R4}(O_2)=0$ (i.e. near p_{50}), $\Delta G^0_{T1R4}(O_2) = -1/4*\log(L_4)$, $\Delta G^0_{T2R4}(O_2) = -2/4*\log(L_4)$ etc. Thus the cooperativity n is related to L_4.

As illustrated by the energy diagrams (SI Fig. 12*c*), at the oxygen pressures that dictate n and p_{50}, the location of states like R_0 that is governed by L_0 and K_R separately (and not the combination $L_0*K_R^4$) are unimportant as their probability is negligible. This demonstrates the minor effect of these parameters on n and p_{50}. Equivalently this shows that from measurements that are mostly around intermediate levels of oxygenation it is very difficult to extract the value of these parameters separately.

Analytic Derivation of *n* and p_{50} as a Function of the MWC Parameters

As can be appreciated from SI Fig. 11, the two most prevalent states of hemoglobin are T_0 and R_4. We can get a first order approximation of p_{50} by assuming that only these two

states are occupied (this is equivalent to taking the limit L_0-->inf, L_4-->0 while keeping L_{T0R4} finite). The saturation level Y is then given by:

$$Y = \frac{(\frac{p}{K_R})^4}{(\frac{p}{K_R})^4 + L_0} \rightarrow\ p_{50} \cong L_0^{1/4} K_R = L_{T0R4}^{1/4} = l_{T0R4} (= L_4^{1/4} K_T = l_4 K_T)\,,$$

where we used the definition that at p_{50}, $Y = 0.5$. We thus arrive at the first-order approximation of the relationship between p_{50} and L_{T0R4}.

A second-order approximation (we use the terms first and second-order to rank the level of approximation and not in the strict meaning of the degree in a Taylor expansion) will take into account all the T states (T_0 to T_4) and the fully oxygenated R_4 state. We neglect the states R_0 to R_3, effectively deleting the R branch of the MWC reaction scheme except for R_4. This can be thought of as taking the limit K_R-->0 and L_0-->∞ while keeping their product $L_0 * K_R^4 = L_{T0R4}$ finite. This simplification is motivated by the observation that hemoglobin parameters are in the domain where abs(log(L_0))<<abs(log(L_4)) (see discussion in the energy level description section). The saturation level including all T states (T_0 to T_4) and R_4 is then:

$$Y = \frac{(\frac{p}{K_R})^4 + L_0 \cdot \frac{p}{K_T} \cdot (1 + \frac{p}{K_T})^3}{(\frac{p}{K_R})^4 + L_0 \cdot (1 + \frac{p}{K_T})^4}.$$

Iteratively applying our result from the first order approximation $\frac{p_{50}}{K_T} \cong l_4$, we derive a second order approximation for p_{50}:

$$p_{50} \cong (1 + l_4)^{3/4} (1 - l_4)^{1/4} l_{T0R4}\,.$$

For evaluating p_{50} the correction term is small as l_4<<1 and thus $(1 + l_4)^{3/4} (1 - l_4)^{1/4} \cong 1$ as can be appreciated from inspecting Fig. 3*c*, where a good fit is observed for the first-order approximation. Nonetheless, the second-order approximation is required for the evaluation of *n*. The cooperativity *n* is the derivative at p_{50} in the Hill plot, i.e. of log(Y/(1-Y) versus log(p).

$$\frac{Y}{1-Y} = \frac{\frac{1}{L_0}(\frac{p}{K_R})^4 + \frac{p}{K_T} \cdot (1 + \frac{p}{K_T})^3}{(1 + \frac{p}{K_T})^4 - \frac{p}{K_T} \cdot (1 + \frac{p}{K_T})^3} = \frac{\frac{1}{L_4} x^4 + x \cdot (1 + x)^3}{(1 + x)^3} = \frac{x^4}{L_4 (1 + x)^3} + x$$

where we denote $x \equiv \frac{p}{K_T}$.

Around p_{50}, $\frac{Y}{1-Y} \approx 1$, and therefore we can use $\log\left(\frac{Y}{1-Y}\right) = \frac{Y}{1-Y} - 1$ because $\log(1+\varepsilon) \approx \varepsilon$ when $\varepsilon \to 0$.

