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HYDROGEN ION TITRATIONS OF THE ANODIC AND CATHODIC HAEMOGLOBIN COMPONENTS OF THE EUROPEAN EEL *ANGUILLA ANGUILLA*

COLIN J. BRAUNER* AND ROY E. WEBER

Danish Center for Respiratory Adaptation, Department of Zoophysiology, Aarhus University, Building 131, 8000 Aarhus C, Denmark

*Present address: McMaster University, Department of Biology, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4J9 (e-mail: braunerc@mcmail.cis.mcmaster.ca)

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Summary

H^+ titrations were conducted on the separated haemoglobin components of eel *Anguilla anguilla* in both the oxygenated and deoxygenated states. In anodic haemoglobin, the addition of GTP, and to a lesser extent Cl^-, increased the magnitude of the Haldane effect and shifted its maximum value into the *in vivo* pH range. Of the 22 histidine residues in the anodic component, only approximately seven were titratable, presumably the β-chain residues at positions 41, 97, 109 and 146 (helical positions C7, FG4, G11 and HC3, respectively).

In cathodic haemoglobin, a small negative Haldane effect was observed at pH values between 6.8 and 8.5 which disappeared in the presence of GTP (molar ratio 3:1 GTP:haemoglobin tetramer). GTP had virtually no effect on the buffer value at fixed oxygenation status, and the lowest buffer value was observed at *in vivo* pH values. No titratable histidine residues were observed in the cathodic component, indicating that all 14 histidines in this component are buried.

We conclude that the anodic component, which constitutes two-thirds of the haemoglobin in the eel, plays the predominant role in CO_2 transport and pH homeostasis *in vivo*.

Key words: *Anguilla anguilla*, haemoglobin, eel, Haldane effect, buffer value, anodic haemoglobin, cathodic haemoglobin, CO_2 transport, CO_2 excretion.

Introduction

Haemoglobin heterogeneity within individuals is a common occurrence in the blood of teleost fishes. In 77 genera of teleost fish sampled in the Amazon, only 8 % of the species possessed a single haemoglobin component, and on average four electrophoretic components were observed per species (Fyhn *et al.* 1979). The functional implications of haemoglobin multiplicity in fish have been extensively studied. Apart from having anodic components with normal Bohr and Root effects, many teleosts have cathodic components, which have high O_2 affinities, small, often reversed, Bohr effects and no Root effects; the cathodic components may therefore safeguard O_2 uptake under hypoxic and acidotic conditions (Weber, 1990). In contrast, little is known about the role that different components play in hydrogen ion buffering and in the transport and excretion of CO_2.

In general, there is an inverse relationship between the magnitude of the Haldane effect and the buffer value of the haemoglobins found in vertebrates. Teleost fishes such as eel, tench, carp and to a lesser extent rainbow trout are characterised by large Haldane effects and a low haemoglobin buffer value at fixed oxygenation status, the reverse being observed in elasmobranchs (Jensen, 1989). The two possible combinations permit equally viable but quite different strategies in terms of CO_2 transport and excretion (Jensen, 1991).

The eel (*Anguilla anguilla*) possesses only two haemoglobin components, one anodic and one cathodic (Pelster and Weber, 1990). It is not known whether the inverse relationship between the magnitude of the Haldane effect and the haemoglobin buffer value observed in whole blood exists within individual haemoglobin components in species exhibiting haemoglobin heterogeneity. This was examined by conducting H^+ titrations (which permit simultaneous assessments of the Haldane effect and buffer value) on isolated and purified haemoglobin components of eel. The molecular bases for the titration characteristics were interpreted in relation to the amino acid sequences that have recently been obtained for both haemoglobin components of eel (Fago *et al.* 1995, 1997). Specifically, the number of titratable histidine residues and their specific locations within the protein moeity were determined in both components.

As in other teleosts, red cell ATP and GTP (NTP) concentrations in eels decrease during exposure to hypoxia, increasing haemoglobin O_2-affinity; GTP is the more potent cofactor (Weber *et al.* 1976). The presence of GTP at a molar GTP:haemoglobin tetramer (Hb_4) ratio of 2:1, as found *in vivo*

(Weber *et al.* 1976), dramatically increases the magnitude of the Haldane effect in the anodic haemoglobin component (Breepoel, 1981*b*). Titrations were also conducted in the presence of GTP at a GTP:Hb_4 ratio of 3:1 (which represents saturating conditions; Breepoel *et al.* 1981*b*) to shed light on the relative roles of the individual components in CO_2 transport and excretion *in vivo*.

Materials and methods

Animal acquisition and care

European eels (*Anguilla anguilla*) were purchased from a local fish farm and maintained in the Zoophysiology Department at Aarhus University in running fresh water at 15 °C. Fish were fed twice a week to satiation and kept for 2 months prior to blood sampling. Blood was drawn from the caudal vein into heparinized syringes. Blood samples from several individuals were pooled and kept on ice.

Preparation of haemoglobin solutions

Red cells were washed three times with 5 vols of cold 0.9 % NaCl and lysed with 3 vols of ice-cold 0.05 mol l^{-1} Tris buffer (pH 7.6). Cell debris was removed by centrifugation at 14 000 revs min^{-1} for 10 min. All remaining procedures were conducted at 4 °C. The haemoglobin solution was repeatedly dialysed against 20 mmol l^{-1} Tris buffer (pH 8.04) over an 18 h period. Haemoglobin components were separated by ion-exchange chromatography on a Sephacel DEAE column (2 cm×20 cm) and eluted with a linearly increasing (0–0.3 mol l^{-1}) NaCl gradient in 20 mmol l^{-1} Tris buffer (pH 8.04). The eluted fractions were collected in 1 ml samples for absorption measurements at 540 nm and for chloride concentration measurements using a Radiometer CMT1 chloride titrator. The elution yielded two main components, one positively charged (cathodic) and the other negatively charged (anodic). Haemoglobin fractions within each component were pooled as indicated in Fig. 1. Each component was dialysed against three changes of distilled water over the following 12 h and then passed repeatedly through a mixed-bed resin (Amberlite IRN-150L monobed mixed resin) to remove ions, checked by conductivity measurements. The concentrated stripped haemoglobin components were separated into 1 ml samples that were frozen at −80 °C until titrations were conducted.

