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Review

Identifying the conformational state of bi-liganded haemoglobin

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Abstract. While most researchers agree on the global features of cooperative ligand binding to haemoglobin (Hb), the internal mechanisms remain open to debate. This is not due to inaccurate measurements, but is rather a consequence of the cooperative ligand binding that decreases the equilibrium populations of the partially liganded states and makes observation of the transitions between these substates more difficult. For example, the equilibrium population of the doubly liganded tetramers is typically less than 5% of the total Hb. As a result many models with widely varying mechanisms may fit the oxygen equilib-rium curve, but may not be consistent with observations of other parameters, such as ligand-binding kinetics or subunit association equilibria. The wide range of methods and models has led to divergent conclusions about the properties of specific substates. One notable debate concerns the properties of the doubly liganded forms. The simple two-state model predicts a shift in the allosteric equilibrium based on the number of ligands bound, but not on their distribution within the tetramer. From studies of dimer-tetramer equilibria of various pure and hybrid forms, it was concluded that a tetramer with two ligands bound on the same $\alpha\beta$ dimer (species 21, an asymmetric hybrid) shows an enhanced tetramer stability, similar to singly liganded Hb, relative to the other three types of doubly liganded tetramers which resemble the triply liganded forms [Ackers et al. (1992), Science **255:** 54–63]. The implications of this model and the relevant experiments will be reviewed here.

Key words. Haemoglobin; hybrids; cooperativity; allosteric transition; ligand kinetics.

Introduction

One of the most fascinating aspects of the Hb molecule is the way the subunits work together to bind ligands. When an oxygen molecule binds to one subunit, the other subunits are altered, and their own affinity for oxygen may be modified. This cooperativity leads to more efficient oxygen delivery due to a steeper binding curve (fig. 1), that is, a larger change in oxygen saturation for the same change in oxygen partial pressure. The internal mechanism for this transfer of information is a current subject of debate that we address in this review.

The classical two-state or Monod-Wyman-Changeux (MWC) model [1] imposes that the allosteric equilibrium depends on the number of ligands bound, but

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not on the arrangement amongst the subunits. This switchover can be depicted graphically as a balance between the two conformations with a 'weighting factor' determined by the number of ligands (fig. 2).

The MWC model requires the Hb tetramers to switch between two conformations, of high and low ligand affinity, with the constraint that all four subunits within the same tetramer share the same conformation. As a mechanical analogy, imagine that as many as four ligands can be charged onto a small dump truck, whose platform can flip between two levels: a 'down' position that securely holds the ligands with a high affinity when the platform is weighted by enough ligands, and an 'up' position with zero or one ligand (fig. 3). The truck will leave the loading dock (lungs) with four ligands that hold the platform in the down position. After three ligands are delivered, the platform will spring up to a 'low-affinity' position to dump the remaining ligand. This analogy is meant to stress the feature that all four binding sites within a given 'tetramer truck' have the same affinity. Like the MWC model for an Hb tetramer, the entire system takes on one of two positions, and each ligand makes the same contribution (weight) towards switching the conformation. There is a continual switching between the two positions; an energetically favoured position means that the protein spends most of its time in that conformation. An equilibrium coefficient near unity (for example with two ligands) would mean that the platform spends nearly equal amounts of time in the two positions. As for the MWC model, the switchover from the empty up position to the loaded down position depends on the number of ligands bound (that is the total weight), but not on their arrangement.

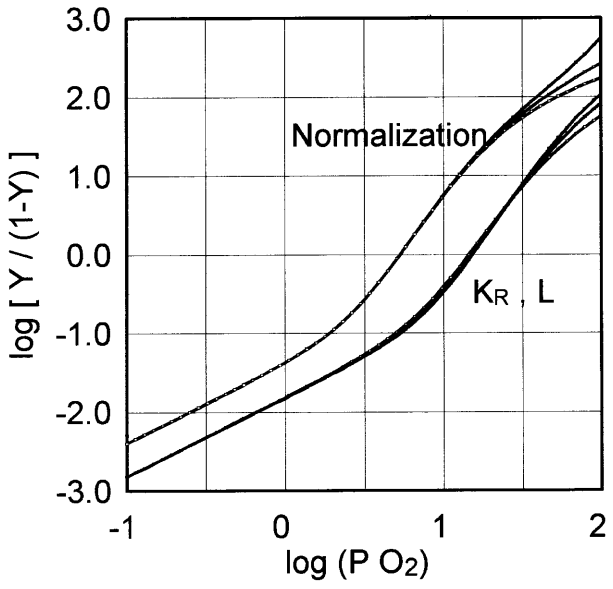

Figure 1. OECs presented as Hill plots. Two simulations are presented: curves typical of stripped Hb (but with 100 mM NaCl) at 25 °C (OEC on the left, with $K_T = 25$ mmHg, $K_R = 0.3$ mmHg, log(L) = 4.9) and a curve for Hb + DPG (right: $K_T = 66$ mmHg, $K_R = 0.6$ mmHg, log(L) = 5.5. Different simulations using the MWC model are superposed, with the condition that the simulations do not differ by more than 1% in Y over the entire curve. This is meant to show the precision of the parameters: for example (curve on left), the normalization factor was changed ±0.2%; (curve on right) K_R was varied ±a factor of 2 (0.3, 0.6 and 1.2 mmHg) and L was changed to maintain the same P_{50}.

An alternative mechanism was more recently proposed for the internal switching. Based on dimer-tetramer equilibria studies, a model was described involving a change in oxygen affinity within the dimeric halves [2, 3]. Within this scheme, the binding of the first ligand greatly increases the affinity for binding a second ligand to the same dimer. To compare with the analogy above, one would now consider the truck with two adjacent platforms, each with a capacity of two ligands (fig. 3). A ligand loaded on either half will hold only that half down and allow a partner to be loaded in the same half at a higher affinity. For both models the first ligand to arrive sees an empty platform in the up (low-affinity) position. However, in this scheme, the second ligand to arrive will see a down position for the singly liganded half, and the up position for the other (empty) half. The second ligand therefore has a preferred binding to complete saturation of the down half; in this case, the third ligand would then see available sites in a low affinity up position for the empty half (fig. 3). If one ligand binds to each half, then both halves drop to the down position, and a subsequent third ligand would see only high-affinity sites (one on each half, not shown in fig. 3). This model thus predicts a dependence on the specific arrangement (symmetry) of the ligands and a cooperativity within the dimer element. The symmetry rule (SR) would then imply a low deoxylike ligand affinity for the '21' Hb hybrid with one fully loaded platform (dimer) and one empty platform, whereas the other three doubly liganded forms have a ligand on each platform and present only down platforms as for the triply and fully liganded forms.

Both the MWC and SR models can simulate most of the equilibrium data on ligand binding, and exceptions can be found for both. They are in agreement on a cooperative ligand binding; they differ on the properties of the partially liganded substates. Unlike the MWC model, the SR model makes a distinction between doubly liganded tetramers depending on whether the two ligands are bound within the same dimer or not.

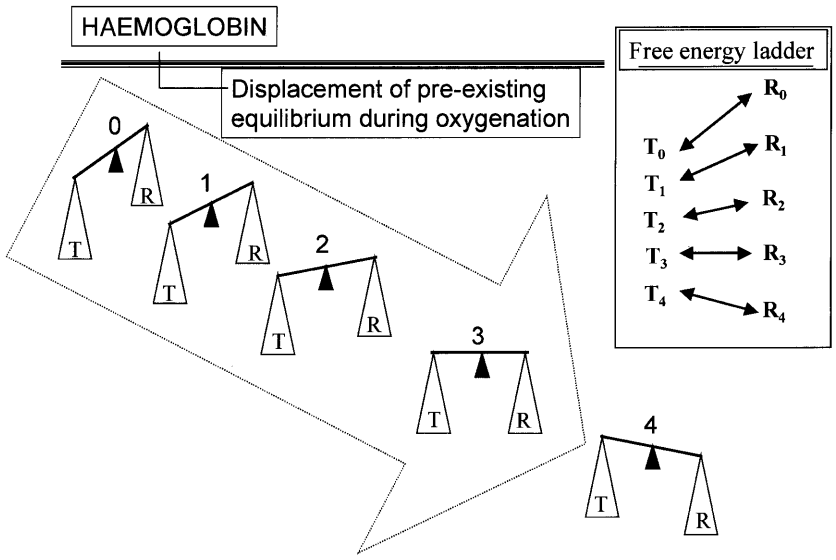

Figure 2. Energy diagram for the Hb substates within a two-state framework. For the simple MWC model, the allosteric equilibrium depends on the number of ligands per tetramer, but not on their distribution within the tetramer. The switchover point is variable in the MWC scheme, depicted here at three ligands.

Experimental observations

There is certainly no lack of data for the interaction of ligands with haemoglobin [4–10]. The large optical signals and ease of preparation of this protein have made it one of the most studied biomolecules. The hundreds of natural mutants and diverse spectroscopic techniques that can be applied have also made it a model system for studying ligand binding, cooperativity and subunit interactions. The use of chemically modified porphyrins, different metal atoms, various ligands and external effectors has produced a large database on the structure [11, 12] and function [13–25] of Hb, and corresponding theoretical aspects [26–31].

Despite the wealth of data, certain questions remain difficult to answer. This is due to the cooperative nature of the ligand binding. In a highly cooperative system, the initial and final states account for most of the substate population. The intermediate substates will be present in much lower proportions relative to a noncooperative occupation of binding sites (fig. 4). Thus the study of the partially liganded forms requires observation of small populations which may have a short lifetime.

Alternatively, modifications can be introduced to stabilize the intermediate species, such as the use of more stable ligands, cross-linked tetramers, or other external constraints. If there were only one deviation from a simple model, it could probably be determined without complication; however, there is never just one additional parameter. In addition to the new phenomenon in question, one needs to take into account simultaneously the difference between the α and β chains, the presence of dimers, and a small fraction of oxidized subunits. Thus while the equilibrium curves can be simulated with only three parameters with the MWC model, a more detailed description immediately leads to a large number of parameters.

