HEMOGLOBIN ALLOSTERY

Experiments, theories and problems

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HEMOGLOBIN REVERSIBLY BINDS OXYGEN

How do we study this reaction?

1) We can measure free and bound oxygen by gas manometric methods (this requires high Hb concentration and the destruction of the sample).

2) We can measure free oxygen by gas manometric or potentiometric methods (or total oxygen by volumetric methods) and the oxygen saturation of Hb by calorimetry or by absorbance spectrophotometry

$$A = \varepsilon_{HbO_2}[HbO_2] + \varepsilon_{Hb}[Hb] =$$
$$= \varepsilon_{HbO_2}[HbO_2] + \varepsilon_{Hb}([Hb]_t - [HbO_2]) =$$
$$= \varepsilon_{Hb}[Hb]_t + \Delta\varepsilon[HbO_2]$$

$$[HbO_2] / [Hb]_t = (A / [Hb]_t - \varepsilon_{Hb}) / \Delta\varepsilon$$

Some features of Hb made it a "model" protein for the study of ligand binding:

1) it is abundant and easy to purify;

2) it contains 0.335% iron: this easily lead to the determination of its minimum MW at 16,700 (by Zinoffski already in 1885);

3) it binds oxygen: since gases can be easily measured its binding capacity could also be estimated early (at 1.34 mL/g by Huffner in 1884), leading to a Fe:O_2 stoichiometric ratio of 1:1;

4) its functional properties are astonishingly complex (= interesting).

As Christian Bohr discovered more than a century ago, hemoglobin binds oxygen cooperatively, i.e. its affinity for the gas increases with its fractional saturation.

Adair demonstrated in 1925 that hemoglobin is a tetramer and binds four molecules of oxygen; thus cooperativity implies that the first molcule of the gas is bound with lower affinity than the fourth.

Conclusion: at least two energy states of the macromolecule must exhist, one with low, the other with high oxygen affinity.

A convenient visual representation of the O_2 binding isotherm is the Hill plot, which directly derives from the mass law:

$$Hb + nO_2 \rightleftharpoons Hb\,(O_2)_n$$

$$K = \frac{[Hb\,(O_2)_n]}{[Hb]\,[O_2]^n}$$

$$\frac{[Hb\,(O_2)_n]}{[Hb]} = K\,[O_2]^n$$

$$\log \frac{Y}{(1-Y)} = \log K + n \log [O_2]$$

$$\text{with } Y = \frac{[Hb\,(O_2)_n]}{[Hb\,(O_2)_n] + [Hb]}$$

As noticed by Hill, cooperativity implies that the "extreme" ligation states, Hb and $Hb(O_2)_4$ are over-represented, whereas the ligation intermediates, HbO_2, $Hb(O_2)_2$ and $Hb(O_2)_3$, are under-represented (indeed the Hill equation assumes ligation intermediates to be absent altogether).

(the population of ligation intermediates in a non-cooperative tetramer would be purely statistical and would obey a binomial distribution:

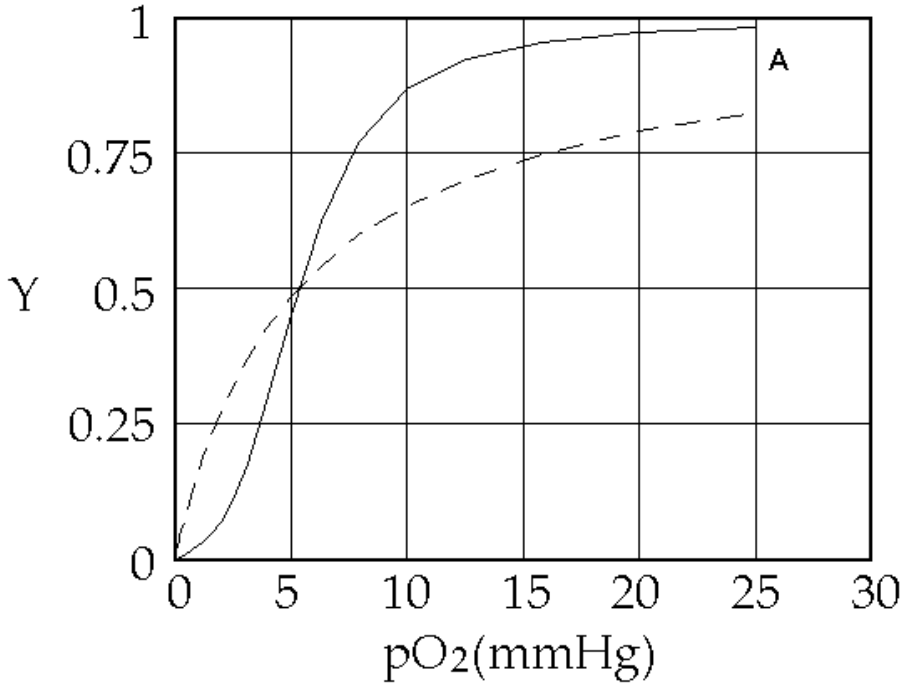
$$1 = [\ Y + (1\text{-}Y)\ ]^4$$

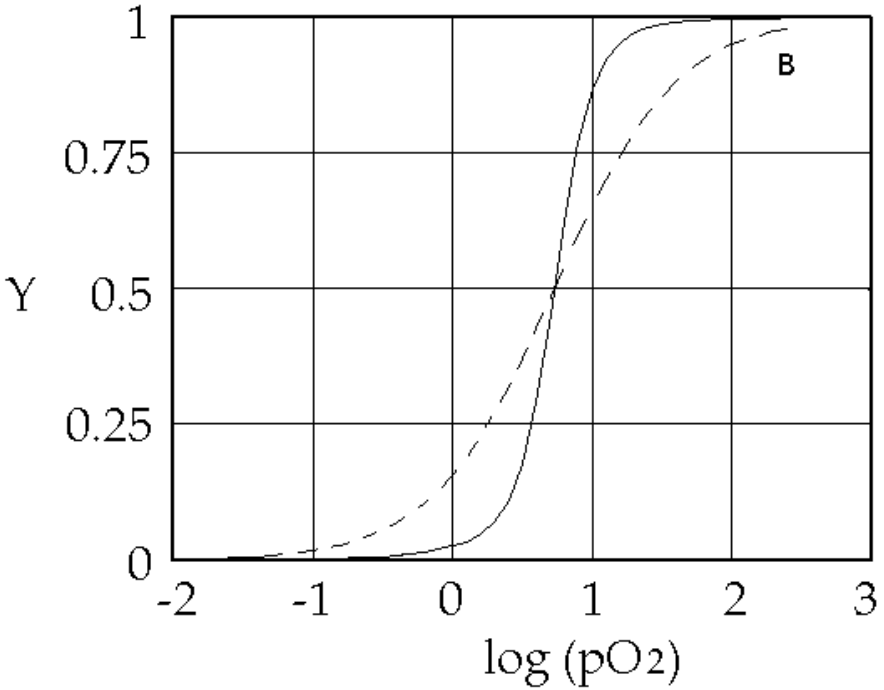
with Y^4 fully liganded tetramers, $4\ Y^3(1\text{-}Y)$ triply liganded tetramers ...)

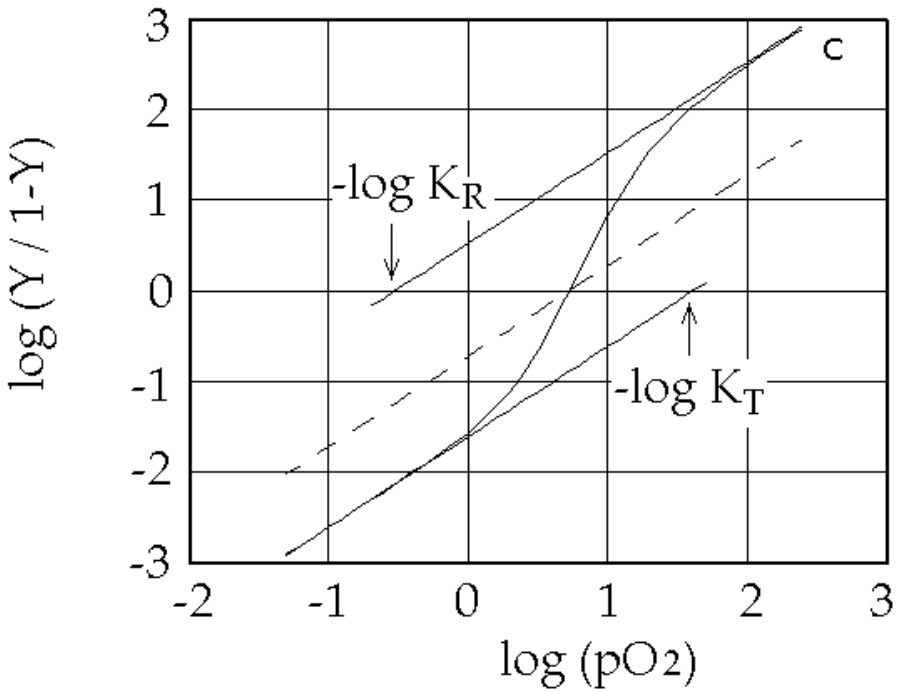
Hill's original theory, developed in 1913, is obsolete and there is no point in revieweing it, except for historical reasons.