Titrations

In each experiment, the concentrated stripped haemoglobin sample was thawed and diluted to a final concentration of 50 μmol l^{-1} Hb_4. Haemoglobin concentration was measured after conversion to cyanomethaemoglobin using a millimolar extinction coefficient of 11 at 540 nm. The haemoglobin solution was then equilibrated with either pure oxygen or pure nitrogen (>99.9965 %) for 2 h in a titration vessel thermostatted at 15 °C. Titrations were conducted starting at the isoionic point of the haemoglobin solution by the automated addition of 10 μl of freshly prepared and carbonate-free 0.01 mol l^{-1} NaOH (assayed with potassium hydrogen phthalate) or 0.01 mol l^{-1} HCl (assayed with NaOH) using a Radiometer Titralab automated titration system with data acquisition. The pH of the haemoglobin solution was measured using a Radiometer combined pH electrode (GK 2321C) connected to the Radiometer Titralab and recorded 5 min after the addition of titrant, by which time the pH had stabilised. Each complete titration curve for the anodic component consisted of four separate titrations; addition of HCl or NaOH from isoionic pH in samples equilibrated with O_2 or N_2. For the cathodic component, only HCl was added from the isoionic point in O_2 and N_2 because of its high isoionic pH.

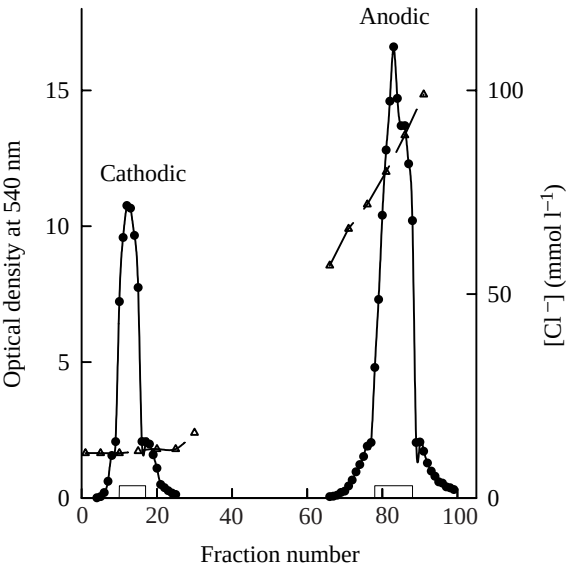

Fig. 1. Separation of the cathodic and anodic haemoglobin components of eel blood by anion-exchange chromatography. Circles, optical density measured at 540 nm; triangles, Cl^- concentration. The box at the base of each peak indicates the fractions pooled in each component.

Titrations were conducted in triplicate (yielding virtually identical results) for the anodic and cathodic components under four different conditions: concentrated stripped haemoglobin diluted with (i) distilled water (stripped); (ii) distilled water with GTP (guanosine 5′-triphosphate sodium salt) at a molar ratio of 3:1 relative to the tetrameric haemoglobin (GTP:Hb_4); (iii) KCl to a final concentration of 0.1 mol l^{-1}; and (iv) KCl to a final concentration of 0.1 mol l^{-1} KCl and GTP at a GTP:Hb_4 molar ratio of 3:1. Titrations were also conducted on GTP solutions alone. The buffer value of the GTP/solvent solution was subtracted from that of the haemoglobin components as described in the text. Prior to the titrations, methaemoglobin levels were measured according to the method of Benesch *et al.* (1973), and samples were discarded if values greater than 5 % were measured.

Results

The haemolysates resolved into two well-defined peaks

during ion-exchange chromatography and consisted of approximately 32 % cathodic and 68 % anodic haemoglobin (Fig. 1).

Titration of the anodic component

The titration curves for the anodic haemoglobin component are shown in Fig. 2, in which Z_H (the net charge of the protein) is plotted as a function of pH in the presence of 0.1 mol l^{-1} KCl in the absence (Fig. 2A) and in the presence (Fig. 2B) of GTP. The traces are drawn to the same scale with zero net proton charge as the reference point (Tanford, 1962). The fixed acid Haldane effect (ΔZ_H; the number of protons released per Hb_4 oxygenated at constant pH) can be calculated from the distance between the oxygenated and deoxygenated curves at constant pH. ΔZ_H is illustrated in Fig. 3 for stripped haemoglobin and for haemoglobin in 0.1 mol l^{-1} KCl in the absence and presence of saturating levels of GTP (3:1 molar ratio of GTP:Hb_4). The maximum fixed acid Haldane effect (referred to simply as the Haldane effect from this point onwards, unless otherwise indicated) increased from 2.1 at pH 6.3 in stripped haemoglobin to 2.9 at pH 6.9 in 0.1 mol l^{-1} KCl (see Fig. 3). The presence of GTP further elevated ΔZ_H to a maximum value of 4.6 at pH 7.3. The trace for the Haldane effect in the presence of GTP without KCl was not included in this figure because it was identical to that of GTP with KCl. At low and high pH values (pH<5.3 and pH>8.1 for stripped haemoglobin, pH<5.9 and pH>7.9 for haemoglobin + KCl, and pH<6.4 and pH>8.2 for haemoglobin + KCl + GTP), a small negative Haldane effect was observed.