Equilibrium properties

For the analysis of any complex system involving several parameters, a large set of independent types of data is required. The more observations of different phenomena, the better the resolution of the internal mechanism. Also, there must be some agreement on the critical data sets and the precision of the measurements. Many proposed models satisfy one data set, but are disproven with a complementary experiment. While there is general agreement that Hb makes an allosteric transition and displays a high level of cooperativity, determination of the actual populations and transitions between substates is another question.

The oxygen equilibrium curves (OECs, fig. 1) show that the first ligand binds with low affinity and initial cooperativity (slope on the Hill plot) near unity; subsequent ligand binding passes through a maximum cooperativity of over 3 near half saturation, and binding of the last ligand occurs with high affinity and a return to a slope

near unity. The experimental techniques allow a resolution of less than 1 part in 1000; however, other factors often impose a minimum error. While the oxygen partial pressure at half saturation (P_{50}) and the slope n_{50} are well defined, the slopes of the asymptotes are highly dependent on the normalization values and small amounts of impurities. One generally has to accept an error of about 0.5% due to sample stability (oxidation), impurities and the fact that the change in absorption may not be the same for each subunit or conformational state. It is not generally appreciated that an error of this size can make significant changes in the shape of the curves. The slope of the asymptotes can easily change from 1 to 1.5 with a shift in normalization of only a few parts per 1000 [31]. The implications of these errors are shown in figure 1.

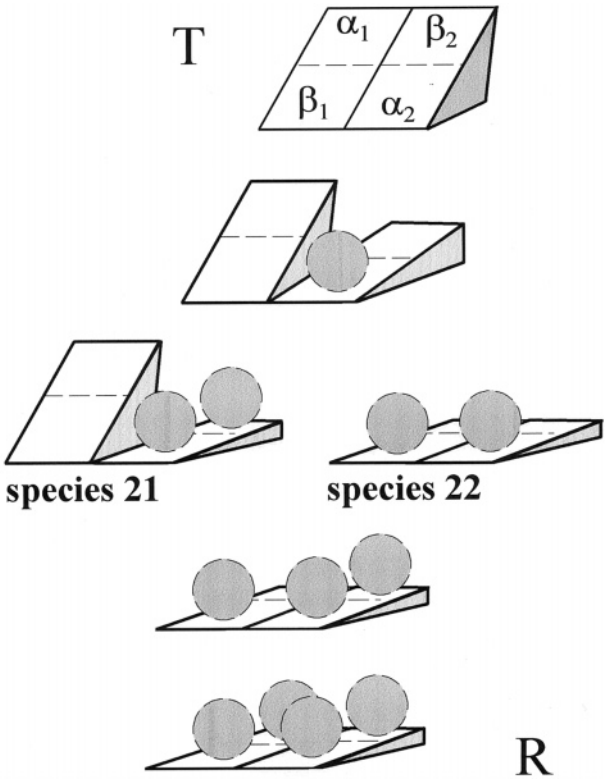

Figure 3. Mechanical analogy for ligand binding to Hb. Imagine a microscopic dump truck capable of transporting four ligands, with platform(s) having two positions. The classical two-state model (not shown) would have a single platform in equilibrium between the 'up' (low affinity) or 'down' (high affinity) position depending on the number of ligands bound, but not on their distribution. For the symmetry rule shown above, the truck has two adjacent platforms capable of transporting two ligands each. When empty (top), the platforms are in the up position of low ligand affinity. Loading of a ligand will lower the platform and allow binding of a second ligand to the same platform (dimer) with a higher affinity. Binding of three or four ligands (bottom) insures that both platforms are in the high-affinity down position. The position of the platforms depends on the arrangement of the ligands. The preferred binding of the second ligand is to complete saturation of the singly bound platform, whereas the other doubly liganded forms with one ligand on each platform (only species 21 and 22 shown) are less probable.

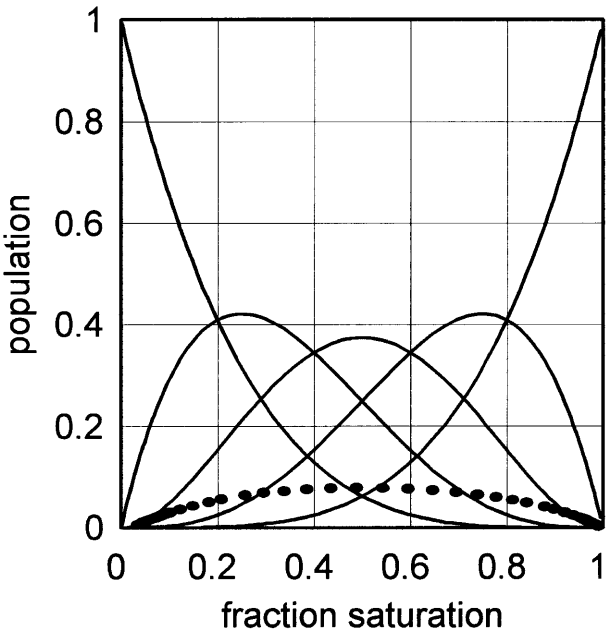

Figure 4. Fraction of species with 0 to 4 ligands bound vs. the fraction ligand saturation. A random (binomial) distribution shows a high population of partially liganded forms, for example, 37% of forms with two ligands at 50% saturation. The high cooperativity of ligand binding to Hb results in a suppression of these forms (● ●) with an overall tendency to switch between the forms with 0 and 4 ligands. Note that for conditions typical of Hb, the maximum population of doubly liganded forms is only about 5% of the total (simulation with MWC model for conditions as for fig. 1).

Also, a change in a small percentage of the sample may produce an amplified effect in a multisubunit system. For example, 2% oxidized subunits may imply nearly 8% of tetramers with one oxidized subunit; generally one needs to know the distribution to correctly account for these imperfections. The obvious conclusion is that fitting the OECs is a necessary but not sufficient condition for selecting a model. Any attempts to introduce a new model must consider these additional perturbations, individually as well as their combined effects.

Compensation of parameters

Models based on the slopes of the asymptotes require an even higher precision. As demonstrated by Imai [7], the data between 99 and 99.9% saturation may be critical for determining certain parameters. Yet the nor-

malization of the OECs will drastically change the data and slope of the asymptote in this saturation region. One example is the α-cooperon model, which imposed the extreme condition of zero oxygen affinity for β chains in the T-state; this condition implies an upper asymptote of slope 2 [32]. Despite this major difference, the OECs can still be fitted to within experimental errors. Such a shift in the slope of the upper asymptote is sensitive to a small change in the normalization factor (fig. 1). This example shows how large differences in certain microscopic parameters can be accommodated or compensated for in simulations of the OECs.

There may still remain a problem of compensation between the fitting parameters. Even the simple MWC model with only three parameters can display a high degree of compensation between parameters. Thus reports of excellent simulations and low sigma or error values do not imply an accurate value of the parameters. One also needs to consider the cross-correlation of the parameters. For the Hb-oxygen binding curves, one can often compensate a change in K_R by a change in L (fig. 1). This effect is more pronounced when the switchover point is above 2.5. Consider an example with $i_s = 3$: if 50% of the triply liganded form is T-state, one will not observe an R-state affinity of the last (fourth) ligand. This 'T-state contamination' of the upper asymptote leads to an error of over 100% in the value of K_R [33].

Thus the signal-to-noise ratio is not the only index to be considered for the data. Instead of improving the resolution of the experiment, a different approach to the problem may provide a more direct and reliable measurement of the parameter in question. This is especially true for complex biological systems, where the error in the reproducibility of the sample itself is often higher than the error of the experimental apparatus. An index of 'information to noise' may be more important than signal to noise.

It is practically impossible to assign an observed value to the pure tetramer form. One can make measurements as a function of each type of 'problem', such as percent dimer or oxidation to obtain the signature of that feature. This will allow a first-order correction to better observe the true binding curve.

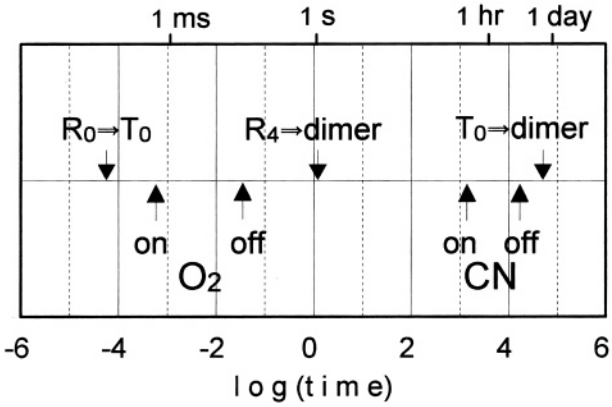

Figure 5. Time-scale of Hb transitions. As required for the physiological function, oxygen binding is relatively rapid; shown here is the off-rate from the fully liganded form, and the on-rate to deoxy Hb for air-equilibrated samples. Note that binding of the ligand CN to metHb occurs on a much slower scale (on-rate for 10 μM free CN, off-rate calculated assuming an affinity of 10^{-6} M). Absorption signals and effector binding indicate a rapid R to T transition (<100 μs), whereas the high stability of the deoxy Hb requires about 10 h for the tetramer to dimer dissociation.

MWC model

Perhaps the simplest description of the binding curve is the MWC or two-state model [1]. In this scheme a tetramer can exist in one of two conformations, the high-affinity R (or relaxed) form or the low-affinity T (tense) state; all four subunits must take on the same properties. The entire curve is described given the instrinsic ligand affinites for the two tetramer conformations, K_R and K_T, and the allosteric equilibrium coefficient of the deoxy form $L = L_0 = T_0/R_0$. The allosteric equilibrium at each ligation level is:

$$L_i = Lc^i = T_i/R_i \tag{1}$$

where c is the ratio of the affinities $c = K_R/K_T$. The MWC model is a stringently defined scheme with only three parameters to describe the oxygenation curve; on the other hand there is no restriction as to the saturation level for the allosteric switchover point $i_s = -\log(L)/\log(c)$.

