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R. J. Feuerlein · R. E. Weber

Oxygen equilibria of cathodic eel hemoglobin analysed in terms of the MWC model and Adair's successive oxygenation theory

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Abstract Allosteric effects of erythrocytic NTP and proton concentrations on cathodic eel Hb were investigated by precise measurement of Hb-O_2 equilibria (including extreme saturation values) and analysis in terms of the MWC two-state model and the Adair four-step oxygenation theory. Stripped cathodic Hb shows a reverse Bohr effect and high sensitivities to ATP and GTP that extend to high pH values (> 8.5). A decrease in pH raises K_T and lowers the allosteric constant L, compared to opposite effects in 'normal' Bohr effect Hbs. Phosphates even at low concentrations (GTP/Hb = 0.5) annihilate the reverse Bohr effect. GTP exerts a greater effect than ATP due to greater changes in K_T and L, and NTP slightly reduces K_R. In the absence of NTP, about 1.1 protons are released on deoxygenation at pH 8.15 (where most protons are released), indicating a pK value of the reverse Bohr group of approximately 8.2 (higher in oxy-Hb and lower in deoxy-Hb). The pH and NTP dependence of the Adair association constants and calculated fractional populations of Hb molecules in different oxygenation stages show that NTP effectors stabilise the T structure and postpone the T-R transition, whereas protons in the absence of NTP have the opposite effect. A molecular mechanism for the reverse Bohr effect is suggested.

Key words Cathodic components · Hemoglobin · Oxygen-binding · Eel, *Anguilla*

Abbreviations *DPG* 2,3 diphosphoglycerate · *FPLC* fast protein liquid chromatography · *Hb* hemoglobin · *HEPES* *N*-2-hydroxymethyl-piperazine-*N'*-2-ethanesulfonic acid · K_T and K_R O_2 association equilibrium constants of Hb in the deoxy- and oxy-states, respectively · k_1, k_2, k_3 and k_4 'Adair' affinity constants for binding of the four O_2 molecules to Hb · L allosteric constant · *NTP* nucleoside triphosphate · P_m medium O_2 pressure · n_{50} Hill's cooperativity coefficient at P_{50} · P_{50} half-satutarion O_2 tension · *TRIS* tris(hydroxymethyl)aminomethane

R.J. Feuerlein · R.E. Weber (✉)
Department of Zoophysiology, Institute of Biological Sciences, University of Aarhus, DK-8000 Aarhus C, Denmark

Introduction

The oxygen-binding reaction of vertebrate Hb is modified by heterotrophic interactions with protons and inorganic anions like Cl^- and organic phosphates. Unlike mammals, fish Hbs exhibit high degrees of molecular and functional heterogeneity. Fish Hbs can be categorized into two major groups: (I) the anodic Hbs with pronounced Bohr effects (reduced O_2 affinity at low pH), Root effects (reduced oxygen capacity or extreme reduction in O_2 affinity at low pH) and phosphate effects (reduced O_2 affinity in the presence of organic phosphates), and (II) the cathodic Hbs with no or little Bohr, Root and phosphate effects (Weber 1990). Having higher O_2 affinities and lower Bohr effects, the cathodic Hbs may be better adapted for O_2 transport under hypoxic, hypercapnic or acidotic conditions than the anodic ones from the same species (Hashimoto et al. 1960; Binotti et al. 1971; Weber et al. 1975; Weber 1990). In fish blood the most common phosphate modulator of erythrocytic O_2 affinity is ATP (Rapoport and Guest 1941; Wood and Johansen 1972), which in some species concurs with GTP (Geoghegan and Poluhowich 1974; Lykkeboe et al. 1975; Weber et al. 1975; Bartlett 1978; Feuerlein and Weber 1994). As demonstrated in studies on eel, GTP appears to be the dominating modulator of O_2 affinity since its erythrocytic concentrations show greater changes in response to changes in environmental conditions and metabolic requirements than NTP, and since it exerts a greater depressant effect on Hb-O_2 affinity of fish Hb at the same concentration, which appears to free the

latter cofactor for more primitive energy-catering commitments in the metabolically active fish red cells (Weber et al. 1975, 1976; Johansen et al. 1976; Jensen and Weber 1985).

The greater GTP than ATP sensitivity of fish Hb-O_2 affinity correlates with studies in molecular modelling showing that only two amino acid substitutions are required to convert a mammalian, DPG-sensitive Hb to one that is stereochemically complementary to NTP, and indicates that GTP forms an additional hydrogen bond with carp Hb compared to ATP (Perutz and Brunori 1982; Gronenborn et al. 1984), which neatly accounts for the observed difference in the effects of these two cofactors on the oxygen affinity of fish Hb (Weber and Lykkeboe 1978).

Cathodic eel Hb shows a reverse Bohr effect (increasing affinity at low pH), no Root effect and a large NTP effect (Gillen and Riggs 1973; Weber et al. 1976; Pelster and Weber 1990). This contrasts with the intensively studied cathodic Hb from trout (trout I) that lacks Bohr, Root and phosphate effects (Brunori 1975; Pelster and Weber 1990). A reverse Bohr effect combined with a phosphate effect seems to be common in tropical teleost species and may represent an adaptation to oxygen-poor water (Brunori et al. 1979; Bunn and Riggs 1979; Garlick et al. 1979; Martin et al. 1979; Weber and Wood 1979).

Earlier studies have investigated the allosteric effects of ATP, GTP and proton concentrations on tench Hb, which predominantly consists of one anodic component that shows strong Bohr, Root and NTP effects (Jensen and Weber 1982; Weber et al. 1987). This study analyses precise O_2 equilibrium data from cathodic eel Hb in terms of Monod, Wyman and Changeux's (1965) two-state model for allosteric transitions and Adair's (1925) successive oxygenation theory. To our knowledge fish Hbs combining a reverse Bohr effect with a pronounced phosphate effect have not previously been analyzed in this way.

Materials and methods

Eels (*Anguilla anguilla*) weighing 284 ± 26 g were obtained from a local pisciculturist and kept at 15 °C in a 1-m^3 tank with running fresh water for at least 2 weeks before blood sampling. Blood was drawn from the caudal vessels into heparinised syringes. The red blood cells were washed three times in 0.9% NaCl and hemolysed by addition of four volumes of 0.1 mol·l^{-1} TRIS buffer pH 7.5. After centrifugation (10 min at 14000 rpm), the hemolysate was stripped from organic phosphates by gel filtration on a 58 × 2 cm column of Sephadex G-25 Fine gel. The suspending buffer was then changed to 20 mmol·l^{-1} TRIS (pH 8.0 at 20 °C) (buffer A) by elution through a column containing Sephadex G-25 Medium gel.