The area between the ΔZ_H curves and ΔZ_H=0 (Fig. 3) was measured to determine the Bohr group recruitment during the addition of Cl^- and GTP. Compared with stripped haemoglobin, the addition of Cl^- increased the area by 5 %, and further addition of GTP increased it by 26 %. Thus, both Cl^- and GTP increased the magnitude of the alkaline Haldane effect, increased the pH at which the maximum Haldane effect was observed and reduced the pH range over which the Haldane effect was observed.

Titration of the cathodic component

Titration curves for cathodic haemoglobin in 0.1 mol l^{-1} KCl in the absence and presence of GTP are shown in Fig. 4. In the absence of GTP, a negative Haldane effect is seen above pH 6.8 (deoxygenated haemoglobin is more acidic than oxygenated haemoglobin at constant Z_H), while below pH 6.8 the Haldane effect is positive but small. Fig. 5 illustrates the magnitude of the Haldane effect over the pH range 5.3–8.5 for the different conditions. At all pH values measured, KCl slightly increased ΔZ_H (or reduced the magnitude of the negative Haldane effect), while GTP virtually eliminated any Haldane effect.

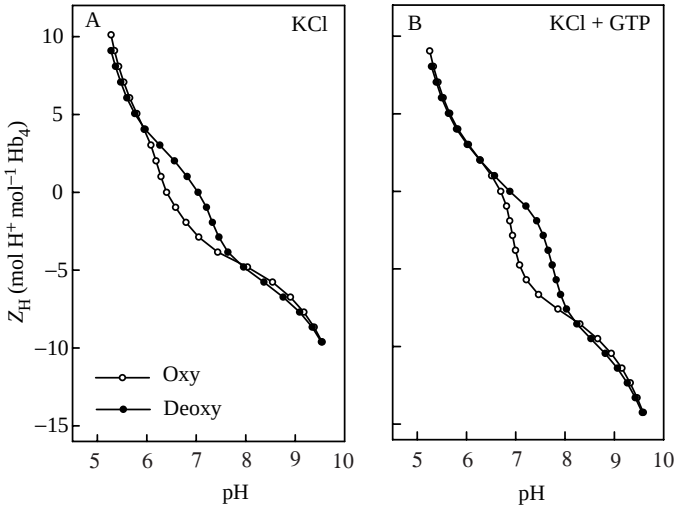

Fig. 2. Titration curves for Z_H (net H^+ charge) as a function of pH for the anodic eel haemoglobin in (A) 0.1 mol l^{-1} KCl and (B) 0.1 mol l^{-1} KCl plus GTP (GTP:Hb_4 molar ratio of 3:1). Open symbols, oxygenated haemoglobin; filled symbols, deoxygenated haemoglobin; temperature, 15 °C.

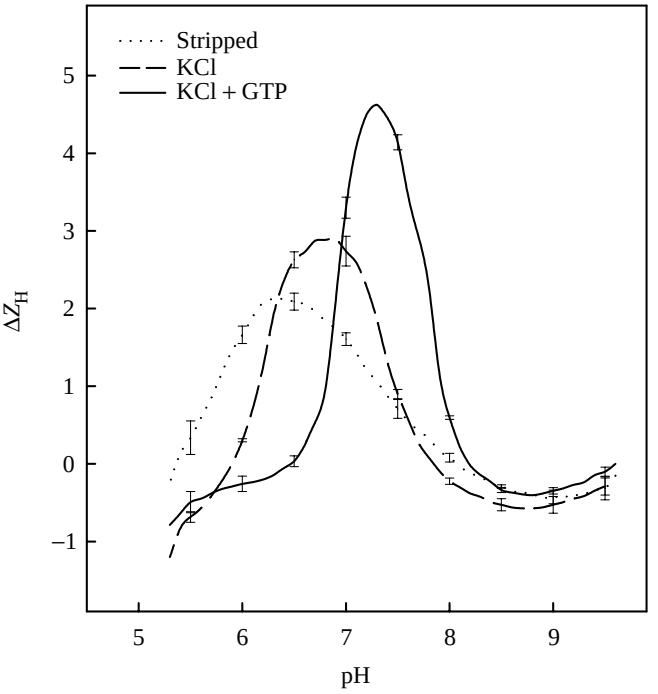

Fig. 3. The fixed acid Haldane effect (ΔZ_H; the number of protons released per Hb_4 oxygenated at constant pH) as a function of pH in the eel anodic component for stripped haemoglobin (dotted line), for haemoglobin in 0.1 mol l^{-1} KCl (broken line) and for haemoglobin in 0.1 mol l^{-1} KCl plus GTP (GTP:Hb_4 molar ratio of 3:1; solid line). ΔZ_H was calculated from the vertical distance between titration curves for oxygenated and deoxygenated haemoglobins at intervals of 0.1 pH unit. Each curve represents the mean of three complete titrations with the S.E.M. indicated by the vertical bars every 0.5 pH unit. Values for haemoglobin with GTP in the absence of KCl are identical to those for haemoglobin in 0.1 mol l^{-1} KCl with GTP (solid line) and have been omitted for clarity.