The saturation of the ligand binding sites is given by:

$$Y = \frac{Lc\alpha(1+c\alpha)^3 + \alpha(1+\alpha)^3}{L(1+c\alpha)^4 + (1+\alpha)^4} \tag{2}$$

with $\alpha = [\text{ligand}]/K_R$. The partially liganded fractional populations are given by:

$$\text{MWC } f_i = [{}^N_i]\alpha^i(1+Lc^i)/[(1+\alpha)^4 + L(1+c\alpha)^4] \tag{3}$$

with the statistical factor $[{}^N_i] = N!/[i!(N-i)!]$. The analogous fractions for a random (binomial) distribution (fig. 4) are:

$$\text{binomial } f_i = [{}^N_i]Y^i(1-Y)^{4-i} \tag{4}$$

Time-scale

Typical oxygen-binding reactions occur on the μs-ms time-scale (fig. 5). For air-equilibrated samples, oxygen

binds to Hb in less than 1 ms; dissociation from the oxy form requires only 20 ms (at 37 °C), and dissociation from the T-state form will be even faster. The partially oxygenated tetramers are therefore continually exchanging ligands between subunits and oscillating between the allosteric states. Rather than being able to isolate and study these partially oxygenated forms, one can be sure that a given tetramer will change its state within 100 ms. Only the deoxy form can be incubated for longer periods of time. For this reason there has been much research based on time-resolved methods to observe the transients, use of more stable hybrids to trap the intermediates and low-temperature techniques to slow down the reactions.

The present review focuses on whether there is a difference between the doubly liganded forms. As seen in figure 4, one might claim that the discussion concerns forms representing only 5% of the total population. This is true, but the properties of these small populations will reveal the underlying mechanism of the overall protein cooperativity. Considering the large database for Hb using many techniques with excellent signals, if one cannot answer fundamental questions with this model system, imagine the freedom of interpreting data from colourless proteins.

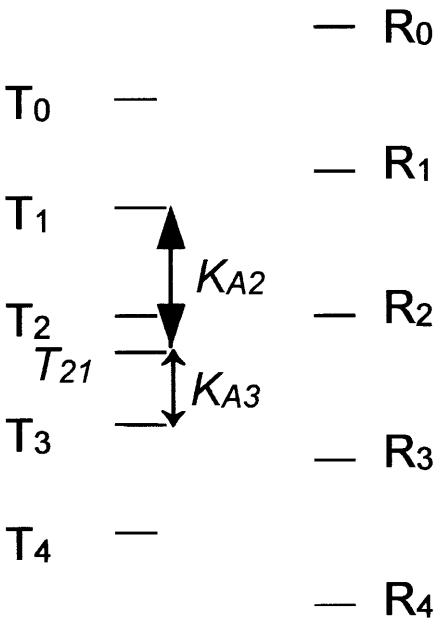

Figure 6. Energy scheme. The MWC models imposes a fixed binding energy for all transitions within the same allosteric state R or T. The SR scheme effectively moves the asymmetric species 21 to a more stable (lower) energy; the affinity for the second ligand (K_{A2}, for the transition T_1 to T_{21}) is then higher, whereas the affinity for the third ligand (K_{A3}, transition T_{21} to T_3) becomes lower.

Symmetry rule (SR) model

One can in principle deduce the energy or affinity of a reaction without directly measuring that parameter. From the closure relation (conservation of energy), it is sufficient to determine the values for the other reaction steps that complete a closed loop. For example, the distance between the fourth and fifth floor of a building could be determined by measuring the distances [fourth to sixth] and [fifth to sixth], or by measuring the transit time at a known velocity. Any other complicated pathway could be used to determine the value of such a 'state' function, but the relevant problems of measurement and accumulated errors must be accounted for.

The method employed by the laboratory of Ackers is such a strategy [2, 3, 34–37]. Direct measurements are made of the dimer-tetramer equilibria which depend on the allosteric conformation [38]; one can then deduce other parameters, such as oxygen binding, that are dependent on these same conformations. The large change in $K_{42} = [\text{dimer}]^2/[\text{tetramer}]$ upon ligation allows a classification by tetramer stability of the 10 possible tetramer species (table 1). In principle this information can then be combined with an oxygen affinity for a reference state to deduce the ligand-binding properties of all forms.

Hybrids with CN-metHb subunits were used to provide a more stable liganded form. For example, ferrous α chains can be mixed with CN-met β chains to form a symmetric doubly liganded hybrid. Other species such as the asymmetric hybrid, composed of a deoxy dimer and a liganded dimer, can only be prepared as a mixture in equilibrium with two parent forms: by mixing deoxy Hb with CN-metHb, the exchange of dimers will produce the [dimer-deoxy/dimer-CN] asymmetric hybrid (species 21). Due to the slow dissociation of deoxy Hb tetramers (fig. 5), a long incubation time is needed.

The original experiments measured the tetramer dissociation rate by observing the absorption change after mixing the Hb samples with haptoglobin [2], a natural plasma protein that specifically binds the dimers with very high affinity. These results indicated that the symmetric hybrids dissociated within seconds as for triply or fully liganded Hb. The asymmetric hybrids (species 21 and 22), measured as a mixture with parent forms, showed a slower kinetic phase characteristic of singly liganded Hb [2].

Developments of low-temperature isoelectric focusing (IEF) allowed a better quantification of the relative amounts of each species [8, chap. 12; 39–42]. To obtain distinct peaks in the IEF experiments, parent Hbs of different types are required. If HbA is used as one parent, then HbS or HbC can be used as the otherparent, since they have distinct pI values. In a typical

experiment CN-metHbA is mixed with deoxy HbS and incubated for 60 h. After equilibrium is reached, the system must be stabilized to avoid a reequilibration of the dimers: the sample is quenched by dilution in a solvent at low temperature, equilibrated under CO. The IEF reveals three peaks corresponding to pure HbA and HbS, and an intermediate peak for the hybrid [dimer-A/dimer-S].

Based on this technique, the authors relocated species 22 with the R-state family as for the symmetric species 23 and 24, and suggested that the original results with haptoglobin were due to an impurity form [41]. A high population (over 45%) of the asymmetric species 21, in equilibrium with the parent forms, supports the original report of an enhanced stability of this tetramer relative to the other doubly liganded tetramer forms. The 21 hybrid behaved more like the singly liganded tetramers in terms of the dimer-tetramer equilibrium (table 1), and was therefore classed as a T-like species.

An extra stability of species 21 would make it the most probable doubly liganded form, binding oxygen with a higher affinity relative to deoxy Hb; this effect of a higher oxygen affinity was characterized as a cooperativity within the T-state, and the dominant pathway for ligand binding via species 21 followed the 'symmetry rule'. We will refer to this energy scheme as the SR model.

The data for CN hybrids have been extended to other metal hybrids and different solvent conditions. Use of Zn-Fe hybrids allowed an indirect determination of the energy scheme for oxygen binding; the separation of species 21 by IEF proved difficult and an alternate energy pathway was employed [37]. While the calculated increase in oxygen affinity to form species 21 was not as large as the factor of 170 for CN-met samples (see table 1), the shift in energy still predicts a preferred pathway for ligand binding: the SR scheme [3]. The shift in energy is also highly dependent on the solvent pH and the metalloporphyrin used to form the hybrids. The asymmetric effect is weak (twofold or less) for use of cobalt-porphyrins or at pH 9; the total cooperativity of ligand binding is also reduced under these conditions [3, 35].

A shift in the stability of species 21 implies new ligand affinities (fig. 6). The MWC model imposes a fixed spacing within each (R or T) conformation; moving a single substate changes the distance (ligand affinity) with its nearest neighbours. At a specific shift of a factor near 1/c (of order 100 as observed for the CN-met hybrids), the second ligand binds with an R-state affinity. At other values of the asymmetric effect, the binding energies for the second and third ligands would not correspond to R or T states; the SR model would then predict four distinct ligand affinities.

Three consequences can be formulated for the SR model. (i) A shift in energy stabilizes the asymmetric hybrid (species 21) relative to the other doubly liganded form and predicts a preferred binding pathway. (ii) This implies a higher affinity for the second ligand relative to the first (cooperativity within the T-state), resulting in an initial switchover point of 1.5. (iii) Species 21 should be stable and behave like a T-state tetramer and therefore show a lower affinity for the third ligand.

Maximum cooperativity

One of the fundamental characteristics of oxygen binding to Hb is cooperativity. While various binding schemes may propose a different number of substates and interactions, the overall cooperativity is one of the necessary conditions to test the validity of a model. The standard index is the Hill coefficient n, the slope of the binding curve in the Hill plot (fig. 1). Generally, the maximum slope n_{max} occurs near 50% saturation, but this depends on the switchover point. A symmetric, or bell-shaped curve of n vs. Y occurs only for a switchover point exactly at two ligand bound ($Y = 0.5$); in this case $n_{max} = n_{50}$, the cooperativity at 50% saturation.

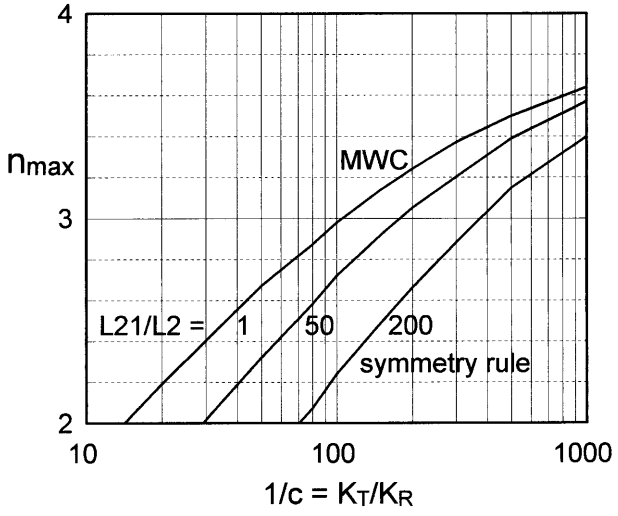

Figure 7. Modification of the cooperativity of ligand binding by introducing a change in the asymmetric effect within the two-state model. A special parameter L21 was used to provide an enhanced stability of the 21 asymmetric hybrid [dimer-deoxy/dimer-oxy]. As L21 increases, cooperativity decreases, since the affinity for binding of the second ligand increases, whereas the reverse occurs for the third ligand, leading to a T-R-T-R sequence of events. However, this loss of cooperativity can be compensated for by an increase in the ratio of the binding of the first and last ligands (either a higher ratio K_R/K_T or the introduction of quaternary enhancement to increase the affinity of the fourth ligand).