The cathodic Hb fraction was isolated by ion-exchange chromatography using a FPLC system (Pharmacia, Uppsala, Sweden) consisting of a Mono Q HR 5/5 anion-exchange column, a Mono S HR 5/5 cation-exchange column, a rheodyne 9125 manual injector with 1-, 2- and 5-ml loops, a 2248 HPLC/FPLC solvent pump, a binary mixing chamber, a VWM 2141 double wavelength detector and HPLC manager and Nelson integration software. Hb samples (20 mg) were applied to a Mono Q HR 5/5 anion-exchange column. The column was eluted at 1 ml·min^{-1}. Buffer B [15 mmol·l^{-1} TRIS (pH 8.0 at 20 °C) + 0.5 mol·l^{-1} NaCl] was increased linearly from 0% at 5 min to 30% at 18 min and linearly to 100% between 18 and 20.5 min. The column was then eluted with buffer B for 5 min, whereafter this buffer was decreased linearly from 100% at 25.5 min to 0% at 28 min. These times were not corrected for the time taken for the buffer to reach the column through the loop.

The cathodic fraction was collected and suspended in a 10 mmol·l^{-1} HEPES buffer (pH 7.65 at 20 °C) (new buffer A) by passage through a Sephadex G-25 Medium. Cathodic Hb samples (2 mg) were then injected into a Mono S HR 5/5 cation-exchange column, and the column was eluted at 1 ml·min^{-1} with buffer B [10 mmol·l^{-1} HEPES (pH 7.65 at 20 °C) + 10 mmol·l^{-1} NaCl] increasing linearly from 0% at 3 min to 35% at 23 min and linearly to 100% during the next 2 min. After 5 min buffer B was decreased linearly from 100% at 30 min to 0% at 32 min. To obtain sufficient material for analyses, samples from several separations were pooled. The cathodic fractions were concentrated by ultrafiltration (Millipore ultra filter PLGC 025 00 with MW cut-off point of 10000) and dialysed for at least 18 h against three changes of 0.01 mol·l^{-1} HEPES containing $5 \cdot 10^{-4}$ mol·l^{-1} EDTA (pH 7.4 at 20 °C). No oxidation was evident spectrophotometrically. The Hb was frozen in separate tubes at − 80 °C [which does not affect the oxygenation properties of the Hb (Fago et al. 1995)] and the tubes thawed on the day of Hb-O_2 equilibria determination.

O_2 equilibria of Hb solutions were recorded in the presence of 0.1 mol·l^{-1} NaCl and 0.1 mol·l^{-1} HEPES buffer [which does not influence oxygenation via perturbation of the free Cl-concentration as may occur with (Bis)TRIS buffers (Weber 1992)] at a final Hb concentration of 0.1 mmol·l^{-1}, which is high compared to that where dimer formation occurs in fish Hbs (Edelstein et al. 1976). The equilibria were measured using a modified gas diffusion chamber coupled to cascaded Wösthoff pumps for mixing pure (> 99.998%) N_2 with air or pure O_2 (Weber 1981; Weber et al. 1987). Values of pH were measured with Radiometer BMS Mk 2 and PHM 72 equipment (Copenhagen, Denmark) after equilibrating the samples to air at the same temperature at which oxygenation equilibria were recorded. Cl^- was added as KCl and assayed by coulometric titration (Radiometer CMT 10). ATP and GTP were administered by adding known volumes of standard ATP or GTP solutions, assayed using Sigma (St Louis, Miss., USA) enzymatic test chemicals.

Extended Hill plots (log([oxyHb]/[deoxyHb]) vs. log PO_2) were obtained from precise O_2 equilibria measurement including extreme saturation values. Absorbances at full saturation were estimated from absorbance versus $1/PO_2$ plots extrapolated to $1/PO_2 = 0$. In a few cases extra measurements were made in the high saturation end of the curve with amplified recording sensitivity. The data were fitted to the two-state model of Monod et al. (1965) and to the Adair equation and the parameters were evaluated as described (Adair 1925; Ferry and Green 1929; Imai 1973, 1982).

The MWC equation relates the Hb-O_2 saturation (Y) to the partial pressure of O_2 (p), the O_2 association constants for the tense (T) and relaxed (R) states (K_T and K_R), and the allosteric constant (L):

$$Y = \frac{LK_Tp(1 + K_Tp)^3 + K_Rp(1 + K_Rp)^3}{L(1 + K_Tp)^4 + (1 + K_Rp)^4} \quad (1)$$

The Adair equation relates the Hb-O_2 saturation (Y) to the partial pressure of O_2 (p) and the association constants for the four successive oxygenation steps (k_1, k_2, k_3 and k_4):

$$Y = \frac{k_1p + 3k_1k_2p^2 + 3k_1k_2k_3p^3 + k_1k_2k_3k_4p^4}{1 + 4k_1p + 6k_1k_2p^2 + 4k_1k_2k_3p^3 + k_1k_2k_3k_4p^4} \quad (2)$$

The MWC and Adair equations were fitted to data in the form of log $[Y/(1\text{-}Y)]$ versus log PO_2 with no weighting of data points. The fits were performed with a commercial program (Fig. P from Biosoft) employing the method of Levenberg-Marquardt.

The median O_2 pressure (P_m) was calculated from the Adair constants as:

$$P_m = (k_1 \cdot k_2 \cdot k_3 \cdot k_4)^{-0.25}$$

The free energy of heme-heme interaction (ΔG) was calculated from the MWC parameters as:

$$\Delta G_{41} = 2.303\ RT \log \frac{(L+1)(Lc^4+1)}{(Lc+1)(Lc^3+1)} \quad (3)$$

where $c = K_T/K_R$, and from the Adair parameters as:

$$\Delta G_{41} = 2.303\ RT \log(k_4/k_1) \quad (4)$$

The n_{max} and $P_{n\,max}$ values were calculated as described by Tyuma et al. (1973), except that the $\Delta\log p$ intervals were 0.01 instead of 0.1.