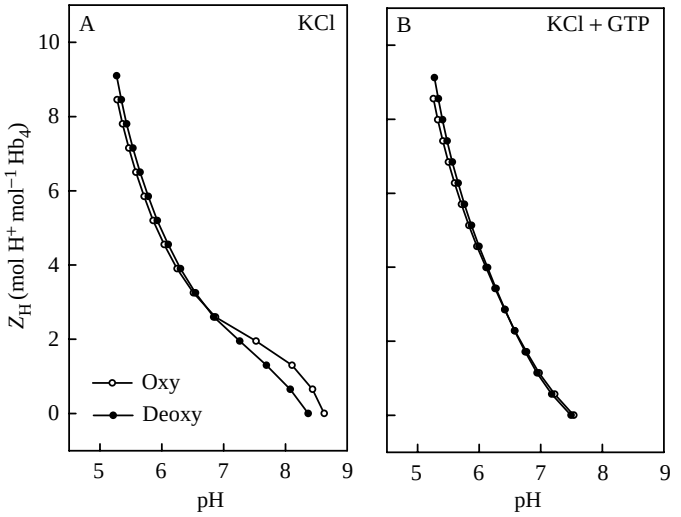

Fig. 4. Titration curves for Z_H as a function of pH for the cathodic eel haemoglobin in (A) 0.1 mol l^{-1} KCl and (B) 0.1 mol l^{-1} KCl plus GTP (GTP:Hb$_4$ molar ratio of 3:1). See legend to Fig. 2 for further details.

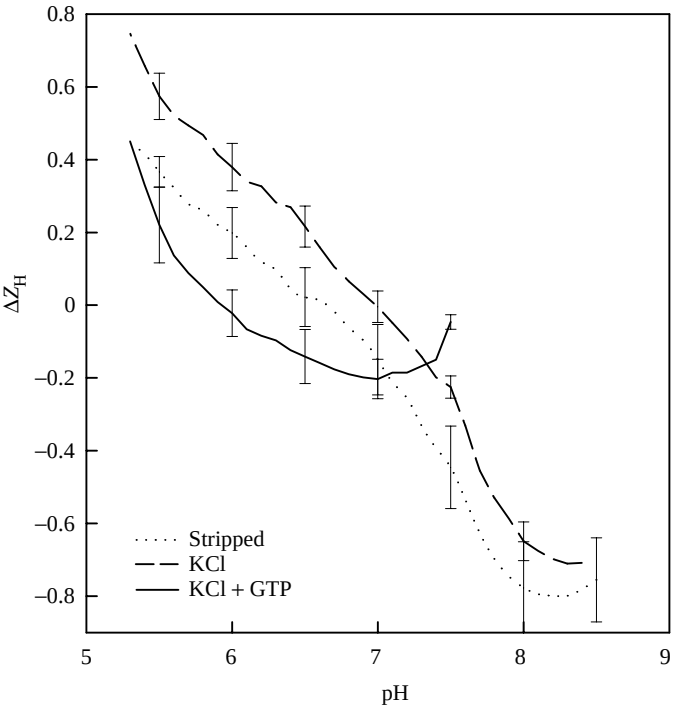

Fig. 5. The fixed acid Haldane effect (ΔZ_H; the number of protons released per Hb$_4$ oxygenated at constant pH) as a function of pH in the eel cathodic component for stripped haemoglobin (dotted line), for haemoglobin in 0.1 mol l^{-1} KCl (broken line) and for haemoglobin in 0.1 mol l^{-1} KCl plus GTP (GTP:Hb$_4$ molar ratio of 3:1; solid line). See legend to Fig. 3 for further details.

Buffer value of the anodic component

The buffer values of the anodic haemoglobin at constant oxygenation status (mol H$^+$ mol^{-1} tetramer pH unit^{-1}) were derived from the slope between adjacent points on the titration curve relating Z_H to pH (Fig. 6A). In the presence of KCl, the buffer value in the pH range 5.5–9 showed maxima at pH 6.2 in oxygenated haemoglobin and at pH 7.3 in deoxygenated haemoglobin. The additional presence of GTP increased the maximum haemoglobin buffer value and shifted this maximum to a higher pH, the shift being greater in oxygenated (to pH 6.9) than in deoxygenated (to pH 7.7) conditions.

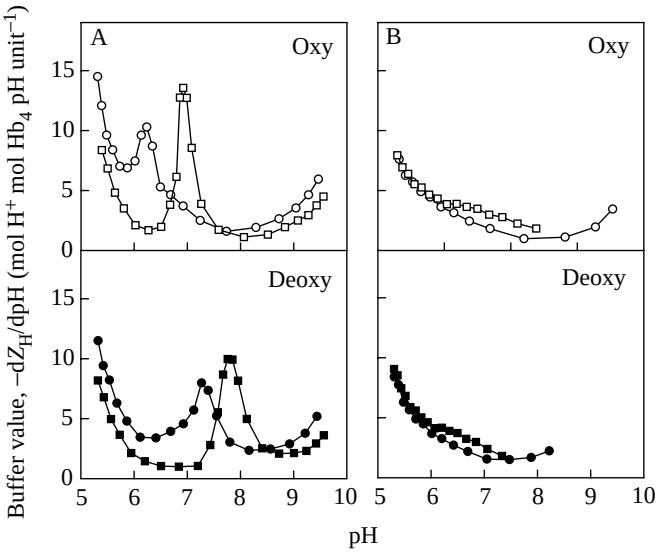

Fig. 6. Buffer value ($-dZ_H/dpH$; mol H$^+$ mol^{-1} Hb$_4$ pH unit^{-1}) as a function of pH in (A) anodic haemoglobin and (B) cathodic haemoglobin in 0.1 mol l^{-1} KCl in the presence (squares) and absence (circles) of GTP at a GTP:Hb$_4$ molar ratio of 3:1 for oxygenated (upper panels) and deoxygenated (lower panels) conditions. The buffer value of GTP alone was subtracted from respective traces.

When the data for the anodic haemoglobin are expressed as the inverse of the buffer value compared with Z_H (differential titration), two well-resolved peaks are obtained, particularly in the deoxygenated condition (Fig. 7A). The distance between these two sharp peaks indicates the number of titratable groups that exist in the neutral pH range (de Bruin and van Os, 1968; Janssen *et al.* 1970, 1972). This value was 9 for the anodic haemoglobin (Fig. 7A).

