Evolution of vertebrate haemoglobins: Histidine side chains, specific buffer value and Bohr effect

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Abstract

This review highlights the use of analytical tools, recently developed in the comparative method of evolutionary biology, for the study of haemoglobin (Hb) adaptation. It focuses on the functional consequences of a previously largely ignored structural feature of Hb, namely the degree and positional specificity of histidine (His) substitution in Hb chains. The importance of His side chains for hydrogen ion buffering, blood CO2 transport capacity and the molecular mechanism of the Bohr effect in vertebrate Hbs is discussed. Using phylogenetically independent contrasts, a significant correlation between the specific buffer value of Hb and the number of predicted physiological buffer groups from Hb sequence data is shown. In a new result, the evolution of the number of physiological buffer groups in 77 vertebrate species is reconstructed on a phylogenetic tree. The analysis predicts that teleost fishes, passeriform birds and some snakes have independently evolved a much-reduced specific buffer value of Hb, possibly for enhancing the efficiency of an acid load to change oxygen affinity via the Bohr effect. This analysis demonstrates how in comparative physiology analysis of genetic databases in an evolutionary framework can identify candidate species for further experimental in vitro and whole animal studies.

Keywords: Haemoglobin; Evolution; Bohr effect; Buffer value; Histidine; Vertebrates; Adaptation; Fish; Bird; Reptile; Phylogenetically independent contrast

1. Introduction and perspective

Haemoglobin (Hb) is arguably the most studied of all proteins. Indeed, the molecular analysis of Hb has been the test-bed for many contemporary ideas and concepts in biology, particularly in understanding crystallographic structure and structure–function relationships of proteins, ligand binding, structural transitions between conformers, allosteric interactions and many more. As a respiratory pigment, Hb plays a well-appreciated role in the carriage of O2 from respiratory organs to deep tissues in larger, complex animals. Throughout the last century blood O2 binding characteristics have been described for numerous vertebrates...
and studies on isolated Hbs have gradually revealed the powerful modulation of O₂ binding by a suite of intracellular allosteric modifiers such as CO₂, hydrogen ions, organic phosphates and chloride (e.g. Bohr et al., 1904; Barcroft, 1928; Margaria and Green, 1933; Benesch and Benesch, 1967; Rollema et al., 1975).

Because Hb functions at the interface between organisms and their environment, it is ideally suited to study physiological adaptation (Powers, 1980). From early comparative studies onwards, researchers have noted an apparent fit between blood respiratory properties and the habitat, lifestyle or energy demand of species (Krogh and Leitch, 1919; Root, 1931; Hall et al., 1936; Schmidt-Nielsen and Larimer, 1958; Lenfant and Johansen, 1972; for a summary of the early literature see Prosser, 1950). These interspecific differences in whole blood O₂ transport characteristics – and also in the intrinsic properties of Hb and their modulation by allosteric modifiers – have often been interpreted as adaptations. However, in their classic essay Gould and Lewontin (1979) have argued that a mere fit between a functional characteristic of an animal and its environment is insufficient as evidence for adaptation (for a recent discussion see Hochachka and Somero, 2002). Physiologists often use the term adaptation loosely and sometimes include phenotypic adjustments to different environmental conditions when discussing adaptation. Here the discussion is restricted to adaptation in the Darwinian sense, whereby a feature may be regarded as an adaptation for some function if it has become prevalent or is maintained in a population (or species, as an adaptation for some function if it has become

Recognising adaptation can be particularly difficult in the context of Hb function, as illustrated by the point that in altitude hypoxia a priori both decreased and increased Hb O₂ affinities can be interpreted as adaptive (see Weber, 1995). This is due to the opposing demands on Hb function in arterial and venous blood, such that any change in O₂ affinity, towards higher or lower values, can be interpreted as beneficial, either for increased O₂ loading at respiratory surfaces or for increased O₂ unloading in tissues. Since the respiratory properties of Hb in two species are unlikely to be exactly the same, claims for adaptation are easily made whenever Hb O₂ affinity differs between species.

Demonstrating that natural selection is acting on a specific function may be difficult (but see Goodman et al., 1975; Czelsunia et al., 1982). In the absence of such information, claims of adaptation should minimally be supported by showing that the suspected feature is somehow correlated with the function for which it is supposed to be an adaptation. One way of establishing this is to reconstruct the evolution of the feature and of the respective function on a phylogenetic tree (Brooks and McLennan, 2002). This allows a historical analysis of whether the feature evolved before, after, or at the same time as the function for which it is supposed to be an adaptation. Alternatively, it can be checked whether the loss of the particular function in a close relative of species A is associated with a loss of the presumed adaptive feature. This may then be taken as an indication that the feature is maintained in species A because of this function. If these kinds of associations are even found more than once and independently in distantly related species groups, correlated evolution, which is consistent with adaptation, may be suspected (see, e.g. Block et al., 1993; Berenbrink et al., 2005). There have been some classic attempts at correlating differences in Hb function between species with evolutionary changes in lifestyle or habitat (e.g. Bunn, 1980; Perutz, 1983). However, until recently a rigorous conceptual framework and reliable information on evolutionary relationships of many groups were missing, such that testing for correlated evolution as a possible indicator of adaptation was often difficult, except for fortunate cases such as high altitude adaptation in geese, where an increased O₂ affinity evolved by convergent evolution in the Hbs of both Andean and Himalayan bar-headed geese relative to their respective close lowland relatives (Jessen et al., 1991). Recent advances in evolutionary biology have provided new comparative methods for testing the adaptive significance of specific physiological features (see Harvey and Pagel, 1991; Garland and Carter, 1994; Brooks and McLennan, 2002; Garland et al., 2005). These methods have been developed mainly for use with morphological traits, but there are some powerful demonstrations of their use within the physiological arena, ranging from evolution of endothermy in fishes (Block et al., 1993), the diving response in seals and sea lions (Hochachka and Mottishaw, 1998), to the O₂ concentrating mechanism in fish eyes and swimbladders (Berenbrink et al., 2005; for more examples, see Garland et al., 2005).
This review draws on (1) the rich earlier literature on comparative Hb function in vertebrates, (2) the continually increasing amount of Hb sequence information in genetic databases, and (3) recent progress in understanding evolutionary relationships among vertebrates, and combines these sources to exemplary analyse aspects of Hb function and adaptation from the molecular to the whole organism level. It introduces some of the tools of the so-called ‘comparative method’, as developed in evolutionary biology, and illustrates how evolutionary reconstruction methods can identify candidate species on which independent experimental tests for adaptational hypotheses may be conducted. As an example of the additional insights to be gained by adopting an evolutionary viewpoint in comparative physiological studies, this review explores the evolution and functional consequences of a previously largely ignored structural feature of Hb, namely the degree and positional specificity of histidine (His) substitution in Hb chains, which has important consequences for the physiological mechanism of the Bohr effect and blood CO2 transport.