The switch over point i_s was calculated as:

$$i_s = \frac{-\log L}{\log c} \quad (4)$$

The four Adair constants were evaluated from the MWC parameters as:

$$k_1 = \frac{K_R + LK_T}{1+L}$$

$$k_2 = \frac{K_R^2 + LK_T^2}{K_R + LK_T}$$

$$k_3 = \frac{K_R^3 + LK_T^3}{K_R^2 + LK_T^2}$$

$$k_4 = \frac{K_R^4 + LK_T^4}{K_R^3 + LK_T^3}$$

The extent of asymmetry of the Hill plots was estimated from the symmetry factor W (Roughton et al. 1955):

$$W = (k_1 \cdot k_4)/(k_2 \cdot k_3) \quad (5)$$

Fractional oxygen saturation was calculated from the Adair constants (Imai 1982). The number of Bohr protons liberated on oxygenation at the individual oxygenation stages was derived as:

$$\Delta H_i^+ = \delta\log k_i/\delta\mathrm{pH} \quad (6)$$

and the total number of Bohr protons per tetramer (number of protons taken up on deoxygenation) as:

$$\Delta H_t^+ = -4(\delta\log P_m)/\delta\mathrm{pH} \quad (7)$$

The difference between pK of the reverse Bohr group in oxy- and deoxy-Hb was calculated as

$$\Delta pK = 2\log\left(\frac{1+\nu}{1-\nu}\right) \quad (8)$$

where

$$\nu = 2(\Delta\log P_m/\Delta\mathrm{pH})$$

Compared to Wyman's (1948) formulation that assumes the implication of four oxygen-linked groups per tetramer, the introduction of the factor 2 in this equation gives the ΔpK values if only two such groups per tetramer were involved.

The two pK values were estimated from the relation $pH_{max} = 0.5(pK_1 + pK_2)$, where pH_{max} is the pH of the maximal Bohr effect (Wyman 1948).

The metHb concentration was estimated from the decrease in the absorbance difference between oxy- and deoxy-Hb during the course of O_2 equilibrium determination. Little metHb formation was observed except in the presence of high ATP concentrations (in which case ΔA fell by 25% during the experiment). To minimize any effect of metHb formation, the deoxy end of the curve was measured first. Given that the R state affinities are relatively constant (see Results) metHb formation will not significantly influence the results.

Results

When chromatographed on a Mono Q HR 5/5 anion-exchange column eel Hb separated into major cathodic and anodic fractions that constituted approximately 40 and 60% respectively (Fig. 1A). On rechromatography (see Materials and methods) the cathodic fraction separated into a major fraction C_2 (approximately 90%), a minor one C_1 (7–8%) and a few trace fractions (Fig. 1B). The C_1 and C_2 fractions were collected for Hb-O_2 equilibrium measurements. On

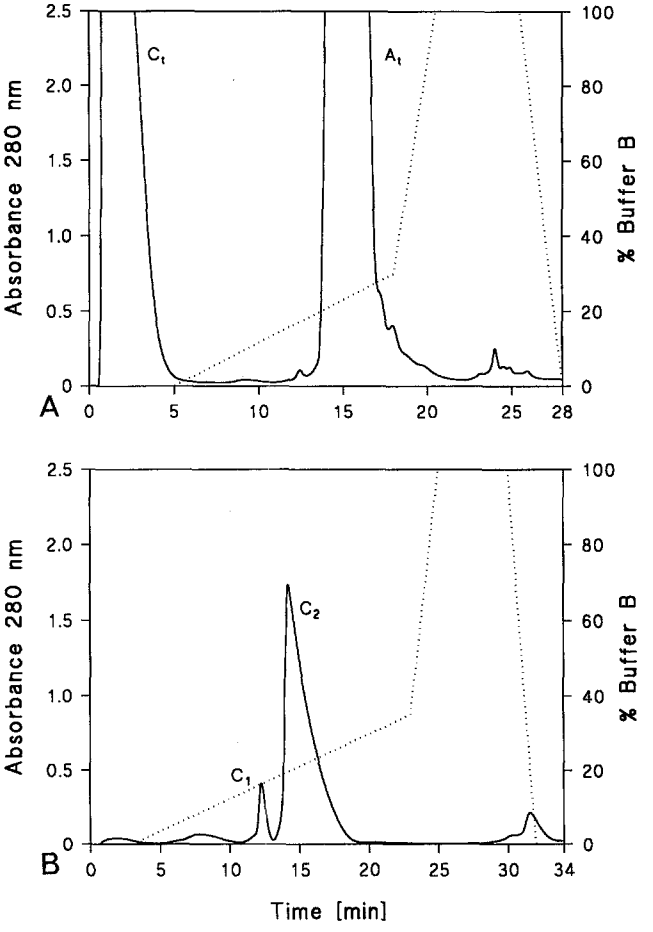

Fig. 1A, B Chromatograms showing separation of cathodic and anodic eel Hb (**A**) and cathodic eel Hb components (**B**) by FPLC. The Hb resolved into major cathodic and anodic fractions and the cathodic fraction resolved into major and minor Hb fractions and smaller fractions. *Dotted lines* indicate the programmed buffer gradient profile. Other separation conditions as described in the text

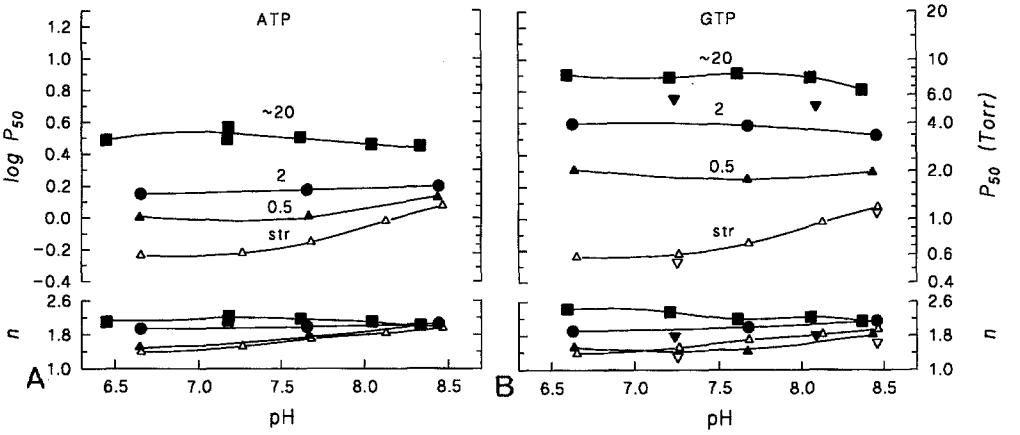

Fig. 2A, B Oxygen affinity of major cathodic eel Hb (C_2) as a function of pH at different ATP/Hb molar ratios (**A**) and GTP/Hb ratios (**B**). △, stripped Hb; ▲, NTP/Hb = 0.5; ●, NTP/Hb = 2; ■, NTP/Hb ≥ 20. Oxygen equilibria were measured in 0.1 mol·l^{-1} HEPES buffer and 0.1 mol·l^{-1} KCl at 15 °C. Tetrameric Hb concentration, 0.1 mmol·l^{-1}. ▽ and ▼, minor component (C_1) in the absence of NTP and in the presence of GTP/Hb ≥ 20, respectively, tetrameric Hb concentration, 0.017 mmol·l^{-1}

rechromatography of C_2 the trace fractions reappeared (not shown), indicating they are formed through changes occurring during the separation.

