Simulations of equilibrium and kinetic data for hemoglobin valency hybrids

Response to Cordone et al.

The authors have described a more general model to fit oxygen equilibrium curves (OEC) for partially oxidized hemoglobin (Hb). In our work concerning these valency hybrids, we used a two-state model (4 parameters), which allowed for ferrous and ferric ligands to shift the allosteric equilibrium by different factors: c and m, respectively. The analysis of Cordone et al. (8 parameters) provides two main extensions: the inclusion of the dimer-tetramer equilibria, and four parameters c_j to include a dependence of c on the number (j) of ferric subunits within a Hb tetramer.

Overall, there is a general agreement between the two models. Both indicate that low spin ferric ligands, such as CN^-, make a contribution similar to the binding of an oxygen molecule (we obtain $m/c = 1$, they report $m/c_0 = 1.5$ or 1.1 for a weighted average of c_0, c_1, and c_2), while high spin ligands such as F^- (or water) show a smaller shift towards the R-state ($m/c = m/c_0 = 3.5$). The main difference in the physical interpretation of the data concerns their value of $c_3 = 1$, which seems to lead to a breakdown of the two-state model since for triply cyano-metHb it predicts $K_T = K_R$.

As the authors indicate, the distribution of substates may be different when the dimers are accounted for. Since research on the internal mechanism for the allosteric transition in Hb requires a consideration of the nature of these substates, their model can potentially help provide a higher resolution description of this protein.

For the kinetic studies, the dimers can be treated as a static fraction of "R-like" Hb, since the dimer-tetramer equilibration is slow compared with the ms ligand recombination. For these kinetic studies, we are dealing with the distribution for Hb with four ligands shown in their Fig. 2 *b* (we used CO as the ferrous ligand). Calculating 18% dimers for 60 μM total heme with a dissociation constant of $K_{42} = 2.5$ μM, they suggest that our need for a 10% dimer contribution is evidence supporting their model; actually for these kinetic studies at 95% metHb we used 200 μM total heme which corresponds to ~10% dimers, as observed. However these values refer to the usual fraction dimer for fully liganded (R-state) Hb; of interest here is 95% met Hb, and specifically for the flash signal one needs the fraction of ferrous subunits as dimers. We applied their model and obtained a dimer contribution of <3%; the flash signal is dominated by the triply CN form, which is just the state they claim is "special". Their parameters place $Hb(CN)_3$, with or without the ferrous ligand, as over 80% T-state ($Lm^3 = 4.3$). Note that we do not observe such a large T-state contribution to the kinetics (Fig. 1). Simulations indicate that inclusion of dimers alone (with the usual c values) does not significantly change the distribution; the parameter $c_3 = 1$ enhances the T-state and the relative amplitude of F3 (their Fig. 2 *b*). Their model actually predicts a decrease in the dimer contribution to our kinetic results; the 10% contribution we required is thus compatible with the usual binomial distribution.

For the OEC, we used progressively higher [Hb], in order to maintain a large oxygen binding signal; 300 μM was used for samples above 75% metHb. Cordone et al. are correct in pointing out that during the deoxygenation process the exchange of dimers will change the distribution. Note that this process involves kinetic parameters, since the dimer-tetramer equilibrium for the deoxy species may require hours. The new distribution (their Fig. 2 *a*) resembles that for the binding of oxygen; the partially liganded states are decreased in amplitude because CN is treated allosterically by assuming the two-state R-T equilibria as for oxygen (only with m instead c).

According to Cordone et al., triply cyano-met Hb tetramers, despite being 80% T-state have an R-state oxygen affinity, because $c_3 = 1$ implying that ${}^3K_T = {}^3K_R$. Consider this triply met species alone; since there is only one ferrous subunit per tetramer, equilibrium curves will necessarily be noncooperative. There is no information available to separate the affinity (K_R and K_T) from the allosteric (L) parameters; a family of values of these parameters can simulate the observed affinity. Both groups are in agreement that the data should be simulated as a series of curves.

