The Journal of Experimental Biology 201, 1073–1084 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JEB1522

SELF-ASSOCIATION, COOPERATIVITY AND SUPERCOOPERATIVITY OF OXYGEN BINDING BY HEMOGLOBINS

AUSTEN F. RIGGS*

Department of Zoology, University of Texas, Austin, TX 78712, USA

*e-mail: riggs@uts.cc.utexas.edu

Accepted 28 January; published on WWW 24 March 1998

Summary

Cooperative ligand binding by tetrameric vertebrate hemoglobins (Hbs) makes possible the delivery of oxygen at higher pressures than would otherwise occur. This cooperativity depends on changes in dimer–dimer interactions within the tetramer and is reflected in a 50 000-fold increase in the tetramer–dimer dissociation constant in human Hb upon oxygenation at pH 7.4, from approximately 2×10^{-11} mol l^{-1} to approximately 10^{-6} mol l^{-1}. Hbs that undergo such ligand-dependent changes in association are widespread in non-vertebrates, where the mechanisms are very different from those in vertebrates. Oligomeric Hbs have been identified in organisms in five phyla (molluscs, echinoderms, annelids, phoronids and chordates) that dissociate to subunits upon oxidation of the heme iron and reassociate with the binding of ferric iron ligands such as CN^-, N_3^- or NO_2^-. Thus, the valence and ligand state of the heme iron control the stability of a critical subunit interface. The broad distribution of this phenomenon suggests a common mechanism of communication between heme and interface that may be almost universal among non-vertebrate Hbs. This interaction may be similar to that known for the homodimeric Hb of the mollusc *Scapharca inaequivalvis*.

Although muscle tissue Hbs or myoglobins (Mbs) are usually monomeric, with non-cooperative O_2 binding, the radular muscles of gastropod molluscs and chitons have homodimeric Mbs that bind O_2 cooperatively. Cooperative non-muscle tissue Hbs have also been identified. These include the neural Hb of the nemertean worm *Cerebratulus lacteus* and the Hb of the diving beetle *Anisops assimilis*, which exhibit deoxygenation-dependent self-association of monomers that is associated with high Hill coefficients. Calculations suggest that the 2–3 mmol l^{-1} concentration of Hb on a heme basis in the brain of *Cerebratulus* should substantially extend the time as an active predator in an anaerobic or hypoxic environment. Oxygen from the Hb of *Anisops* is delivered to a gas bubble and thereby controls the buoyant density.

Many Hbs of amphibians, reptiles, birds and some embryonic mammals exhibit a further 'supercooperativity' of O_2 binding which depends on reversible deoxygenation-dependent tetramer–tetramer association to form an assemblage with a very low affinity for O_2. This phenomenon results in steeper O_2-binding curves than exhibited by tetramers alone. The increased cooperativity should result in an increase in the amount of O_2 delivered to the tissues and should be especially valuable for avian flight muscles.

Key words: oxygen binding, hemoglobin, cooperativity, supercooperativity, self-association, dimer, tetramer, invertebrate, vertebrate.

Introduction

Hemoglobins that bind ligands cooperatively are widely distributed in organisms from bacteria to mammals. Hill (1913) was the first to deduce the essential feature of cooperativity: the binding of the first ligand makes it easier to bind a second one. Although the mechanisms for cooperative oxygen binding within tetrameric vertebrate Hbs have been extensively studied for almost a century and are now relatively well-understood (Perutz *et al.* 1987; Perutz, 1989; Ackers *et al.* 1992; for a recent discussion of the issues, see also Ackers *et al.* 1997), it is now clear that different mechanisms are dominant in Hbs of non-vertebrates. Indeed, the mechanisms established in vertebrates have not so far been found in any non-vertebrate Hb. Cooperativity in tetrameric $\alpha_2\beta_2$ Hbs of mammals depends on oxygenation-dependent changes in the iron-proximal histidine bond that produce a chain of conformational perturbations that lead to a weakening shift of the $\alpha^1\beta^2$ interface between two dimers the subunits of which are joined together at the $\alpha^1\beta^1$ interface. However, this interface does not change appreciably during the oxygenation of mammalian Hbs. Oxygenation-dependent weakening of both these interfaces occurs frequently in the Hbs of reptiles and amphibians so that the oxygenated species often include an equilibrium mixture of tetramers, dimers and monomers (reviewed by Brittain, 1991). The Hbs of most birds and of some reptiles and amphibians are characterized by a further deoxygenation-dependent self-association of tetramers. These tetramer–tetramer complexes have a greatly lowered oxygen affinity. Partial oxygenation results in dissociation to tetramers

of higher oxygen affinity. This oxygenation-dependent increase in oxygen affinity is a second mechanism of cooperativity that is superimposed on the cooperativity of O_2 binding *within* the tetramer. It is this phenomenon that we term 'supercooperativity'. Such self-association of deoxygenated tetramers should be greatly enhanced by the molecular crowding and the excluded-volume effect within red cells (Zimmerman and Minton, 1993). Such enhancement should result in a further increase in the supercooperativity of O_2 binding within red cells and an increased value of the Hill coefficient. This coefficient, n_H, commonly used as an overall measure of cooperativity, is proportional to the statistical variance of the distribution of ligands among the various species of Hb (Edsall and Gutfreund, 1983). Thus, the higher the Hill coefficient, the more non-random is the distribution. The concentration of Hb in the solvent available to it within a mammalian red cell is approximately $335\,g\,l^{-1}$ or $5\,mmol\,l^{-1}$ tetramer. The molecules, almost in contact and close to the limit of solubility, occupy a volume fraction similar to that in a crystal (Perutz, 1946). Under these crowded conditions, the excluded volume effect has major consequences. The apparent lack of self-association of tetramers of mammalian Hbs has led to the suggestion (Riggs, 1976) that there should be a general selective advantage against Hbs having complementary surfaces unless the interaction leads to an advantageous change in physiological properties. Although the evidence for supercooperativity is compelling, many of the early results are difficult to interpret because spurious apparent supercooperativity can be produced by incomplete oxygenation.

The homodimeric structures of deoxy and oxy Hbs in the circulating erythrocytes of the clam *Scapharca inaequivalvis* show a cooperative mechanism that is completely different from that of vertebrate Hbs (Royer *et al.* 1990). The subunit contacts in vertebrate Hbs are dominated by contacts involving the C, G and H helices, whereas the E and F helices are on the external surface of the tetramer (Perutz *et al.* 1987). In contrast, the subunit contacts in the dimeric *Scapharca* Hb involve the E and F helices, so that the hemes are in almost direct contact. The cooperative mechanism in this Hb involves only small changes in the local environment of hemes, and the global conformational changes that characterize vertebrate Hbs are completely absent.

The goal of this brief review is to evaluate these diverse processes of ligand-dependent dissocation/association found in the hemoglobins of many organisms. There are two key questions to be addressed. First, how widespread is the '*Scapharca*' mechanism among non-vertebrate Hbs? Second, how important are the tetramer–tetramer interactions responsible for 'supercooperativity'?

Bacteria

Expression of the homodimeric Hb of the bacterium *Vitreoscilla* sp., first called cytochrome *o*, increases some 50-fold when the culture is made hypoxic ($P_{O_2} \leqslant 15$ mmHg; 1 mmHg=0.1333 kPa) (Tyree and Webster, 1978; Wakabayashi *et al.* 1986). The X-ray structure of the homodimer reveals that the intersubunit contacts between parts of the E and H helices are very different from those of other known oligomeric Hbs (Tarricone *et al.* 1997). The Hb has been reported to bind CO with high cooperativity (Hill coefficient, $n_H \approx 2$). Cytochrome *o* (=Hb) from *Acetobacter suboxydans* has also been reported to bind CO cooperatively (Hill $n_H \approx 1.9$) (Daniel, 1970). However, the quantitative importance of cooperative intracellular Hbs in O_2 delivery has not yet been assessed. It could be that a primary function of some microbial Hbs is not in oxygen delivery but rather in sensing oxygen stress (Bunn and Poyton, 1996) or as a terminal oxidase (Wittenberg and Wittenberg, 1990).

It should be mentioned here that CO-equilibrium measurements can give a spurious cooperativity under some conditions if a gas phase is present. Thus, beef heart cytochrome oxidase has been reported to bind CO cooperatively with a Hill coefficient of 1.26 (Wald and Allen, 1957), but this appears to be an artifact that resulted from the lack of sufficient time for equilibration. Measurement of CO binding by soybean legHb using *exactly* the same tonometry revealed an apparent Hill coefficient of 2.0, but this value was shown to be the result of an inadequate 10 min equilibration time; 60–120 min was required because the rate of transfer of the introduced CO from gas phase to solution is limited by the very low P_{CO} used in the experiments (Imamura *et al.* 1972).