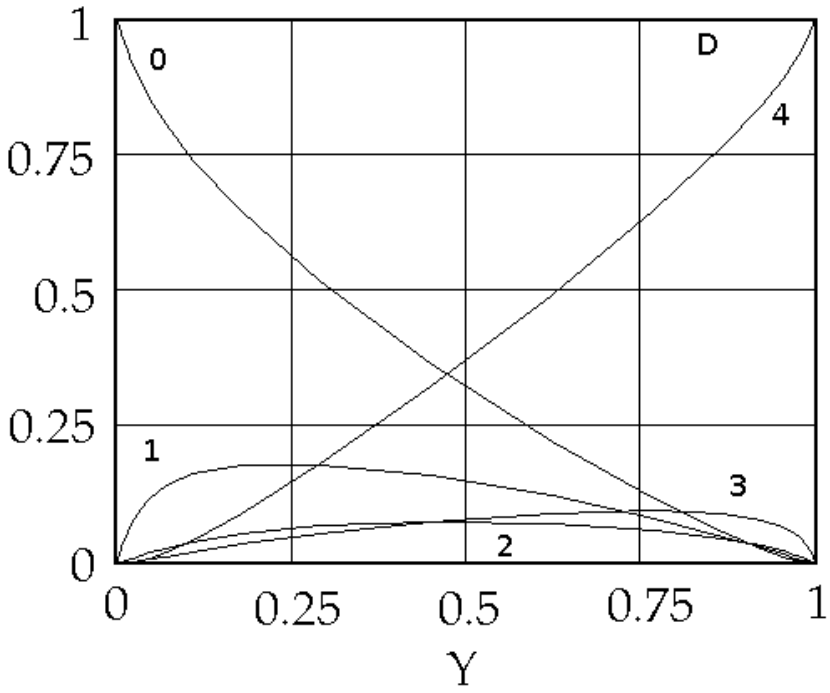
Suffice it to say that the Hill plot of the O_2 binding isotherm of Hb has two n=1 asymptotes, not anticipated by Hill.

What matters to the present discussion is that a one-energy-state hemoprotein (e.g. Mb or the β_4 homotetramer) presents n=1 over the whole O_2 binding isotherm, and each asymptote of the Hill plot of Hb corresponds to one energy state. Thus Hb cannot have less than two.


A
Y
1
0.75
0.5
0.25
0
0
5
10
15
20
25
30
pO2(mmHg)


B
Y
1
0.75
0.5
0.25
0
-2
-1
0
1
2
3
log (pO2)


C
log (Y / 1-Y)
3
2
1
0
-1
-2
-3
-log K_R
-log K_T
-2
-1
0
1
2
3
log (pO2)


D
0
4
1
3
2
1
0.75
0.5
0.25
0
0
0.25
0.5
0.75
1
Y

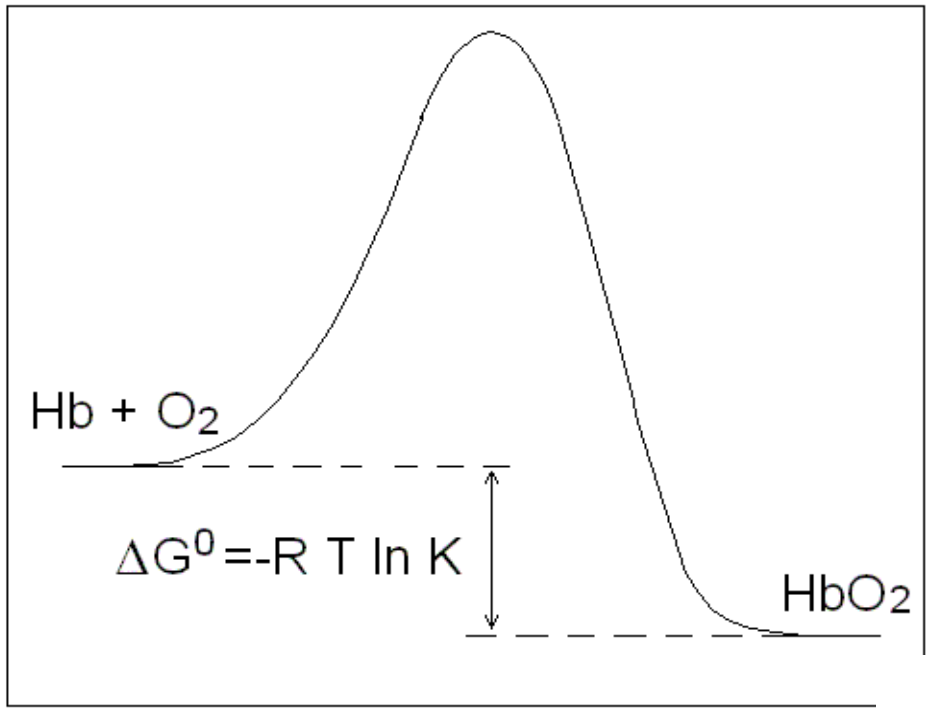
What do we mean with the terms "one-energy-state" and "two-energy-states"?

We must recall that the equilibrium constant of a chemical reaction is associated to a free energy change by the relationship:

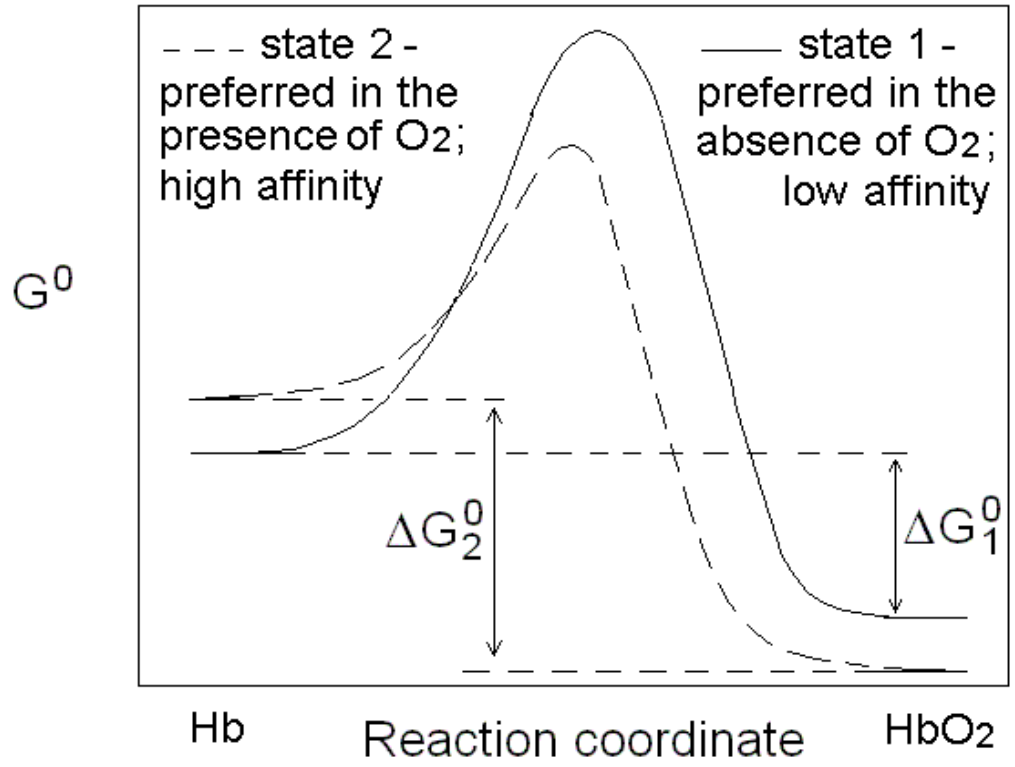
$$\Delta G^0 = -R\ T\ \ln K$$

If the binding isotherm of Hb requires (at least) two equilibrium constants, it is associated to (at least) two ΔG^0; thus either or both the reactant or the product must vary their energy state G^0.

A one-energy-state system



A two-energy-state system



Cooperativity is a functional concept.

However, we saw that it implies (at least) two energy states of the macromolecule, and it is hard to imagine that these two states may have exactly the same structure.

Adair wrote a model-free four-states treatment to describe the binding of four oxygen molecules to one Hb tetramer.

Linus Pauling hypothesized the first structural model of cooperativity. His model had structural implications, in the form of intersubunit weak bonds that could be formed or broken during oxygenation. These structural features could not be confirmed by crystallography.

WHICH STATES FOR Hb ?

Under dissociating conditions (e.g. urea) or upon separation of the constituent α and β polypeptide chains, oxygen affinity increases.

Thus the high affinity state of Hb corresponds to the state of its isolated subunits and should be considered its *resting* or *fundamental* energy state.