Buffer value of the cathodic component

In contrast with the buffer value for the anodic component, that of the cathodic component at constant oxygenation status (Fig. 6B) showed no peak in the measured pH range and a minimum buffer value near pH 7.5 in both the oxygenated and deoxygenated haemoglobins. In addition, only a small increase in the haemoglobin buffer value was induced by GTP in the pH range between 6 and 7.5. Also, the differential titration revealed only one well-resolved peak in contrast with the two observed in anodic haemoglobin (Fig. 7C,D), indicating that there are no titratable groups within the neutral pH range in the cathodic haemoglobin component. Titrations were conducted beyond the pH values reported in Fig. 7; however, no second peak was found (data not shown).

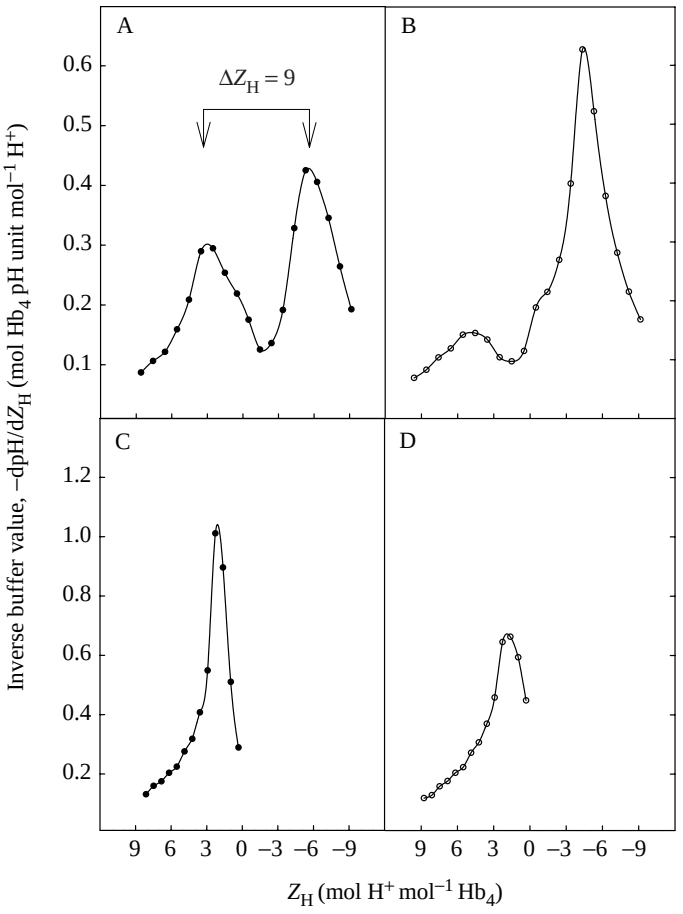

Fig. 7. Differential titration curve (–dpH/dZ_H, the inverse of buffer value, *versus* Z_H) of (A) deoxygenated and (B) oxygenated anodic haemoglobin of eel in 0.1 mol l^{-1} KCl and (C) deoxygenated and (D) oxygenated cathodic haemoglobin of eel in 0.1 mol l^{-1} KCl. The horizontal distance between inflection points indicates the number of titratable groups within the neutral pH range (see Discussion for further explanation).

Discussion

The haemoglobin system in eel *A. anguilla* consists of one-third cathodic and two-thirds anodic component (Fig. 1; Pelster and Weber, 1990; Fago *et al.* 1995). The H^+ titrations of these components reveal pronounced differences that correlate well with molecular data and indicate distinctive roles in buffering. While the anodic component possessed a large Haldane effect and a low buffer value, the cathodic component exhibited a low Haldane effect and low buffer value. These data indicate that the H^+ equilibria of the anodic haemoglobin component are the more important for H^+ buffering and CO_2 transport.

Haldane effect

Anodic component

The Haldane effect (which is thermodynamically equivalent to the Bohr effect; Wyman, 1964) arises from changes in the pK of specific ionizable groups that are linked to haemoglobin oxygenation (Imai, 1982). In mammalian haemoglobin, the Bohr effect is divided into two regions over the pH range between 5 and 10. Whereas an increase in proton concentration decreases the oxygen affinity (alkaline Bohr effect) in the alkaline pH range, it increases the oxygen affinity below pH 6.5 as a result of an uptake of protons during oxygenation (acid Bohr effect). While the alkaline Bohr effect was large in anodic eel haemoglobin relative to that in human haemoglobin, the acid Bohr effect was not observed in the stripped component because of the presence of a Root effect (Pelster and Weber, 1990), which represents a drastic reduction in haemoglobin O_2-affinity at low pH. In the presence of GTP, however, the titrations reveal a slight acid Bohr effect below pH 6.4 (Fig. 3).

In human haemoglobin, proton binding to His β146(HC3) (the histidine residue occurring at the 146th position of the β-subunit and at helical position HC3), which forms a salt bridge with Asp β94(FG1), accounts for approximately 50 % of the Bohr effect (Perutz *et al.* 1969; Kilmartin and Wootton, 1970; Shih *et al.* 1984). Although His β146(HC3) is conserved in eel anodic haemoglobin, Asp β94(FG1) is replaced by Glu (Fago *et al.* 1997), which may still form a salt bridge with His β146(HC3) and contribute substantially to the Bohr effect (Chien and Mayo, 1980*b*). In carp haemoglobin, removal of His β146(HC3) reduces the magnitude of the Bohr effect by half (Parkhurst *et al.* 1983). The magnitude of the Haldane effect is determined by the number of Bohr groups and their pK shifts, so that additional groups must be involved or the same groups must contribute more in anodic eel haemoglobin to account for the much larger Haldane effect compared with that of human haemoglobin (Imai, 1982). Additional groups that may be involved are discussed by Fago *et al.* (1997).