Invertebrates and primitive vertebrates

Cooperative tissue hemoglobins

Although most Mbs are non-cooperative monomers, the radular muscles of several gastropods and chitons contain cooperative dimeric Mbs (Manwell, 1958; Terwilliger and Read, 1971; Geraci *et al.* 1977; Smith *et al.* 1988) with Hill coefficients of 1.2–1.6. None of these dimers has been reported to undergo ligand-dependent subunit dissociation. In contrast, the notonectid diving beetles have cooperative Hbs that do have this property. The Hb of the notonectid backswimmer *Anisops assimilis* self-associates extensively upon deoxygenation (Wells *et al.* 1981), from largely monomeric (approximately 17 kDa) HbO_2 to an equilibrium mixture of dimers, trimers, tetramers and hexamers at 75% deoxygenation. The overall effect of this ligand-dependent association is that the O_2 equilibrium changes from being non-cooperative at low concentrations to highly cooperative at $300\,mg\,ml^{-1}$, with a maximal n_H value near 6.0. Oxygen released from the Hb into a gas bubble provides a mechanism by which the buoyant density is regulated (Miller, 1966).

The nemertean worm *Cerebratulus lacteus* has Hb in circulating red cells, in neural tissue and in the body wall muscles (T. L. Vandergon, C. K. Riggs, T. A. Gorr, J. M. Colacino and A. F. Riggs, in preparation). Although the red cell Hb has not yet been studied, both the neural and body wall Hbs bind O_2 with high cooperativity *in vivo* and *in vitro* (neural Hb $n_H \approx 2.6$–3.0, body wall Hb $n_H \approx 2.1$–2.2) even though the

ligated Hbs are monomeric. This finding indicates deoxygenation-dependent self-association, probably at least to tetramers. Calculations show that the neural Hb, present at 2–3 mmol l^{-1} heme concentration, could function as an oxygen store to prolong the activity of this predator in anoxic muds where it searches for prey. Even under normoxic conditions, the neural and body wall Hbs could augment O_2 delivery during bursts of activity.

Mollusca

Cooperativity in Scapharca *Hb: a predominant mechanism in invertebrates?*

The red cells of the arcid clam *Scapharca inaequivalvis* have two Hbs, homodimeric Hb I and heterotetrameric Hb II, both of which bind O_2 cooperatively by mechanisms completely different from those used by the $\alpha_2\beta_2$ tetrameric Hbs of vertebrates (Chiancone *et al.* 1981; Ikeda-Saito *et al.* 1983). The essential features of the *Scapharca* mechanism will be summarized here because of the finding that some of the properties are shared by many Hbs in organisms of diverse phyla. Royer *et al.* (1990) have shown that the subunit interface of the homodimeric Hb I is formed by the E and F helices so that the two hemes are in direct contact *via* their propionic acid groups through a set of 17 symmetrically ordered water molecules (Royer *et al.* 1996, 1997). Ligand binding to the heme causes the iron atom to move into the heme plane, as it does in vertebrate Hbs. Associated changes in heme shape squeeze a phenylalanine (residue 97) out of the heme pocket into the interface to contact the other subunit. These changes disrupt the 17 ordered water molecules, and six are released. The hemes move further into the pocket so that the propionic acid groups of each subunit move away from those of the other subunit. These local changes in the heme pocket and the adjacent interface are the processes that are responsible for cooperative O_2 binding. The importance of water molecules in the mechanism is underlined by the finding that mutation of interface residue Thr72→Val causes a 40-fold increase in O_2 affinity, although the change involves the loss of only two hydrogen bonds and two water molecules (Royer *et al.* 1996).

The crystal structure of the heterotetrameric Hb II of *Scapharca* (Royer *et al.* 1995) shows that the structure of the heterodimers within the tetramer is very similar to that of the homodimeric Hb I, suggesting that O_2 binding to the heterodimer within the tetramer is also cooperative. Oxygen binding by Hb I is characterized by a Hill coefficient, n_H, of 1.5. The higher cooperativity of O_2 binding by the tetrameric Hb II (n_H=2.1) reflects either the additional interactions across the dimer–dimer interface or the association of deoxygenated tetramers. A striking difference between Hbs I and II is that O_2 binding by Hb I has no heterotropic effects, i.e. the equilibrium is unaffected by allosteric factors such as protons or anions. In contrast, tetrameric Hb II self-associates upon deoxygenation, probably to tetramers of tetramers (Chiancone *et al.* 1981), a process that is pH-dependent and linked to anion binding (Boffi *et al.* 1990).

Although ferrous dimeric Hb I and tetrameric Hb II do not dissociate appreciably, oxidation results in dissociation of Hb I to monomers and of Hb II to dimers (Spagnuolo *et al.* 1988). These dissociations are marked by hemichrome formation in which the sixth coordination position of the iron is occupied by an endogenous ligand, presumably the distal histidine. The dissociation is reversed by addition of ligands such as NO_2^- to the ferric iron. Thus, the association states of Hbs I and II are both valence- and ligand-state-dependent. This property, initially thought to be unique to *Scapharca*, turns out to be widespread and occurs in the Hbs of animals in at least five phyla including the chordates. Examples are discussed below.

Annelida

Zhu *et al.* (1996*b*), following the earlier work of Ascoli *et al.* (1978), have shown that the extracellular Hb of the annelid *Lumbricus terrestris* behaves upon oxidation exactly as described for the Hb of the mollusc *Scapharca*. *Lumbricus* Hb has four major kinds of globin chains: chains *a*, *b* and *c* (forming a disulfide-linked trimer) and chain *d*. The CO–ferrous trimer self-associates extensively to assemblages as large as $(abc)_{10}$, and CO chain *d* is largely tetrameric (d_4),but oxidation causes the trimer assemblages to dissociate completely to $(abc)_2$ and d_4 to dissociate to d_2. Addition of CN^- reverses these dissociations completely. The *abc* trimer and chain *d* associate to form $(abcd)_4$, a principal subunit, which, together with non-globin structural units (‘linkers’), form the 4.1 MDa, approximately 200 polypeptide, assemblage of the Hb. Partial oxidation causes the Hb to shed subunits, a property that accounts for the wide variation in reports of the molecular mass. Just as in *Scapharca* Hb, the oxidation of the heme causes a conformational change of the E helix that results in hemichrome formation. This shift is associated with a weakening of a critical subunit interface that results in dissociation.

Echinodermata

The intracellular Hbs of *Caudina arenicola* (Bonaventura and Kitto, 1973; Mitchell *et al.* 1995) are dimeric as oxy Hbs and associate to tetramers and larger assemblages upon deoxygenation. The ligand-linked dissociation appears to be completely responsible for the observed cooperativity. The Hbs dissociate to monomeric hemichromes upon oxidation, just as in *Scapharca*, and cyanide causes reassociation. The crystal structures of *Caudina* Hbs (Mitchell *et al.* 1995) reveal that the interface in the dimer is similar to that in *Scapharca*, although the contact residues differ.

Phoronida

The ligated intracellular Hbs of *Phoronopsis viridis* are completely dimeric, but dissociate to monomers upon oxidation and reassociate to dimers upon cyanide addition (Garlick *et al.* 1979). The oxygen equilibrium has low cooperativity (n_H=1.2) at a Hb concentration of 125 μmol l^{-1} heme.

Chordata

The lampreys and hagfish are the most primitive living

vertebrates, and their Hbs have properties that sharply distinguish them from those of all other vertebrates. Although cooperative O_2 binding (n_H=1.2) was found in lamprey Hb by Wald and Riggs (1951), it was not recognized because it was thought that the Hb was always monomeric. The Hbs of lampreys are monomeric at pH 7 when ligated with CO or O_2, but self-associate to dimers and tetramers upon deoxygenation (Briehl, 1963; Behlke and Scheler, 1970; Andersen, 1971; Andersen and Gibson, 1971; Dohi *et al.* 1973; Brittain *et al.* 1989). The ligand-dependent dissociation from dimers and tetramers with low O_2 affinity to high-affinity monomers is responsible for the cooperativity (n_H=1.2–1.4). The release of protons accompanying the dissociation accounts completely for the pH-dependence of O_2 binding. Oxidized Hbs of the lampreys *Lampetra fluviatilis* (Behlke and Scheler, 1970) and *Petromyzon marinus* (Qiu, 1997) are monomeric, but addition of metHb ligands causes reassociation to dimers at low pH, as in many invertebrate Hbs.

The pH-dependent dissociation of lamprey Hb within red cells will increase the osmotic pressure and alter the acid–base balance. These topics are reviewed in detail by Nikinmaa *et al.* (1995).

Hagfish Hbs also self-associate upon deoxygenation, but the level of cooperativity is lower than in lamprey Hbs. Bannai *et al.* (1972) found that none of the isolated components of the Hb of the hagfish *Eptatretus burgeri* showed significant cooperativity, but discovered that upon deoxygenation two components formed a hybrid tetramer that exhibited significant cooperativity. Evidence for such subunit interaction has also been shown for the Hb of the hagfish *Myxine glutinosa* (Fago and Weber, 1995). The combination of unlike chains to produce a cooperative complex might be thought of as a precursor of the cooperative $\alpha_2\beta_2$ tetramers of higher vertebrates, but there is no structural evidence that the interfaces are similar. If the interfaces resemble those suggested for the lamprey, the cooperative mechanism would be totally different.