Upon association of the unliganded subunits to unliganded tetramers some structural change occurs that diminishes (constrains) the oxygen affinity. This was recognized by Wyman as the "quaternary constraint" of oxygen affinity

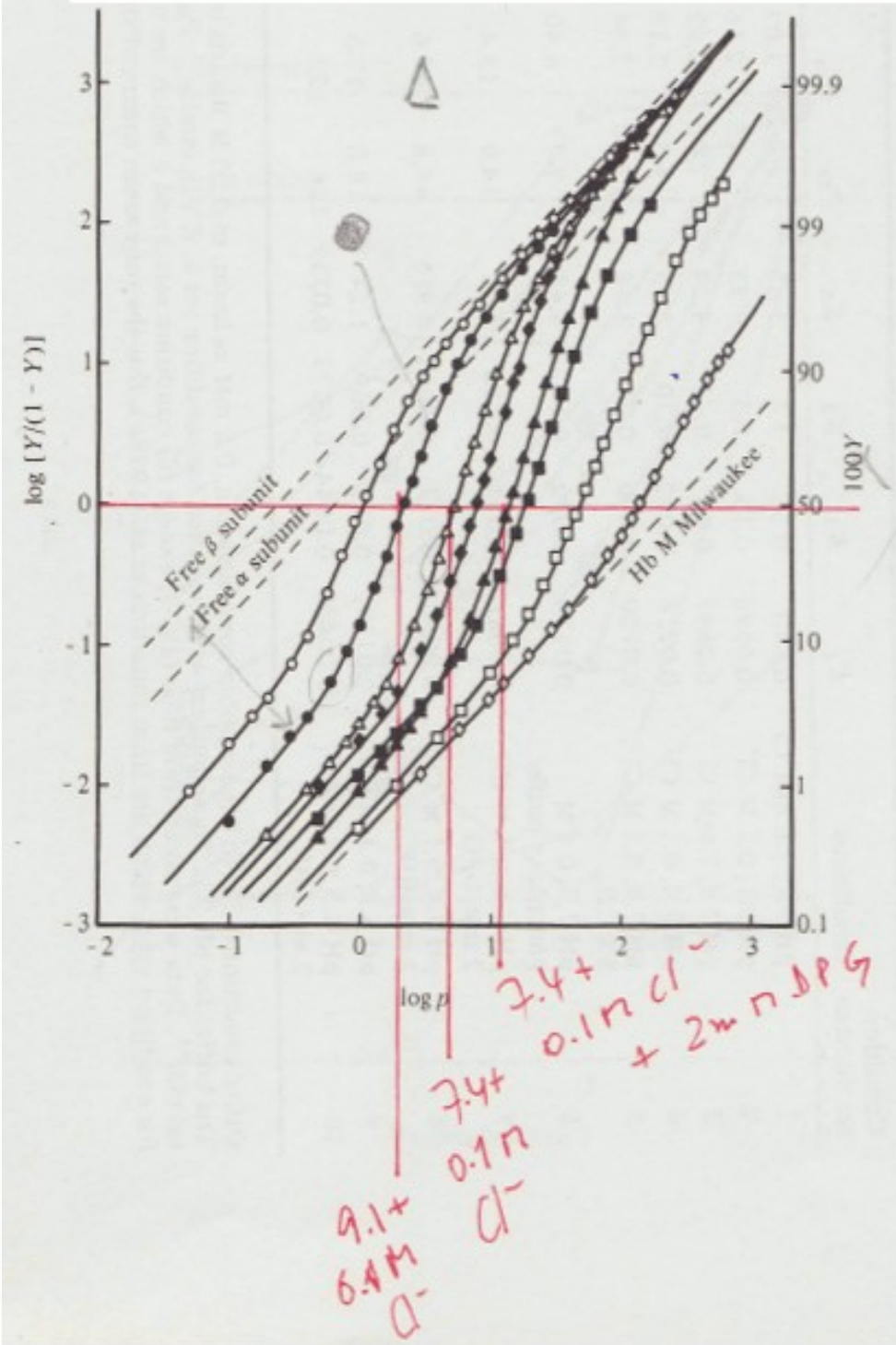


Fig. 6.2. Hill plots of oxygenation of human adult haemoglobin (Hb A) under representative sets of effector conditions. Temperature, 25 °C; haemoglobin concentration, 0.6 mM on haem basis; in 0.05 M Bis-tris buffer for pH 7.4 and pH 6.5, and in 0.05 M Tris buffer for pH 9.1. ○, pH 9.1, 2.6 mM Cl^-; ●, pH 9.1, 0.1 M Cl^-; △, pH 7.4, 0.1 M Cl^-; ▲, pH 7.4, 0.1 M Cl^-, 2 mM DPG; ♦, pH 7.4, 0.1 M Cl^-, 5% CO_2; □, pH 7.4, 0.1 M Cl^-, 2 mM IHP; ■, pH 6.5, 0.1 M Cl^-; ◇, pH 6.5, 0.1 M Cl^-, 2 mM IHP. The plots for condition sets pH 7.4, 7 mM Cl^- and pH 7.4, 0.1 M potassium phosphate are omitted here to avoid overcrowding. Points were experimentally observed and solid lines were calculated from the estimated values of the Adair constants (Table 6.2). The oxygenation data were taken from Imai (1979) except for the data for plot (♦) which were from Imaizumi *et al.* (1978*b*). The plots for free α and β subunits are from Tyuma *et al.* (1971*a*) and the plot for Hb M Milwaukee is from unpublished data of Imai *et al.* Reproduced from Imai (1978) with additional data.

QUATERNARY CONSTRAINT

Mims et al. (JBC 1983, 258, 14219-14232) report the following values for the oxygen affinities of the α, β subunits in their isolated state and in T- and R- state Hb tetramers:

isolated α subunits	1.79 μM^{-1}
isolated β subunits	3.75 μM^{-1}
α subunits in ^{R}Hb	4.92 μM^{-1}
β subunits in ^{R}Hb	2.81 μM^{-1}
α subunits in ^{T}Hb	0.016 μM^{-1}
β subunits in ^{T}Hb	0.0047 μM^{-1}

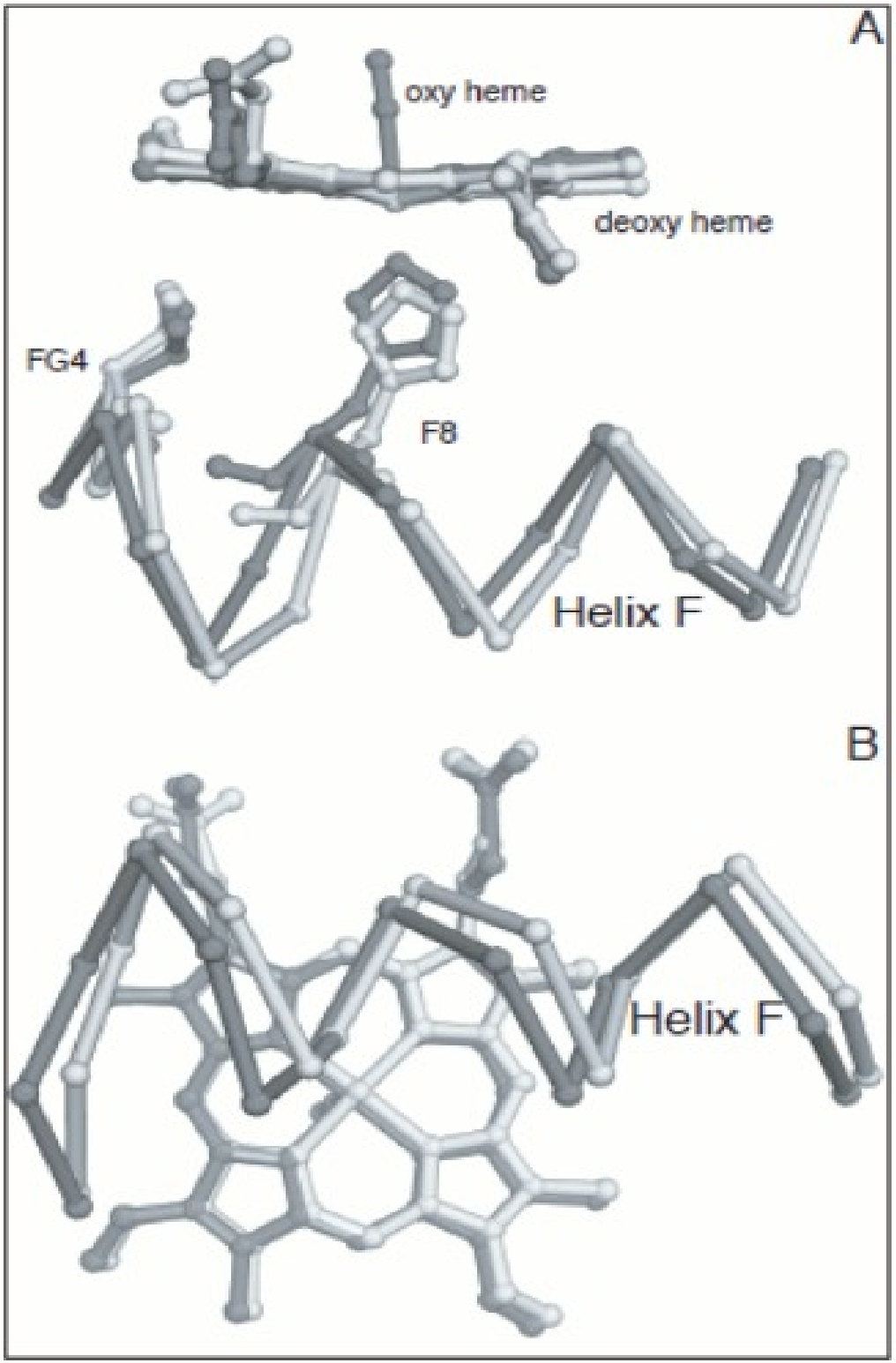
This table, and the preceding figure, clearly show that Hb cooperativity is due to a progressive release of the constraint imposed by the quaternary assembly.