2. Role of His side chains for Hb function

The uncharged α-imidazole side chain of His reversibly binds hydrogen ions with a pkₐ value that depends on the local protein environment, temperature and solvent composition (Tanford, 1962; Reeves, 1972). Unlike ionisable side chains of the 19 other main biological amino acids, the pkₐ values of His in proteins typically encompass the range between ca. pH 6.0 and 8.0 (Lukin and Ho, 2004; Fig. 1). Hence, next to the N-terminal Cα-NH₂ groups, whose pkₐ may vary between 7.25 and 8.0 in human HbA (Van Beek and De Bruin, 1980), His side chains are the major buffer groups, which contribute to hydrogen ion buffering at physiological pH in globins and other polypeptides. Notably, hydrogen ions are passively distributed across the red cell membrane of most vertebrates via the Jacobs–Stewart cycle, which involves a powerful anion exchange protein (band 3) that equilibrates acid–base equivalents across the red blood cell membrane (Hladky and Rink, 1977). Therefore, changes in plasma pH due to, e.g., the release of lactic acid from tissues, are readily transferred to the microenvironment

Fig. 1. pkₐ values of histidine side chains on the surface of liganded and unliganded human HbA (HbCO and DeoxyHb, respectively) at 29 °C in 0.1 M HEPES and 0.1 M NaCl in D₂O. Values were estimated by ¹H nuclear magnetic resonance techniques (Fang et al., 1999; Lukin and Ho, 2004). Histidines are labelled by positional number in the respective α- or β-globin chain and by the helical nomenclature. The two haeme-associated histidines in each globin chain [α58 (E7), α87 (F8), β63 (E7), β92 (F8)] and the two α chain histidines buried in the contact sites within each αβ dimer [α103 (G10), α122 (H5)] do not buffer at physiological pH and are omitted (Goodman et al., 1975; Perutz, 1990). Upper and lower dashed lines encompass pkₐ values within one pH unit of the estimated intracellular red blood cell pH of ectothermic vertebrates at the measurement temperature (pH 7.1, solid horizontal line). Corresponding values in HbCO and deoxyHb are connected by lines. An increase or decrease in the pkₐ of a given histidine upon transition from liganded HbCO (similar to oxyHb) to unliganded deoxyHb indicates a positive or negative contribution to the overall magnitude of the (normal or alkaline) Bohr effect, respectively. See text for further details.
of Hb inside the red blood cell, where they can affect Hb function.

Hydrogen ion binding by Hb is physiologically important for two reasons. It increases blood CO₂ transport capacity, since the equilibrium of the CO₂ hydration reaction that yields bicarbonate and hydrogen ions is shifted towards the formation of bicarbonate by buffering of free hydrogen ions. Hence, in the presence of non-bicarbonate hydrogen ion buffers a given $P_{CO₂}$ is associated with a higher total CO₂ concentration than in the absence of non-bicarbonate buffers. Consequently, a given arterio-venous $P_{CO₂}$ difference is associated with a higher arterio-venous total CO₂ difference, and hence CO₂ carrying capacity. Second, hydrogen ion binding by specific His side chains is responsible for a substantial part of the fixed acid (CO₂-independent) Bohr effect at physiological pH in human and other vertebrate Hbs. Although these two functions may influence each other, they are discussed separately to begin with.

2.1. His side chains and the mechanism of the Bohr effect in vertebrate Hbs

2.1.1. pH-dependency of Hb O₂ affinity: the Bohr effect

The Bohr effect of Hb has been the subject of several reviews, including a series of publications occasioned on the centenary of its first description in 1904 by Christian Bohr, August Krogh and Karl Hasselbalch (Bohr et al., 1904; Kilmartin and Rossi-Bernardi, 1973; Riggs, 1988; Bonaventura et al., 2004; Giardina et al., 2004; Jensen, 2004). The molecular mechanism for the Bohr effect has been studied intensely over the past decades. According to the classical two-state allosteric model (see Perutz, 1990), hydrogen ion binding to specific Bohr groups above pH 6.0 stabilises the low O₂ affinity T(ensed)-state of the Hb tetramer over the high affinity R(elaxed)-state of the Hb tetramer over the high affinity R(elaxed)-state (alkaline or normal Bohr effect). This reduces the overall O₂ affinity of Hb and shifts the $P_{O₂}$ for half-maximal Hb O₂ binding ($P_{50}$) towards higher values, thereby facilitating O₂ unloading in acidic tissues. Conversely, higher pH values at respiratory surfaces, where CO₂ is released, decrease $P_{50}$ and facilitate Hb O₂ loading. The magnitude of the Bohr effect is expressed in one of two ways, either as the Bohr coefficient, $\phi$, or as the number of hydrogen ions taken up per tetramer upon a full change from oxy to deoxyHb, $\Delta H^+$ (or Bohr protons). Both parameters are equivalent according to the linkage equation (Wyman, 1964). In case of symmetrical O₂ equilibrium curves, the Bohr coefficient $\phi$ can be described by the change in log $P_{50}$ upon a change in pH:

$$\phi = \frac{\delta \log P_{50}}{\delta \text{pH}} \quad (1)$$

The number of Bohr protons, $\Delta H^+$, is determined by hydrogen ion titration as the difference in bound hydrogen ions at fixed pH between fully oxygenated (or carbon monoxide saturated) R-state Hb and fully deoxygenated T-state Hb. $\Delta H^+$ usually refers to Hb tetramers and is related to the Bohr coefficient $\phi$ by:

$$\Delta H^+ = -4\phi \quad (2)$$

Thus, the higher $\Delta H^+$, the greater the number of additional hydrogen ions bound by deoxyHb relative to oxyHb, and the larger the decrease of log $P_{50}$ per unit increase in pH.

The magnitude of the Bohr coefficient of a given Hb may be affected by a multitude of factors, including the concentrations of chloride ions, species-specific organic phosphates and CO₂. It is further influenced by temperature and the actual pH range under investigation (see Giardina et al., 2004). In addition, the influence of any one of these factors may vary between species. For instance, deoxygenated human HbA at physiological values of temperature, pH and chloride concentration, and in the absence of organic phosphates, binds maximally two additional hydrogen ions as compared to fully oxygenated Hb (Siggaard-Andersen, 1975). This contrasts with four or more Bohr protons in many fish Hbs under the same conditions (Jensen and Weber, 1985; Jensen, 1989, 2001; Berenbrink et al., 2005).

2.1.2. Limitations of functional studies on isolated Hb

At this point the relevance of studies on isolated Hb for the understanding of in vivo blood respiratory properties may be questioned. Biological systems show emergence, implying that the functional properties of an integrated system cannot simply be predicted from the study of its isolated components. Whilst studies on isolated Hb in solution have undoubtedly provided valuable insights, they often ignore the context of inte-
grated function of Hb at the cellular and whole animal level. Although in some cases the O_2 binding curves of whole blood can be recreated in purified Hb solutions by inclusion of all known allosteric modifiers in the appropriate amount (e.g. Dahms et al., 1972), it is often inappropriate to infer whole blood O_2 transport characteristics from studies of Hb in solutions alone.