In conclusion, oligomeric Hbs have been identified in five phyla (chordates, echinoderms, phoronids, molluscs and annelids) that dissociate upon oxidation and reassociate with the addition of a ferric heme ligand. This finding points to a common mechanism that links oxidation of the heme iron to the weakening of a critical subunit interface. We suggest that the same pathway is used for communication between heme and the subunit interface during cooperative O_2 binding. The crystal structures of both *Caudina* and *Scapharca* Hbs reveal that the E/F helices form the dimer interface. We infer that the critical interface is likely to be similar, although not identical, in all these Hbs. This phylogenetic perspective suggests that the most common cooperative mechanism in the animal kingdom may be that of *Scapharca* Hb.

Vertebrates

Tetramer–tetramer association and supercooperativity

Avian Hbs and bloods have been the most extensively studied. Many studies of chicken blood have reported very steep O_2 binding curves with Hill coefficients above 4 at high levels of oxygenation (for references, see Cobb *et al.* 1992; Vorger, 1994). Examples of such equilibria (Lapennas and Reeves, 1983*a*,*b*) are shown in Fig. 1. These data show that embryonic red cells of the chicken have steeper O_2 equilibria than those of the adult; both are much steeper than that of human red cells. In contrast, most *in vitro* studies of Hb solutions do not exhibit this property. This puzzling difference has not been fully resolved, but may depend in part on differences in concentration and allosteric factors. Such data can be produced by deoxygenation-dependent self-association of tetramers. However, erroneously high Hill coefficients can also result from incomplete saturation with oxygen, as emphasized by Lapennas *et al.* (1981) and Vorger (1987, 1994). The superposition of the artifactual effects of incomplete saturation on the real effects of tetramer–tetramer self-association makes analysis of some of the early data difficult. Nevertheless, the evidence for tetramer–tetramer interactions is compelling. We will consider first the general consequences of linkage (Wyman, 1964; Wyman and Gill, 1990) between oxygenation and dissociation of tetramer–tetramer complexes. We neglect the dissociation of tetramers to dimers in the discussion, but one should recognize that real data do exhibit ligand-dependent dissociation of tetramer to dimer at low concentrations as well. The deoxygenation-linked self-association of tetramers can be described in terms of a thermodynamic cycle (Fig. 2)

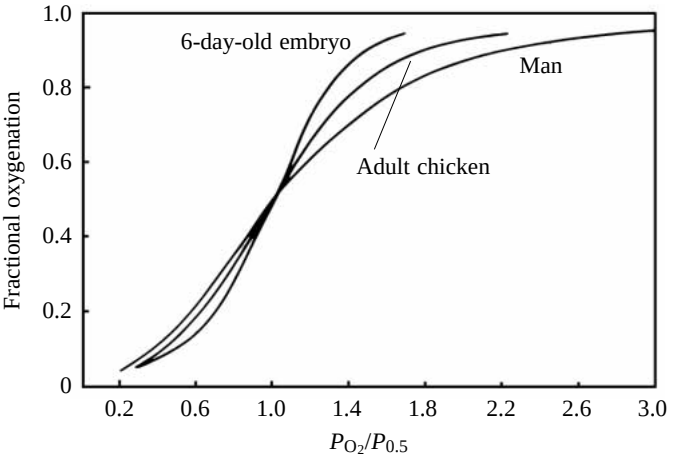

Fig. 1. Oxygen equilibrium of blood from adult and 6-day-old embryos of the chicken and from man. The data have been normalized to the same P_{O_2} value for 50 % saturation to emphasize the differences in shape. The experiments with chicken blood used a P_{O_2} of 650 mmHg (1 mmHg=0.1333 kPa) to achieve full saturation (adapted from Lapennas and Reeves, 1983*b*). The maximal Hill coefficient for the 6-day-old embryo is 6.5 at 85 % saturation and approximately 4.1 for the adult blood at 80 % saturation (Lapennas and Reeves, 1983*a*).

In this cycle, Hb refers to the tetramer, all K values and standard free energy values (ΔG^o values) refer to the overall equilibrium association processes. Conservation of energy

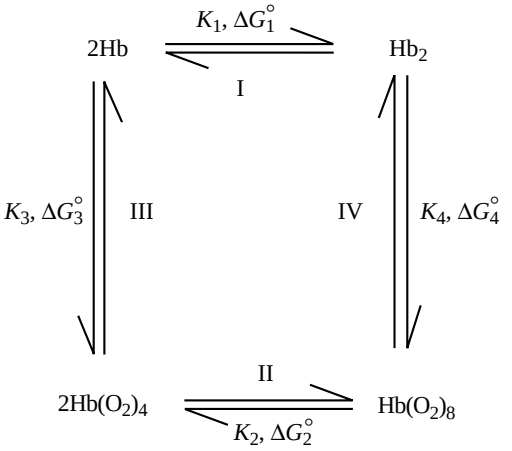

Fig. 2. A thermodynamic cycle describing deoxygenation-linked self-association of Hb tetramers. In this cycle, Hb refers to the tetramer, all dissociation constant (K) values and standard free energy (ΔG^o) values refer to the overall equilibrium association processes. For further details, see text.

requires that $\Delta G_1^o+\Delta G_4^o=\Delta G_2^o+\Delta G_3^o$. Consequently, $K_1K_4=K_2K_3$ or $K_1/K_2=K_3/K_4$. This means that if $K_1>K_2$ then, necessarily, $K_3>K_4$. Cobb *et al.* (1992) showed by sedimentation velocity analysis that tetramers of component D of chicken Hb self-associate upon deoxygenation to dimers of tetramers in the absence of organic phosphates, but that component A does not measurably self-associate under these conditions. Self-association could not be detected in either component when oxygenated. Thus, for component D, in the cycle outlined above, $K_1 \gg K_2$ and $K_3 \gg K_4$, i.e. the Hb tetramer must have a much higher oxygen affinity than that of Hb_2 (octamer). During oxygenation, the equilibria will shift from low-affinity octamers to higher-affinity tetramers. This shift will necessarily be associated with a rise in the Hill coefficient. Moreover, this rise will be dependent on total protein concentration. Thus, oxygen equilibria of Hbs at low concentrations would be governed primarily by the cooperative O_2 binding *within* tetramers (process III in Fig. 2). Process I (Hb_2 formation) would be insignificant, but would become increasingly important as the concentration increases. Although no studies have yet been made of the concentration-dependence of oxygen binding by chicken Hb, we can ask whether the published studies of oxygen binding have been performed at protein concentrations sufficiently high to make the effect of tetramer–tetramer interactions experimentally observable. This question can be addressed as follows. For process I, $2Hb \rightleftharpoons Hb_2$, the equilibrium dissociation constant K_{Diss} is given by:

$$\frac{\alpha^2 C_T}{1-\alpha} = K_{Diss}.$$

The mass fraction of tetramer (Hb), α, is determined by C_T, the total mass concentration of the Hb. Cobb *et al.* (1992) give the values K_{Assoc}= 0.39 $cm^3\,mg^{-1}$ or K_{Diss}=2.56 $mg\,cm^{-3}$ at

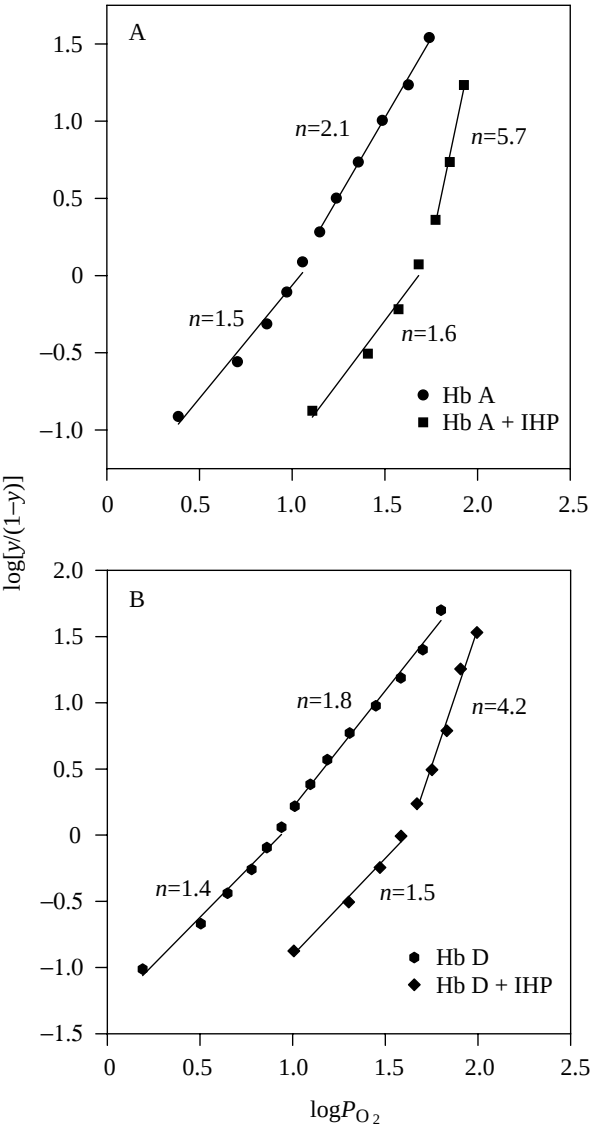

Fig. 3. Oxygen equilibria of components of chicken Hb. (A) Component A, (B) component D. The plots are derived from the original data used for Fig. 2 of Isaacks *et al.* (1976). IHP, inositol hexaphosphate; y is fractional oxygenation. Conditions are given in Table 1.