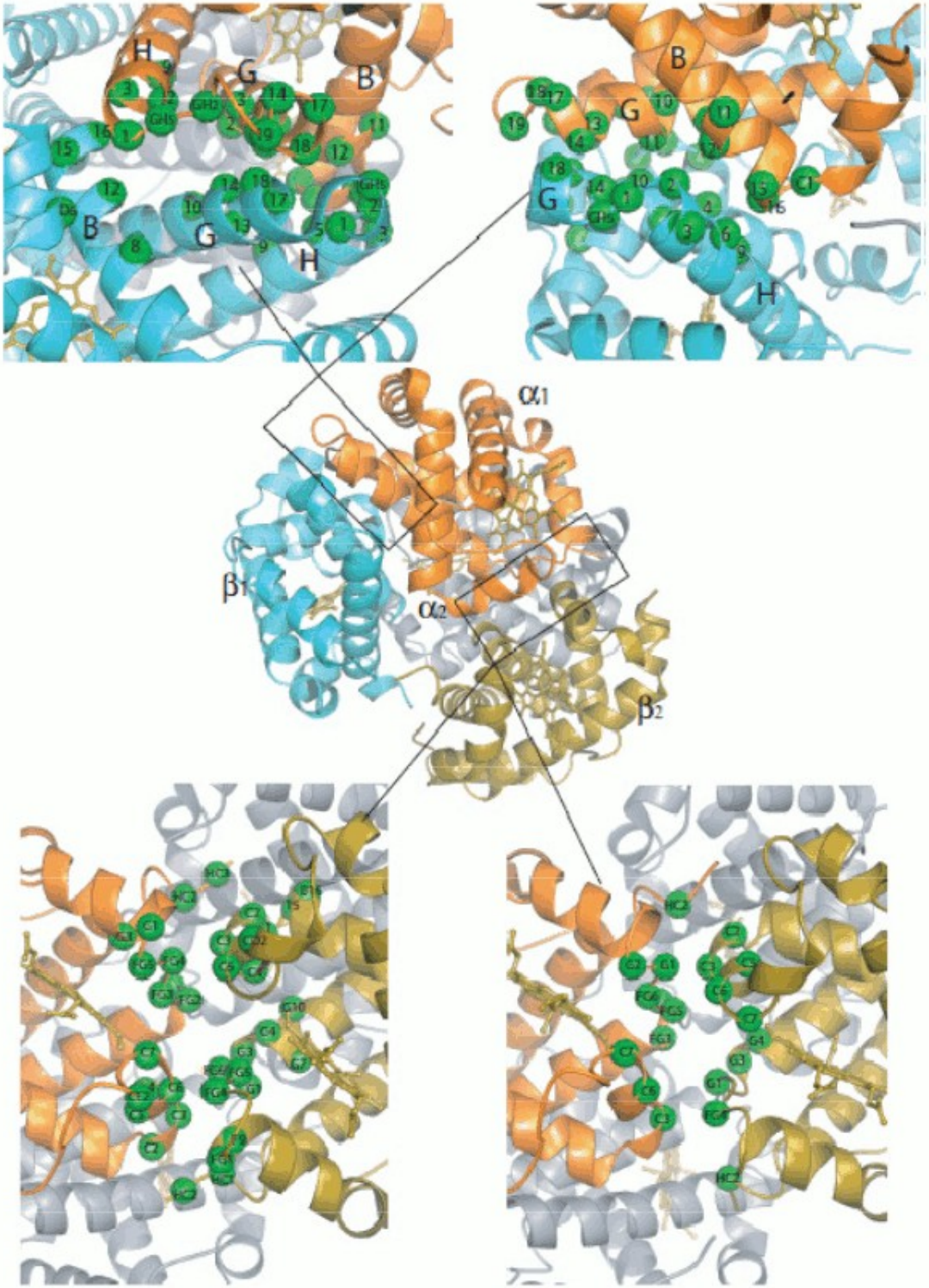
MONOD'S TWO STATE MODEL: THE MINIMAL INTERPRETATION OF COOPERATIVITY

In 1963 Jacques Monod introduced the structural concept of allostery (literally "other shape") to describe a protein stable in two different conformations.

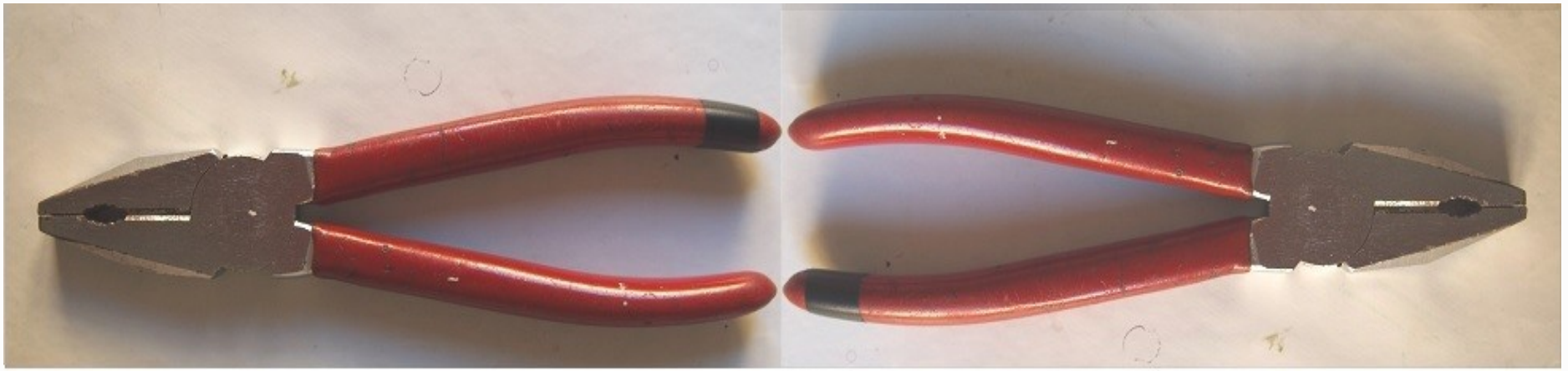
In 1965 Monod, Wyman and Changeux demonstrated that allostery can provide a structural basis for cooperativity, provided that the cooperative macromolecule were a "symmetric oligomer". The Hb tetramer is a symmetric dimer of $\alpha\beta$ heterodimers, and was used as an example of the two-state model.

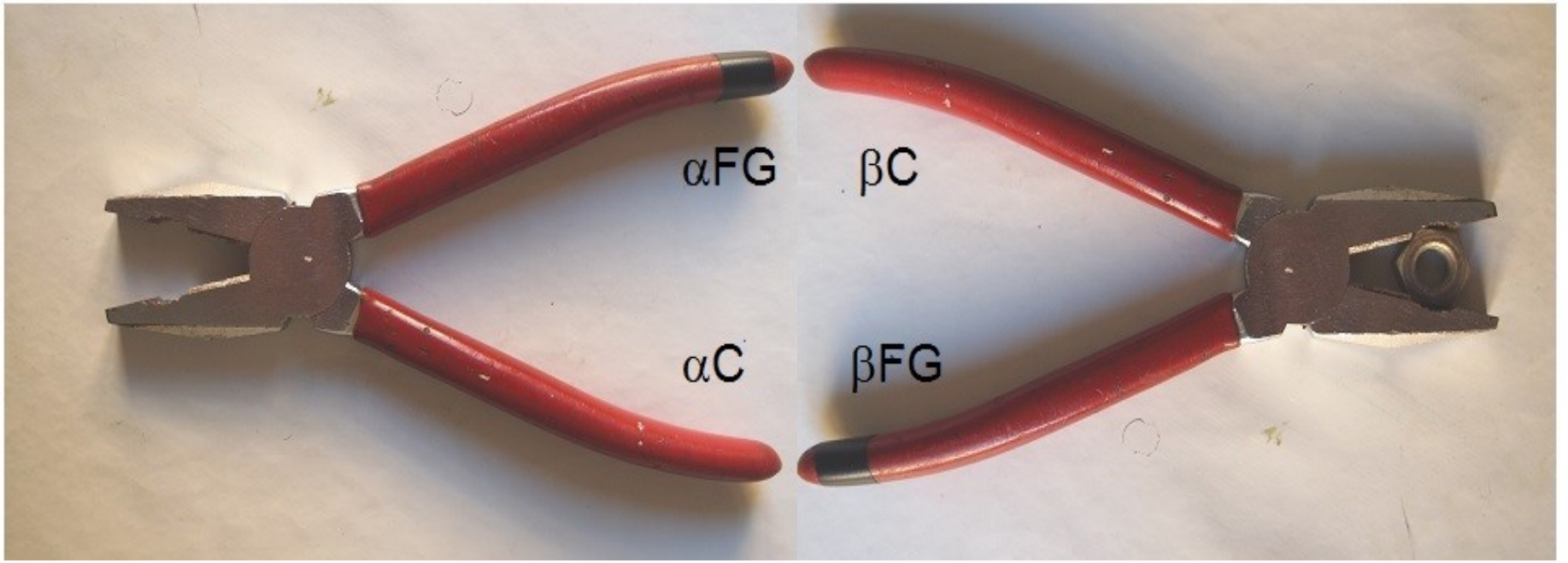
In what follows I shall use the MWC model as a reference frame to describe some experiments carried out on Hb.