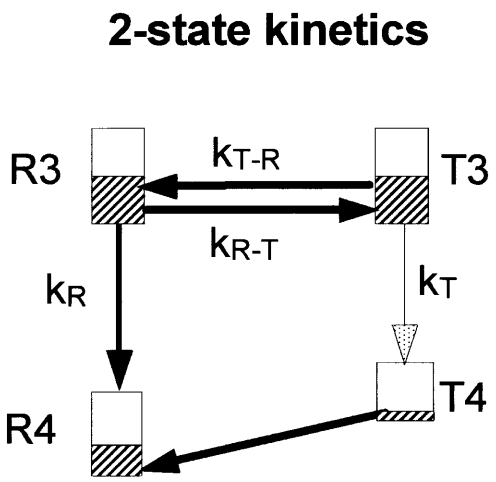

Figure 8. Mechanical analogy for kinetics, considering only four substates R_3, T_3, R_4 and T_4. If the R-T equilibration is rapid (thicker lines depict faster rates), one will observe a single phase since both R_3 and T_3 will drain via R_3 to R_4; the observed rate is then the rapid rate (k_R) modulated by the equilibrium fraction saturation of that substate. If the R-T kinetics are slow (case not shown), one will observe distinct phases for the ligand binding $R_3 \rightarrow R_4$ and $T_3 \rightarrow T_4$.

Higher values of n require larger ratios of the intrinsic R and T, or first and fourth, ligand affinities (fig. 7). Also, a larger Hill coefficient is observed when the switchover point is near 2. The maximum possible value of n cannot exceed the number of ligands involved, that is, 4 for the Hb tetramer or 2 for a cooperative dimeric system [42]. To fit the high observed cooperativity of Hb of over 3, high values of the allosteric equilibrium parameter L ($>10^4$) and the ratio of affinities 1/c (>100) are required; the maximum population of doubly liganded forms would be about 5% for this case.

For the MWC model the value and position of the n_{max} is completely variable between the theoretical limits of 1 and 4. Values below 1 could be a sign of anticooperativity, but two independent molecules with different affinities may also show a slope near zero at the intermediate saturation range, where the high affinity site is already liganded and the low affinity site is at a low saturation level.

Starting with the MWC framework, one can assign a value of L to each specific substates to study the effect of changing the allosteric equilibrium for a given species. To simulate an asymmetric effect, one can vary the value of L21 relative to its normal MWC value $L2 = Lc^2$. As can be seen in figure 7, increasing L21/L2 leads to lower cooperativity. That is, for the same values of K_R and K_T, the SR would lead to lower cooperativity [43]; equivalently, a higher ratio $c = K_R/K_T$ is needed to obtain the same cooperativity and apply the SR at the same time. For example, to obtain the observed value for Hb tetramers of $n = 3.2$, a ratio of affinites over 500 is required. Note also that the ratio of affinities between CO and oxygen is about 120 for the T-state and 240 for the R-state [44]; thus any condition imposed on the value of 1/c for oxygen would imply an increase of a factor of 2 for CO.

The R and T notation is misleading in the SR framework; it is a carryover from the MWC model where there are only two conformations. With the SR model, the shift in energy of species 21 implies a higher affinity for the second ligand relative to the first (T-state) ligand. In the special case of a large shift, as reported for the deoxy/CN hybrids, the affinity for the second ligand is nearly that of the R-state, over 100 times higher than that for the first. One then has an R-state affinity for ligand binding between two tetramers that are both classed as 'T-state'. That is, the transition from a singly liganded (T-state) tetramer to the 'T-like' species 21 has an R-like affinity as for the binding of the fourth ligand. Furthermore, a shift in energy which leads to a decrease in ΔG in one direction (between singly and doubly liganded tetramers) necessarily increases the distance in the other direction (between doubly and triply liganded forms, see fig. 6); thus binding a ligand to species 21 to form a triply liganded tetramer occurs with a lower (T-like) affinity. It is thus wrong to refer to 'the' T to R transition within the framework of the SR, because the ligands bind with a sequence of T-R-T-R affinities. This scheme necessarily has a lower cooperativity of ligand binding relative to the MWC model (for the same ratio of K1 to K4).

The lower cooperativity, due to the enhanced stability of species 21, can be compensated for with a higher ratio of K_T/K_R (fig. 7). This obviously imposes additional constraints on the observed affinities. The SR also implies a significant cooperativity at low saturation values, since the high affinity for the second ligand (transition from singly liganded to species 21) implies an initial switchover point of 1.5.

Evidence for quaternary enhancement (QE). Based on data of OEC vs. Hb concentration, it was reported that the fully liganded conformation of Hb tetramers has a higher affinity for oxygen than the dimers [45–47]. This would imply that association of dimers to tetramers provokes a quaternary effect; specifically, the affinity for the fourth liganded is augmented, while the 'R-state' affinity for the third ligand is considered similar to that of the dimers. The increased affinity for the fourth ligand is critical for the discussion of maximum cooperativity. In the simulations presented in figure 7, the ratio K_R/K_T was varied, but the maximum cooperativity could also be increased by incorporating the quaternary

enhancement, that is, increasing the affinity for the fourth ligand to increase the ratio of K_4 to K_1.

The QE is thus intimately related to the SR. The SR rule alone, with the standard values of the affinities ($K_R = K_{dimers} = K_T/100$) would lead to unacceptably low values of the cooperativity [43, see fig. 7]. Only with a higher K_R/K_T or K_4/K_1 can the SR be applied while maintaining a high maximum cooperativity. For this reason the equilibrium and kinetic studies concerning the QE will also be discussed.

A shift towards lower PO_2 values can be seen in the Hb OECs, as the total protein concentration is lower. This is due to the increased fraction of high-affinity, noncooperative dimers. Since the signal for oxygen binding to dimers is not spectrally distinct from that for tetramers, separation of the dimer and tetramer properties involves a simulation of the overall data sets at different protein concentrations. According to the authors of the original study [45], valid OECs are not feasible for samples below 0.5 μM in total haem (about 50% dimer for the liganded state under standard conditions) due to the low signal amplitude.

The amount of dimers changes along the saturation curve, being near zero for the deoxy forms. Since the actual amount of dimers cannot be observed, the QE parameter is included in a model-dependent fashion. Concentrations on the millimolar range are feasible using thin cells, and the OECs for predominantly tetramer solutions can be measured. However, as demonstrated with the simulations in figure 1, the precision in the value for the last ligand from equilibrium curves is not as high as for the first ligand.

Separation of species at low temperature

The low-temperature IEF techniques have allowed a trapping of the partially liganded intermediates [48, 49]. The low temperature quenching decreases the rate for dimer or ligand interchange, and the IEF then allows a separation and determination of the relative populations. This technique provides experimentally determined populations for comparison to the model-dependent predictions. Since ligand binding is cooperative, the populations are expected to be low relative to a random distribution of ligands among the four sites. The results confirm the low equilibrium populations, with maximum values of less than 5% for the doubly liganded (with CO) tetramers [48].

Based on dimer-tetramer studies, CO and oxygen binding show the same energy scheme for all 10 substates to within experimental errors [36]. For the low-temperature IEF studies with CO as ligand, the sum of the population of species 21 and 22 (separation of these two forms was not possible) was similar to the sum of the populations of the symmetric hybrids 23 plus 24 [39]. Thus there cannot be a large enhancement of the population of species 21 relative to the other doubly liganded forms. Overall the difference between the two symmetric hybrids (alpha vs. beta) is similar to the difference between the symmetric and asymmetric hybrids.

In a more recent study, the contributions of the species 21 and 22 were estimated [49]; they reported a ratio of $[21]/[22] = 1.5$. These data would support a modest asymmetric effect, but it is far from the value of 170 for CN-met hybrids [3] that predict a major change in the binding pathway.

Mixed metal Co/Fe hybrids

Rather than using tightly bound ligands to obtain stable hybrids, one can also modify the metal atom of the porphyrin [50–60]. Since certain metals do not bind oxygen or CO, one can obtain model systems for the deoxy conformations. Cobalt porphyrin is a useful form, since it has a much lower affinity for oxygen and does not bind CO.

In addition, cross-linking of Hb tetramers can be used to stabilize the tetramer and prevent dimer exchange [50, 53]. This cross-linking technology has proved useful for preparing blood substitutes based on Hb solutions. The cross-linked tetramers may show moderate changes in the ligand affinity and allosteric transition, but still show a fully functional, cooperative Hb tetramer. In this way one can stabilize the asymmetric hybrid to allow its isolation for subsequent study of ligand-binding properties. Using this method, the full family of Hb tetramer species were prepared [53].

Studies of the oxygen binding to the partially liganded species showed a similar behaviour for tetramers with the same number of ligands. Specifically, species 21 showed an R-like ligand binding, similar to the other doubly liganded and triply liganded forms. Larger differences were observed for the α, β chains than for the asymmetric vs. symmetric hybrids [53].

The dimer-tetramer equilibria studies on hybrids involving cobalt porphyrins also showed a much lower asymmetric effect [35], with the enhanced affinity for species 21 of about a factor of 2. The studies of oxygen binding [53] and dimer-tetramer equilibria could thus be considered in agreement that the asymmetric effect is weak for the iron/cobalt hybrids.