This compensation problem is already evident for ferrous Hb: they use $\log(L) = 7.3$, while we used $\log(L) = 6$ for the same data (pH 6.5 Bis Tris); they used $\log(L) = 5$ for their data at pH 7. The main difference is at the triply liganded level; because this species is never highly populated the OEC is not sensitive to changes in the $R_3 - T_3$ equilibrium. There have been claims that the population of T_3 is zero, but such conclusions are sensitive to small changes in the data and the normalisation of the OEC. In this case, Cordone et al. predict a difference of nearly a factor of 100 in T_3/R_3 between their two simulations (both for ferrous Hb under similar conditions). This value (T_3/R_3) is generally poorly determined from the OEC alone and one cannot draw conclusions about the allosteric equilibrium of Hb with three (ferrous or ferric) ligands.

According to their parameters, $Hb(CN)_3$ and $Hb(CN)_3(O_2)$ are both 80% T-state and yet the observed oxygen affinity is not T-state, but R-state. But what does it mean to be in the T-state if it does not show T-state properties? Somehow their model started within a two-state framework, predicted a large fraction of T-state material for triply cyano-metHb (with or without oxygen), and then seems to eliminate the T-state oxygen affinity by use of a special parameter c_3 which forces ${}^3K_T = {}^3K_R$. Yet this species is still given a T-state conformation when calculating the dimer-tetramer equilibrium which leads to the enhancement of the population of this species (F3 in their Fig. 2 *b*). How can the same tetramer display T-like properties of K_{42} and an R-state oxygen affinity? We feel this arrives from the impossibility of separating the allosteric and affinity parameters for the triply liganded species.

One method of enhancing the amount of T-state is by addition of strong effectors. For samples that were 90% cyano-metHb, we observed a shift in p50 of a factor of 45 by adding both IHP and L345, which we interpret as a shift towards the T-state. According to their model (with $c_3 = 1$), these data would have to be interpreted as a factor of 45 change in the R-state affinity.

The kinetic data show a similar trend. While the amount of

slow (T-state) recombination is low for triply cyanometHb, the addition of IHP and L345 increases the fraction slow to 0.55 (Fig. 1). The observed rate of the slow phase corresponds to that of ferrous Hb with the same effectors, a rate 200 times slower than the R-state with these effectors. The only way to account for all the data is by maintaining distinct R and T state affinities at all ligation and oxidation levels.

The authors have not demonstrated how each new parameter changes the simulation. While they claim an "excellent" fit with four additional parameters, there is no discussion of the compensation between the parameters. It is disturbing that they state "the experimental data is not available", when in fact there was no request for the data; the data is certainly available to all.

If the departure from the two-state framework is attributed to the linear geometry of the Fe-CN bond, then should this effect ($c_3 = 1$) also apply to CO binding? They report this effect for aquometHb ($c_3 = 0.5$), yet the argument for the linear geometry would not apply. If c depends on the number of ferric ligands, then can one rule out an inverse effect (that is a dependence of m on the number of oxygen molecules bound)? Many other factors may also contribute at the same level of analysis: differences between the alpha and beta chains, a dependence of K_{42} on the oxidation level, etc.

In conclusion we agree with their model concerning the inclusion of dimers, but have doubts about the value for c_3. We presented four types of data: equilibrium and kinetic; with and without effectors. They fit only one type (equilibrium data without effectors) which has the least amount of information concerning the parameter c_3.

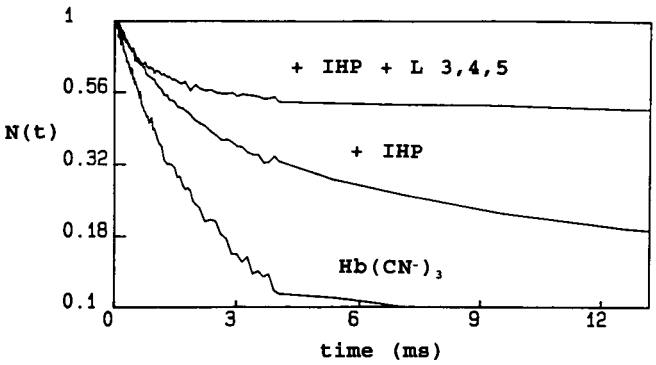

FIGURE 1 CO recombination kinetics to 95% cyano-met Hb (200 μM in heme) at 25°C, pH 6.5 Bis Tris, equilibrated with 0.1 atm CO. $N(t)$ is the fraction of ferrous hemes without ligand. The dominant CO rebinding signal is for triply cyano-met tetramers. The addition of the effectors IHP and L345 induces a large increase in the fraction slow, characteristic of rebinding to T-state Hb.

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