A
oxy heme
deoxy heme
FG4
F8
Helix F
B
Helix F


H
G
B
B
G
H
B
G
H
α1
β1
α2
β2

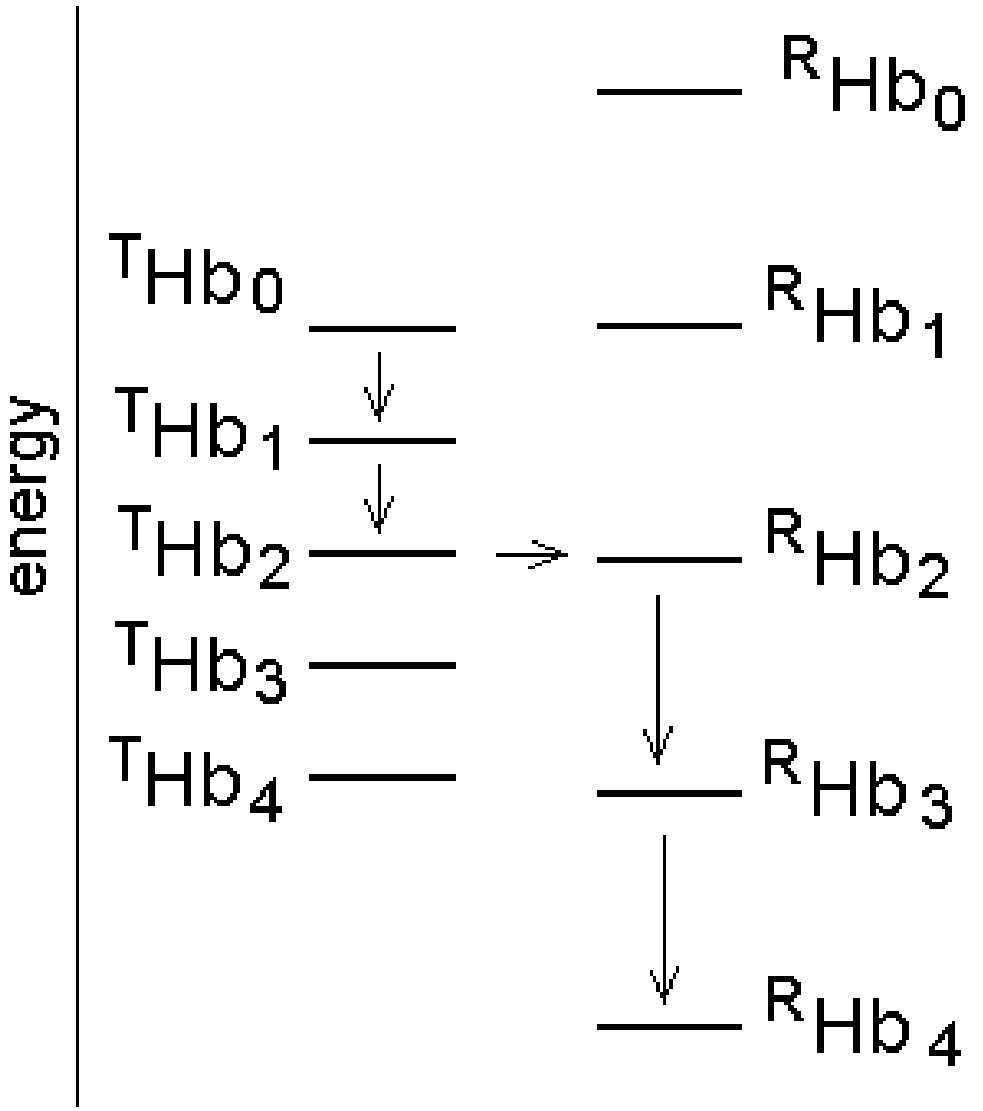
The importance of symmetry: a practical example.

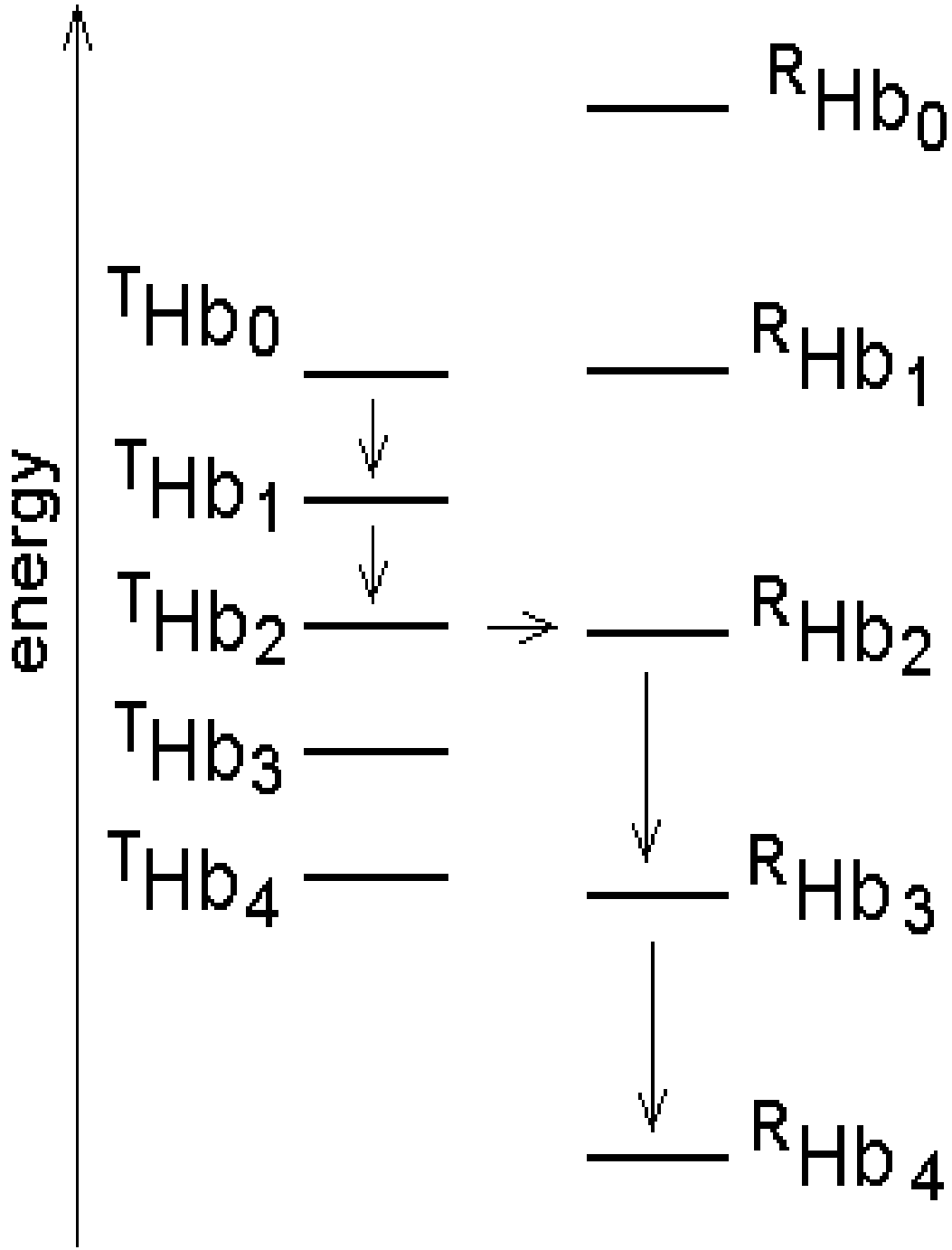




TWO STRUCTURES - TWO ENERGY STATES

The Monod, Wyman and Changeux (MWC) model postulates that Hb is allosteric, i.e. stable in either of two alternative structures called R and T, having different oxygen affinity.
The structures correspond to energy states and the T structure, having low oxygen affinity is more stable than the R in the absence of the gas.




energy
$^{R}Hb_0$
$^{T}Hb_0$
$^{R}Hb_1$
$^{T}Hb_1$
$^{T}Hb_2$
$^{R}Hb_2$
$^{T}Hb_3$
$^{T}Hb_4$
$^{R}Hb_3$
$^{R}Hb_4$

The T and R structures / states, in themselves, are non-cooperative: cooperativity arises as a consequence of the oxygen-linked structural transition from the T to the R state.

The R- and T- structures coexhist in equilibrium in the presence and in the absence of oxygen, and the following relationships apply:

$$[^{T}Hb_0] / [^{R}Hb_0] = L_0$$

$$[^{T}Hb_1] / [^{R}Hb_1] = L_0 K_R / K_T = L_1$$

$$[^{T}Hb_n] / [^{R}Hb_n] = L_0 (K_R / K_T)^n = L_n$$

(in the original formulation K_R and K_T are for the dissociation equilibrium and $K_R << K_T$; $L_0 >> 1$ and $L_4 << 1$).

The oxygen affinity of Hb in the MWC model is the weighted average of the (constant) affinities of ^{R}Hb and ^{T}Hb; as the oxygen saturation increases, the relative population of ^{R}Hb also increases at the expenses of that of ^{T}Hb and the average oxygen affinity increases.