In the absence of organic phosphates the Hb shows a pronounced reverse Bohr effect ($\phi = \frac{\Delta \log P_{50}}{\Delta \mathrm{pH}} = +0.29$ between pH 7.7 and 8.5) which extended to high pH values (> 8.5, Fig. 2). ATP or GTP strongly decreased oxygen affinity in the whole pH range investigated, GTP having the greater effect. The NTP effect is manifested even at high pH (8.5), which is unusual. ATP and GTP have large effects at NTP/Hb ratios of 0.5–2.0, which cover physiological values, indicating that changes in the NTP/Hb ratio would be an effective mechanism for regulating O_2 affinity. The reverse Bohr effect is annihilated by the phosphates, even at a GTP/Hb ratio of only 0.5, indicating that it is operative only at GTP/Hb ratios that are much lower than physiological values (Wood and Johansen 1972; Geoghegan and Poluhowich 1974; Weber et el. 1976; Kono and Hashimoto 1977; Leray 1979) (Fig. 2A, B). The functional properties of the minor cathodic component C_1 are similar to those of the major cathodic C_2 (both showing a reverse Bohr effect and a strong GTP effect; Fig 2B).

Hill plots of precise O_2 equilibrium curves of the major cathodic eel Hb (C_2) at different pH and NTP/Hb ratios are presented in Figs. 3 and 4. The curves shown were fitted to the MWC equation, and fit the experimental points well in all cases. Interestingly the reverse Bohr effect correlates with an increase in K_T and a fall in L as pH decreases (Fig. 3 and Table 1), which is exactly opposite to the pH response seen in human Hb, where K_T decreases and L increases (Imai and Yonetani 1975). The K_R values remain practically constant as with human Hb. The decrease in the binding constant c occurring in parallel with the rise of the free energy of heme-heme interaction ΔG_{41} as pH is increased indicates the formation of additional bonds that favour the deoxy (T) structure as pH is raised. This

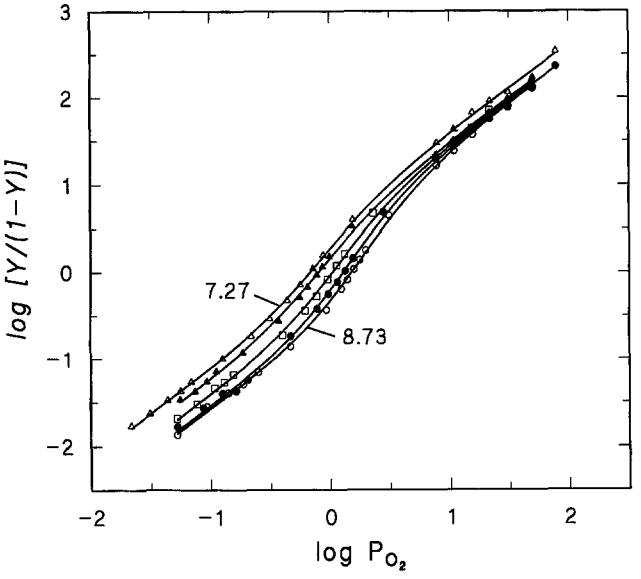

Fig. 3 Hill plots of oxygenation equilibria of stripped cathodic eel Hb. △, pH 7.27; ▲, 7.69; □, 8.13; ●, 8.46; ○, 8.73. Other details as in Fig. 2

contrasts with human Hb where such bonds are formed in deoxy Hb as pH is lowered (Perutz 1970). The magnitudes of the switch over point i_s (Table 1) indicate that at pH 7.7 the main transition from the T to the R conformation occurs upon binding of the second and third O_2 molecule. However, when the pH is either raised or lowered the switch over appears to be delayed further towards the third oxygen bound.

GTP at a GTP/Hb molar ratio of about 20 lowers the oxygen affinity and obliterates the reverse Bohr effect (Fig. 4A and Table 1). As shown, the control mechanism appears to be a lowering of K_T and an increase in L, whereas K_R remains relatively constant, which is similar to the response of human Hb to DPG (Tyuma et al. 1973). This results in a lowering of c and a doubling of the free energy of heme-heme interaction

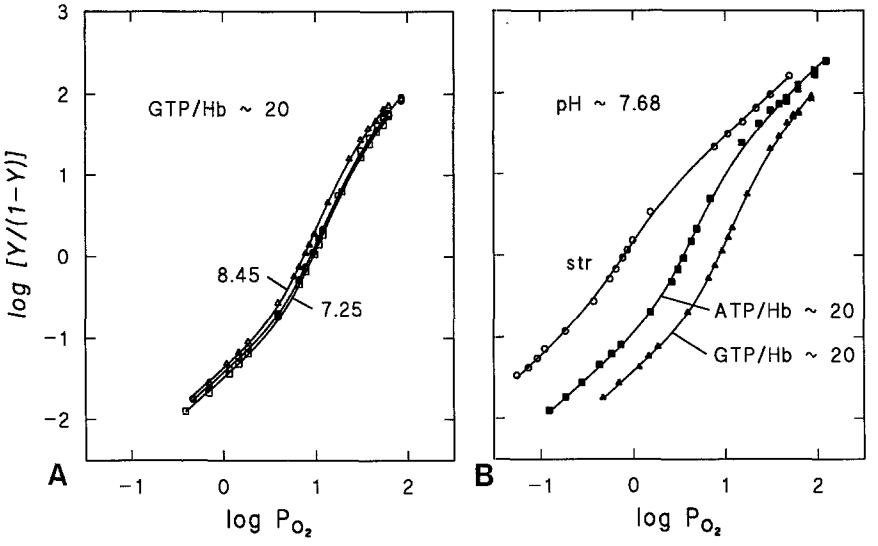

Fig. 4 **A** Hill plots of cathodic eel Hb at GTP/Hb ratios ≥ 20. □, pH 7.25; ○, 7.67 △, 8.45. **B** Hill plots of cathodic eel Hb, ○, stripped (pH 7.69); ■, ATP/Hb ratio ≥ 20 (pH 7.68); ▲, GTP/Hb ratio ≥ 20 (pH 7.67). Other details as in Fig. 2