Thus, studies on mammalian red blood cells have led to the assumption that hydrogen ions are generally passively distributed across the red blood cell membranes of vertebrates, such that changes in whole blood extracellular pH cause directly proportional changes in intracellular pH. This has duly led to the distinction between an intracellular and extracellular Bohr effect, depending on which pH is chosen for the basis of the calculation of the Bohr coefficient (see Jensen, 2004). However, comparative studies in several vertebrate groups in the last two decades have revealed that in many species Hb function is regulated in vivo by secondarily active ion transporters in the red blood cell membrane, which change intracellular pH and cell volume (Nikinmaa, 1992; Berenbrink and Bridges, 1994). Probably the best studied of these transport systems is the adrenergically activated Na^+/H^+ exchanger in teleost fishes that protects the intracellular Hb from, e.g. exercise-induced acidosis (Berenbrink et al., 2005). Therefore, in the presence of endogenous adrenergic agonists, in vivo blood O_2 binding characteristics may deviate considerably from what can be predicted on the basis of studies on isolated Hb alone.

Another example is the preferential and reversible binding of human deoxyHb to components of the cytoskeleton in an O_2-dependent manner, displacing several glycolytic enzymes and possibly also affecting membrane transport proteins. This has led to the suggestion of Hb as an O_2 sensor that modulates red blood cell metabolism and membrane ion transport, which in turn may affect Hb O_2 binding by a feedback mechanism (Motais et al., 1987; Giardina et al., 1995; Barvitenko et al., 2005).

The foregoing two responses vary between vertebrate groups, indicating a largely unexplored link between integrated Hb function and evolutionary history. Thus, the adrenergically activated Na^+/H^+ exchanger, which elevates intracellular red blood cell pH and thereby increases Hb O_2 affinity via the Bohr effect, evolved only in advanced teleost fishes (Berenbrink et al., 2005). On the other hand, significant oxygenation–dependent interaction of Hb with band 3, the major integrating centre of red blood cell membrane proteins, may be insignificant in fishes under in vivo conditions whereas it is likely to occur in birds and mammals (Weber et al., 2004). This is supported by the finding that, in contrast to humans, oxygenation–dependent changes in membrane ion transport activity are not associated with bulk changes in O_2 saturation of Hb in rainbow trout red blood cells (Berenbrink et al., 2000).

Hence experiments on isolated Hb are often insufficient to fully account for the respiratory characteristics of whole blood. However, they allow a much better mechanistic analysis of the respiratory characteristics of whole blood, because each of the allosteric modulators of Hb can be controlled separately. This allows comparative analyses of the relative magnitude by which separate mechanisms contribute to, e.g., the overall Bohr effect in a given species. Referring back to conditions in isolated red blood cells and to whole blood in vivo may then allow insights into the evolution of blood respiratory characters, which would otherwise remain obscured.

2.1.3. Insights into the evolution of whole blood O_2 transport properties from studies on isolated Hb

This point is illustrated by comparing whole blood O_2 transport characteristics in endothermic birds and mammals. At physiological pH, P_{CO_2}, and temperature, mammalian and avian whole blood exhibit similar sized Bohr effects (Powell, 2000). This may be due to inheritance from their last common amniote ancestor. Closer analyses of isolated human and goose Hbs suggest that the overall Bohr effect in the two animals is the sum of several components, which differ quantitatively and qualitatively. Birds and mammals have evolved elevated concentrations of different organic phosphate compounds in their red blood cells, which significantly increase the Bohr coefficient (Kilmartin and Rossi-Bernardi, 1973; Rollema and Bauer, 1979). However, the increased Bohr coefficient afforded by the mammalian organic phosphate 2,3-bisphosphoglycerate (BPG), is partly counteracted by specific CO_2 binding of Hb at physiological P_{CO_2} (Kilmartin and Rossi-Bernardi, 1973). In contrast, the enhancing effect of the bird-specific organic phosphate myoinositol 1,3,4,5,6-pentaphosphate (IPP) on
the Bohr effect is unaffected by physiological $P_{CO_2}$ (Petschow et al., 1977).

There are further differences in the Bohr effect between goose and human Hb in the absence of organic phosphates and CO$_2$. At physiological chloride concentrations the isolated major Hb component of goose blood has a 40% smaller Bohr effect than human HbA (Rollema and Bauer, 1979). This is not merely a quantitative but also a mechanistic difference. The Bohr effect of goose Hb under this condition is largely chloride-dependent and reduced to about 15% when chloride is decreased from 100 to 1 mM (Rollema et al., 1975). Hence the functional analysis of Hb in solution suggests that the similar whole blood Bohr effect in birds and mammals may have been independently acquired by different mechanisms during evolution.

This illustrates the value of including functional characterisations of isolated Hbs for an integrative and evolutionary understanding of blood respiratory physiology. The following discussion of the role of His side chains for the mechanism and evolution of the Bohr effect is limited to isolated Hb in the absence of CO$_2$ and organic phosphates. This is due to insufficient comparative data on the effects of these allosteric modifiers and not meant to imply that CO$_2$ and organic phosphates play only a minor role in the overall Bohr effect.

2.1.4. How many amino acids contribute to the Bohr effect of Hb in jawed vertebrates?

The identity of the amino acids responsible for the Bohr effect in human HbA and in the Hbs of other species has been a matter of some debate. Because of their $pK_a$ values, His side chains are the likely sites for the binding of Bohr protons. Stabilisation of the T-state of Hb by preferential hydrogen ion binding in deoxyHb requires that the $pK_a$ values of His involved in the Bohr effect increase upon deoxygenation. Earlier studies favoured the view of a few key Bohr groups, whose nature was assumed to be basically the same in all tetrameric Hbs of vertebrates (Perutz, 1983). X-ray crystallography studies of T-state human HbA indicate that the C-terminal His146β forms a salt bridge with Asp94β of the same globin chain. This salt bridge, which is broken in the R-state, raises the $pK_a$ value of His146β, such that this group contributes to about one half of the maximal alkaline Bohr effect of human HbA in 0.1 M chloride solution. The remaining part of the Bohr effect was ascribed to preferential binding of chloride to deoxygenated Hb at a site close to the N-terminal C$_a$-NH$_2$ group of Val1α. This was estimated to increase the $pK_a$ of this NH$_2$ group from 7.25 in oxyHb to 8.0 in deoxyHb, which is consistent with the ca. 50% reduction of the total Bohr effect in the absence of chloride (Van Beek and De Bruin, 1980).

The strong deoxygenation of certain fish Hbs caused by low pH even in the presence of high O$_2$ concentrations, which is known as the Root effect, was regarded by Perutz and Brunori (1982) as an enhanced version of the normal or alkaline Bohr effect of human Hb, chiefly brought about by a single amino acid substitution. Thus, serine 93β, which is substituted by cysteine in human HbA, was thought to additionally stabilise the salt bridge of His146β in the T-state of fish Hbs, raising its $pK_a$ even further and causing the increased Bohr effect (Perutz and Brunori, 1982).