The binding polynomial for ^{R}Hb and ^{T}Hb (using $^{R}Hb_0$ as the reference species) are as follows

$$[^{R}Hb]_t = [^{R}Hb_0]\,(1 + [O_2] / K_R)^4$$

$$[^{T}Hb]_t = [^{R}Hb_0]\;L_0\;(1 + [O_2] / K_T)^4$$

and for the total Hb content of the sample:

$$[Hb]_t = [^{R}Hb_0][(1 + [O_2] / K_R)^4 + L_0\;(1 + [O_2] / K_T)^4]$$

At the same time, Max Perutz solved the first crystallographic structures of oxy- and deoxy- Hb. In the essence, the Hb tetramer can be described as two identical pseudo-symmetric heterodimers, each made up of an α and a β subunit. The subunits are small globular proteins whose structure is made up of 8 helical segments named A through H.

The heterodimer interface is formed by the contacts αB - βH; αG - βG; αH - βB

Deoxy- and oxy-Hb differ because of the position and orientation of the $\alpha\beta$ dimers with respect to each other. The allosteric structural change can be described as a sliding and rotation of the $\alpha_1\beta_1$ dimer with respect to the $\alpha_2\beta_2$. The allosteric interface is contributed by the contacts αC - βFG and αFG -βC

Perutz structures seemed to beautifully confirm Monod's hypotheses: e.g. they clearly showed that the oligomer had a (pseudo-) symmetric structure in both allosteric states and that the interfaces were isologous and highly symmetric.

Successive structural data, collected over fifty years, provided further support to the MWC model:
1) the structures of $^{T}Hb_0$, $^{T}Hb_4$, and $^{R}Hb_4$ could all be solved, and we have plausible models of $^{R}Hb_0$ (e.g. the β_4 homotetramer).
2) Ligation intermediates were mimicked using mixed metal hybrid Hbs; none of them had a structure different from those of ^{R}Hb or ^{T}Hb.
3) Crystals of ^{T}Hb bind oxygen non-cooperatively and with low affinity, whereas crystals of ^{R}Hb display lack of cooperativity and are highly reactive.

STRUCTURE DEGENERACY OF $^{R}HbO_2$ and ENERGY DEGENERACY OF ^{T}Hb

Two observations seem to contrast with the premises of the MWC model. In my opinion, however, the discrepancy is only apparent and easily reconciled with the model.

1) More structures have been described for the R-state of human (and other) Hb(s). These are called R2, R3 … The R-state is thus structurally degenerate

2) More energy states (i.e. oxygen affinities) have been found for the T-state of human (and other) Hb(s). The T-state is thus energetically degenerate.

STRUCTURE DEGENERACY OF THE R-STATE

We observed that the structural degeneracy of the R-state corresponds to quaternary conformations that do not constrain the allosteric interface (Bellelli and Brunori, BBA 2011, 1262-1272).

This observation is consistent with the expectation that the structural basis of quaternary constraint is very specific and that any unconstrained structure will be R-like and high affinity: thus structure degeneration in the R-state does not contradict any fundamental axiom of the MWC model and instead confirms that the energy states of Hb are fundamentally two: constrained (T-like) or unconstrained (R-like or subunit-like).

Interatomic distances at the cooperative $\alpha_1\beta_2$ interface

Hb derivative	PDB code	αC6-FG4	βC6-FG4	αFG4-βFG4
Human deoxy (T)	2HHB	9.73	9.66	31.74
Human deoxy (T)	2DN2	9.71	9.66	31.76
Human αNi βFeCO (T)	1NIH	9.88	9.73	31.67
Human oxy (T)	1GZX	9.70	9.84	31.78
Trout Hb1 deoxy (T)	1OUT	9.88	9.53	32.73
Tuna deoxy (T) pH 7.5	1V4W	9.68	9.15	33.12
Tuna deoxy (T) pH 5.5	1V4X	9.44	9.19	32.95
Human oxy (R)	2DN1	10.38	9.91	29.05
Human CO (R)	2DN3	10.38	10.04	29.25
Human CO (R2)	1BBB	10.28	10.49	28.58
Human CO (R3)	1YZI	10.4	10.94	27.49
Horse deoxyBME (R)	1IBE	10.11	10.27	29.91
Horse CO BZF (R)	1IWH	10.61	9.86	29.79
Trout Hb1 CO (R)	1OUU	9.92	9.96	31.05
Tuna Hb CO (R)	1V4U	9.75	10.22	28.9
Sperm whale deoxy Mb	1A6N	10.80		
Sperm whale CO Mb	1MBC	10.69		

αC6-FG4 is the distance (in A) between the Cαs of residues at topological positions C6 and FG4 in the same α subunit, according to Val93α1 in human Hb); βC6-FG4 is the distance (in A) between the Cαs of residues at topological positions C6 and FG4 in the Hb); αFG-βFG4 is the distance (in A) between the Cαs of residues at topological positions αFG4 and βFG4 in the same α1β1 dimer (e.g., Val (in A) between the heme irons of the two β subunits of the same tetramer; p_{50} is the O_2 partial pressure (in mm Hg) required to achieve Experimental conditions for the determination of p_{50} for O_2: (a) lower asymptote of the O_2 binding isotherm of HbA at pH 7.4, [13]). (b) The Ni-Fe mixed metal hybrid Hbs that contain a Ni protoporphyrin IX in one type of chain and the heme in the other

ENERGY DEGENERACY OF THE T-STATE

It was observed by K. Imai (and others) that the oxygen affinity of ^{T}Hb changes very significantly in the presence of allosteric effectors (e.g. pH, chloride, 2,3-DPG).

This was not anticipated by the MWC model that postulated the allosteric effectors only to bias the allosteric constant L_0 (an effector binding preferentially to ^{T}Hb would increase L_0, one binding preferentially to ^{R}Hb would decrease this parameter).

Perutz's structures of Hb, however, showed that the cooperative $\alpha_1\beta_2$ interface is not the only possible site where constraints can be exerted: further constraints can be exerted to some extent independently of the R and T structures (e.g. at the C-termini of each chain).

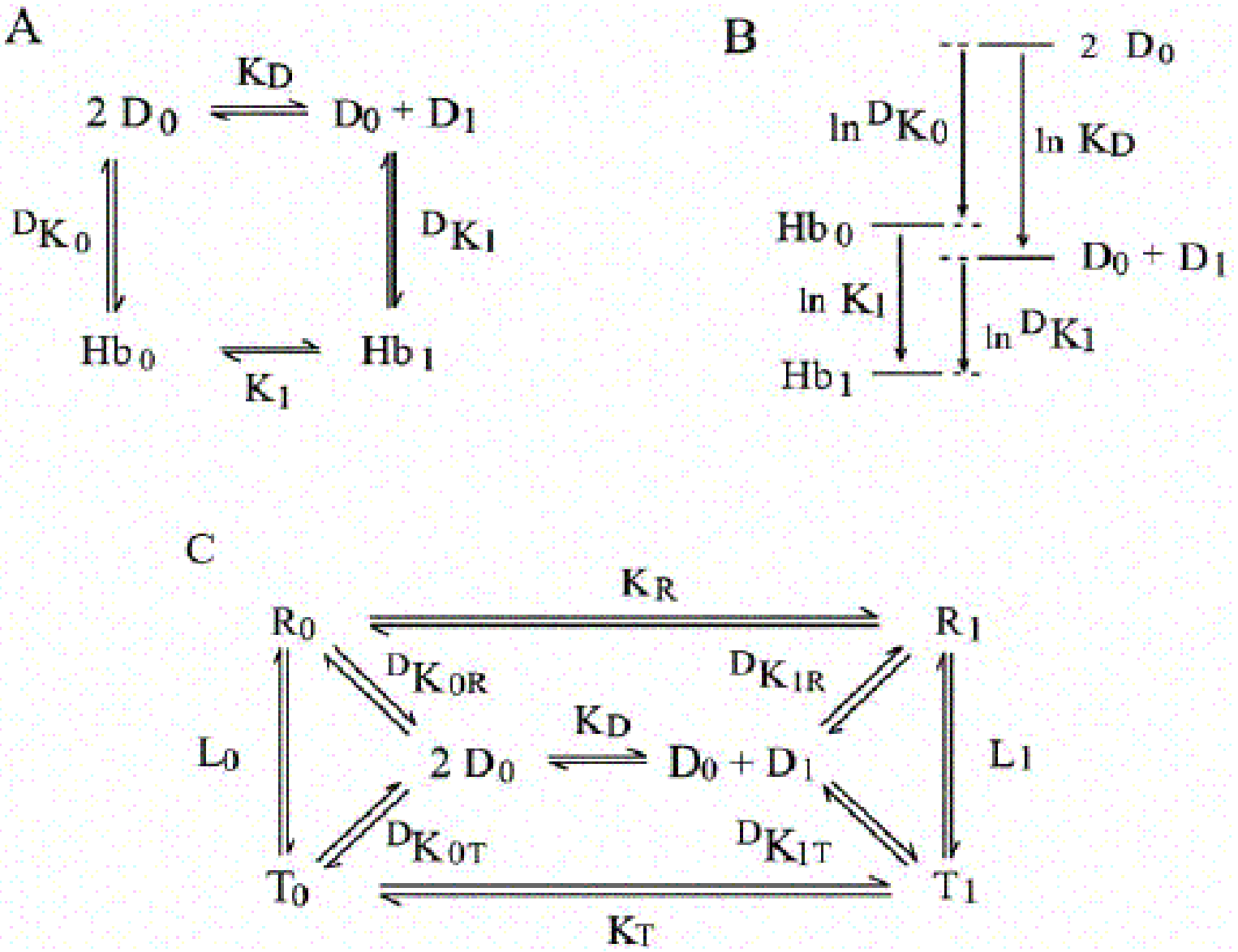
DISSOCIATION INTO DIMERS AND THE MWC MODEL

An astonishing success of the MWC model is its ability to describe (perhaps with some approximation) the relationship between oxygenation and dissociation into dimers.