We thus have

$$\log\left(\frac{Y}{1-Y}\right) \approx \frac{x^4}{L_4(1+x)^3} + x - 1 \ .$$

Differentiating to get the slope:

$$\frac{d\log\left(\frac{Y}{1-Y}\right)}{d\log(p)} = \frac{d\log\left(\frac{Y}{1-Y}\right)}{dx}\frac{dx}{dp}\frac{dp}{d\log p} = \frac{L_4(1+x)^4 + x^3(4+x)}{L_4(1+x)^4}\frac{1}{K_T}xK_T = \frac{xL_4(1+x)^4 + x^4(4+x)}{L_4(1+x)^4}$$

The cooperativity n (also denoted n_{50} as we analyze the cooperativity at half maximum) is by definition:

$$n = \frac{d\log\left(\frac{Y}{1-Y}\right)}{d\log(p)}$$

evaluated at p_{50}. We use the second order approximation for the half saturation point $p_{50} \cong (1+l_4)^{3/4}(1-l_4)^{1/4} l_{T0R4}$ and therefore $x \cong (1+l_4)^{3/4}(1-l_4)^{1/4} l_4$ to arrive at:

$$n \cong \frac{\left(4 + l_4 \cdot (1+l_4)^{3/4} \cdot (1-l_4)^{1/4}\right) \cdot (1+l_4)^3 \cdot (1-l_4)}{\left(1 + l_4 \cdot (1+l_4)^{3/4} \cdot (1-l_4)^{1/4}\right)^4} + l_4 \cdot (1+l_4)^{3/4} \cdot (1-l_4)^{1/4} = f(l_4).$$

which shows that under these assumptions n is a function of only L_4. To appreciate the validity of this approximation we refer to Fig. 3*b* where we see that for most data sets this relationship explains most of the variability in the values of n.

A Taylor expansion for small values of l_4:

$$n \cong 4 - 6 \cdot l_4 - l_4^2 + 81/4 \cdot l_4^3 - 263/8 \cdot l_4^4 + O(l_4^5) \ ,$$

where the terms with order 5 and higher affect the cooperativity by <1% for the characteristic value of $L_4=10^3$.

Heterotropic Effects in the MWC Framework

The MWC model was originally used to describe both homotropic effects (the cooperative binding of oxygen) and heterotropic effects (binding of effectors such as protons, DPG, etc.). Under the original model the binding of effectors does not change the affinities K_R and K_T and only changes L_0 (and thus L_4). However, it was shown previously that the binding of effectors does affect the affinities as also seen in this study. It should thus be understood that we use the MWC model as a model for the cooperative

binding of oxygen and for extracting the effects of other effectors, but do not make the original assumption of constant affinities under binding of effectors.

Alternative Models to the MWC Model

More complex models have been discussed in the literature (19-23). These models are usually elaborations of the two-state concept that use more degrees of freedom (free parameters) and can provide a more detailed description of hemoglobin function, especially with respect to its kinetic behavior. Given that the MWC characterizes the saturation curve quite well, a significant advantage for using the MWC model over other models in this study is that we are already at the limit of the number of parameters that can be reliably deduced from existing measurements of the saturation curve. The parameters of more advanced models cannot be determined from the available measurements of most organisms and were thus not used here. The alternative sequential KNF model (24) was found to be less suitable in describing hemoglobin (22, 23), especially following the result that oxygen binding in crystals of hemoglobin that are in the T state showed perfectly non-cooperative binding (25).
Some authors suggested the importance of inclusion of the dimer-tetramer dissociation (26) and self-association of tetramers to create super-cooperativity (27). Inclusion of these effects requires the addition of more free parameters to the models that cannot be constrained with existing experimental data.

Physiological Considerations

Physiological Ranges

The standard physiological conditions under which human hemoglobin is studied is a temperature of 37^{O}C, pH of 7.4, a partial pressure of 40 mmHg CO_2, 2 mM DPG, and 0.1 M Cl^-. These also tend to be the standard conditions for other mammals though some differences occur especially in terms of DPG. In studying the properties of hemoglobin, researchers have varied the levels of different effectors that interact with hemoglobin and change its binding properties. The natural physiological effectors are known to be DPG, pH (protons), and CO_2 as well as effectors that are relatively stable under the prevailing physiological conditions (chloride ions) and artificial effectors not present *in vivo* (Bezafibrate, denoted BZF). When studying the physiological parameter range we are clearly interested only in the effectors that vary *in vivo*. Most experimental measurements used extreme conditions well beyond what can be expected *in vivo* (e.g. no DGP, no Cl^-, pH of 6.5 or 9.1). It is difficult to clearly determine what to include as the relevant physiological ranges. We choose the pH range of 6.8-7.6 to be included with constant DPG, CO_2, and Cl^-. In SI Figs. 7 and 8 we present the data from ref. 1 with the extreme case of including or omitting these effectors completely, and also the data of ref. 28 (SI Fig. 9) where a realistic range of pH, CO_2, and DPG was explored but where the raw data is absent and only inferred Adair constants are available.

Effective OEC

It should be noted that some of the physiological effectors such as pH may vary along the path of the circulatory system. Thus, the pH in the tissues where oxygen is being downloaded will be lower (more acidic) than in the lungs. Thus, in practice it may be that there is an effective saturation curve (29), composed of a hybrid between several saturation curves, each reflecting one "static" condition. This has the implication that a pure p_{50} change as a function of pH (as for example under strenuous activity) will translate into an effect also on the cooperativity *n*.

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