Table 1 MWC parameters (±SE) of cathodic eel Hb-O_2 equilibria and their dependence of ATP, GTP (NTP/Hb ≥ 20) and proton concentrations

Buffer	pH	K_T mmHg	K_R mmHg	L	c	ΔG_{41} kJ·mol^{-1} per site	i_s
0.1 mol·l^{-1} HEPES,	7.266	0.70 ± 0.03	5.4 ± 0.3	163 ± 44	0.13	4.17	2.50
0.1 mol·l^{-1} KCl	7.685	0.50 ± 0.02	3.7 ± 0.2	73 ± 15	0.14	4.25	2.15
	8.131	0.35 ± 0.02	3.5 ± 0.2	174 ± 46	0.10	5.05	2.24
	8.463	0.27 ± 0.01	3.3 ± 0.2	313 ± 69	0.08	5.63	2.28
	8.727	0.26 ± 0.01	3.6 ± 0.2	708 ± 175	0.07	5.74	2.50
+ GTP	7.248	0.032 ± 0.001	1.7 ± 0.1	5 ± 1.4·10^4	0.02	8.92	2.71
+ GTP	7.671	0.037 ± 0.001	2.2 ± 0.2	1 ± 0.3·10^5	0.02	8.92	2.81
+ GTP	8.447	0.042 ± 0.001	2.5 ± 0.2	8 ± 2.7·10^4	0.02	9.04	2.77
	7.685	0.50 ± 0.02	3.7 ± 0.2	73 ± 15	0.14	4.25	2.15
+ ATP	7.681	0.097 ± 0.004	2.9 ± 0.2	9 ± 2.5·10^3	0.03	7.54	2.66
+ GTP	7.671	0.037 ± 0.001	2.2 ± 0.2	1 ± 0.3·10^5	0.02	8.92	2.81

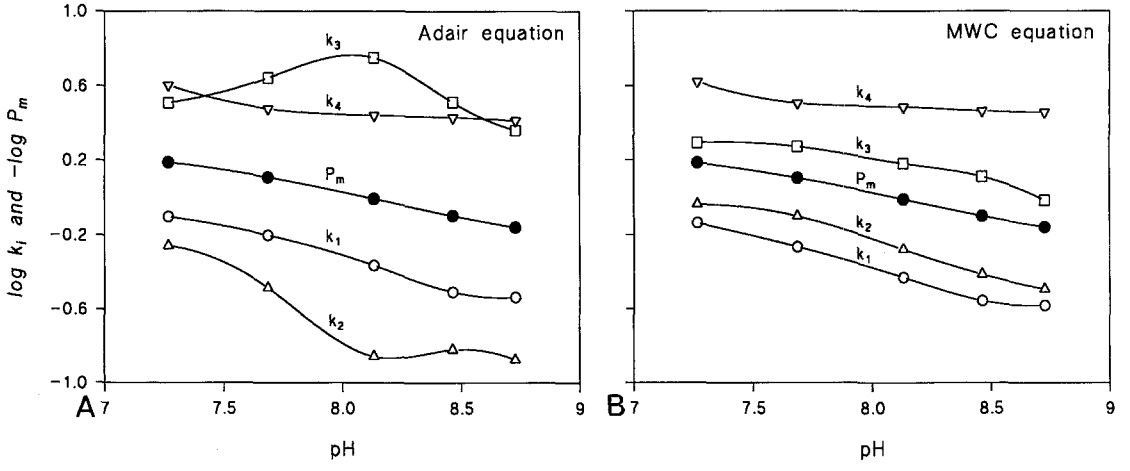

Fig. 5A, B pH dependence of the P_m values and Adair constants in the absence of NTP: **A** fitted to the Adair equation; **B** calculated from the MWC parameters. Other details as in Fig. 2

ΔG_{41}. The switch-over (i_s) is delayed until binding of the third and fourth oxygen molecules (Table 1). The greater effect of GTP on oxygen affinity is due to greater changes in K_T and L than those induced by ATP. The ΔG_{41} value in the presence of ATP is intermediate to those obtained in the absence of phosphates and in the presence of GTP (Fig. 4B and Table 1). The greater suppressive effect of GTP than ATP on oxygen affinity is therefore attributable to a greater stabilisation of deoxyHb by the former effector.

Analysis of the reverse Bohr effect in terms of the Adair theory indicates apparent anti-cooperativity in

Table 2 Adair parameters (±SE) of cathodic eel Hb-O_2 equilibria and their dependence on ATP, GTP (NTP/Hb ≥ 20) and proton concentrations

	Adair						MWC				
Buffer	pH	k_1	k_2	k_3	k_4	ΔG_{41}	k_1	k_2	k_3	k_4	ΔG_{41}
0.1 mol·l^{-1} HEPES,	7.266	0.79 ± 0.03	0.55 ± 0.11	3.2 ± 0.7	4.0 ± 0.1	3.89	0.73	0.92	1.96	4.17	4.17
0.1 mol·l^{-1} KCl	7.685	0.63 ± 0.02	0.33 ± 0.09	4.4 ± 1.2	3.00 ± 0.07	3.74	0.54	0.79	1.87	3.21	4.25
	8.131	0.43 ± 0.02	0.14 ± 0.07	5.6 ± 3	2.77 ± 0.09	4.45	0.37	0.53	1.52	3.07	5.05
	8.463	0.31 ± 0.01	0.15 ± 0.06	3.2 ± 1.4	2.7 ± 0.1	5.18	0.28	0.39	1.29	2.92	5.63
	8.727	0.29 ± 0.01	0.13 ± 0.04	2.3 ± 0.8	2.60 ± 0.09	5.25	0.26	0.32	0.96	2.87	5.74
+ GTP	7.248	0.031 ± 0.001	0.047 ± 0.008	0.08 ± 0.02	1.55 ± 0.17	9.41	0.03	0.03	0.13	1.33	8.92
+ GTP	7.671	0.040 ± 0.001	0.018 ± 0.006	0.26 ± 0.09	1.28 ± 0.08	8.29	0.04	0.04	0.11	1.54	8.92
+ GTP	8.447	0.042 ± 0.002	0.04 ± 0.01	0.14 ± 0.04	1.84 ± 0.19	9.08	0.04	0.04	0.14	1.82	9.04
	7.685	0.63 ± 0.02	0.33 ± 0.09	4.4 ± 1.2	3.00 ± 0.07	3.74	0.54	0.79	1.87	3.21	4.25
+ ATP	7.681	0.112 ± 0.004	0.02 ± 0.01	2.2 ± 1.8	1.98 ± 0.06	6.88	0.10	0.11	0.37	2.26	7.54
+ GTP	7.671	0.040 ± 0.001	0.018 ± 0.006	0.26 ± 0.09	1.28 ± 0.08	8.29	0.04	0.04	0.11	1.54	8.92