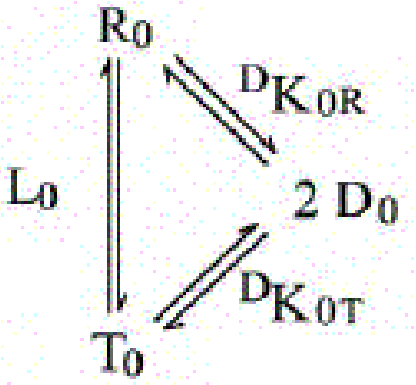
It had long been known that liganded Hb easily dissociates into $\alpha_1\beta_1$-type dimers having high oxygen affinity. The K_d is in the low micromolar range. Unliganded Hb dissociates some 10^4 to 10^5 times less readily.

Unliganded dimers have high oxygen affinity, consistent with the quaternary constraint being localized at the $\alpha_1\beta_2$ interface (which is disrupted by dissociation).

DISSOCIATION INTO DIMERS AND THE MWC MODEL



We consider first the dissociation into dimers for unliganded Hb:



we define:

$$^{R}Hb_0 \rightleftharpoons 2\,\alpha\beta \;;\; {}^{D}K_{R,0} = [\,\alpha\beta\,]^2 / [\,^{R}Hb_0]$$

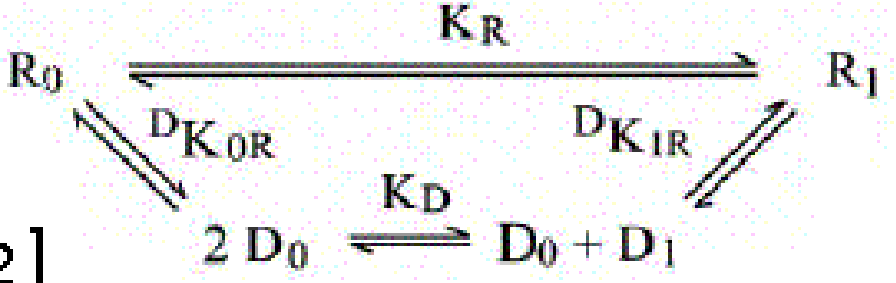
$$^{T}Hb_0 \rightleftharpoons 2\,\alpha\beta \;;\; {}^{D}K_{T,0} = [\,\alpha\beta\,]^2 / [\,^{T}Hb_0]$$

and we easily obtain:

$$\frac{{}^{D}K_{R,0}}{{}^{D}K_{T,0}} = \frac{[\,\alpha\beta\,]^2\ [\,^{T}Hb_0]}{[\,\alpha\beta\,]^2\ [\,^{R}Hb_0]} = L_0$$

The MWC model predicts that unliganded T-state Hb is more stable than R-state Hb to dimerization by a factor that equals the allosteric constant L_0.

We consider next the oxygenation of R-state Hb in relation to its dimerization :



we define:

$$K_R = [^RHb\,][O_2] / [^RHbO_2]$$

$$K_D = [D]\,[O_2] / [DO_2]$$

$$^DK_{0R} = [D]^2 / [^RHb\,]$$

$$^DK_{1R} = [D]\,[DO_2] / [^RHbO_2]$$

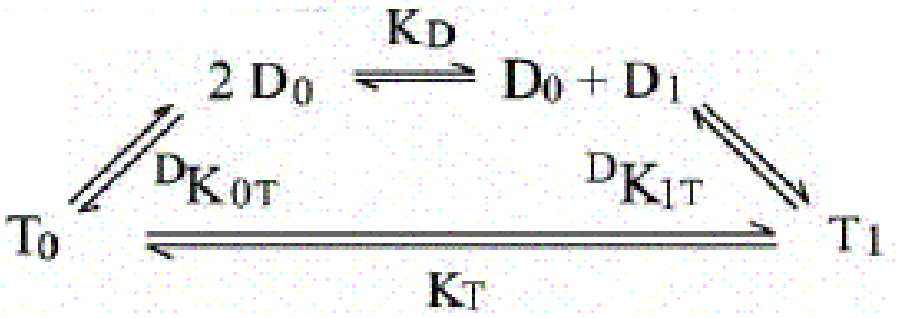
we next remark that:

$$K_R\,^DK_{0R} = K_D\,^DK_{1R}$$

since the $\alpha\beta$ dimers have the same O_2 affinity as ^RHb (i.e. $K_R = K_D$), it follows that $^DK_{0R} = {}^DK_{1R}$

i.e. dimerization of ^RHb is independent of ligation

We consider next the oxygenation of T-state Hb in relation to its dimerization :



we define:

$$K_T = [{}^T\mathrm{Hb}\,][O_2] / [{}^T\mathrm{HbO_2}]$$

$$K_D = [D]\,[O_2] / [DO_2]$$

$$^DK_{0T} = [D]^2 / [{}^T\mathrm{Hb}\,]$$

$$^DK_{1T} = [D]\,[DO_2] / [{}^T\mathrm{HbO_2}]$$

we next remark that:

$$K_T\,{}^DK_{0T} = K_D\,{}^DK_{1T}$$

since the $\alpha\beta$ dimers have higher O_2 affinity than ^{T}Hb (i.e. $K_T > K_D$), it follows that $^DK_{0T} < {}^DK_{1T}$
i.e. dimerization of ^{T}Hb increases with ligation

Measurement of the dimer-tetramer equilibrium for the different ligation intermediates allows one to reconstruct the whole energy landscape of oxygenation (this approach was pioneered by Gary K. Ackers, who emphasized some small deviations from the predictions of the MWC model)

To carry out this experiment one needs:

1) to prepare the separated α and β subunits in complex with some slowly dissociating ligand (e.g. the cyano-met derivative);

2) to mix each with the partner subunit in the unliganded state;

3) to add haptoglobin to the mixture (which contains cyano-met / deoxy hybrid tetramers) and to record the time course of dissociation into dimers (by stopped flow) under the assumption that the combination is unchanged

Free energies of cooperativity as determined from the rate constants of dissociation into dimers for three analogues of ligation intermediates (from Ackers 1998 Adv. Protein Chem. 51,185-253)

0:1	$\alpha_1^0\beta_2^0$ $\beta_1^0\alpha_2^0$	none	0 k cal / mol	0	0
1:1	$\alpha_1^X\beta_2^0$ $\beta_1^0\alpha_2^0$	$\alpha_1^X\beta_2^0$ + $\alpha_1^0\beta_2^0$ $\beta_1^0\alpha_2^X$ $\beta_1^0\alpha_2^0$	3.1	2.9	1.5
1:2	$\alpha_1^0\beta_2^0$ $\beta_1^X\alpha_2^0$	$\alpha_1^0\beta_2^X$ + $\alpha_1^0\beta_2^0$ $\beta_1^X\alpha_2^0$ $\beta_1^0\alpha_2^0$	3.3	3.7	2.0
2:1	$\alpha_1^X\beta_2^0$ $\beta_1^X\alpha_2^0$	$\alpha_1^X\beta_2^X$ + $\alpha_1^0\beta_2^0$ $\beta_1^X\alpha_2^X$ $\beta_1^0\alpha_2^0$	3.1	3.4	2.1
2:2	$\alpha_1^X\beta_2^X$ $\beta_1^0\alpha_2^0$	$\alpha_1^X\beta_2^0$ + $\alpha_1^0\beta_2^X$ $\beta_1^0\alpha_2^X$ $\beta_1^X\alpha_2^0$	6.4	6.6	3.0
2:3	$\alpha_1^X\beta_2^0$ $\beta_1^0\alpha_2^X$	none	6.1	6.8	3.1
2:4	$\alpha_1^0\beta_2^X$ $\beta_1^X\alpha_2^0$	none	6.4	6.2	3.2
3:1	$\alpha_1^X\beta_2^X$ $\beta_1^X\alpha_2^0$	$\alpha_1^X\beta_2^X$ + $\alpha_1^0\beta_2^X$ $\beta_1^X\alpha_2^X$ $\beta_1^X\alpha_2^0$	6.3	6.5	3.0
3:2	$\alpha_1^X\beta_2^0$ $\beta_1^X\alpha_2^X$	$\alpha_1^X\beta_2^X$ + $\alpha_1^X\beta_2^0$ $\beta_1^X\alpha_2^X$ $\beta_1^0\alpha_2^X$	6.2	6.5	3.1
4:1	$\alpha_1^X\beta_2^X$ $\beta_1^X\alpha_2^X$	none	6.0	6.9	2.5

THE STRUCTURE AND AFFINITIES OF LIGATION INTERMEDIATES

Two fundamental tenets of the MWC model are:

1) the two states/structures are always present in solution, irrespective of the presence or the absence of oxygen or other ligands;

2) ligation intermediates adopt either the R or T conformation and none else.