The presence of 0.1 mol l^{-1} Cl^- slightly increased the Haldane effect and the pH at which the maximum value was observed in the anodic component. In human haemoglobin, α1Val accounts for approximately 25 % of the alkaline Bohr effect in the presence of Cl^-. As in other fish haemoglobins (Farmer, 1979), the N-terminal residues of the α-chains are acetylated in *A. anguilla* and in the American eel *A. rostrata* (Fago *et al.* 1997; Gillen and Riggs, 1973) and are therefore unavailable to contribute to the Bohr effect. Another group that has been proposed to contribute to the Cl^--induced Bohr effect in vertebrate haemoglobins is Lys β82(EF6) (Perutz *et al.* 1980), which is conserved in both the anodic and cathodic eel components (Fago *et al.* 1995, 1997). GTP similarly increased both the Haldane effect and the pH at which it was maximal. The absence of cumulative effects of GTP and Cl^- (Fig. 3, data for haemoglobin in the presence of GTP are identical in the presence and absence of KCl; results not shown) suggests overlapping binding sites for the two effectors (e.g. at Lys β82). Apart from this residue, organic phosphates bind to fish haemoglobin at the N-terminal amino group Val(NA1), at Glu(NA2) and at Arg(H21) of the β-chains (Gronenborn *et al.* 1984; Weber and Wells, 1989). All these residues are conserved in anodic eel haemoglobin (Fago *et al.* 1997), explaining the large influence of GTP on the Haldane effect.

Cathodic component

In the absence of organic phosphates, oxygenation of the cathodic haemoglobin at pH values above 6.8 is associated with an uptake of protons, causing a reversed acid Bohr effect. The absence of the normal alkaline Bohr effect correlates with the replacement of His β146(HC3) with Phe (Fago *et al.* 1995). In human haemoglobin, the removal of this histidine inhibits the expression of the normal alkaline Bohr effect and permits expression of a reversed Bohr effect, which suggests that the Bohr groups exhibit a higher affinity for protons in the oxygenated than in the deoxygenated state. The possible molecular mechanisms underlying the reversed Bohr effect are discussed elsewhere (Fago *et al.* 1995).

The most likely groups to contribute to the reverse Bohr effect are the N-terminal amino groups of the β-chains and the imidazole groups of histidines, all of which possess pKa values in the pH range at which the reverse Bohr effect is observed. The five histidine residues in the α-chains are unlikely to contribute because they are not titratable (Fig. 7C,D) and they occur in many fish haemoglobins that do not exhibit a reverse Bohr effect (Fago *et al.* 1995).

The presence of Cl^- slightly increased the Haldane effect (decreased the negative Haldane effect) at all pH values. This effect is probably due to the presence of Lys β82(EF6), which may contribute to the Cl^--dependent Bohr effect in human haemoglobin and is conserved in cathodic haemoglobin. Although Cl^- strongly decreases the oxygen affinity of cathodic eel haemoglobin (Weber *et al.* 1976), Breepoel *et al.* (1981*a*) found no Cl^- effect at concentrations greater than $0.1\,mol\,l^{-1}$. GTP in the presence or absence of Cl^- (results for the latter not shown) virtually eliminates the negative Bohr effect at pH values above 7, as has been observed previously (Weber *et al.* 1976; Fago *et al.* 1995; Feuerlein and Weber, 1996).

Buffer value

Eel haemolysates exhibit a lower buffer value than human haemolysates (Breepoel *et al.* 1980), and titrations reveal a low buffer value for both the anodic and cathodic haemoglobin components (Fig. 6). The buffer value of haemoglobin at fixed oxygenation status is determined by the nature of the groups that exchange protons with the solvent. The groups titrated can be roughly divided into three classes, each distinguished from the others by an inflection point in a titration curve (de Bruin and van Os, 1968; Janssen *et al.* 1970, 1972): (a) the carboxyl groups of aspartic and glutamic acids, which are titrated below pH 5.5, (b) the imidazole groups of histidine and the N-terminal amino groups, which are titrated between pH 5.5 and 9 (neutral pH range), and (c) the groups titrated at pH values above 9. However, the pK values vary depending on the nature of adjacent groups (Paetzel and Dalbey, 1997). The low buffer value in the neutral pH range in teleost fish haemoglobins compared with that in most other vertebrate haemoglobins is correlated with the low total histidine content of the haemoglobin (Jensen, 1989). In eel, the anodic component contains 22 (Fago *et al.* 1997) and the cathodic component contains 14 (Fago *et al.* 1995) histidine residues (Table 1), values much lower than those found in human haemoglobin A (38 residues, Braunitzer *et al.* 1961).

Table 1. *Histidine residues in the α- and β-chains of the anodic and cathodic eel haemoglobins*

Anodic component	Cathodic component
α-chain	
α45(CD3)	α45(CD3)
α58(E7) ------- Distal -------	α59(E7)
α87(F8) ------ Proximal ------	α88(F8)
α103(G10)	α104(G10)
α123(H5)	α124(H5)
β-chain	
β41(C7)	
β63(E7) ------- Distal -------	β63(E7)
β82(F8) ------ Proximal ------	β92(F8)
β97(FG4)	
β109(G11)	
β146(HC3)	

Residues considered to be titratable are printed in bold type.

Not all the histidine residues in the tetramer are available for proton binding since some are buried within the protein moiety. In the anodic component, there appear to be nine titratable groups in the neutral pH range (Fig. 7), as is also observed in carp (Jensen, 1989). Excluding the N-terminal amino groups of the α-chains that are acetylated and those of the β-subunits that are available for titration, this leaves seven titratable histidines per tetramer from a total of 22 histidines (Table 1). Of the 22 histidines in the anodic component, eight (per tetramer) consist of the proximal and distal histidines which ligate with the iron atom of the haem groups and are thus unavailable for proton binding, leaving 14 histidine residues. Thus, of the seven titratable histidine residues per tetramer (Fig. 7), there are 14 that could potentially be titrated in the neutral pH range in the anodic component.