In contrast to the idea of a few key amino acid residues constituting the Bohr effect in all tetrameric vertebrate Hbs, more recently, evidence emerged for a large number of His side chains, which contribute to the Bohr effect in human HbA. In a series of studies using $^1$H NMR and two-dimensional $^1$H and $^{15}$N NMR on native, recombinant, and chemically modified human HbA and its mutants, C. Ho and co-workers have completed the determination and assignment of the $pK_a$ values of all surface His residues in human HbA in the liganded, R-state and the unliganded, T-state conformation (carbonmonoxyHb and deoxyHb, respectively; Fang et al., 1999; see Lukin and Ho, 2004). Fig. 1 gives these $pK_a$ values, which were obtained at 29°C in the presence of 0.1 M chloride and in the absence of allosteric organic phosphate modulators. His are labelled by their positional number in the respective α- or β-globin chain and by their helix annotation. His with $pK_a$ values in oxy and deoxy Hb at the given pH. For tetrameric Hb comprised of two identical αβ dimers, the individual contribution of a Bohr site $\Delta H^+_i$ is given by two times the difference in fractional occupation of the hydrogen ion binding...
site between T-state deoxyHb and R-state oxyHb:

$$\Delta H_i^+ = 2 \left( \frac{[H^+]}{[H^+] + K_{ai}^T} - \frac{[H^+]}{[H^+] + K_{ai}^R} \right)$$  \hspace{1cm} (3)$$

or

$$\Delta H_i^+ = 2 \left( \frac{10^{-pH}}{10^{-pH} + 10^{-pK_{ai}^T}} - \frac{10^{-pH}}{10^{-pH} + 10^{-pK_{ai}^R}} \right)$$  \hspace{1cm} (4)$$

where $K_{ai}^T$ and $K_{ai}^R$ are the acid dissociation constants of an individual (i) site in T-state and R-state Hb, respectively. (Note that Eq. (3) given here differs from the respective Eq. (2) misprinted in Lukin and Ho, 2004.)

Using Eq. (4) and $pK_a$ values from Fig. 1, it can be shown that the sum of the contributions of all His amounts to close to 90% of the total alkaline Bohr effect measured in human HbA in the presence of 0.1 M chloride. This suggests that no other, non-His site such as the $C_\alpha$-NH$_2$ group of Val146 plays a major role in the Bohr effect of human HbA. The reduction of the Bohr effect in the absence of chloride may then be explained by differential interaction of chloride with His $pK_a$ values in oxy and deoxyHb. In the absence of chloride, when this putative interaction is lacking, the differences between $pK_a$ values would be diminished and hence also the Bohr effect. This is supported by the known influence of anions on the $pK_a$ of specific His side chains (Busch et al., 1991; Sun et al., 1997).

The analysis of individual His contributions to the overall Bohr effect still provides support for the terminal His of $\beta$ chains His146$\beta$ as a predominant Bohr group in human HbA. This group shows the largest increase in $pK_a$ upon deoxygenation (Fig. 1) and the above calculation indicates that it is responsible for about 63% of the maximal alkaline Bohr effect of human HbA in the presence of 0.1 M chloride. This is in line with comparative data on some amphibian and fish Hbs, which lack the terminal His in their $\beta$ chains and show a greatly diminished or even reversed Bohr effect (e.g., great crested newt minor Hb component, Kleinschmidt et al., 1988; eel cathodic Hb, Fago et al., 1995). Similarly, chemical removal of the terminal $\beta$His in carp Hb reduces the Bohr effect by ca. 50% (Parkhurst et al., 1983).

In further contrast to a universal role of a few key amino acid side chains in the mechanism for the Bohr effect in tetrameric vertebrate Hbs, several more recent studies indicate that the role of His146$\beta$ in the molecular mechanism of the Bohr effect differs between the tetrameric Hbs of vertebrate. Thus, X-ray crystallographic data on two teleost fishes with strong Bohr and Root effect Hbs shows that the salt bridge in deoxyHb, which elevates the $pK_a$ of His146$\beta$ relative to the oxy form in human HbA, is not formed in these Hbs (Ito et al., 1995; Yokoyama et al., 2004). This implies a much-reduced role for this group in the Bohr effect. In addition, the terminal $C_\alpha$-NH$_2$ groups of the $\alpha$ chains are blocked and therefore not titratable in teleost Hbs; therefore a specific chloride-dependent mechanism that could elevate the $pK_a$ of these groups is also lacking. Moreover, teleosts generally possess a reduced number of titratable surface His (Jensen, 1989; Berenbrink et al., 2005), suggesting that a major part of the large Bohr effect in teleosts may be due to non-His sites. Indeed, two such sites have been recently suggested from X-ray crystallographic T- and R-state structures of tuna Hb (Yokoyama et al., 2004).

Interestingly, the salt bridge that raises the $pK_a$ of His146$\beta$ in human deoxyHb and is responsible for more than 60% of the Bohr effect in the presence of 0.1 M chloride is also not formed in goose deoxyHb (Liang et al., 2001). This may explain the above-mentioned 40% lower Bohr effect in goose Hb compared to human HbA under the same conditions. The contribution of other His residues to the Bohr effect may also differ in goose and human Hb. Sequence alignment of human and avian Hbs shows that His$98\alpha$, which contributes positively to the alkaline Bohr effect in human HbA ($pK_a^T > pK_a^R$), is missing in Hbs of geese and most other birds. Similarly, His$77\beta$, which actually diminishes the overall alkaline Bohr effect ($pK_a^T < pK_a^R$), is also missing in most bird Hbs.

The above comparison of mammalian, avian and teleost fish Hbs suggests that several different His and non-His sites contribute to a varying degree to the overall Bohr effect in different species. These differences in mechanism are in line with the idea that the Bohr effect may have evolved separately in teleosts, mammals and, perhaps, birds. Independent evidence for this comes from an evolutionary reconstruction of the overall magnitude of the Bohr effect on a vertebrate phylogeny (Berenbrink et al., 2005). Without any supposition of underlying mechanisms, the results of the latter study suggest a low Bohr effect of about one Bohr proton in the last common ancestor of today’s jawed verte-
brates, a condition retained by living elasmobranchs. This was followed by two separate increases in the magnitude of the Bohr effect. One increase occurred in the ray-finned fishes (a group including teleosts) and the other in amniotes (reptiles, birds and mammals). This surprising finding fits with the distinct differences in the magnitude and molecular mechanisms of the Bohr effect between human and teleost fish Hbs, which were outlined above.