pH 7.5 in the absence of organic phosphates, where K_{Assoc} is the association constant. Table 1 gives the mass concentrations of Hb used in various O_2 equilibrium measurements of chicken Hb together with calculated values of α on the basis that K_{Diss}=2.56 $mg\,cm^{-3}$. These calculations suggest that the measurements by Isaacks *et al.* (1976) (chicken Hb) and Vorger (1994) (pigeon Hb) were made at concentrations too low to expect to see clear evidence for tetramer–tetramer interactions. However, re-examination of the data of Isaacks *et al.* (1976) is instructive. Fig. 3 shows Hill plots for Hbs A and D calculated by J. E. Knapp, M. A. Oliveira, Q. Xie, S. R. Ernst, A. F. Riggs and M. R. Hackert (in preparation) from the data used for Fig. 2 of Isaacks *et al.* (1976). These data provide an explanation for the apparent paradox that the Isaacks data

Table 1. *Estimated extent of dissociation, α, of tetramer–tetramer complexes of some avian hemoglobins*

		Temperature (°C)	pH	Protein concentration (mg ml^{-1})	α	P_{50} (mmHg)[1]	n[2]	Reference
Chicken[3]	Component A	37	7.4	1.7	0.69	36.4	2.3	Isaacks *et al.* (1976)
	Component D	37	7.4	1.7	0.69	27.9	2.3	Isaacks *et al.* (1976)
Chicken[4]	Component A	37	6.9	200	0.11	81	2	Baumann *et al.* (1987)
	Component D	37	6.9	200	0.11	68	3	Baumann *et al.* (1987)
Pigeon[5]	Stripped	25	7.4	2.0	0.66	9.3	2.4	Vorger (1994)
	+IPP	25	7.4	2.0	0.66	16.4	2.9	Vorger (1994)

[1]Partial pressure of O_2 at 50 % oxygenation.
[2]Value of the Hill coefficient provided by authors.
[3]50 mmol l^{-1} bis-Tris, pH 7.4, P_{CO_2} = 40 mmHg, 77 mol ATP per mol Hb tetramer.
[4]Buffer: 'simulated physiological conditions', pH 6.9, 3 mol ATP per mol Hb tetramer.
[5]50 mmol l^{-1} Tris, 100 mmol l^{-1} NaCl, pH 7.4, molar ratio IHP/ Hb tetramer = 67.
IHP, inositol hexaphosphate; IPP, inositol phentaphosphate.

show high Hill coefficients for the oxygen equilibria of both Hbs A and D in the presence of inositol hexaphosphate (IHP) but not in the absence of IHP, whereas Cobb *et al.* (1992) found that deoxy Hb D formed dimers of tetramers and deoxy Hb A showed no detectable self-association. The simplest explanation appears to be (1) that the oxygen equilibria were performed at concentrations where the deoxy Hb would be largely tetrameric rather than octameric and (2) that IHP binding shifts the equilibrium in favor of octamers. This may mean that IHP is bound more tightly to octamers than to tetramers and that, since IHP binding to chicken Hb is pH-dependent (Brygier *et al.* 1975), the Hill coefficient should also be pH-dependent. Alternatively, if the deoxygenated Hb consisted of an equilibrium mixture of R-state and T-state forms, IHP would shift the equilibrium towards T-state forms and so the proportion of Hb_2 would increase.

A striking feature of the O_2 equilibria in Fig. 3 is that the curves, appear biphasic in the presence of IHP but not in its absence. Could the elevated Hill coefficients at high degrees of oxygenation in Fig. 3 be merely the artifactual consequence of the low oxygen affinity resulting from IHP binding? This might result in less than 100 % saturation being taken as full saturation. Fig. 4 shows simulated Hill plots on the assumption that the value n_H is either 2.0 or 3.0 and that 89 % oxygenation, corresponding to 102 mmHg (Hb D data), is used as if it were 100 % oxygenation. Although the data of Fig. 4 resemble those of Isaacks *et al.* (1976) (Fig. 3), a major difference is apparent: the lack of saturation produces only a 1.5-fold increase in the mean Hill coefficient between the lower and upper parts of the curves in the simulated data, whereas Fig. 3 shows a 2.8- to 3.5-fold increase. The maximal P_{O_2} used in the experiments was 265 mmHg, indicating that the high n_H values cannot be attributed completely to inadequate saturation and therefore must reflect the known presence of dimers of tetramers in deoxy chicken Hb D (Cobb *et al.* 1992).

Vorger (1994) has made very careful O_2 binding studies of both blood and Hb from the pigeon, which has only component A whereas most birds possess both components A and D. The oxygen equilibria for blood and Hb solutions were very similar and showed no evidence for supercooperativity: the maximal Hill coefficient of 3.35 (pigeon blood) is close to the maximal value of 3.38 obtained for human Hb (Doyle *et al.* 1992). Vorger (1994) suggests that supercooperativity is absent in pigeon blood because it requires two different components. However, the data of Isaacks *et al.* (1976) (see Fig. 3) show evidence for high cooperativity in isolated single components of chicken Hb. The measurements of solutions of pigeon Hb were made at too great a dilution to ensure detection of supercooperativity were it present. The lack of n_H values greater

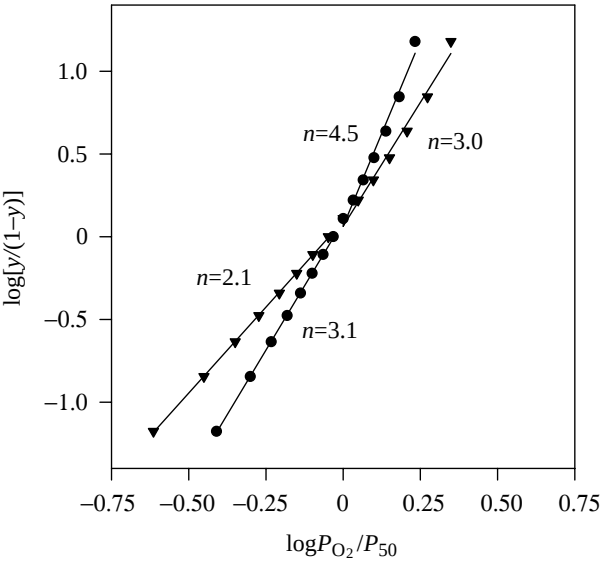

Fig. 4. Simulation of the effect of incomplete saturation. Two sets of data were created, one with the Hill coefficient n_H=2.0 (triangles) and the other with n_H=3.0 (filled circles). The degree of saturation used to define '100 %' oxygenation in both sets of data is 89 %. See text and legend to Fig. 3 for details.

than 4 in pigeon blood, however, is strong evidence against the presence of supercooperativity. This result might conceivably have arisen from depletion of the organic phosphates within the red cells either before or during the measurements.

The data of Baumann *et al.* (1987) in Table 1 are not consistent with those of Isaacks *et al.* (1976). The data of Baumann *et al.* (1987) were obtained at a protein concentration of 200 mg ml^{-1} so that virtually all of the deoxy Hb should have been associated to dimers of tetramers, but the n_H values do not show this. Two possible reasons for this can be suggested: (1) the lengthy technique used generates up to 25 % metHb (Baumann *et al.* 1982), which would decrease n_H, and (2) Isaacks *et al.* (1976) used a P_{CO_2} of 40 mmHg, whereas Baumann *et al.* (1987) used no CO_2. The known preferential binding of CO_2 to T-state Hb would increase the amount of both Hb_T and $(Hb_T)_2$ so that n_H should be elevated. The discrepancy between the two sets of data underlines the need to re-examine this system to define the conditions required for supercooperativity.

Mammals

Holland and associates have found very high cooperativity of O_2 binding in the red cells of embryonic marsupials (Holland *et al.* 1988, 1994; Calvert *et al.* 1994) and rabbits (Holland and Calvert, 1995). They used a Hemo-Scan instrument modified as described by Lapennas and Lutz (1982) to ensure that the recorded P_{O_2} accurately corresponded to that of the chamber. They found that full saturation was achieved by air (P_{O_2}≈150 mmHg); raising the P_{O_2} to 400 mmHg did not change the final absorbance significantly (Holland *et al.* 1994). Furthermore, they measured O_2 binding in both directions: oxygenation and deoxygenation gave indistinguishable results (Holland *et al.* 1988). Fig. 5 shows the results for the brushtail possum. The overall pattern corresponds closely to that reported by Lapennas and Reeves (1983*a*,*b*) for chicken blood (see Fig. 1), which shows a biphasic Hill plot with n_H values greater than 4 above 50 % oxygenation. The blood for these studies was obtained from newborn marsupials which at birth are still embryonic, as reflected in the fact that neural connections between eye and brain have not been made. Whole embryonic rabbit blood gave similar biphasic Hill plots with high cooperativity in the upper half of the curves. The increase in the Hill coefficient is approximately 240 % between the lower and upper halves of the curve, a result similar to that in the data of Isaacks *et al.* (1976) for chicken Hb. Hemolysis by addition of distilled water abolished the high cooperativity, but it is unclear why this occurred. Dilution, a decrease in organic phosphate levels and the loss of intracellular buffering could be partly responsible.