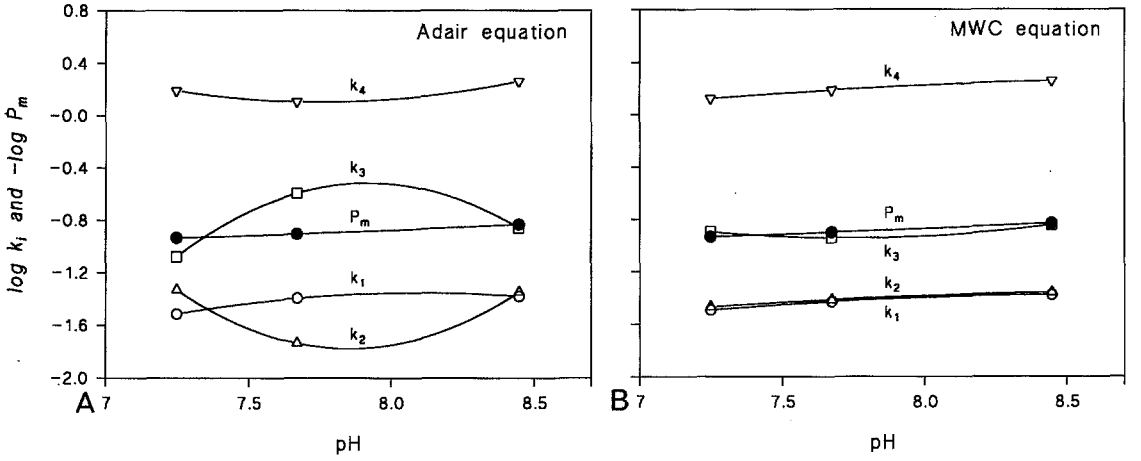

Fig. 6A, B pH dependence of the P_m values and Adair constants at a GTP/Hb molar ratio ≥ 20: **A** fitted to the Adair equation; **B** calculated from the MWC parameters. Other details as in Fig. 2

binding the second and fourth O_2 molecules in the absence of NTP ($k_2 < k_1 < k_4 < k_3$), which may reflect subunit heterogeneity. However, the k_i values derived from the MWC parameters show the usual cooperative pattern (i.e., $k_1 < k_2 < k_3 < k_4$; Fig. 5, Table 2). In the presence of GTP the k_i values derived from Adair and MWC analyses are similar, showing high cooperativity for binding the third and fourth oxygen molecules (Fig. 6, Table 2). In the presence of ATP essentially only the third oxygen molecule appears to be bound cooperatively when analyzed according to Adair, whereas the third and the fourth molecules are cooperatively bound when analyzed according to the MWC model (Table 2). The ΔG_{41} values derived from Adair and MWC analyses showed general agreement, although the Adair values were slightly lower in the absence of phosphates (Table 2).

The calculated fractional populations of the molecules in the various stages of oxygenation and their dependence on proton and NTP concentrations for representative data sets (high and low pH and in the absence and presence of ATP and GTP) are shown in Fig. 7A–E. For comparison the fractional distribution in a non-cooperative Hb is shown in Fig. 7F. At pH 7.7 the different oxygenation species ($Hb(O_2)_i = f_i$) show high degree of symmetry around $Y = 0.5$ (Fig. 7A). Raising of the pH to 8.7 (Fig. 7B) increases f_0 and f_4 at the expense of f_3. The same pattern is evident with ATP (Fig. 7C) and GTP (Fig. 7D) added. The apparent anti-cooperativity in binding the second oxygen to the stripped Hb seen with the Adair scheme is associated with low f_2 values (Fig. 7A) compared to the fractions calculated from the MWC parameters (Fig. 7E).

The P_{50} and P_m values are very similar. However, in the presence of NTP P_{50} values are slightly higher than P_m, correlating with higher symmetry factors (Table 3). The n_{50} values are also similar to the n_{max} values in the absence of NTP, but slightly lower than n_{max} values in the presence of ATP or GTP. Given the small differences, P_{50} and n_{50} are reasonable estimates of P_m and n_{max} (Table 3).