The study by Berenbrink et al. (2005) focused on evolution of Hb function in ray-finned fishes and investigated only a few amniotes. Analysis of more tetrapod species is necessary in order to better resolve the evolution of the overall Bohr effect magnitude in amniotes. Still open questions are: is the lower Bohr effect in some bird Hbs relative to mammals in the absence of organic phosphates and CO2 a legacy from a common ancestor before the divergence of birds and mammals, after which point the Bohr effect in mammals underwent an additional increase? Or did the Bohr effect increase separately in birds and mammals, but to a different degree? Alternatively, is the lower Bohr effect in birds due to a secondary reduction? Future studies on Hb of basal mammalian lineages and on avian Hbs and their close living relatives, crocodilians and turtles, together with evolutionary reconstructions, may provide the answer.

2.2. His side chains and the specific buffer value of vertebrate Hbs

2.2.1. Interspecific differences in His content and specific buffer value of vertebrate Hbs

Independent of their role in the Bohr effect, His side chains are important for hydrogen ion buffering and increased CO2 transport capacity. According to the special proximity of His pK_a values to physiological pH, it is conceivable that the sum of the titratable His residues on the surface of the \( \alpha_2 \beta_2 \) Hb tetramer and of the N-terminal \( C_\alpha-NH_2 \) groups is directly related to the specific buffer value of Hb, \( \beta_{Hb} \). The latter is expressed as the number of hydrogen ions per Hb tetramer (Hb4), which is released upon a unit increase in pH at constant oxygenation.

As comparative data on the amino acid composition and primary \( \alpha \) and \( \beta \)-globin sequences of a range of vertebrate Hbs began to accumulate, Riggs (1970) pointed out that the Hb of some fishes contained only half as many His residues as found in human and lungfish Hbs. The significance of this observation for \( \beta_{Hb} \) had not been fully realised until Jensen (1989) showed that \( \beta_{Hb} \) was only half as high in trout and carp Hb as compared to shark and mammalian Hb. In the same study an inverse relationship was noted in the four species between the magnitudes of \( \beta_{Hb} \) and the fixed acid Bohr effect (Jensen, 1989). These interspecific differences in hydrogen ion buffer properties and the Bohr effect of Hb have been linked to different CO2 transport strategies in teleost fishes versus sharks and air breathing mammals (Jensen, 1989; Jensen et al., 1998a; Nikinmaa, 1997).

However, not all His residues have a pK_a close enough to physiological pH (within about one pH unit) in order to significantly affect hydrogen ion buffering in vivo. The pH of arterial blood in normoxic, normocapnic and resting animals decreases with increasing body temperature (Reeves, 1972) and at constant body temperature varies with evolutionary position (see Ultsch and Jackson, 1996). At an acclimation temperature of 29 °C, the value at which the His side chain pK_a values of human HbA have been determined (Fig. 1), an average of about pH 7.6 can be calculated from the equation given by Ultsch and Jackson (1996), which is based on 81 ectothermic vertebrate species. Fewer studies have addressed the temperature dependence of pH inside red blood cells, but using the respective formula based on 13 ectothermic vertebrates yields an intracellular red blood cell value of about pH 7.1 under the same conditions (Ultsch and Jackson, 1996). The lower intracellular pH is caused by the passive equilibration of acid–base equivalents across the red cell membrane according to a Donnan distribution together with a higher net negative charge of impermeable intracellular solutes relative to plasma (Hladky and Rink, 1977; for review see Jensen, 2004). Depending on the magnitude of the Bohr effect and blood buffer value, intracellular pH in deoxygenated blood at constant \( P_{CO_2} \) is usually higher than in oxygenated blood. However, in venous blood in vivo this is more or less compensated by higher \( P_{CO_2} \) levels, such that intracellular pH of red blood cells in mixed venous blood can be assumed to be close enough to pH 7.1 for the current purpose. Accordingly, the relative importance of individual His side chains for hydrogen ion buffering at physiological pH inside red blood cells can be estimated by how closely their pK_a values match 7.1.
Using the values for human HbA in Fig. 1, clearly the $pK_a$ of His143β (H21) is too low to contribute significantly at physiological pH in both deoxyHb and carbonmonoxyHb, which is structurally similar to oxyHb. The same holds true for His45α (CD3) in deoxyHb. The conserved proximal and distal haeme-linked His in vertebrate Hbs and two $\alpha$ chain His in the $\alpha\beta$ dimer contact region are not considered titratable under physiological conditions (Goodman et al., 1975; Perutz, 1990) and therefore not included in Fig. 1. Thus, of the total 38 His in the $\alpha_2\beta_2$ tetramer of human HbA (10 in $\alpha$ and 9 in $\beta$-chains), only 24 and 22 His are titratable at physiological pH in oxygenated and deoxygenated HbA, respectively. Adding to this number the 4 N-terminal Cα-NH$_2$ groups per Hb tetramer yields 28 and 26 physiological buffer groups in oxy- and deoxyHb, respectively.

2.2.2. Predicting the number of physiological buffer groups in vertebrate Hb from globin sequence data

Due to the large and continuously increasing number of Hb amino acid and nucleotide sequences it is possible to identify other vertebrate groups, which have evolved large differences in the number of physiological buffer groups in their Hb. Fig. 2 shows $\beta_{HB}$ values from a wide range of vertebrates as a function of the estimated number of physiological buffer groups. Values for $\beta_{HB}$ were taken from the literature and refer to deoxygenated Hb in the absence of organic phosphate modulators at temperatures, chloride concentrations and pH values relevant to the respective in vivo situations. Estimates of physiological buffer groups are based on the assumption that His residues in a given species have similar $pK_a$ values as His at homologous positions in human HbA. It is further assumed that in each species, His residues that occupy external, solvent accessible positions in the X-ray crystallography models of human HbA are titratable at physiological pH, whereas His residues at internal positions are not (see Berenbrink et al., 2005).

As evident in Fig. 2, $\beta_{HB}$ is about four-fold higher in smooth hound and spiny dogfish, members of the basal jawed vertebrate lineage of elasmobranchs, than in the ancient jawless lamprey lineage. This is consistent with a key physiological innovation during vertebrate evolution, the acquisition of a powerful red blood cell anion exchange protein (band 3) in all jawed vertebrates studied so far (Nikinmaa, 1997; Jensen et al., 2001). As mentioned earlier, band 3 allows for rapid equilibration of acid–base equivalents across the erythrocyte membrane via the Jacobs–Stewart cycle, thereby recruiting the substantial intracellular Hb reservoir as a buffer for extracellular acid-loads associated with, e.g. elevated physical activity. Lamprey red blood cells have very little anion exchange activity and their Hb consists of a single globin chain that is monomeric when oxygenated and forms dimers and tetramers upon deoxygenation.

Fig. 2. Correlation between measured specific buffer value of Hb ($\beta_{HB}$) and the number of predicted physiological buffer groups in the composite Hbs of selected adult vertebrates. Physiological buffer groups were calculated from Hb sequence information as the number of un-blocked N-terminal Cα-NH$_2$ groups per Hb tetramer yields 28 and 26 physiological buffer groups in oxy- and deoxyHb, respectively.