Fish

The Hb of an elasmobranch, the spiny dogfish *Squalus acanthias*, is tetrameric only in the deoxy form; it dissociates reversibly to dimers upon oxygenation (Fyhn and Sullivan, 1975). The adult Hb used in these studies was treated with iodoacetamide to prevent irreversible polymerization by disulfide formation. Organic phosphates (ATP and GTP) greatly reduce the O_2 affinity as in other Hbs, and also enhance the cooperativity of O_2 binding (Weber *et al.* 1983). This is the expected result because organic phosphates bind preferentially to tetrameric deoxy Hbs. Dissociation of the tetramer destroys the binding site for GTP or ATP so that enhanced cooperativity produced by organic phosphates results directly from the decreased tetramer–dimer dissociation in the presence of ATP or GTP.

The tetrameric oxy Hbs of teleost fish, in contrast, are extremely resistant to dissociation to dimers (Edelstein *et al.* 1976): the tetramer to dimer dissociation constant is 100-fold lower than that of human Hb. However, sedimentation analysis

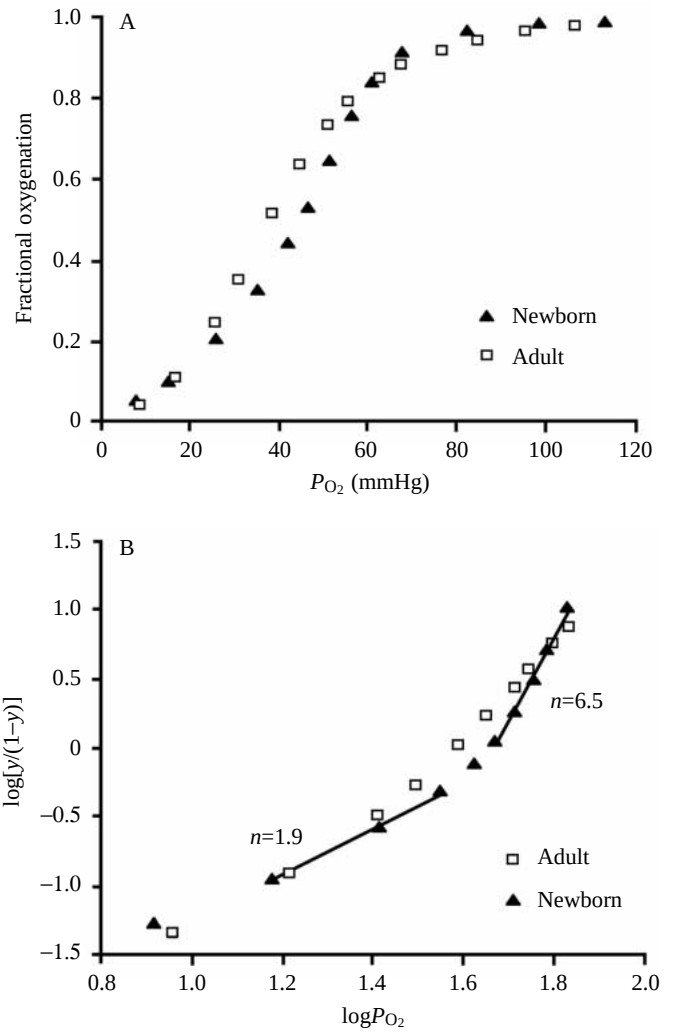

Fig. 5. Oxygen equilibria of blood from newborn brushtail possum and its mother, adapted from Figs 1 and 2 of Calvert *et al.* (1994). The same data are used for both A and B. Equilibria were measured in the presence of P_{CO_2}=42 mmHg at 37 °C. The *n* values shown are for the newborn blood; the *n* values for adult blood increase from 2.9 (lower part) to 3.6 (upper part); *y*, fractional oxygenation. For further details, see legend to Fig. 3.

of deoxy carp Hb indicates a weak self-association to dimers of tetramers with a dissociation constant of $\approx 22\,mg\,ml^{-1}$ (Atha and Riggs, 1982). Deoxy Hb in the red cells should be at least 75 % associated on this basis. The *in vivo* effect of tetramer–tetramer interaction should therefore be substantial. The linked effect of such association on O_2 binding has not yet been investigated.

Amphibians

The Hbs of amphibians display a complex array of dissociation/association processes that change during development. Elli *et al.* (1970) showed by sedimentation velocity experiments that the Hbs of the frog *Rana esculenta* and the axolotl *Axolotl mexicanum* both polymerize reversibly upon deoxygenation. The deoxygenation-linked assembly of axolotl Hb was found to be extremely sensitive to pH: a rise in pH from 6.5 to 7.0 caused almost complete dissociation. This suggests a very large pH-dependence of O_2 binding (Bohr effect), but this linkage has yet to be studied. In contrast, the corresponding assembly of Hb of the frog *Rana esculenta* is independent of pH, and the Hb of the newt *Triturus cristatus* does not self-associate at all.

The ligand-dependent dissociation behavior of the Hbs of the tadpole and adult bullfrog *Rana catesbeiana* have been studied extensively. The adult hemoglobin has two major components, B and C, present in the molar ratio 1:2. Component C polymerizes by forming intermolecular disulfide bonds, but this can be prevented by reaction of available sulfhydryl groups with iodoacetamide. The more interesting association occurs upon deoxygenation of a 1:2 mixture of components B and C which forms a mixed trimer of tetramers, BC_2 (Aggarwal and Riggs, 1969; Tam and Riggs, 1984). Analysis of sedimentation velocity profiles reveals that the association can be satisfactorily described in terms of two steps, $B+C \rightleftharpoons BC$ and $BC+C \rightleftharpoons BC_2$, in which the second C is bound more tightly than the first, indicating significant cooperativity (Tam et al., 1993). The tightness of the BC_2 assemblage increases strongly with a decrease in temperature, which suggests that the interactions are not primarily hydrophobic but electrostatic, Van der Waals and hydrogen bonding. Model building has suggested a possible explanation for this cooperativity: the first C binds to B at one site, whereas the second C binds to both C and B (Smith *et al.* 1993). This deoxygenation-linked association is reflected in the strong dependence of the O_2 equilibrium on protein concentration (Tam and Riggs, 1984). Thus, the O_2 affinity decreases and the Hill coefficient increases as the concentration of Hb rises. The n_{max} values of the separate B and C components are lower than in mammals, but the 1:2 B:C mixture causes n_H to reach values similar to those in mammals. This indicates that BC_2 formation may serve to raise cooperativity close to the value in mammals rather than to create 'supercooperativity'. The very high Hill coefficients (n_H>4) reported earlier (Tam and Riggs, 1984) may have resulted from incomplete saturation with O_2, as previously discussed.

The formation of BC_2 also makes an important contribution to the Bohr effect because the assembly process is pH-dependent. The dissociation of BC_2 within the red cell would be accompanied by a threefold increase in the osmotic pressure if compensating ionic changes did not occur. These cellular changes have yet to be explored.

The Hbs of the tadpole of the bullfrog *Rana catesbeiana* display ligand-linked dissociation of tetramers to dimers and to monomers that is strongly pH-dependent (Atha *et al.* 1979). These dissociations are partly reflected in the pH-dependence of cooperativity. However, the cooperativity as measured by n_H decreases from 2.5 to 1.5 between pH 7 and pH 8, where very little dissociation of the tetramer occurs. So this drop in cooperativity with pH must be intrinsic within the tetramer and not a function of dissociation. Cooperativity of O_2 binding is modulated not only by pH but also by organic phosphates (Watt and Riggs, 1975), which raise the cooperativity because they bind only to tetramers. These studies emphasize the fact that the O_2 equilibria are concentration-dependent and that measuring this dependence is essential for establishing physiologically relevant data.

Bårdgard *et al.* (1997) have recently made very careful measurements of O_2 binding and aggregation of the adult Hb from the European frog *Rana temporaria*. A single component accounts for 80–90 % of the Hb. This Hb associates upon deoxygenation and the O_2 equilibria are strongly concentration-dependent, as with the Hb of the bullfrog. They obtained data up to a P_{O_2} of 760 mmHg and estimated full saturation by extrapolation. The maximal values of the Hill coefficient, n_H, never exceeded 3.0 even at very high Hb concentrations. The data were analyzed in terms of the two-state Monod–Wyman–Changeux model. Deoxygenation-dependent tetramer–tetramer association suggests, however, that the number of states required to describe the system is likely to be greater than two.