The single maximum in the differential titration curve of the cathodic component (Fig. 7C,D) indicates that there are no titratable groups in the neutral pH range for this component (Breepoel *et al.* 1981*a*); thus, none of the 14 histidine residues in this component (Fago et *al.* 1995) is titratable. It may be possible to determine which histidines in the anodic component are titratable if it can be assumed that the titratabilities of histidine residues found at the same location in the two components are equivalent. On this basis, the histidines at β41(C7), β97(FG4), β109(G11) and β146(HC3) in the anodic component, i.e. eight per tetramer, would be available for titration (Table 1). Together with the two β-chain N-terminal residues, this indicates the presence of 10 titratable groups, which agrees closely with the value of nine groups as determined in Fig. 7.

The addition of GTP to the anodic component increased the maximal buffer value observed and the pH at which it was manifested (Fig. 6). The implied increase in apparent pK of

haemoglobin in both the oxygenated and deoxygenated condition indicates that GTP binds to both forms. Although the affinity for organic phosphates is generally much higher for deoxygenated than for oxygenated haemoglobin (Garby *et al.* 1969), there is evidence for GTP binding to oxygenated haemoglobin in tench (Weber *et al.* 1987) and for IHP binding to oxygenated haemoglobin in carp (Chien and Mayo, 1980*a*,*b*). The raised pK in the presence of GTP increased the pH at which the Haldane effect was observed (Fig. 3). The reduced difference between pK values for oxygenated and deoxygenated haemoglobin resulted in the narrower pH range over which the Haldane effect was observed (Fig. 3).

In conclusion, in the titrations conducted in the present study, GTP was present at a 3:1 molar excess compared with haemoglobin tetramers, which is not much greater than that measured *in vivo* (2:1 GTP:Hb_4; Weber *et al.* 1976). These data provide insight into the roles of the two components *in vivo*. A dominant aspect with respect to CO_2 transport and pH homeostasis of the blood is that the Haldane effect is very large in the anodic component but virtually absent from the cathodic component. Although both haemoglobins have low buffer capacities relative to those of air-breathing vertebrates, that of the anodic component is always equal to or greater than that of the cathodic component, which is probably due to the specific histidine residues at β41(C7), β97(FG4), β109(G11) and β146(HC3). Thus, the inverse relationship between the magnitude of the Haldane effect and the buffer value observed in the whole blood haemolysates from various vertebrates (Jensen, 1989) does not extend to the individual components in eel. Since the cathodic component constitutes only approximately one-third of the total haemoglobin, it will make only a minor contribution to the buffer value of the blood.

Haemoglobin multiplicity in fish is thought to permit a division of labour between the various haemoglobin components with respect to O_2 transport (Weber, 1990). This also appears to apply to CO_2 transport and pH homeostasis, which in the eel is accomplished predominantly by the anodic component.

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References

Benesch, R. E., Benesch, R. and Yung, S. (1973). Equations for the spectrophotometric analysis of hemoglobin mixtures. *Analyt. Biochem.* **55**, 245–248.

Braunitzer, G., Gehring-Müller, R., Hilschmann, N., Hilse, K., Hobom, G., Rudloff, V. and Wittmann-Liebold, B. (1961). Die Konstitution des normalen adulten Humanhämoglobins. *Hoppe-Seyler's Z. physiol. Chem.* **325**, 283–286.

Breepoel, P. M., Kreuzer, F. and Hazevoet, M. (1980). Studies of the hemoglobins of the eel (*Anguilla anguilla* L.). I. Proton binding of stripped hemolysate; separation and properties of two major components. *Comp. Biochem. Physiol.* **65**A, 69–75.

Breepoel, P. M., Kreuzer, F. and Hazevoet, M. (1981*a*). Studies of the hemoglobins of the eel (*Anguilla anguilla* L.). II. Proton binding of a component with a negative Bohr effect. *Comp. Biochem. Physiol.* **69**A, 225–230.

Breepoel, P. M., Kreuzer, F. and Hazevoet, M. (1981*b*). Studies of the hemoglobins of the eel (*Anguilla anguilla* L.). III. Proton and organic phosphate binding to the Root effect hemoglobin component. *Comp. Biochem. Physiol.* **69**A, 709–712.

Chien, J. C. W. and Mayo, K. H. (1980*a*). Carp hemoglobin. I. Precise oxygen equilibrium and analysis according to the models of Adair and of Monod, Wyman and Changeux. *J. biol. Chem.* **255**, 9790–9799.

Chien, J. C. W. and Mayo, K. H. (1980*b*). Carp hemoglobin. II. The alkaline Bohr effect. *J. biol. Chem.* **255**, 9800–9806.

De Bruin, S. H. and van Os, G. A. J. (1968). Determination and interpretation of the differential titration curves of proteins. The differential curves of bovine methemoglobin, CO-hemoglobin and serum albumin. *Rec. Trav. Chim. Pays-Bas* **87**, 861–872.

Fago, A. E., Bendixen, H. and Weber, R. E. (1997). The anodic hemoglobin of *Anguilla anguilla*. Molecular basis for allosteric effects in a Root-effect hemoglobin. *J. biol. Chem.* **272**, 15628–15635.

Fago, A., Carratore, V., DiPrisco, G., Feuerlein, R. J., Sottrup-Jensen, L. and Weber, R. E. (1995). The cathodic hemoglobin of *Anguilla anguilla*. Amino acid sequence and oxygen equilibria of a reverse Bohr effect hemoglobin with high oxygen affinity and high phosphate sensitivity. *J. biol. Chem.* **270**, 18897–18902.