Linear regression analysis: $\gamma = 3.47 + 0.31x$ $r^2 = 0.86, p < 0.001$

Physiological buffer groups (mol (mol Hb$_{A2}$)$^{-1}$)
linked His and, in addition, part of the N-terminal Cα-NH₂ groups are chemically blocked, leaving just about two physiological buffer groups per tetramer. This contrasts with 18 and 30 predicted physiological buffer groups in smooth hound and spiny dogfish, respectively. A relatively large number of physiological buffer groups and an elevated $\beta_{Hb}$ are also characteristic for lungfish and tetrapods (Fig. 2). Conceivably, after the evolution of band 3 and increased acid-base permeability of the red blood cell membrane, any mutation resulting in an increase of surface His residues and thereby providing additional hydrogen ion buffering and CO₂ transport capacity would be under positive selection pressure and lead to fixation.

Fig. 2 also indicates two contrasting conditions within the higher jawed vertebrates after their divergence from elasmobranchs. A high $\beta_{Hb}$ linked to increased surface His residues in lungfish and tetrapods, and a low $\beta_{Hb}$ linked to low His residues in teleosts. The physiological significance of this observation is poorly investigated. One speculation is that the prevalence of high buffer values in tetrapods and lungfish is related to the evolution of air breathing and the associated increase in blood $P_{CO₂}$, which upon hydration confers a pronounced acid load to the animal. In water-breathing teleosts the selection pressure for high Hb buffer values seems to have relaxed, if not reversed. Of particular significance is the presence in these animals of exquisitely pH-sensitive Hb isoforms with strong Bohr and Root effects, which, on localised acidification within the gas secretory glands of the swimbladder and retina, cause unloading of O₂, even at high $P_{O₂}$ (Berenbrink et al., 2005). This response may be impeded by high intracellular buffer values. An additional significant factor is the presence of the adrenergically activated Na⁺/H⁺ exchanger in red blood cells of many teleosts, which on stress hormone activation causes a substantial increase in intracellular pH. Again this response may be impeded by high intracellular buffer values (Nikinmaa, 1997; Berenbrink et al., 2005).

Despite the limited number of investigated species, and their widely different phylogenetic positions, which include members of all traditional vertebrate classes, conventional linear regression indicates a highly significant correlation between $\beta_{Hb}$ and the estimated number of physiological buffer groups ($p<0.001$) and a relatively narrow 95% confidence interval (Fig. 2, dashed lines).

2.2.3. Phylogenetically independent contrasts, physiological buffer groups and specific buffer values of Hb

However, this kind of statistical analysis can substantially overestimate the significance of a correlation, because it essentially treats species as independent data points (Garland et al., 2005). It neglects that species are related in a hierarchical fashion and may have experienced different times of independent evolutionary history. In other words, analyses of species data by conventional linear regression in most cases overestimate the statistical degree of freedom to an extent, which varies with the nature of the underlying species tree. For example, the four data points for the teleosts (eel, carp, trout and tuna) should really carry less weight in linear regression analysis, because the fossil record indicates that they diverged only relatively recently from each other, about 130–150 million years ago (Mya). Hence, they were able to evolve independently from each other for a shorter period of time than for example spiny dogfish and smooth hound, or chicken and alligator, whose lineages both diverged already about 240 Mya (Benton, 1993; Harland et al., 1990).

Phylogenetically independent contrast analysis corrects for these inadequacies in correlation analysis of species data by explicitly taking the phylogenetic relationships of species into account (for a recent review see Garland et al., 2005). As expected, the statistically more appropriate analysis by phylogenetically independent contrasts yields a lower correlation coefficient and a broader 95% confidence interval (Fig. 2, solid lines). However, the relationship between $\beta_{Hb}$ and the estimated number of physiological buffer groups is still highly significant ($p<0.01$).

2.2.4. Reconstructing evolution of physiological buffer groups on a vertebrate phylogeny

The above relationship suggests that calculation of physiological buffer groups from primary sequence data can be taken as a first guideline to search for differences in $\beta_{Hb}$ among vertebrate Hbs. Appendix A lists the number of estimated physiological buffer groups of tetrameric, adult Hb in 77 species of jawed vertebrates, as obtained mainly from the SwissProt database. In case of multiple Hb isoforms, the weighted average of
Fig. 3. Reconstructed evolution of number of physiological buffer groups per Hb tetramer in adult jawed vertebrates. Values are colour-coded. For living species, they were calculated from Hb sequence data as in Fig. 2 and are listed in Appendix A. Values for ancestral species at the branch points of the circular tree were calculated by linear parsimony using MacClade 4 (Maddison and Maddison, 2000; Berenbrink et al., 2005). Whenever two values were equi-parsimonious for a given ancestral species, the higher value is shown (MAXSTATE option in MacClade 4). The composite phylogenetic tree was assembled from several recent molecular and morphological studies and must be regarded as provisional because of uncertainties in some groups. See text for further details.
the major isoforms (≥10%) is reported. Fig. 3 gives a colour-coded graphical presentation of this information together with a hypothesis over the evolutionary relationship between species. Before a more detailed discussion of Fig. 3, a few words about the underlying phylogenetic tree are necessary.

2.2.5. **A new view of evolutionary relationships within vertebrates**

The past decade has seen major improvements in our understanding of the evolutionary relationships among vertebrates, due to the continually increasing amount of DNA sequence data and the development of new, rigorous methods for analysing both anatomical and molecular sequence data. This new view of vertebrate relationships deviates in some cases considerably from the traditional way of grouping vertebrates. Major changes include the interordinal relationships within placental mammals (Madsen et al., 2001; Murphy et al., 2001), the position of turtles within reptiles (Kumazawa and Nishida, 1999), and the relationships within teleosts (Miya et al., 2003). There are still some contentious issues, which, when eventually resolved, may change parts of the phylogenetic tree in Fig. 3 and the following analysis which is based upon it. These concern the identity of the most basal group of placental mammals (Amrine-Madsen et al., 2003), the position of tree shrews among Laurasiatheria (Madsen et al., 2001; Murphy et al., 2001), the closest living relative of snakes within Squamata (Lee, 2005), and the details of the interordinal relationships within elasmobranchs, advanced birds and advanced teleosts (Winchell et al., 2004; Cracraft, 2001; Miya et al., 2003). However, these questions are currently actively pursued and progress so far promises to provide a solid framework that will allow the use of phylogenetic methods to reconstruct the evolution of Hb function in all major groups of vertebrates.