Reptiles

Blood and Hbs of two species of garter snake *Thamnophis* sp. have been reported to bind O_2 with high cooperativity, with n_H>4 (Berner and Ingermann, 1988; Sode, 1991). Could this finding have resulted from their use of air to achieve full saturation? If their reports of n_H values of approximately 5 reflect the true properties of the oxygen equilibrium, then close to full saturation could be reached with air; if it is wholly an artifact, then full saturation would not have been reached. The analysis given for chicken Hb is applicable here (see Fig. 3). The Sode data show a Hill coefficient ratio (n_{upper}/n_{lower}) of approximately 4 for whole blood. Comparison with the simulation in Fig. 3 suggests that the whole-blood data do reflect the presence of supercooperativity that disappears upon hemolysis. This difference is not clearly evident in the data of Berner and Ingermann (1988), where the rise in n_H with oxygenation is only slightly greater than that shown in the simulations in Fig. 4. We conclude that supercooperativity may be present in whole snake blood, but its presence in Hb solutions is uncertain.

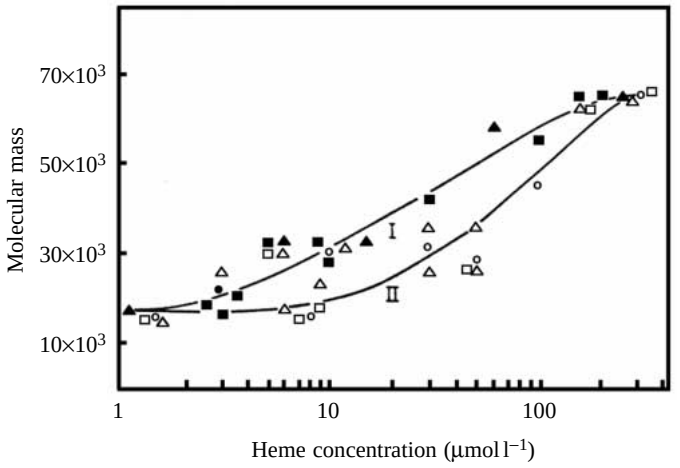

Fig. 6. Results of sedimentation equilibrium experiments on the CO-Hbs of the turtle *Phrynops hilarii* from Reischl *et al.* (1984). The data for components I (filled symbols) and II (open symbols) are shown. Triangles, pH 6.5; circles, pH 7.7; squares, pH 8.6, at 20 °C.

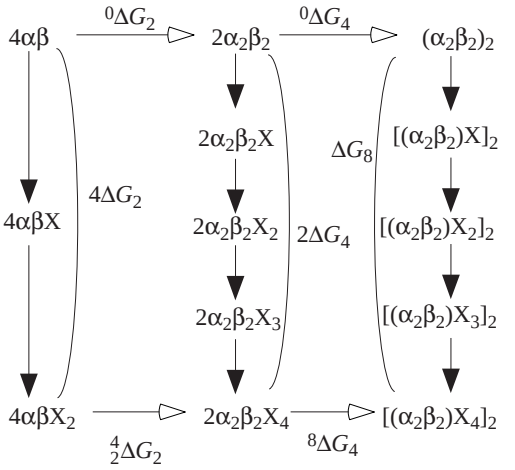

Fig. 7. Linkage system for a tetrameric $\alpha_2\beta_2$ hemoglobin that both dissociates to $\alpha\beta$ dimers and associates to $(\alpha_2\beta_2)_2$ octamers. The difference $2\Delta G_4$ minus $4\Delta G_2$ will give the cooperative free energy, as defined by Ackers *et al.* (1992), and ΔG_8 minus ΔG_4 will give the additional cooperative free energy resulting from tetramer–tetramer interaction. ΔG, standard free energy. The species with odd numbers of ligands are omitted in the oxygenation of the octomer (right column).

Extensive studies by Focesi and associates on the Hb of the Brazilian water snake *Liophis miliaris* are of particular interest here because oxygenation is accompanied by a large increase in the tetramer to dimer dissociation constant in the presence but not in the absence of ATP (Matsuura *et al.* 1987; Focesi *et al.* 1990). The deoxy Hb is an equilibrium mixture of dimers and tetramers in the absence of ATP. These data indicate that ATP shifts the equilibrium to mostly tetramers because it binds only to them. Cooperativity of O_2 binding is low (n_H≈1.0–1.2) in the absence of ATP, but in its presence cooperativity is pronounced (n_H≈1.9–2.0). Amino acid sequence analysis (Matsuura *et al.* 1989) indicates that the $\alpha^1\beta^2$ dimer–dimer contacts are weakened by two β chain substitutions which eliminate two electrostatic interactions: Glu(G3)→Val and Glu(CD2)→Thr.

Many tetrameric turtle Hbs dissociate readily not only to dimers but also to monomers with dilution (Sullivan and Riggs, 1967; Reischl *et al.* 1984). An example of the concentration-dependence of the molecular mass is shown in Fig. 6. Hill plots of oxygen binding data are biphasic, with n_H increasing from 1.8 to 2.8. This shift must reflect the pronounced dissociation properties shown in Fig. 6, but it may also include a contribution from incomplete oxygenation. The quantitative linkage between dissociation and oxygenation has not yet been investigated.

Conclusions

This brief survey attempts to answer the two questions asked in the Introduction: how widespread is the *Scapharca* mechanism among non-vertebrate Hbs, and how important are tetramer–tetramer interactions in vertebrate Hbs? The conclusion is reached that the *Scapharca* mechanism may be the predominant process in invertebrate Hbs. Surprisingly, it may also occur in a primitive vertebrate, the lamprey. Thus, lamprey Hb may be considered as an invertebrate Hb in a vertebrate.

Ligand-linked subunit dissociation/association processes occur in many diverse Hbs. Although, mammalian Hbs usually dissociate only to dimers, Hbs of numerous lower vertebrates dissociate to both dimers and monomers. Strong evidence for tetramer–tetramer association of some deoxy Hbs has been presented. The overall linked relationships for a dimer–tetramer–octamer system required for a complete description of chicken Hb are shown in Fig. 7. The Monod–Wyman–Changeux (MWC) two-state model has been used extensively to describe the cooperative processes within the tetramer (from $\alpha_2\beta_2$ to $\alpha_2\beta_2X_4$ in Fig. 7). Ackers *et al.* (1992, 1997) have been able to determine the detailed linkage relationships for the dimer–tetramer system in human Hb, but addition of tetramer–tetramer interactions adds a daunting new complexity. However, determination of the weight-average molecular mass as a function of the degree of oxygenation would make the task tractable. The X-ray structure of deoxy chicken Hb will be needed for understanding the process. For some of the systems that exhibit tetramer–tetramer interactions, the gain in cooperativity may well be not to create 'supercooperativity' (i.e. Hill coefficients greater than 4), but rather to raise an intrinsically low cooperativity of tetrameric Hbs.

The author is grateful to Yang Qiu and James Knapp for valuable discussions. I thank Russell Isaacks for making available the original data used for the construction of Fig. 3. Current work in the author's laboratory has been supported by NSF grant MCB-9723825.

References

ACKERS, G. K., DOYLE, M. L., MYERS, D. AND DAUGHERTY, M. A.

(1992). Molecular code for cooperativity in hemoglobin. *Science* **255**, 54–63.

ACKERS, G. K., PERRELLA, M., HOLT, J. M., DENISOV, I. AND HUANG, Y. (1997). Thermodynamic stability of the asymmetric doubly-ligated hemoglobin tetramer ($\alpha^{+CN}\beta^{+CN}$)($\alpha\beta$): Methodological and mechanistic issues. *Biochemistry* **36**, 10822–10829.

AGGARWAL, S. J. AND RIGGS, A. (1969). The hemoglobins of the bullfrog, *Rana catesbeiana*. I. Purification, amino acid composition and oxygen equilibria. *J. biol. Chem.* **244**, 2372–2383.

ANDERSEN, M. E. (1971). Sedimentation equilibrium experiments on the self-association of hemoglobin from the lamprey *Petromyzon marinus*. *J. biol. Chem.* **246**, 4800–4806.

ANDERSEN, M. E. AND GIBSON, Q. H. (1971). A kinetic analysis of the binding of oxygen and carbon monoxide to lamprey hemoglobin. *J. biol. Chem.* **246**, 4790–4799.

ASCOLI, F., ROSSI-FANELLI, M. R., CHIANCONE, E., VECCHINI, P. AND ANTONINI, E. (1978). Studies on erythrocruorin. VI. Ferric derivatives of earthworm erythrocruorin. *J. molec. Biol.* **119**, 191–202.

ATHA, D. H. AND RIGGS, A. F. (1982). Conformational transitions in carp hemoglobin at low pH. Evidence for aggregation of tetramers. In *Hemoglobin and Oxygen Binding* (ed. C. Ho), pp. 269–275. New York: Elsevier Press.

ATHA, D. H., RIGGS, A., BONAVENTURA, J. AND BONAVENTURA, C. (1979). Hemoglobins of the tadpole of the bullfrog, *Rana catesbeiana*. pH dependence of ligand binding and subunit dissociation equilibria and kinetics. *J. biol. Chem.* **254**, 3393–3400.

BANNAI, S., SUGITA, Y. AND YONEYAMA, Y. (1972). Studies on hemoglobin from the hagfish *Eptatretrus burgeri*. *J. biol. Chem.* **247**, 505–510.

BÅRDGARD, A., FAGO, A., MALTE, H. AND WEBER, R. E. (1997). Oxygen binding and aggregation of hemoglobin from the common European frog, *Rana temporaria*. *Comp. Biochem. Physiol.* **117**B, 225–231.