Oxygen binding to species 21

Few direct ligand-binding studies have been made on species 21. One functional study was the oxygen equilibrium binding [7, 61]; this study requires a measurement on species 21 in an equilibrium mixture with the parent oxy and CN forms. Analysis then involves a correction for the OEC of the ferrous parent, whose

relative contribution may change with the fraction ligand saturation; to minimize this correction, a large excess of the CN-metHb parent was used to enhance the fraction of ferrous haems occurring as the hybrid form [61]. Using about 5 mM Hb with over 90% cyanometHb, the OEC was measured for the mixture using the thin-cell method [61].

The extracted oxygen-binding parameters for species 21 indicated a low T-like affinity ($P_{median} = 6$ mmHg) and a high cooperativity 1.94 [61]. This would be consistent with the SR, where species 21 is in a T-state conformation; the first ligand to bind to the hybrid (third ligand overall, taking into account the two CN ligands) would have a T-like affinity and the last ligand an R-like affinity. This result also demonstrates the T-R-T-R sequence of binding affinity to an Hb tetramer based on the SR, mentioned in the section on cooperativity.

While a cooperativity near 2 is not surprising for Hb tetramers, it implies a rather extreme condition for a system with two binding sites. The observed cooperativity of 1.94 is close to the theoretical maximum of 2 and implies a large ratio of affinities for the two ligands. As reported [61], the difference in energy for the two ligands that bind to species 21 is 4 kcal/mol, or over a factor of 1000 in affinity. As indicated for the theoretical analysis, the SR would require a very high ratio of ligand-binding affinities.

Experimental factors such as loss of CN and autoxidation should be considered. Since a reducing system cannot be used for studies of valency hybrids, and low-affinity haemoproteins are known to have higher autoxidation rates [62], a T-like species 21 might show an accelerated oxidation rate. A full spectral analysis of the sample would reveal oxidation or CN loss, although for studies using a single wavelength, the overall change in absorption provides a control. For the study in question [61], the observation wavelength of 415 nm is near the isosbestic point for the transition from CN to aquo metHb, so the dominant signal should be for oxygen binding; however, a transition to aquo-met Hb might favour the low-affinity conformation [63, 64].

The oxygen-binding data for the asymmetric 21 hybrids are quite different from those for the other biliganded hybrids. As mentioned in the study, the simulations of the OEC data for species 21 showed larger residuals than for the studies of the other hybrids [61]. While the analysis takes into account dimer exchange, one also needs to take into account the stability of species 21 and the kinetics for the dimer exchange, since it would lead to a difference in the substate populations. A simulation of the kinetics of the reequilibration of the tetramer species via dimer exchange reveals two limiting cases. If the dissociation of species 21 is slow (>100 s), then the amount of free dimers is low and reequilibration is slow compared with the OEC measurement. On the other hand, if the SR is not valid and species 21 dissociates on the order of a few seconds, then the final stages of the deoxygenation curve would involve an accumulation of the deoxy parent Hb. The exact proportions depend on the relative stability of the tetramers; for values typical of the MWC model (table 1), species 21 would represent less than 10% of the ferrous sites in the final (deoxy) state. The OEC of the mixture does not necessarily measure the properties of species 21; the results of this study are consistent with the SR, but do not offer an independent support of the model.

Crystals or gels of deoxy Hb

The allosteric transition in Hb is such a fundamental property of the protein that preventing it is no easy task. Exposing a crystal of deoxy Hb to an oxygenated solution results in a broken crystal. Strong effectors have also been used to obtain T-like Hb with three ligands, but the fully liganded form still shows R-like properties. Crystals grown in poly(ethylene glycol) (peg) provide a more flexible environment to accommodate small structural changes, allowing the measurement of oxygenation curves [65, 66].

Crystals. The peg crystal of deoxy Hb binds all four ligands with a T-like affinity, showing little apparent cooperativity [65]. The low-oxygen affinity of the Hb crystal is similar to that observed for the binding of the first ligand to Hb in the presence of strong effectors. One advantage of a crystal is the fixed orientation of the haem groups; this permits a separation of the contribution of the α and β chains. The authors reported a small cooperativity within the T-state that counteracts a difference in α, β chain affinity to produce the low apparent cooperativity [66]. This effect was weak compared with the SR prediction of high cooperativity in the T-state, but one could argue that the crystal prevents the cooperativity because it prevents the overall T to R transition.

Gels. An alternative method for trapping the allosteric forms is the use of a gel matrix. The Hb tetramer maintains its conformation of insertion (oxy or deoxy) when incorporated in such gels [67]. This allows studies in a compromise environment, rigid enough to preserve the allosteric conformation, but still flexible enough to demonstrate many solution properties, such as the Bohr effect or the influence of external effectors [68]. Studies in this type of gel show that Hb is still sensitive to pH and effectors as for solution studies, yet they show noncooperative ligand binding. If a gel of oxyHb is prepared, the OECs show a noncooperative binding with the R-state affinity [67, 68].

Effectors. Studies have also been made with Hb in the presence of strong effectors [69], or cross-linked Hb [70], that shift the allosteric equilibrium towards the

low-affinity form. In these cases the low-affinity structure seems favoured for Hb with as many as three ligands, but the fully liganded tetramer still shows R-like properties (such as geminate recombination). The OECs show a low cooperativity and a late switchover point (>3 ligands).

For the crystals, the gels and the strong effectors, the OECs show practically no cooperativity in the lower saturation region (below 50%). This is not predicted by the SR, since the favoured pathway of oxygen binding to species 21 predicts a significant cooperativity of oxygen binding at low oxygen saturation values. While the MWC model easily accommodates a variable switchover point, the SR is rigid in this regard.

The lack of cooperativity of R-state Hb, such as oxyHb gels or symmetric hybrids, is evidence against the QE effect. One needs to consider the normalization of the data (fig. 1) before making conclusions based on the slope (cooperativity) of the asymptotes of the OEC.

Kinetics

Kinetic measurements reveal additional features for the ligand-binding properties and transitions between substates. While equilibrium observations often correspond to an average value, the kinetics may show clearly resolved phases for each state. One advantage of kinetic methods is the higher population of partially liganded substates. While 50% saturation at equilibrium yields less than 5% of doubly liganded Hb, 50% photolysis produces amounts closer to the random distribution (fig. 4) of 37%.

Another advantage of laser photolysis is to rapidly produce substates. If ligand recombination is fast compared with the allosteric transition time, one can study ligand binding to the protein still in the preflash conformation. This is the case for the ns geminate recombination, and this phase can thus serve as a probe of the fraction of Hb in the R-state before photolysis [71]. For high concentrations of oxygen, the bimolecular rate may also exceed that for the allosteric transition; one then detects the rate for ligand binding to the R_4 conformation, which might not be identical to the R_3 conformation. Note that CO and oxygen binding are generally fast compared with the dimer-tetramer interactions; unlike equilibrium studies, the dimers make a static (R-like) contribution to the ms kinetic signals. The association of dimers, which depends on the dimer concentration $k = 10^6\ M^{-1}\ s^{-1}$ requires over 100 ms for total protein concentrations below 1 mM (about 10 μM in dimer.

New pathways may also be probed by kinetic techniques. For example, photoproduction of deoxy Hb reveals that about 100 μs are required before relaxation of R_0 to the stable T_0 deoxy Hb [72]. Kinetics of ligand binding through perturbation techniques provide a different point of view of the system. The kinetics of ligand binding must be compatible with the energy scheme. That is, the kinetically determined equilibrium constant (ratio of rates) should agree with the equilibrium value. This leaves a certain freedom in the values of the rates, but in the case of a two-state model there would be only two distinct values for each rate. One would expect a T-like on-rate to species predominantly in the T-state such as deoxy Hb, and an R-like off-rate from a liganded tetramer such as oxy Hb.

Kinetics of CO binding to Hb

The pioneering studies of rapid mixing experiments and flash photolysis by Gibson did in fact show slow T-like binding of CO to deoxy Hb, and a rapid binding of the final ligand, demonstrating the kinetic analogue of the equilibrium data [73]. At high dissociation levels that produce deoxy or singly liganded Hb, more of the slow (T-like) phase was observed. At low photolysis levels, one could isolate the reaction for the binding of the fourth ligand, which occurs with a higher rate. It is now generally accepted that the difference in the R- and T-state affinities is expressed mainly in the on-rates for CO, whereas the opposite is true for oxygen or NO binding. The flash method further demonstrated that the R to T transition was rapid relative to the ms CO rebinding, a necessary condition to observe the T-like bimolecular recombination. Any states that require a

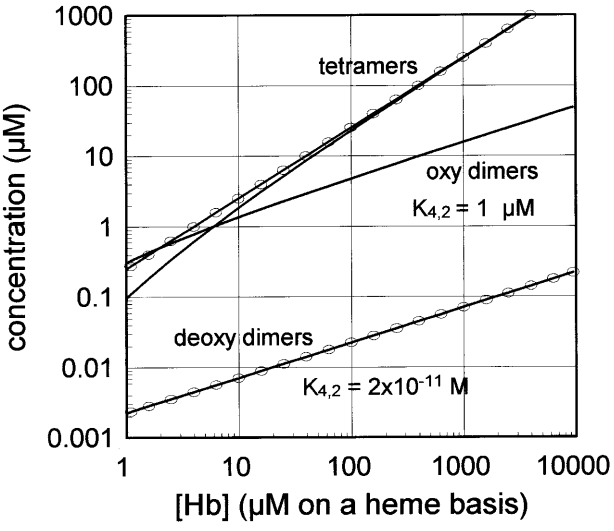

Figure 9. Concentration of tetramers and dimers for two values of K_{42}. Independent calculations were made for $K_{42} = 1$ μM, typical of oxy Hb, and 2×10^{-11} M as for deoxy Hb (○-○-○).