Farmer, M. (1979). The transition from water to air breathing: effects of CO_2 on hemoglobin function. *Comp. Biochem. Physiol.* **62**A, 109–114.

Feuerlein, R. J. and Weber, R. E. (1996). Oxygen equilibria of cathodic eel hemoglobin analysed in terms of the MWC model and Adair's successive oxygenation theory. *J. comp. Physiol.* **165**, 597–606.

Fyhn, E. H., Fyhn, H. J., Davis, B. J., Powers, D. A., Fink, W. L. and Garlick, R. L. (1979). Hemoglobin heterogeneity in Amazonian fishes. *Comp. Biochem. Physiol.* **62**A, 39–66.

Garby, L., Gerber, G. and Verdier, C. H. (1969). Binding of 2,3-diphosphoglycerate and adenosine triphosphate to human haemoglobin A. *Eur. J. Biochem.* **10**, 110–115.

Gillen, R. G. and Riggs, A. (1973). Structure and function of the isolated hemoglobins of the American eel (*Anguilla rostrata*). *J. biol. Chem.* **248**, 1961–1969.

Gronenborn, A. M., Clore, G. M., Brunori, M., Giardina, B., Falcioni, G. and Perutz, M. F. (1984). Stereochemistry of ATP and GTP bound to fish haemoglobins. A transferred nuclear Overhauser enhancement, ^{31}P-nuclear magnetic resonance, oxygen equilibrium and molecular modelling study. *J. molec. Biol.* **178**, 731–742.

Imai, K. (1982). *Allosteric Effects in Haemoglobin*. Cambridge: Cambridge University Press. 275pp.

Janssen, L. H. M., De Bruin, S. H. and van Os, G. A. J. (1970). H^+ titration studies of human hemoglobin. *Biochim. biophys. Acta* **221**, 214–227.

Janssen, L. H. M., De Bruin, S. H. and van Os, G. A. J. (1972). Titration behavior of histidines in human, horse and bovine hemoglobins. *J. biol. Chem.* **247**, 1743–1749.

Jensen, F. B. (1989). Hydrogen ion equilibria in fish haemoglobins. *J. exp. Biol.* **143**, 225–234.

JENSEN, F. B. (1991). Multiple strategies in oxygen and carbon dioxide transport by haemoglobin. In *Physiological Strategies for Gas Exchange and Metabolism* (ed. A. J. Woakes, M. K. Greishaber and C. R. Bridges), pp. 55–78. Cambridge: Cambridge University Press.

KILMARTIN, J. V. AND WOOTTON, J. F. (1970). Inhibition of Bohr effect after removal of C-terminal histidines from haemoglobin β-chains. *Nature* **228**, 766–767.

PAETZEL, M. AND DALBEY, R. E. (1997). Catalytic hydroxyl/amine dyads within serine proteases. *Trends biochem. Sci.* **22**, 28–31.

PARKHURST, L. J., GOSS, D. J. AND PERUTZ, M. F. (1983). Kinetic and equilibrium studies on the role of the β-147 Histidine in the Root effect and cooperativity in carp hemoglobin. *Biochemistry* **22**, 5401–5409.

PELSTER, B. AND WEBER, R. E. (1990). Influence of organic phosphates on the Root effect of multiple fish haemoglobins. *J. exp. Biol.* **149**, 425–437.

PERUTZ, M. F., KILMARTIN, J. V., NISHIKURA, N., FOGG, J. H. AND BULTER, P. J. G. (1980). Identification of residues contributing to the Bohr effect of human haemoglobin. *J. molec. Biol.* **138**, 649–670.

PERUTZ, M. F., MUIRHEAD, H., MAZZARELLA, L., CROWTHER, R. A., GREER, J. AND KILMARTIN, J. V. (1969). Identification of residues responsible for the alkaline Bohr effect in haemoglobin. *Nature* **222**, 1240–1243.

SHIH, T.-B., JONES, R. T., BONAVENTURA, J., BONAVENTURA, C. AND SCHNEIDER, R. G. (1984). Involvement of His HC3(146)β in the Bohr effect of human hemoglobin. *J. biol. Chem.* **259**, 967–974.

TANFORD, C. (1962). The interpretation of hydrogen ion titration curves of protein. *Adv. Protein Chem.* **17**, 69–165.

WEBER, R. E. (1990). Functional significance and structural basis of multiple hemoglobins with special reference to ectothermic vertebrates. In *Comparative Physiology; Animal Nutrition and Transport Processes*; 2, *Transport, Respiration and Excretion: Comparative and Environmental Aspects*; II, *Blood Oxygen Transport: Adjustment to Physiological and Environmental Conditions*, vol. 6 (ed. J. P. Truchot and B. Lahlou), pp. 58–75. Basel, Switzerland: Karger.

WEBER, R. E., JENSEN, F. B. AND COX, R. P. (1987). Analysis of teleost hemoglobin by Adair and Monod–Wyman–Changeux models. Effects of nucleotide triphosphates and pH on oxygenation of tench hemoglobin. *J. comp. Physiol.* **157**, 145–152.

WEBER, R. E., LYKKEBOE, G. AND JOHANSEN, K. (1976). Physiological properties of eel haemoglobin: hypoxic acclimation, phosphate effects and multiplicity. *J. exp. Biol.* **64**, 75–88.

WEBER, R. E. AND WELLS, R. M. G. (1989). Hemoglobin structure and function. In *Lung Biology in Health and Disease; Comparative Pulmonary Physiology: Current Concepts*, chapter 10 (ed. S. C. Wood and C. Lenfant), pp. 279–310. New York: Marcel Dekker.

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