Point 1 has already been addressed, and the structures of $^{T}Hb_0$, $^{T}Hb_4$ and $^{R}Hb_4$ have been determined, together with several mimics of $^{R}Hb_0$(e.g. BME-reacted Hb or the β_4 homotetramer).

We address now point 2.

THE STRUCTURE AND AFFINITIES OF LIGATION INTERMEDIATES

Ligation intermediates are too unstable and attain too low population to be directly characterized. However T. Yonetani pioneered a technique to produce their mimics, in the form of mixed metal hybrid Hbs.

The procedure is as follows:

1) the heme is extracted from Hb, to yield the globin;

2) the globin is reconstitued with a protoporphyrin IX carrying a metal other than Fe (e.g. Ni);

3) the metal substituted α and β subunits are separated;

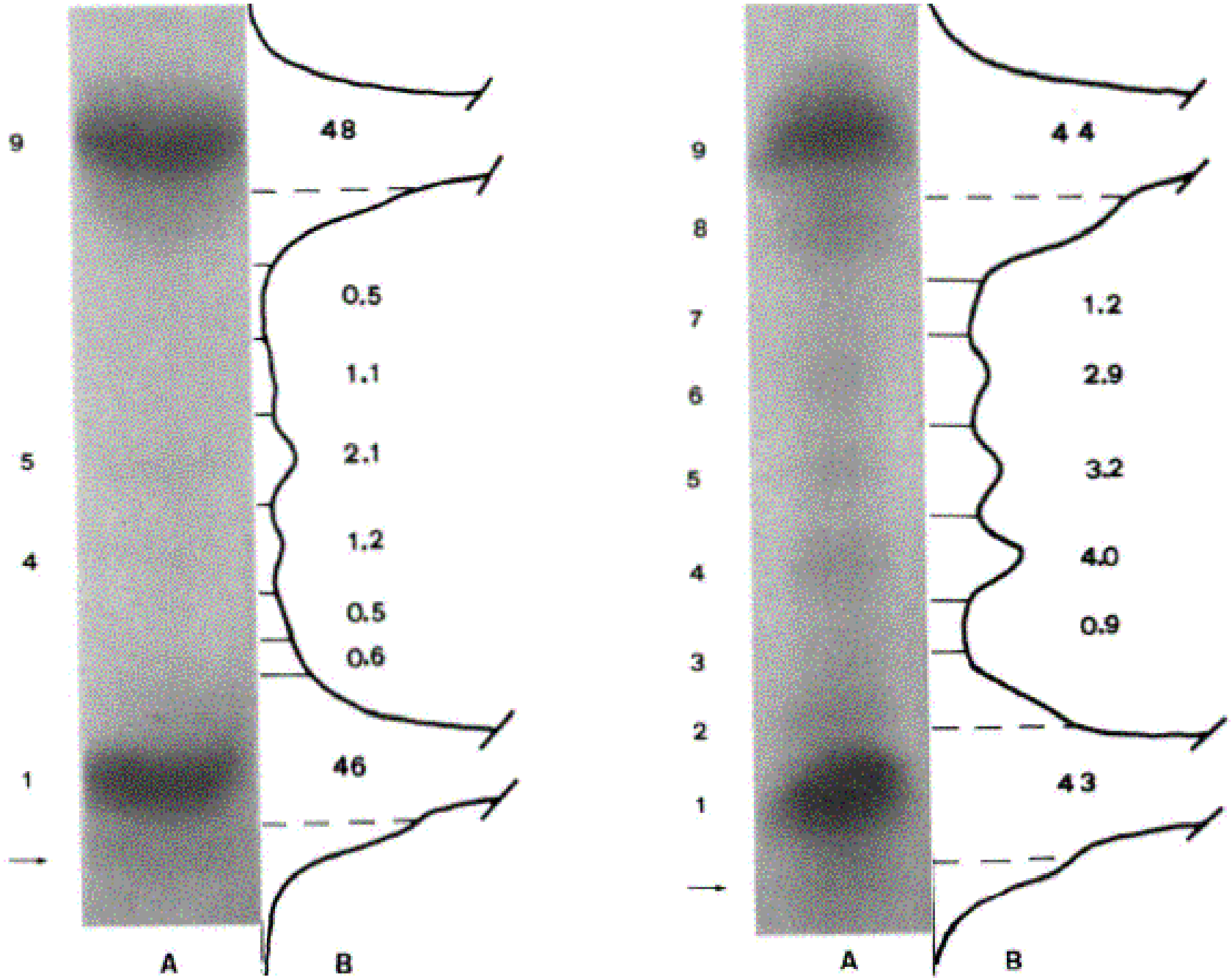
4) tetrameric mixed metal hybrid Hbs are formed by mixing the metal substituted subunits with normal, iron-containing partner subunits.

DIRECT DETERMINATION OF THE CONCENTRATION OF LIGATION INTERMEDIATES

M. Perrella devised an experimental method to determine the realtive concentrations of ligation intermediates. They anaerobically mixed Hb and HbCO and allowed the solution time to reach its equilibrium condition. Then the mixed with a ferricyanide solution at -30°C.

Under these conditions the unliganded heme iron is rapidly oxidized to the ferric state, whereas the CO bound iron remains reduced.

Isolelectric focusing is then used to resolve the mixture, taking advantage of the additional positive charge(s) conferred by the oxidized iron atoms.



Two isoelectrofocusing runs by Perrella et al. 1983 JBC, 258, 4511-4517.

TABLE I

Identification of the hemoglobin components of Fig. 5

Component No.	Species or hybrid	Parent species of hybrid
1	$(\alpha_2^{CO}\beta_2^{CO})$	
2	$(\alpha^{CO}\beta^{+})(\alpha^{CO}\beta^{CO})$	$(\alpha_2^{CO}\beta_2^{CO})$; $(\alpha_2^{CO}\beta_2^{+})$
3	$(\alpha_2^{CO}\beta_2^{+})$	
4	$(\alpha^{+}\beta^{CO})(\alpha^{CO}\beta^{CO})$	$(\alpha_2^{CO}\beta_2^{CO})$; $(\alpha_2^{+}\beta_2^{CO})$
5	$(\alpha^{+}\beta^{+})(\alpha^{CO}\beta^{CO})$	$(\alpha_2^{CO}\beta_2^{CO})$; $(\alpha_2^{+}\beta_2^{+})$
6	$(\alpha^{+}\beta^{+})(\alpha^{CO}\beta^{+})$	$(\alpha_2^{CO}\beta_2^{+})$; $(\alpha_2^{+}\beta_2^{+})$
7	$(\alpha_2^{+}\beta_2^{CO})$	
8	$(\alpha^{+}\beta^{+})(\alpha^{+}\beta^{CO})$	$(\alpha_2^{+}\beta_2^{CO})$; $(\alpha_2^{+}\beta_2^{+})$
9	$(\alpha_2^{+}\beta_2^{+})$	

How are Perrella's measurements of ligation intermediates correlated with MWC parameters?

The first observation is that the MWC model assumes perfect symmetry and this implies statistical population of equally liganded intermediates (e.g. in the case of the monoliganded intermediate one expects that [$\alpha\alpha^{CO}\beta_2$] = [$\alpha_2\beta\beta^{CO}$]

Indeed the MWC model allows one to calculate the sum of equally liganded intermediates, with the formula that one may derive from the binding polynomial:

$$[Hb_n] / [Hb]_t = \frac{[O_2] (1 + Lc^n) / K_R^n}{[(1 + [O_2] / K_R)^4 + L_0(1 + [O_2] / K_T)^4}$$

with $c = K_R / K_T$

Table 5. Population of HbCO Intermediates at 48% Saturation, as Quantified by Cryoelectrofocusing [93,94]

Intermediate	Relative Abundance	Two-State Model	Perrella and Di Cera	Non Cooperati
$\alpha_1\alpha_2\beta_1\beta_2$ (Hb_0)	0.418	0.415	0.408	0.0625
$\alpha_1^{CO}\alpha_2\beta_1\beta_2$	0.024			
$\alpha_1\alpha_2\beta_1^{CO}\beta_2$	0.049			
Hb_1	0.073	0.066	0.056	0.250
$\alpha_1^{CO}\alpha_2^{CO}\beta_1\beta_2$	0.01			
$\alpha_1^{CO}\alpha_2\beta_1^{CO}\beta_2 + \alpha_1^{CO}\alpha_2\beta_1\beta_2^{CO}$	0.029			
$\alpha_1\alpha_2\beta_1^{CO}\beta_2^{CO}$	0.014			
Hb_2	0.053	0.015	0.047	0.375
$\alpha_1^{CO}\alpha_2^{CO}\beta_1^{CO}\beta_2$	0.038			
$\alpha_1^{CO}\alpha_2\beta_1^{CO}\beta_2^{CO}$	0.047			
Hb_3	0.085	0.092	0.081	0.250
$\alpha_1^{CO}\alpha_2^{CO}\beta_1^{CO}\beta_2^{CO}$ (Hb_4)	0.37	0.415	0.408	0.0625