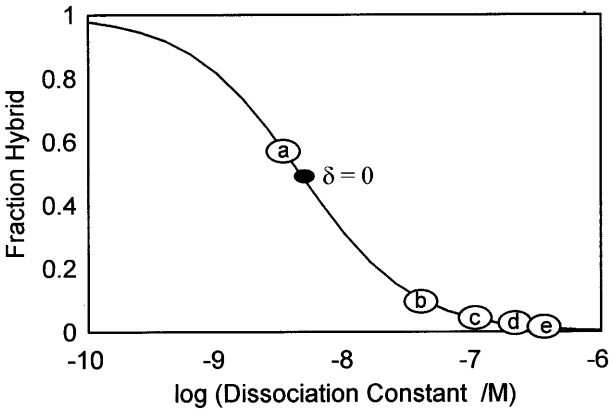

Figure 10. Asymmetric effect. Various observed or calculated values for the properties of the asymmetric hybrid (species 21): (a) deoxy/CN asymmetric hybrids [3], (b) deoxy/CN hybrids at short equilibration times [92, 96], (c) deoxy/oxy hybrids determined from studies with Fe/zinc hybrids [37], (d) a factor of 2 shift to higher stability, based on kinetics of CO binding to asymmetric hybrids [76–78], or populations of substates by low-temperature IEF and CO kinetics [39, 49], (e) MWC model [43]. The $\delta = \Delta G_{21,obs} - 1/2(\Delta G_{01} + \Delta G_{41}) = 0$ is the case of energetic additivity of the hybrid relative to the parent forms.

long time to form will not be observed. Thus the flash technique provides complementary data to equilibrium studies; in addition, a different set of populations can be produced.

CO binding kinetics to symmetric hybrids. The association of CO to symmetric valency hybrids was studied by stopped-flow mixing [74, 75]. They observed a majority (70%) of rapid R-like kinetics for CO binding to the symmetric hybrids [74], and noted that the remaining 30% occurring with the slow (T-like) rate was not compatible with a rapid equilibrium between the R-T states. When the R-T transition process is rapid compared with ligand binding, one should observe a single kinetic phase with a rate for the R-state modulated by the proportion of R-state.

To help visualize this phenomenon, consider the analogy presented in figure 8. For simplicity we consider a two-state (R and T) system and limit the description to the binding of the fourth ligand. Thus ligand binding occurs as R_3 to R_4, or T_3 to T_4, in parallel with the allosteric equilibrium R_i-T_i. Each substate can be considered as a container, where the level of liquid represents the fraction saturation. Ligand binding then occurs as a leak from the container for triply liganded Hb to the container below (for Hb with four ligands); a larger rate corresponds to a bigger hole in the container (wider transition line). Similarly, the allosteric transition depends on the rates between the R and T substates. Remember that the ligand binding (vertical transition in fig. 8) provides a large spectroscopic signal, whereas the allosteric transition is difficult to observe. Consider as an initial condition that all the liquid is in containers R_3 and T_3.

If the R_3-T_3 (horizontal) transitions are very slow, each substate will drain independently to the substate below of the same conformation. One will observe distinct kinetic phases, with amplitudes corresponding to the initial individual populations.

At the opposite extreme, if the R-T transitions are rapid compared with ligand binding (the case depicted in fig. 8), the liquid in the R_3-T_3 complex will empty via the faster pathway (which is $R_3 \rightarrow R_4$ for CO binding). A single kinetic phase will be observed, with the observed rate being that of the R-state rate modulated by the fraction population in the R-state. Thus for an equal mixture of R_3 and T_3 in rapid exchange, the T_3 container empties via the transition $T_3 \rightarrow R_3$ followed by R-state binding, and one simply observes a single phase with half the R-state rate.

Kinetics of CO binding to asymmetric hybrids

As mentioned in the previous sections, the asymmetric hybrids cannot be isolated for equilibrium studies. They can be studied as an equilibrium mixture with the parent forms, but this introduces an additional parameter for the stability of species 21, which is in fact one of the critical parameters in question. In this section we discuss the kinetic methods to produce the hybrids by selectively removing ligands from fully liganded Hb; ligand binding is used as a probe of the hybrid, paying particular attention to the delay time between production of the hybrid and the subsequent study of the ligand-binding properties. Too short a delay time and one will simply observe the properties of the original allosteric conformation. Too long an incubation, and the system will return to an equilibrium condition. We group here several results based on flash photolysis or stopped flow that were used to generate and study CO association to species 21, but differing in the incubation time of the hybrid.

Photoproduced asymmetric hybrids. The photolysis technique can be used to produce a significant amount of the asymmetric hybrids. By mixing HbCO with CN-metHb, the exchange of dimers leads to formation of CO/CN hybrids in stoichiometric proportions [36]. These mixtures can then be photodissociated to produce the deoxy/CN hybrids [76]. Under conditions of a large excess of CN-metHb, most of the CO dimers will be paired with a CN dimer. At high photodissociation levels of the CO/CN hybrid, the main CO-rebinding signal will be for binding to the asymmetric species.

According to the SR, species 21 should behave like the singly liganded tetramers, and therefore show slow (T-like) CO binding. The opposite was observed: as the

amount of CN-met-Hb was increased to enhance the fraction CO signal due to asymmetric hybrids, the fraction of slow phase decreased [76]. These experiments indicate an R-like binding of CO to the asymmetric hybrids. A similar result was observed for CO/NO [77] or CO/azide [78] hybrids.

Two conditions are necessary for observing T-like kinetics after photolysis of an R-state Hb. The R to T transition must be faster than the ligand rebinding to the R-state (1 ms for 10% CO), and the allosteric equilibrium should be greatly shifted towards the T-state (over 90% T-state). A small or intermediate fraction ($<50\%$) of T-state in rapid equilibrium with the R-state would not provide T-like kinetics; as explained in the previous section with the aid of figure 8, the alternate pathway via the R-state would lead to R-like kinetics.

Since T-like kinetics for CO binding to species 21 was not observed, either species 21 does not behave like singly liganded Hb, or more time is needed for the allosteric transition. While studies have shown the R-T transition to occur on a sub-ms time-scale [72], one could imagine that the transition for species 21 is anomalously slow.

Two experimental difficulties for the flash photolysis method should be mentioned. Dithionite cannot be used to remove oxygen, since it would reduce the CN-metHb sites. Since oxygen has a higher on-rate and lower quantum yield, its presence can increase the apparent R-like behaviour. Analysis of the kinetics at different wavelengths would help detect this problem. A second shortcoming is incomplete photodissociation of the CO-bound haems. Longer light pulses are more efficient since they can redissociate ligands that rebind rapidly via the geminate phase. With the standard 10-ns lasers, only about two-thirds of the sites can be dissociated; the exact yield depends strongly on the temperature. Both of these problems are eliminated in the stopped-flow technique, using dithionite to remove oxygen from one dimer.

Stopped flow. To consider if a longer time is necessary for the R to T transition of species 21, the stopped-flow method is useful. Double-mixing techniques allow a variable incubation time between the first mixture and the subsequent analysis step [79–81]. An oxy/X hybrid (syringe 1, where X is a stable ligand such as CO, azide or CN) can first be mixed with dithionite (syringe 2) to remove the oxygen; in a second mixing step CO is added (syringe 3) to study CO binding to the vacated sites:

Syringe 1 $Hb(O_2)_4 + Hb(O_2)_2(X)_2 + Hb(X)_4$

Syringe 2 dithionite

$$\text{— (mix 1)} \rightarrow Hb + Hb(X)_2 + Hb(X)_4$$

Syringe 3 buffer/CO

$$\text{— (mix 2)} \rightarrow Hb(CO)_4 + Hb(CO)_2(X)_2 + Hb(X)_4$$

CO binding to deoxy/azido-met hybrids. In a recent study, a solution of oxyHb + azido-metHb was prepared to form liganded O_2/azide hybrids [78]. This hybrid solution was mixed by stopped flow with dithionite to form deoxy/ferric-azide hybrids (and some parent deoxy Hb); the dissociation of azide requires at least 100 s in the presence of a few mM dithionite. After a delay, the deoxy/azide hybrids were then mixed with the CO solution. The kinetics show a mixture of rapid and slow CO binding; after correction for the deoxy parent, there would be less than 20% of the slow phase for CO binding to the hybrids [78]. This is similar to the results with flash photolysis, but with an increased delay after production of the asymmetric hybrid from about 1 ms (for the flash photolysis measurements) to 200 ms.

Note that relative to CN, use of azide as met ligand occurs as a mixture of high- and low-spin ferric forms. The high-spin forms are thought to be shifted more towards the T-state, relative to low-spin ligands [63–64].

Effect of incubation time (2–11 s): deoxy/CO hybrids. An even longer delay was used in a study where CO was used as the second ligand (X) in the initial solutions; excess CO was removed to avoid CO binding before the final mixture [79]. Species 21 was designated isomer III or I3 [79] or as isomer V [10, chap. 20]. These studies did not detect a large difference between the various hybrids. For both the symmetric and asymmetric forms, about 20% slow (T-like) kinetics was observed for CO binding to the deoxy/CO hybrids [79].

As the delay before the second mixing (addition of CO) was increased to 11 s, the fraction of slow (T-like) phase increased to about 40% [79]. Three explanations can be considered for this transition:

1) A slow R to T transition of species 21. This would require a time coefficient of about 10 s, nearly a million times slower than that observed transition times for photodissociated Hb [72]. A slow conversion was also proposed for the kinetics of CO binding to symmetric hybrids [74]. Such a slow transition, compared with ms oxygen binding (fig. 5), would not have much physiological effect.

2) Disproportionation by rearrangement of the CO ligands to form more deoxy Hb [79]. This would require a high CO off-rate from species 21 of about 0.1/s, compared with dissociation from the liganded (R-state) conformation of rate 0.01/s. This explanation would present a conflicting set of parameters, with a stable T-like species 21, a T-like CO off-rate, but at the same time a rapid R-like CO binding to species 21.