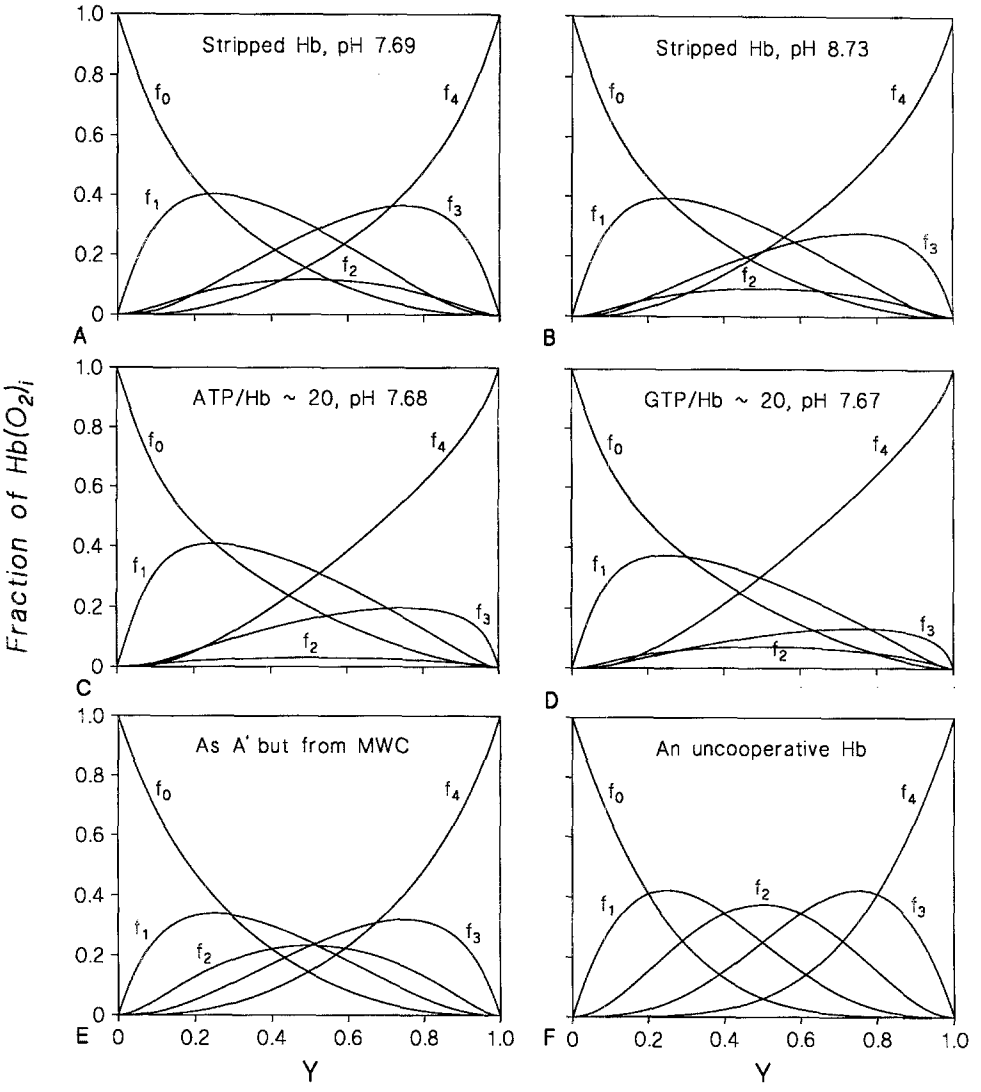

Fig. 7A–F Fractional populations of Hb molecules in different stages of oxygenation (f_i, where i = 0, 1, 2, 3 or 4 bound O_2 molecules) calculated from the Adair parameters (**A–D**). **A** stripped Hb at pH 7.69; **B** stripped Hb at pH 8.73; **C** Hb in the presence of ATP at an ATP/Hb molar ratio ≥ 20 at pH 7.68; **D** Hb with GTP at a GTP/Hb ratio ≥ 20 and at pH 7.67; **E** as **A** but calculated from the MWC parameters; **F** an uncooperative Hb. Other details as in Fig. 2

Table 3 Oxygen equilibrium parameters of cathodic eel Hb and their dependence of ATP, GTP (NTP/Hb ≥ 20) and proton concentrations

Buffer	pH	P_{50} mmHg	n_{50}	$P_{n,max}$ mmHg	n_{max}	P_m mmHg	W
0.1 mol·l^{-1} HEPES,	7.266	0.68	1.73	0.79	1.75	0.65	1.78
0.1 mol·l^{-1} KCl	7.685	0.80	1.87	0.84	1.82	0.78	1.32
	8.131	1.05	1.98	1.14	2.02	1.02	1.53
	8.463	1.30	1.92	1.40	2.08	1.25	1.71
	8.727	1.52	2.09	1.76	2.09	1.44	2.46
+ GTP	7.248	9.33	2.29	12.45	2.45	8.59	12.25
+ GTP	7.671	8.78	2.35	11.61	2.56	8.01	11.07
+ GTP	8.447	7.44	2.36	9.77	2.49	6.78	12.33
	7.685	0.68	1.73	0.84	1.82	0.78	1.32
+ ATP	7.681	3.63	2.34	4.52	2.48	3.31	5.93
+ GTP	7.671	8.78	2.35	11.61	2.56	8.01	11.07

The number of Bohr protons released upon oxygenation estimated from the slope of the log k_i (or P_m) versus pH curves, is shown in Fig. 8. In the absence of NTP, the reverse Bohr effect indicates Bohr proton binding to the Hb upon oxygenation. The minimum ΔH_i^+ and ΔH_1^+ values are at approximately pH 8.15 and pH 8.21, respectively, indicating that the Bohr group responsible for the reverse Bohr effect has a pK near 8.2 (lower in deoxy Hb and higher in oxy Hb) (Fig. 8). When GTP is present the small normal Bohr effect indicates that protons are taken up on deoxygenation (Fig. 8). In the absence of NTP the Hb molecules bind about 1.1 protons on oxygenation (at pH 8.15). Using the equations for calculating ΔpK (Eq. 8) this indicates that only two

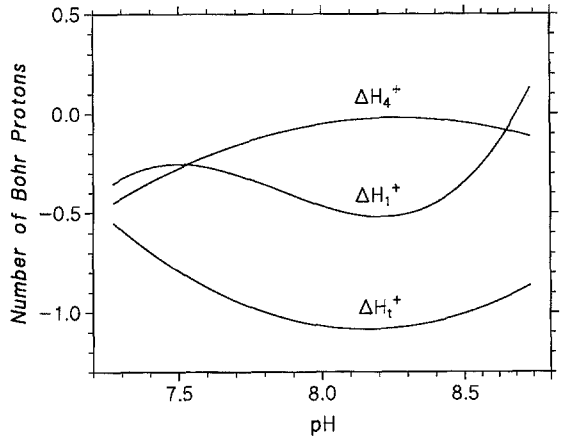

Fig. 8 Number of protons released on oxygenation of Hb as a function of pH in the absence of NTP (calculated from Fig. 5A). ΔH_1^+, ΔH_4^+ and ΔH_t^+, the number of protons released upon binding of the first, the last and all O_2 molecules, respectively, to the Hb molecule. Other details as in Fig. 2

groups (with approximate pK values of 8.7 in oxy-Hb and 7.6 in deoxy Hb) may account for the reverse Bohr effect in the absence of phosphates (Fig 8).

Discussion

The Bohr effect normally facilitates unloading of oxygen in the acid tissues. Some fish Hbs (e.g., trout Hb I) are known to lack Bohr and phosphate effects, and thus remain capable of binding oxygen at low pH (Weber and Jensen 1988; Weber 1990). The fact that the reversed Bohr effect seen in stripped cathodic eel Hb is obliterated by GTP at a GTP/Hb ratio of only 0.5 indicates physiological similarity to trout I (lacking a Bohr effect over a wide pH range). Cathodic eel Hb, in contrast to trout Hb I, displays high ATP and GTP sensitivities (Fig. 2), whereby the oxygen affinity of this "emergency" Hb can be modulated in response to red cell NTP levels (Weber and Jensen 1988; Weber 1990). We find similar oxygen-binding properties in the minor cathodic Hb (C_1) as in the major component (C_2). Since the Hb samples analyzed were obtained from pooled blood samples, it is unknown whether the occurrence of different cathodic components represents polymorphism.