BAUMANN, R., FISCHER, J. AND ENGELKE, M. (1987). Functional properties of primitive and definitive red cells from chick embryo: oxygen-binding characteristics, pH and membrane potential and response to hypoxia. *J. exp. Zool.* (Suppl.) **1**, 227–238.

BAUMANN, R., PADEKEN, S. AND HALLER, E. A. (1982). Functional properties of embryonic chicken hemoglobins. *J. appl. Physiol.* **53**, 1439–1448.

BEHLKE, J. AND SCHELER, W. (1970). Der Einfluss von Liganden auf den Assoziationsgrad des Desoxy-Hämoglobins der Flussneunaugen (*Lampetra fluviatilis* L.). *FEBS Lett.* **7**, 177–179.

BERNER, N. J. AND INGERMANN, R. L. (1988). Molecular basis of the difference in oxygen affinity between maternal and fetal red blood cells in the viviparous garter snake *Thamnophis elegans*. *J. exp. Biol.* **140**, 437–453.

BOFFI, A., VECCHINI, P. AND CHIANCONE, E. (1990). Anion-linked polymerization of the tetrameric hemoglobin from *Scapharca inaequivalvis*: characterization and functional relevance. *J. biol. Chem.* **265**, 6203–6209.

BONAVENTURA, J. AND KITTO, G. B. (1973). Ligand-linked dissociation of some invertebrate hemoglobins. In *Comparative Physiology* (ed. L. Bolis, K. Schmidt-Nielsen and S. H. P. Maddrell), pp. 493–507. Amsterdam: North-Holland Publishing Co.

BRIEHL, R. W. (1963). The relationship between the oxygen equilibrium and aggregation of subunits in lamprey hemoglobin. *J. biol. Chem.* **238**, 2361–2366.

BRITTAIN, T. (1991). Cooperativity and allosteric regulation in non-mammalian vertebrate haemoglobins. *Comp. Biochem. Physiol.* **99**B, 731–740.

BRITTAIN, T., O'BRIEN, A. J., WELLS, R. M. G. AND BALDWIN, J. (1989). A study of the role of subunit aggregation in the expression of cooperative ligand binding in the haemoglobin of the lamprey *Mordacia mordax*. *Comp. Biochem. Physiol.* **93**B, 549–554.

BRYGIER, J., DEBRUIN, S. H., VAN HOOF, P. M. K. B. AND ROLLEMA, H. S. (1975). The interaction of organic phosphates with human and chicken hemoglobin. *Eur. J. Biochem.* **60**, 379–383.

BUNN, H. F. AND POYTON, R. O. (1996). Oxygen sensing and molecular adaptation to hypoxia. *Physiol. Rev.* **76**, 839–885.

CALVERT, S. J., HOLLAND, R. A. B. AND GEMMELL, R. T. (1994). Respiratory properties of the neonatal blood of the common Brushtail Possom (*Trichosurus vulpecula*). *Physiol. Zool.* **67**, 407–417.

CHIANCONE, E., VECCHINI, P., VERZILI, D., ASCOLI, S. AND ANTONINI, E. (1981). Dimeric and tetrameric hemoglobin from the mollusc *Scapharca inaequivalvis*. *J. molec. Biol.* **152**, 577–592.

COBB, J. A., MANNING, D., KOLATKAR, P. R., COX, D. J. AND RIGGS, A. F. (1992). Deoxygenation-linked association of a tetrameric component of chicken hemoglobin. *J. biol. Chem.* **267**, 1183–1189.

DANIEL, R. M. (1970). The electron transport system of *Acetobacter suboxydans* with particular reference to cytochrome o. *Biochim. biophys. Acta* **216**, 328–341.

DOHI, Y., SUGITA, Y. AND YONEYAMA, Y. (1973). The self-association and oxygen equilibrium of hemoglobin from the lamprey, *Entosphenus japonicus*. *J. biol. Chem.* **248**, 2354–2363.

DOYLE, M. L., LEW, G., DEYOUNG, A., KWIATKOWSKI, L., WIERZBA, A., NOBLE, R. W. AND ACKERS, G. K. (1992). Functional properties of human hemoglobins synthesized from recombinant mutant β-globins. *Biochemistry* **31**, 8629–8639.

EDELSTEIN, S. J., MCEWEN, B. AND GIBSON, Q. H. (1976). Subunit dissociation in fish hemoglobins. *J. biol. Chem.* **251**, 7632–7637.

EDSALL, J. T. AND GUTFREUND, D. (1983). *Biothermodynamics*, 201. New York: John Wiley & Sons.

ELLI, R., GIULIANI, A., TENTORI, L., CHIANCONE, E. AND ANTONINI, E. (1970). The hemoglobin of Amphibia. X. Sedimentation behaviour of frog, triton and axolotl hemoglobins. *Comp. Biochem. Physiol.* **36**, 163–171.

FAGO, A. AND WEBER, R. E. (1995). The hemoglobin system of the hagfish *Myxine glutinosa*: aggregation state and functional properties. *Biochim. biophys. Acta* **1249**, 109–115.

FOCESI, A., OGO, S. H. AND MATSUURA, M. S. A. (1990). Dimer–tetramer transition in hemoglobins from *Liophis miliaris*. II. Evidence with the stripped proteins. *Comp. Biochem. Physiol.* **96**B, 119–122.

FYHN, U. E. H. AND SULLIVAN, B. (1975). Elasmobranch hemoglobins: dimerization and polymerization in various species. *Comp. Biochem. Physiol.* **50**B, 119–129.

GARLICK, R. L., WILLIAMS, B. J. AND RIGGS, A. F. (1979). The hemoglobin of *Phoronopsis viridis* of the primitive invertebrate phylum Phoronida: characterization and subunit structure. *Archs Biochem. Biophys.* **194**, 13–23.

GERACI, G., SADA, A. AND CIROTTO, C. (1977). Cooperative, low-molecular weight dimeric myoglobins from the radular muscle of the gastropod mollusc *Nassa mutabilis* L. *Eur. J. Biochem.* **77**, 555–560.

HILL, A. V. (1913). The combination of haemoglobin with oxygen and with carbon monoxide. *Biochem. J.* **7**, 471–480.

HOLLAND, R. A. B. AND CALVERT, S. J. (1995). Oxygen transport by

rabbit embryonic blood: high cooperativity of hemoglobin-oxygen binding. *Respir. Physiol.* **99**, 157–164.

HOLLAND, R. A. B., CALVERT, S. J., HOPE, R. M. AND CHESSON, C. M. (1994). Blood O_2 transport in newborn and adult of a very small marsupial (*Sminthopsis crassicaudata*). *Respir. Physiol.* **98**, 69–81.

HOLLAND, R. A. B., RIMES, A. F., COMIS, A. AND TYNDALE-BISCOE, C. H. (1988). Oxygen carriage and carbonic anhydrase activity in the blood of a marsupial, the tammar wallaby (*Macropus eugenii*), during early development. *Respir. Physiol.* **73**, 69–86.

IKEDA-SAITO, M., YONETANI, T., CHIANCONE, E., ASCOLI, F., VERZILI, D. AND ANTONINI, E. (1983). Thermodynamic properties of oxygen equilibria of dimeric and tetrameric hemoglobins from *Scapharca inaequivalvis*. *J. molec. Biol.* **170**, 1009–1018.

IMAMURA, T., RIGGS, A. AND GIBSON, Q. H. (1972). Equilibria and kinetics of ligand binding by leghemoglobin from soybean root nodules. *J. biol. Chem.* **247**, 521–526.

ISAACKS, R. E., HARKNESS, D. R., ADLER, J. L. AND GOLDMAN, P. H. (1976). Studies on avian erythrocyte metabolism. Effect of organic phosphates on oxygen affinity of embryonic and adult-type hemoglobins of the chick embryo. *Archs Biochem. Biophys.* **173**, 114–120.

LAPENNAS, G. N., COLACINO, J. M. AND BONAVENTURA, J. (1981). Thin-layer methods for determination of oxygen binding curves of hemoglobin solutions and blood. *Meth. Enzymol.* **76**, 449–470.

LAPENNAS, G. N. AND LUTZ, P. L. (1982). Oxygen affinity of turtle blood. *Respir. Physiol.* **48**, 59–74.

LAPENNAS, G. N. AND REEVES, R. B. (1983*a*). Oxygen affinity and equilibrium curve shape in blood of chicken embryos. *Respir. Physiol.* **52**, 13–26.

LAPENNAS, G. N. AND REEVES, R. B. (1983*b*). Oxygen affinity of blood of adult domestic chicken and red jungle fowl. *Respir. Physiol.* **52**, 27–39.

MANWELL, C. (1958). The oxygen–respiratory pigment equilibrium of the hemocyanin and myoglobin of the amphineuran mollusc *Cryptochiton stelleri*. *J. cell. comp. Physiol.* **52**, 341–352.

MATSUURA, M. S. A., FUSHITANI, K. AND RIGGS, A. F. (1989). The amino acid sequence of the α and β chains of hemoglobin from the snake, *Liophis miliaris*. *J. biol. Chem.* **264**, 5515–5521.