3) Another possibility is dimerization of species 21 followed by random tetramerization. The deoxy Hb would accumulate due to its higher stability. This mechanism was dismissed because the overall data fitting showed a low dimerization rate of species 21 [79]. However, this is

the critical parameter in question, and fitting routines may not be efficient at exploring different pathways. This effect was also observed for use of deoxy/azide hybrids [78], where the ligand disproportionation reaction (2) should not occur on this time-scale. A rapid dimerization of species 21 would not be compatible with the SR, since the high stability of species 21 is a basic tenet of the SR.

CO binding to deoxy/NO hybrids. The stopped-flow experiment of Cassoly [82] is on a still longer time-scale, since samples of oxy/NO hybrids were deoxygenated and kept several minutes before stopped-flow studies with CO. This experiment is effectively the longtime extrapolation of the double-mixing experiment [79], which showed an increase in the fraction slow phase as the delay time increased. The kinetics to the deoxy/NO hybrids showed essentially slow phase; this result supports the SR prediction of slow CO binding to species 21. However, the results would also be compatible with the MWC model if species 21 is not stable and dissociates to reform the deoxy parent. It is thus necessary to know if the hybrids did or did not dissociate, equivalent to the original question concerning the stability of these forms. There was a difference in the absorption spectrum of the deoxy/NO mixture relative to a simple sum of the two parent spectra [82]. This would imply that the sample was not simply the sum of the parent forms.

NO hybrids

Hybrids have been formed using NO as ligand, since it also forms a stable complex. The dissociation of NO from the liganded conformation requires several hours; the difference in allosteric states is expressed in the off-rate for NO, and dissociation from the deoxy conformation requires a few minutes. One of the disadvantages of NO is the chain heterogeneity, as NO binds preferentially to the α chains [83]. Another property is that NO binding to the iron atom may weaken or break the bond between the proximal histidine and the iron atom [84–86]. Thus one may create a situation of a liganded subunit, but with the proximal histidine remaining in a deoxy position.

NO dissociation. The dissociation of NO from asymmetric hybrids has been studied [41]. The hybrids show a rapid off-rate, as for the deoxy conformation. However, the argument presented for a mixture of R-T conformations (fig. 8) applies here as well. A 50-50 mixture of R and T forms in rapid equilibrium will drain via the container with the rapid rate, in this case the deoxy conformation. In the absence of a reference state, one can only conclude that the hybrid is not predominantly in the liganded conformation. The data could be compatible with the roughly equal mixtures of allosteric states.

CO/NO hybrids. Another study involved use of NO hybrids with two or three NO ligands. It is known that Hb-$(NO)_4$ in the presence of inositol hexaphosphate (IHP) can take on the T-state conformation. For example, the sickle cell HbS-NO + IHP will participate in polymer formation as for deoxy HbS [84]. The iron atom is in a pentacoordinated configuration in this case, due to rupture of the iron proximal histidine bond. The haem-haem interaction apparently requires this pathway: ligand binding pulls on the iron atom which brings the proximal histidine with it, which in turn shifts the position of the F-helix. Without the Fe-His bond the information of ligand binding is not transmitted to the other subunits. Thus the allosteric equilibrium may be more dependent on the position of the proximal histidine than the actual ligation state of the iron atom. Normally they are perfectly correlated, but NO binding which ruptures the Fe-His bond leaves the His in the deoxy position, and that is the information conveyed to the neighbouring subunits. In the case of triply NO Hb with IHP, CO binding occurs to the fourth subunit with a slow T-like rate [77]. In fact, there is little geminate recombination, indicating that the $Hb(NO)_3CO$ form is in the deoxy conformation (even before photodissociation).

As mentioned above, mixing of HbCO with HbNO leads to [dimer-CO/dimer-NO] hybrids. Photodissociation of this form produces a species 21 (deoxy dimer/NO dimer); CO recombination is rapid, as for the CO/CN and CO/azide experiments, which is not compatible with the SR.

Addition of IHP to the CO/NO hybrids shifts the allosteric equilibrium towards the T-state, and the CO recombination is then slow; this was also observed for the CO/CN hybrids and the symmetric hybrids. However, a larger fraction slow was observed for the [dimer-CO/dimer-NO] + IHP hybrid than for the analogous symmetric hybrids [77]. This is evidence in favour of a dependence of the allosteric equilibrium on the distribution of the ligands. The shift is in the same direction as predicted by the symmetry rule and thus supports a moderate asymmetric effect.

Dissociation of deoxy/NO asymmetric hybrids. Using the low-temperature IEF technique to quantify the populations of the substates, Perrella et al. followed the amount of this species 21 vs. time [41]. During the first 35 s there was a modest decrease in the amount of the deoxy/NO hybrids. A significant decrease in species 21 after a few minutes was attributed in part to the disproportionation reaction, as evidenced by the formation of the singly and triply NO forms. This result is relevant to the sample preparation of the deoxy/NO hybrids mentioned above. When the Hb is partially T-state, the NO dissociation requires only a few minutes; the deoxy/NO

asymmetric hybrid therefore cannot be considered a stable form on the order of minutes.

Kinetics of ligand binding to deoxy Hb

A fundamental analysis of the Hb ligand-binding pathway is to determine the kinetics of individual binding steps. When deoxy Hb is mixed with a solution of ligand, the binding of all four ligands can be followed. As for flash photolysis studies, CO as ligand has the advantage of showing a large difference in the R and T association rates, and the time-scale for CO binding is suitable for stopped-flow studies (while oxygen binding is often too rapid). Unlike the flash technique, starting with a deoxy sample helps eliminate the dimer contribution. An acceleration of the kinetics after binding a few ligands reveals the switchover point from the slow deoxy Hb to the rapid liganded conformation.

The CO kinetic study shows that the first two ligands bind with similar rate coefficients [10, chap. 25, 49], some 30 times slower than the fourth ligand. This supports the simple two-state model, which predicts a change in on-rate after binding of the second or third ligand in these conditions. The data are not consistent with the SR model, which implies a new affinity for the second ligand. One could argue that the new affinity for species 21 is due to a different off-rate (rather that a change in on-rate), but this would imply a very high CO off-rate, which has never been observed.

A recent study also shows a small difference (factor of 2) in rates for binding of the first and second ligand [49]. In this study combining the CO kinetics and analysis of the substate populations, the relative amounts of doubly liganded forms did not vary by more than a factor of 2 [49]. The difference between the α and β chains was thus comparable to the asymmetric effect.

Quaternary enhancement

Kinetic methods have also been applied to study the quaternary enhancement effect [87–91]. The advantage of the kinetic method is the observation of resolved processes for the different forms, provided the dimer-tetramer reequilibration is slow compared with the ligand binding. Note that this condition is just the opposite of the description of ligand binding to different allosteric conformations (fig. 8) when the R-T reequilibration is fast compared to ligand binding. In the case of dimers and tetramers (fig. 9), the dimer-tetramer reaction is generally slow compared with the ms ligand binding, and discrete phases can be observed.

Distinct rates have been observed for β chains as a function of protein concentration. The monomers show a threefold faster CO association rate compared with β_4 tetramers [87]. There is thus an observable effect on the ligand-binding rates when β chains assemble to form tetramers. No such change was observed for Hb dimers vs. tetramers [88–91]. The binding of the fourth ligand shows the same kinetic properties as the dimer.

Note that since signal averaging can be employed for the kinetic experiments, a better signal to noise can be obtained for the small signals, relative to OEC experiments that require about 1 h. Furthermore, when the kinetic phases are resolved, even a small percentage of a phase with distinct rate will be detected, as opposed to equilibrium studies that show an averaged value.

Electron transfer

It is clear that there are conflicting reports concerning the properties of species 21. The dimer-tetramer equilibrium studies predict special properties of this asymmetric hybrid, whereas most ligand binding studies have not detected these properties. As mentioned above, asymmetric hybrid experiments require a very different time-scale from that for the O_2 or CO kinetics. Any change in sample during the long incubation of over 48 h would certainly invalidate the conclusions based on that method. One such transformation has been suggested: according to Shibayama et al. [92], electron transfer may occur between Hb subunits. That is, the ferrous iron in deoxy subunits loses an electron (to become Fe^{3+}), whereas the oxidized subunits accept the electron and end up in the ferrous state. This would randomize the arrangement of the oxidized subunits within the tetramer and result in a mixture of hybrids and ligation states.

The SR scheme is based essentially on the data that show a high population for species 21 in equilibrium with the deoxy and CN-metHb parents (fig. 10). Fifty percent hybrid is expected for a random mixture of CN-met subunits. If electron transfer occurs which produces a randomizing effect, the high population of hybrid would not reflect the properties of species 21.

The electron transfer was demonstrated by separating the Hb species (HbA and HbC) after incubation and showing that they were no longer fully ferrous or ferric. In addition, there was a loss of CN from the samples leading to formation of aquo-metHb. The loss of CN is plausible, since reduction of the haem iron to the ferrous state greatly reduces its affinity for CN [93], so the CN would dissociate from the newly reduced haem and enter the solvent. If the free CN concentration is low, the slow association of CN ($\tau = 15$ min at 10 μM, see fig. 5) might favour loss to the gas phase over CN rebinding.

Aquomet versus cyanomet Hb. In the response to this proposed mechanism, data were presented confirming the electron transfer from deoxy to aquo-metHb subunits [94]; the authors showed that for samples prepared

with aquo-met subunits, several additional bands appear in the IEF. However, they did not accept electron transfer to CN-met subunits, since the additional bands were not present when excess CN was used to insure CN-met subunits. There is agreement that electron transfer occurs between Hb subunits; the open question concerns the actual rates for the various conformations and ligands. Specifically, the rate of transfer from deoxy to CN-met subunits is a critical parameter for the dimer-tetramer equilibria studies.

While the argument that the IEF will reveal electron transfer by the presence of additional bands is valid for the aquomet subunits [94], it is not the case for CO/CN-bound subunits. CO and met-CN forms migrate to the same pI; a single IEF band could therefore include a mixture of CO- and CN-bound subunits. No results were reported for experiments of separating the two types of Hb (A and S) to determine the fraction oxidized of each, or analysis of the Hb forming the individual IEF bands to determine the fraction CO or CN.