Gillen and Riggs (1973) suggested that the reverse Bohr effect of the cathodic Hb of the American eel could be due to a positive and a negative group in the molecule that move closer to each other, or two positive charges that move apart, upon oxygenation. Our results show that the reverse Bohr effect results from a stabilisation of the T-state as pH is raised, whereas the R-state is not influenced (Fig. 3). The stabilisation of the T-state could be due to additional salt bridges occurring at high pH between a positive charge and a residue which is uncharged at low pH but negatively charged at high pH. Alternatively a destabilisation of the T-state could occur at low pH due to the repulsion of two positively charged groups, of which one is uncharged at high pH.

The observation that CO_2 enhances the reverse Bohr effect in the closely-related American eel (*Anguilla rostrata*), indicates involvement of a CO_2-binding site (Gillen and Riggs 1973). Given that the N-terminal residues of the α-chains of cathodic eel Hb are acetylated (as is common in fish Hbs), CO_2-binding is limited to the N-terminal of the β-chains (Gillen and Riggs 1973; Weber 1990; Fago et al. 1995). The estimate that about 1.1 protons are taken up per tetramer upon oxygenation indicates that only one residue of each of the two α-chains or one of each of the two β-chains need to be involved. The N-terminal of the β-chains thus may account for the whole reversed Bohr effect. The finding that the pK of the group responsible for the reverse Bohr effect is near 8.2 (higher in oxy-Hb and lower in deoxy-Hb) favours this interpretation, since the pK values of the N-termini of the β-chains in cathodic eel carboxy Hb have been estimated to be 8.41 (Breepoel et al. 1981). The destabilisation of the T-state at low pH could thus be due to repulsion between the protonated N-termini of the β-chains or between these termini and the other positive charges at the phosphate binding site that face the central cavity in deoxy-Hb but are external in oxy-Hb (Bonaventura and Bonaventura 1978; Perutz et al. 1980).

The reverse Bohr effect occurring in certain fish Hbs at high pH but not in human Hb A could be due to a rise in the pK of the N-termini of the β-chains caused partly by the substitution of the positively charged β2-His (in human Hb A) by negatively charged glutamate (in fish) [cf. carp Hb; Gillen and Riggs 1972)]. In this way the reverse Bohr effect caused by repulsion of the positive charges at the phosphate binding site is shifted to a higher pH and no longer is masked by Bohr groups responsible for the alkaline Bohr effect. If the N-terminus of the β-chains is responsible for the reverse Bohr effect, its blocking will annihilate the reverse Bohr effect (Kilmartin et al. 1969). Alternatively, the reverse Bohr effect of cathodic eel Hb can be due at least partly to an uncommonly high pK of the 143β residue, which is responsible for half of the acid Bohr effect in human Hb A. Compared to human Hb A, eel cathodic Hb exhibits a β143 His → Lys substitution (Fago et al. 1995).

The organic phosphates ATP and GTP regulate O_2 affinity of the cathodic eel Hb primarily by changing K_T and L, whereas GTP also affects K_R. These effects are similar to those seen in tench Hb (Weber et al. 1987) but different from those in human Hb, where K_R does not change in the presence of DPG (Tyuma et al. 1973; Imai 1973). GTP enhances cooperativity in the cathodic eel Hb as evident from the very low fractional concentrations of Hb molecules with two and three O_2 molecules bound. This means that the second, third

and fourth O_2 molecules bind almost simultaneously. The fall in k_4 and the increase in i_s to almost 3 upon addition of ATP or GTP indicate that the phosphates remain bound to about half of the triligated Hb. The similarity of the ATP and GTP effects in cathodic eel and the anodic tench Hb (at physiological pH) is remarkable since tench Hb possesses a normal Bohr and Root effect, whereas cathodic eel Hb displays a reverse Bohr and no Root effect (Jensen and Weber 1982; Weber et al. 1987; Pelster and Weber 1990).

The free energy of heme-heme interaction is generally lower in fish Hbs than in human Hb, where it approximates 12.6 $kJ \cdot mol^{-1}$ per site in the absence of DPG and 15.2 $kJ \cdot mol^{-1}$ per site in the presence of DPG, pH 7.4, 25 °C and in 0.1 $mol \cdot l^{-1}$ Cl^- (Imai 1982). The ΔG_{41} values of cathodic eel Hb, with and without phosphates are similar to those in Hbs of trout I [8.0 $kJ \cdot mol^{-1}$ per site in the absence of phosphate, pH 6.8, 20 °C and absence of Cl^-; Wyman et al. (1977)], carp [4.6 $kJ \cdot mol^{-1}$ per site in the absence of phosphate and pH 7.65 and 5.3 $kJ \cdot mol^{-1}$ per site in the presence of IHP, pH 7.98, 15 °C and absence of Cl^-; Chien et al. (1980)] and tench [6.1 $kJ \cdot mol^{-1}$ per site in the absence of phosphate and 8.24 $kJ \cdot mol^{-1}$ per site in the presence of GTP, pH 7.32, 15 °C and absence of Cl^-; Weber et al. (1987)].

The free-energy change for the T-R transition in the absence of oxygen ΔG_t [defined as $\Delta G_t = RT \ln L$; Imai (1973)] is low in stripped cathodic eel Hb: 10 $kJ \cdot mol^{-1}$, which corresponds to only two salt bridges (Perutz 1970) or, alternatively, four salt bridges weakened at low pH due to repulsion between two positive charges. It increases to 22 and 28 $kJ \cdot mol^{-1}$, respectively, with ATP and GTP added. This corresponds well with the values for tench Hb [13.1, 28.5 and 24.9 $kJ \cdot mol^{-1}$ for stripped Hb and the presence of ATP and GTP, respectively, calculated from Weber et al. (1987)] but is much less than those for human Hb [20.5 and 48.0 $kJ \cdot mol^{-1}$ in the absence and presence of DPG (Imai 1973)], indicating that fewer salt bridges restrain cathodic eel Hb and tench Hb in the T-state compared to human Hb (Perutz 1970). Part of this reduction is due to the acetylation of the N-terminal of the α-chains in fish Hbs (Gillen and Riggs 1973; Weber 1990), that prevents formation of two of the six salt bridges proposed by Perutz to occur in human Hb (Perutz 1970). Another part may be due to increased repulsion between the positive charges involved in phosphate binding site at neutral pH in the T-state due to an increased pK of the N-termini of the β-chains. This will, however, be counteracted by the β2-His → Glu substitution in fish Hb.

Further work is needed to determine whether blocking of the N-termini of the β-chains of cathodic eel Hb and other selected fish Hbs annihilates the reverse Bohr effect found in some fish Hbs, and whether a reverse Bohr effect occurring in some fish Hbs at high pH correlates with high pK values of the N-termini of the β-chains.

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