MATSUURA, M. S. A., OGO, S. H. AND FOCESI, A. (1987). Dimer–tetramer transition in hemoglobins from *Liophis miliaris*. I. Effect of organic polyphosphate. *Comp. Biochem. Physiol.* **86**A, 683–687.

MILLER, P. L. (1966). The function of haemoglobin in relation to maintenance of neutral buoyancy in *Anisops pellucens* (Notonectidae, Hemiptera). *J. exp. Biol.* **44**, 529–543.

MITCHELL, D. T., KITTO, G. B. AND HACKERT, M. L. (1995). Structural analysis of monomeric hemichrome and dimeric cyanomet hemoglobins from *Caudina arenicola*. *J. molec. Biol.* **251**, 421–431.

NIKINMAA, M., AIRAKSINEN, S. AND VIRKKI, L. V. (1995). Haemoglobin function in intact lamprey erythrocytes: interactions with membrane function in the regulation of gas transport and acid–base balance. *J. exp. Biol.* **198**, 2423–2430.

PERUTZ, M. F. (1946). The composition and swelling properties of haemoglobin crystals. *Trans. Faraday Soc.* **42**B, 187–195.

PERUTZ, M. F. (1989). Mechanisms of cooperativity and allosteric regulation in proteins. *Q. Rev. Biophys.* **22**, 139–236.

PERUTZ, M. F., FERMI, G., LUISI, B., SHAANAN, B. AND LIDDINGTON, R. C. (1987). Stereochemistry of cooperative mechanisms in hemoglobin. *Acct. chem. Res.* **20**, 309–321.

QIU, Y. (1997). The genes and subunit association of hemoglobins in the lamprey, *Petromyzon marinus*. PhD dissertation, University of Texas, Austin, TX.

REISCHL, E., HÖHN, M., JAENICKE, R. AND BAUER, C. (1984). Bohr effect, electron spin resonance spectroscopy and subunit dissociation of the hemoglobin components from the turtle *Phrynops hilari*. *Comp. Biochem. Physiol.* **78**B, 251–257.

RIGGS, A. (1976). Factors in the evolution of hemoglobin function. *Fedn Proc. Fedn Am. Socs exp. Biol.* **35**, 2115–2118.

ROYER, W. E., FOX, R. A., SMITH, F. R., ZHU, D. AND BRASWELL, E. H. (1997). Ligand-linked assembly of *Scapharca* dimeric hemoglobin. *J. biol. Chem.* **272**, 5689–5694.

ROYER, W. E., HEARD, K. S., HARRINGTON, D. J. AND CHIANCONE, E. (1995). The 2.0 Å crystal structure of *Scapharca* tetrameric hemoglobin: cooperative dimers within an allosteric tetramer. *J. molec. Biol.* **253**, 168–186.

ROYER, W. E., HENDRICKSON, W. A. AND CHIANCONE, E. (1990). Structural transitions upon ligand binding in a cooperative dimeric hemoglobin. *Science* **249**, 518–521.

ROYER, W. E., PARDANANI, A., GIBSON, Q. H., PETERSON, E. S. AND FRIEDMAN, J. M. (1996). Ordered water molecules as key allosteric mediators in a cooperative dimeric hemoglobin. *Proc. natn. Acad. Sci. U.S.A.* **93**, 14526–14531.

SMITH, D. J., ZHU, H., KOLATKAR, P. R., TAM, L.-T., BALDWIN, T. O., ROE, B. A., BROYLES, R. H. AND RIGGS, A. F. (1993). The hemoglobins of the bullfrog, *Rana catesbeiana*. The cDNA derived amino acid sequences of the α chains of adult hemoglobins B and C: their roles in deoxygenation-induced aggregation. *J. biol. Chem.* **268**, 26961–26971.

SMITH, S. E., BRITTAIN, T. AND WELLS, R. M. G. (1988). A kinetic and equilibrium study of ligand binding to the monomeric and dimeric haem-containing globins of two chitons. *Biochem. J.* **252**, 673–678.

SODE, F. (1991). Oxygen binding characteristics of whole blood and hemoglobin from the snake *Thammophis sirtalis*. *Comp. Biochem. Physiol.* **100**B, 697–703.

SPAGNUOLO, C., RINELLI, P., COLETTA, M., VECCHINI, P., CHIANCONE, E. AND ASCOLI, F. (1988). Oxidation reaction of *Scapharca inaequivalvis* hemoglobins with nitrite. *Biochim. biophys. Acta* **956**, 119–126.

SULLIVAN, B. AND RIGGS, A. (1967). The subunit dissociation properties of turtle hemoglobins. *Biochim. biophys. Acta* **140**, 274–283.

TAM, L.-T. AND RIGGS, A. F. (1984). Oxygen binding and aggregation of bullfrog hemoglobin. *J. biol. Chem.* **259**, 2610–2616.

TAM, L.-T., MANNING, D., COX, D. J. AND RIGGS, A. F. (1993). The hemoglobins of the bullfrog, *Rana catesbeiana*. Deoxygenation-linked association of tetrameric components B and C to form the trimer BC_2: sedimentation analysis and oxygen equilibria. *J. biol. Chem.* **268**, 26972–26977.

TARRICONE, C., GALIZZI, A., CODA, A., ASCENZI, P. AND BOLOGNESI, M. (1997). Unusual structure of the oxygen-binding site in the dimeric bacterial hemoglobin from *Vitreoscilla* sp. *Structure* **5**, 497–507.

TERWILLIGER, R. C. AND READ, K. R. H. (1971). Oxygen equilibrium studies of the radular muscle myoglobins of the gastropod molluscs, *Buccinum undatum* L. and *Busycon canaliculatum*. *Int. J. Biochem.* **2**, 253–261.

TYREE, B. AND WEBSTER, D. A. (1978). The binding of cyanide and carbon monoxide to cytochrome o purified from *Vitreoscilla*. Evidence for subunit interaction in the reduced protein. *J. biol. Chem.* **253**, 6988–6991.

VORGER, P. (1987). Blood oxygen equilibria and theoretical models. II. A critical estimation. *Comp. Biochem. Physiol.* **88**A, 613–618.

VORGER, P. (1994). Oxygen binding properties of blood and hemoglobin from the pigeon *Columba livia*. *Comp. Biochem. Physiol.* **109**B, 391–406.

WAKABAYASHI, S., MATSUBARA, H. AND WEBSTER, D. A. (1986). Primary sequence of a dimeric bacterial haemoglobin from *Vitreoscilla*. *Nature* **322**, 481–483.

WALD, G. AND ALLEN, D. W. (1957). The equilibrium between cytochrome oxidase and carbon monoxide. *J. gen. Physiol.* **40**, 593–608.

WALD, G. AND RIGGS, A. (1951). The hemoglobin of the sea lamprey, *Petromyzon marinus*. *J. gen. Physiol.* **35**, 45–53.

WATT, K. W. K. AND RIGGS, A. (1975). Hemoglobins of the tadpole of the bullfrog, *Rana catesbeiana*. Structure and function of isolated components. *J. biol. Chem.* **250**, 5934–5944.

WEBER, R. E., WELLS, R. M. G. AND ROSSETTI, J. E. (1983). Allosteric interactions governing oxygen equilibria in the haemoglobin system of the spiny dogfish *Squalus acanthias*. *J. exp. Biol.* **103**, 109–120.

WELLS, R. M. G., HUDSON, M. J. AND BRITTAIN, T. (1981). Function of the hemoglobin and the gas bubble in the backswimmer *Anisops assimilis* (Hemiptera: Notonectidae). *J. comp. Physiol.* **142**, 515–522.

WITTENBERG, J. B. AND WITTENBERG, B. A. (1990). Mechanisms of cytoplasmic hemoglobin and myoglobin function. *A. Rev. Biophys. biophys. Chem.* **19**, 217–241.

WYMAN, J. (1964). Linked functions and reciprocal effects in hemoglobin: a second look. *Adv. Protein Chem.* **19**, 223–286.

WYMAN, J. AND GILL, S. J. (1990). *Binding and Linkage. Functional Chemistry of Biological Macromolecules*. Mill Valley, CA: University Science Books. 330pp.

ZHU, H., HARGROVE, M., XIE, Q., NOZAKI, Y., LINSE, K., SMITH, S. S., OLSON, J. S. AND RIGGS, A. F. (1996*a*). Stoichiometry of subunits and heme content of hemoglobin from the earthworm *Lumbricus terrestris*. *J. biol. Chem.* **271**, 29999–30006.

ZHU, H., OWNBY, D. W., RIGGS, C. K., NOLASCO, N. J., STOOPS, J. K. AND RIGGS, A. F. (1996*b*). Assembly of the gigantic hemoglobin of the earthworm *Lumbricus terrestris*. Roles of subunit equilibria, non-globin linker chains and valence of the heme iron. *J. biol. Chem.* **271**, 30007–30021.

ZIMMERMAN, S. B. AND MINTON, A. P. (1993). Macromolecular crowding: biochemical, biophysical and physiological consequences. *A. Rev. biophys. biomolec. Struct.* **22**, 27–65.