2.2.6. **Convergent evolution of reduced His content in vertebrate Hbs**

In an attempt to initiate this kind of analysis Fig. 3 shows a reconstruction of the evolution of physiological buffer groups in jawed vertebrates by linear parsimony (Maddison and Maddison, 2000; Berenbrink et al., 2005). This method is just one of several related methods, which have been developed for the reconstruction of ancestral values on a phylogenetic tree (Garland et al., 1999; Garland and Ives, 2000). Linear parsimony uses values for a given character in living species at the tips of the underlying species tree and assigns values to each branch point on the tree by minimising the sum of differences along all pairs of connected tip or branch point values (Maddison and Maddison, 2000). This is based on the principle that species tend to inherit their characteristics from their ancestors and therefore evolutionary changes leading to differences in the character of interest are relatively rare. Hence the evolutionary reconstruction, which requires the least overall amount of change along the branches of the tree, is favoured. For a more detailed discussion of this topic see Maddison and Maddison (2000) and Garland et al. (2005).

The results of the evolutionary reconstruction in Fig. 3 suggest that a relatively high number of physiological buffer groups was the ancestral condition of the first jawed vertebrates. This condition is also found in most of the analysed living vertebrates, which show a remarkably similar number of physiological buffer groups of about 24–28 per Hb tetramer. Closer analysis of the aligned sequences reveals that this constancy is not due to the conservation of the same physiological buffer groups in these vertebrates. Rather, the same total number of physiological buffer groups in distantly related species is achieved to a significant extent by the contribution of different physiological buffer groups. Arctic skate, coelacanth and guinea pig all have 28 predicted physiological buffer groups per Hb tetramer, but only half of them or fewer are identical between them.

On several occasions, distinct groups of vertebrates have independently evolved a reduced number of physiological buffer groups. Thus, all teleosts in Fig. 3 possess only a third or even fewer physiological buffer groups than most other vertebrates (5–8 against 24–28, respectively). A second, independent case occurs in the crocodile-bird group, where the three passeriform birds (blackbird, starling, sparrow) possess only a third of the physiological buffer groups (12–15) that are found in crocodilians (34–40), the closest living relatives of birds. Unlike in teleost fishes, a gradual reduction from 34 to 24 and then to 20 buffer groups can be reconstructed in the lineage leading to living Passeriformes.

The correlation between the number of physiological buffer groups and $\beta_{\text{Hb}}$ in Fig. 2 predicts that $\beta_{\text{Hb}}$ in passeriform birds will be significantly lower than
in more ancient bird lineages like ostrich, chicken or pigeon. If true, this will have consequences for the CO\textsubscript{2} transport capacities and for the ability to buffer intracellular acid–base loads in red blood cells of Passeriformes, a group which comprises more than one half of all living bird species. This appears to be the first instance where previously unrecognised, potentially important differences in whole organism physiology of a large animal group are predicted simply from molecular sequence data.

The foregoing discussion illustrates the value of evolutionary reconstructions for identifying suitable study species for independent experimental tests of particular physiological hypotheses. Thus, in the current example, passeriform birds can be identified as candidate species for further tests on the relationship between a reduced number of physiological buffer groups, $\beta$H\textsubscript{b}, and whole organism physiology. Other, less clear-cut reductions in the number of physiological buffer groups occur in some snakes within the Squamata and in the musk shrew, a member of the mammalian radiation of Laurasiatheria. Determination of $\beta$H\textsubscript{b} in these groups will serve as further, independent test cases for a correlation between $\beta$H\textsubscript{b} and the number of physiological buffer groups.

Overall the above analysis indicates that a specific Hb histidine content is characteristic for a given vertebrate group, and that this value differs strikingly between some of the groups. Given that there are only three and four histidines in each of the two $\alpha$- and two $\beta$-globin chains in a Hb tetramer which are conserved in most vertebrates (Berenbrink et al., 2005), the apparent lack of a greater variation within a group seems surprising and not in line with Kimura’s (1979) theory of predominantly neutral evolution of Hb sequences. It rather suggests that the His content in vertebrate Hbs is under some sort of previously unrecognised, but strong selective control that differs between different vertebrate groups.

2.2.7. Strategies to identify the adaptive value of a low or high Hb His content

The low number of titratable surface histidine side chains in passeriform birds and teleost fishes and the likely associated reduction in $\beta$H\textsubscript{b} appear puzzling and beg the question as to their physiological significance. Identifying the direction of evolutionary change is an important tool for understanding selection pressures and adaptation of function. In case of the low $\beta$H\textsubscript{b} and number of physiological buffer groups in teleosts, it is important to know whether this is an ancestral feature that has been passed on from an ancient common ancestor to vertebrates, or whether it has evolved specifically in the teleost lineage. Fig. 3 suggests the latter, but inclusion of lampreys in the data set of Fig. 3 actually opens up the question again, because it leads to an equivocal reconstruction, where a high and low number of physiological buffer groups are equally parsimonious (not shown). Information on the number of physiological buffer groups in the last common ancestor of elasmobranchs, teleosts, lungfishes, coelacanth and tetrapods is important for the strategy to identify the adaptive value (if any) of changes in that number. If teleosts merely inherited a low number of physiological buffer groups from the common ancestor of that group, chances are better to identify any adaptive value of a change in that number by studying elasmobranchs or the lungfishes-coelacanth-tetrapod group, because these would then be the lineages where a change occurred, i.e. an increase in the number of physiological buffer groups. If, however, the ancestral value at the base of jawed vertebrates was high, a reduction must have occurred in a recent common ancestor of teleosts and these would be the study group of choice.

Identifying where on a phylogenetic tree evolutionary changes occurred then allows correlating these with other changes, such as environmental conditions, exercise physiology, acid–base regulation or hypoxia tolerance. Although a correlation is not proof of a causal relationship, searching for this kind of correlation is useful, because it may allow the formulation of adaptive hypotheses, which otherwise would not have come to mind. In the ideal case, experimental tests can then be designed, which either confirm or refute the adaptive hypothesis (Futuyma, 1998).

A case in point is the positive correlation between blood O\textsubscript{2} affinity and habitat altitude in closely related species within several vertebrate groups, which has led to the hypothesis that a high blood O\textsubscript{2} affinity may represent adaptation to life at high altitude. Compelling evidence for this hypothesis comes from experiments on rats in which blood O\textsubscript{2} affinity was increased by carbamoylation of Hb. In contrast to control rats, these animals were able to survive simulated altitudes equivalent to more than 9000 m (Eaton et al., 1974).
2.2.8. Origin and adaptive hypothesis of low His content and specific buffer value in teleost Hbs

The question when the low Hb His content and $\beta_{Hb}$ found in modern teleosts first evolved has been recently addressed in an integrative study on the evolution of a number of blood gas transport parameters in jawed vertebrates (Berenbrink et al., 2005). The study suggests that the reduction in $\beta_{Hb}$ occurred within the early ray-finned fishes, after their divergence from lungfishes and tetrapods, in a common ancestor of today’s teleost fishes. Evolutionary reconstruction suggests that it was preceded by a large increase in the Bohr effect, which coincided – and may have been mechanistically linked – with the origin of the Root effect, the above mentioned extreme form of pH sensitive Hb O2 binding, which prevents even air-equilibrated Hb to become fully saturated with O2 at low pH (Berenbrink et al., 2005). A large Bohr effect not only improves the efficiency of O2 and CO2 transport between respiratory surfaces and tissues, but also provides additional hydrogen ion buffer sites upon Hb deoxygenation in venous blood. This may have allowed the reduction in the number of other physiological buffer groups over evolutionary time without unduly affecting CO2 transport capacity.