Fraction of sites saturated = fraction of time liganded. Even if electron transfer occurs only to aquomet subunits, it cannot be neglected. According to the conditions used and the affinity of metHb for cyanide, and confirmed by controls made with and without a large excess of CN, the oxidized subunits are not more than 97% saturated with CN [78, 94]. Since the dissociation rate of CN is rapid compared with the incubation time, the oxidized haems will be temporarily without CN, and CN association is slow (about 15 min at 10 μM free CN). Equivalently, the fraction of haems not saturated with CN corresponds to the fraction of time spent without ligand. Thus 97% saturation with CN means that 3% of the time (over 1 h) is spent without CN bound. This alone could account for significant electron transfer.

Electron transfer might also explain the biphasic approach to equilibrium of the Hb mixtures, just after deoxygenation [95, 96]. The biphasic kinetics suggest two physical reactions, since a single reaction phase is expected if only the two parent forms and the asymmetric hybrid are involved. The biphasic kinetics thus imply an additional mechanism is involved.

Such a mechanism has previously been demonstrated for other metalloporphyrins [97, 98], and the electron transfer rate could be enhanced by other molecules [99]. A lower rate at elevated pH [96] would indicate a pH dependence of the transfer rate. The electron transfer under conditions which maintain CN-metHb has since been confirmed [78, 96]. Additional electron transfer measurements would be necessary to test the other hybrid systems. In general, new experiments designed to

Table 1. Dimer tetramer assembly free energies of the 10 Hb substates.

Ligation state (Dimer 1)	(Dimer 2)	Species	L = [T]/[R]	τ (s)	K_{42} (μM)	ΔG	ΔG (kcal/mol)	ΔG
			MWC	MWC	MWC	MWC	$Fe/Fe^{+}CN$	Fe/FeO_2
(α_1 β_1)	(α_2 β_2)	**01**	33500	48050	0.000021	−14.4	−14.4	−14.4
(α-X β)	(α β)	**11**	183	264	0.0037	−11.35	−11.3	−11.6
(α β-X)	(α β)	**12**	183	264	0.0037	−11.35	−11.2	−11.6
(α-X β-X)	(α β)	**21**	1	2.9	0.34	−8.7	−11.4	−9.4
(α-X β)	(α β-X)	**22**	1	2.9	0.34	−8.7	−8.3	−7.7
(α-X β)	(α-X β)	**23**	1	2.9	0.34	−8.7	−8.2	−7.6
(α β-X)	(α β-X)	**24**	1	2.9	0.34	−8.7	−8.5	−7.8
(α-X β-X)	(α β-X)	**31**	0.0055	1.4	0.69	−8.3	−8.6	−7.5
(α-X β-X)	(α-X β)	**32**	0.0055	1.4	0.69	−8.3	−8.4	−7.5
(α-X β-X)	(α-X β-X)	**41**	0.00003	1.4	0.7	−8.3	−8.5	−8.1
Cooperativity within dimer 1:			$(\Delta G_{11} - \Delta G_{01}) - (\Delta G_{21} - \Delta G_{11}) =$			0.4	3.2	0.6
						Ratio (no units)		
Ratio of oxygen dissociation constants (pathway 01-11-21)						2	237	2.8

Hb species ij denotes tetramers with i ligands (X). Experimental data are for valency hybrids $Fe/Fe^{+}CN$ and Fe/FeO_2 (derived from Zn/Fe hybrids) [94]. The dimer-tetramer equilibria energies ΔG are in kcal/mol, where 1 kcal = 4184 Joules. The dimer-tetramer molar equilibrium coefficient is given as $K_{42}/(1M) = \exp(\Delta G/RT)$ calculated at 21.5 °C (RT = 0.585 kcal/mol). For the MWC model, L for state 01 was determined from the experimental free energy for deoxy Hb of −14.4 kcal/mol; a reference state for species 41 of −8.3 kcal/mol was used, and 1/c = 183 to provide $Lc^2 = 1$. The lifetime τ is the reciprocal of the tetramer to dimer dissociation rate, assuming an association rate of 10^6 M^{-1} s^{-1} for all types of dimer. The cooperativity 'within dimer 1', refers to the ratio of oxygen dissociation constants for the first ligand (species 01 and 11) to the second ligand (species 11 and 21) in the same dimer, derived from $(\Delta G11 - \Delta G_{01}) - (\Delta G_{21} - \Delta G_{11})$ in kcal/mol, or as the ratio (L0 + 1)(L2 + 1)/(L1 + 1)(L1 + 1) in the MWC scheme.

study specific substates and transitions are necessary to reveal the underlying mechanism of cooperative ligand binding to haemoglobin.

Summary

The SR model, based on dimer-tetramer equilibrium studies, predicts an enhanced stability of species 21. This implies an increased affinity for the second ligand (transition T_1 to species 21). Evidence of this preferred binding pathway would be a higher stability of species 21, a higher affinity for the second ligand, a higher population of species 21 relative to other doubly liganded species at equilibrium, or a low (T-like) affinity for the binding of the third ligand to species 21.

Simulations of oxygen equilibrium curves do not distinguish these features. There is too much freedom for individual parameters to prefer one model over another. It is clear that many microscopic models can provide similar macroscopic results. One can only make restrictive claims about certain parameters such as a minimum ratio of affinities K4 to K1 (fig. 7). From the OEC alone, it seems pointless to attempt to prove the validity of any given model.

Critical to any analysis is whether the species in question is in fact present at the predicted amount. If species 21 is as stable as singly liganded, then T-like properties seem to confirm this hypothesis; if species 21 is not so stable, then the parent deoxy Hb is preferentially formed and one also expects observation of T-like parameters.

An additional problem is intrepreting various experimental data, especially when it is presented in an unfamiliar format. Consider the data presented in table 1; we reproduce here the dimer-tetramer equilibrium ΔG_2 energies for two experimental systems of Ackers and colleagues. For comparison, the same energies were calculated for the two-state model, which imposes the same energy for tetramers with the same number of ligands. In addition, the dimer-tetramer equilibrium coefficient K_{42} and the allosteric equilibrium coefficient L are shown for the MWC model. Comparing the MWC model to the dimer-tetramer equilibrium data for the Fe/Fe^+CN system, the dominant shift is for the 21 species.

The increase in affinity for the second ligand (forming species 21) has been described as a 'cooperativity within the T-state', since the deoxy, singly liganded and species 21 were all classed as T-states [3]. The calculated cooperativity considers the pathway 01-11-21 (see table 1), which considers the ligand affinity without an explicit equilibrium between R and T states. This presents an ambiguity for comparison to the two-state (MWC) model. Considering only the T-states, there is by definition no change in K_T versus the number of ligands bound. However, by the proposed pathway which compares the overall affinity of the second ligand relative to the first, a cooperativity of ligand binding is certainly present in the MWC model whenever significant R-state is present at the doubly liganded level: a factor of 2 is calculated for an equilibrium of 50% ($L2 = 1$) of each conformation (table 1). For the Fe/Fe^+ data, the calculated ratio of oxygen affinities is 237 ($\Delta\Delta G$ of 3.2 kcal/mol); this value was apparently rounded off to 3 kcal/mol when reported as a factor of 170 [3, 94].

Application of this definition (pathway 01-11-21) does not seem to have been uniformly employed; the origin of the reported value of 40 for the $Fe/Fe\text{-}O_2$ (equivalent to the Zn/Fe) is not obvious; Huang et al. [34] indicated an energy of 2.2 kcal/mol (factor of 40), but this value is only the ΔG between 11 and 21 tetramers. It was pointed out (by N. Shibayama) that the correct value based on the reported free energies is $[(14.4 - 11.6) - (11.6 - 9.4)] = 0.6$ kcal/mol or a factor of 2.8, which is consistent with the simple two-state model. Apparently only the Fe/Fe^+CN dimer-tetramer equilibrium data predict a strong cooperativity of the binding of the first two ligands. In fact one can consider that 9 of the 10 energies are in agreement between the MWC and Fe/Fe^+ CN data (previously 8 of 10 species when analysed in 1986 [16]), and the special energy for species 21 is derived from the special experimental conditions involving the long incubation time.

Furthermore, in the Fe/FeO_2 system, rather than a large increase in stability of species 21, the other three doubly liganded tetramers are shifted in the opposite direction relative to the MWC or Fe/Fe^+CN values. This maintains a preference for ligand binding to species 21, but undermines the physical explanation of why species 21 is special. Overall, one can see that the differences between the chains or the model systems employed are as large as the asymmetric effect.

Under certain conditions there is general agreement that the asymmetric effect is weak or nonexistant, such as at high pH, or using hybrids with cobalt-porphyrin. The fact that certain effects are specific to the metal atom or the ligand used again show the danger of using alternate model systems to mimic the ligation state of Hb. Specifically, deoxy cobalt-porphyrin-globin is not as stable as deoxy Hb, and under certain conditions NO bound subunits may behave as unliganded species. In many cases, use of cyano-metHb appears to be a valid model for liganded Hb. The discrepency concerning the SR does not appear to be the choice of cyano-metHb as model, but rather involves the experimental method.

The main evidence for the SR model is the enhanced stability of species 21 deduced from the dimer-tetramer equilibrium experiments involving a long incubation time. Studies of the populations of partially liganded

Hb [39, 41] and ligand binding indicate that the difference between the chains is of the same order of magnitude as the asymmetric effect; kinetics of ligand binding did not show T-like properties for the asymmetric hybrid.

There is thus a major conflict in the results of the dimer-tetramer studies, and the functional studies which show a weak or no asymmetric effect. A systematic error has been proposed for the dimer-tetramer experiments involving a long incubation time: an electron transfer would randomize the oxidized subunits and explain the high population attributed to species 21. If the electron transfer data are valid, the principal evidence for a large asymmetric effect would disappear. As the electron transfer results are quite recent, they should also be critically analysed. In any case, valid conclusions can be obtained only through a combination of experiments, taking into account the various substates involved and the kinetics of transition between the various conformations.

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