This scenario may explain how, after the evolution of a strong Bohr effect, a reduction in physiological buffer groups may have proceeded without negative side effects. It does not, however, explain the apparent strong selection pressure for such a reduction, which seems to exclude His side chains from most external positions on the surface of the tetramer and which is potentially also responsible for the chemical blocking of terminal $\alpha$-NH$_2$ groups in the $\alpha$-globins of modern teleosts by acetylation. As a consequence, regarding physiological buffer groups and $\beta_{Hb}$, teleosts have almost reverted to the situation in lampreys (Fig. 2). Interestingly, lamprey Hb also shows a strong Bohr effect, albeit based on an entirely different mechanism as compared to jawed vertebrates (Jensen, 1999; Heaslet and Royer, 1999; Qiu et al., 2000).

A possible advantage of low $\beta_{Hb}$ after a strong Bohr effect had evolved is the increased efficiency of a given acid load to promote changes in Hb O2 binding via the Bohr effect. Even if the Bohr coefficient (i.e. the change in $P_{50}$ upon a given change in pH) were the same between two Hbs, the Hb with the lower number of physiological buffer groups would need correspondingly less of an acid load to achieve a given change in O2 affinity.

The most extreme reductions of the number of physiological buffer groups or $\beta_{Hb}$ among all vertebrates are found in lampreys and teleost fishes (Figs. 2 and 3). These are also the groups with the strongest Bohr effects (see above) and they are water breathers. This may be significant, because of the differences between water and air as respiratory media. The generally lower O2 content and higher viscosity of water relative to air is likely to require increased ventilation at higher energetic cost in water breathers as compared to air breathers with similar O2 demands. This may place a premium on maximising blood O2 transport efficiency in water breathers by having a large Bohr effect combined with low $\beta_{Hb}$. The high O2 content of air and the relative ease of ventilating air compared to water may allow for a lower Bohr coefficient in air-breathers. Further, the higher bicarbonate buffer value relative to water-breathers (Heisler, 1986) conceivably renders evolutionary reductions in $\beta_{Hb}$ less efficient in reducing whole blood buffer values in air breathers. In addition, the high O2 content in human HbA depends on the contribution of numerous His sites, a reduction in His content in order to lower $\beta_{Hb}$, and thereby increase the efficiency of the Bohr effect may be counter productive, as the loss of some His sites may at the same time decrease the Bohr coefficient.

The independent evolutionary events in teleosts and lamprey, which led to a low Hb His content and $\beta_{Hb}$, and to a large Bohr coefficient in both groups, even show a further parallelism: Lampreys and teleost fishes are the only two vertebrate groups in which pH inside red blood cells is regulated (at least under some circumstances). Both groups express Na$^+$/H$^+$ exchangers in their red blood cell membrane, which elevate intracellular pH and thereby increase the O2 affinity of their strong Bohr effect Hbs (Nikinmaa, 1992). Such a mechanism may be hampered in red blood cells with a high $\beta_{Hb}$ because of the large hydrogen ion transport capacities needed to change intracellular pH (Nikinmaa, 1997; Berenbrink et al., 2005).

3. Future directions

The evolutionary changes in Hb His content and the predicted changes in Hb buffer values have revealed
a new aspect of Hb function in vertebrates. These changes do not appear random and seem to be group specific. Future studies need to establish whether the low number of physiological buffer groups in birds and snakes are indeed correlated with a reduced $\beta_{Hb}$. Identifying more precisely, where on the phylogenetic tree of vertebrates the transitions between high and low $\beta_{Hb}$ occurred, may then allow formulation of adaptive hypotheses, which can be tested. Simultaneous analysis of the magnitude of the Bohr coefficient in these groups may show whether or not, these two parameters are interdependent in birds and snakes, as they appear to be in lampreys and teleost fishes.

Such studies may also shed light on the evolution of the Bohr effect in amniotes and clarify, whether the reduced Bohr effect relative to human HbA in some reptilian and bird Hbs is indeed consistent with an adaptation to diving (see Giardina et al., 2004) or whether it is rather the legacy of a common ancestor and also occurs in non-diving species (Zhang et al., 1996).

Despite some progress, the diversity of red blood cell ion transport mechanisms, which may affect Hb function, in reptiles, birds and non-domesticated mammals is still largely unexplored. However, if pH-regulatory mechanisms existed in the red blood cells of these groups, the present analysis predicts that they are most likely to be found in species whose Hbs possess a low number of physiological buffer groups, such as sparrow, indigo snake and musk shrew (Fig. 3).

Given the importance of blood buffer values for the efficiency of a given acid load to promote the Bohr effect, and given further that a Bohr effect is observed in the respiratory pigments of other animal phyla (Bridges and Morris, 1989), it would also be interesting to investigate how the evolution of the Bohr effect in, e.g. crustacean haemocyanins relates to their specific hydrogen ion buffer values. This analysis may be the more rewarding, as some crustaceans have become more or less terrestrial air breathers and crustacea therefore face the same differences in respiratory media and blood CO$_2$/bicarbonate buffer values as experienced by vertebrate water-breathers and air-breathers (Morris and Bridges, 1994).

These examples demonstrate how, by adopting an evolutionary viewpoint and contemporary conceptual analytical tools, respiratory physiologists can gain additional insights leading to promising new research directions. This includes novel hypotheses, experiments and study species, which otherwise may never have come to mind, even in a research area as intensively investigated for more than a century as Hb and the respiratory function of blood.

Acknowledgements

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Appendix A

Number of physiological buffer groups in the composite Hbs of adult jawed vertebrates as predicted from globin sequence information and titratability or positional information of homologous sites in human HbA. Sources are only referenced by author names when no SwissProt Accession number was available. In case of more than one major $\alpha-$ or $\beta$-globin type being found in the blood of a species, sub-types are indicated in brackets and the number of physiological buffer groups is a weighted average of all chain types occurring at a frequency at or above 10%. Common names correspond to Fig. 3 and are abbreviated for space considerations. See text for further details.
<table>
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<th>Common name</th>
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Note: N. kaouthia refers to Naja kaouthia.

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References


Fang, T.-Y., Zou, M., Simplaceanu, V., Ho, N.T., Ho, C., 1999. Assessment of roles of surface histidyl residues in the molec-
ular basis of the Bohr effect and of β143 histidine in the binding of 2,3-bisphosphoglycerate in human normal adult hemoglobin. Biochemistry 38, 13423–13432.


Margaria, R., Green, A.A., 1933. The first dissociation constant, $pK_a$, of carbonic acid in hemoglobin solutions and its relation to the existence of a combination of hemoglobin with carbon dioxide. J. Biol. Chem. 102, 611–634.


