ALLOSTERIC INTERACTIONS GOVERNING OXYGEN EQUILIBRIA IN THE HAEMOGLOBIN SYSTEM OF THE SPINY DOGFISH, SQUALUS ACANTHIAS

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SUMMARY

The oxygenation-linked, allosteric interactions of erythrocytic organic phosphates and urea with the haemoglobin (Hb), and the functional significance of the Hb multiplicity, were studied in an elasmobranch, Squalus acanthias.

The autochthonous red cell nucleoside triphosphates (NTP) ATP and GTP (guanosine triphosphate) strongly depress O2 affinity of the stripped (cofactor-free) Hb and increase cooperativity in O2 binding. As previously found in teleost Hbs, GTP exerts a greater effect than ATP at the same concentration. Urea, in contrast, increases O2 affinity and depresses cooperativity. It also antagonizes the modulator effectivity of NTP at physiological NTP/Hb concentration ratios.

Deoxygenation of the Hb raises blood pH. This Haldane effect contrasts with earlier findings for Pacific specimens, but accords with the presence of a Bohr effect (\( \phi = \Delta \log P_{50}/\Delta pH \)).

S. acanthias Hb resolves into six main components (three pairs) on the basis of isoelectric point. There is no evidence for radical functional differentiation as found in teleosts with electrophoretically anodal and cathodal Hb components.

The physiological implications of the findings and the possible molecular mechanisms basic to the NTP and urea effects are discussed.

INTRODUCTION

The red cells of teleost fish contain high concentrations of ATP, often in concurrence with guanosine triphosphate (GTP) (Parks et al. 1973; Geoghegan & Poluhowich, 1974; Weber & Lykkeboe, 1978; Bartlett, 1980). These nucleoside triphosphates (NTP) depress the O2 affinity of the haemoglobin (Hb) by allosteric interaction as does 2,3-diphosphoglycerate (DPG) in human red blood cells (Benesch & Benesch, 1967; Chanutin & Curnish, 1967). Changes in their concentrations correlate neatly with adaptive modulation of blood O2 affinity in teleosts in response to changed environmental or metabolic stimuli (reviewed by Weber, 1982). Where

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GTP appears to play the greater role, as evident from its greater depressional effect on O₂ affinity and its lesser inhibition by divalent cations compared to ATP under identical in vitro conditions (Weber, Lykkeboe & Johansen, 1975; Weber & Lykkeboe, 1978).

Very little is known about the effects of erythrocytic cofactors on Hb function in the elasmobranchs, despite a tantalizing variation in type and concentration of potential ligands encountered. The erythrocytic ATP/GTP ratios vary tremendously—from less than unity in the school shark, Galeorhinus australis, to about 6 and 12 in the dogfishes Squalus acanthias and Scyliorhinus canicula, respectively (Coates, Paton & Thompson, 1978; Leray, 1979; Wells & Weber, 1983). Some species, e.g. the ray Narcacion nobiliana and Squalus acanthias, additionally contain small concentrations of inositol pentaphosphate (IPP) (Borgese & Nagel, 1978), the allosteric cofactor common in birds. Importantly, the erythrocytes of marine elasmobranchs also contain high concentrations of urea (near 0.5 M, Browning, 1978) where it serves an osmotic function. Urea, however, also binds to carboxyl groups of proteins like Hb (Krichevskaya, Lukash & Kartasheva, 1973) whereby it may influence its oxygenation properties.

Fish and other ectothermic vertebrates frequently possess multiple Hb systems. Many teleost fish possess anodal Hb components, whose O₂ affinities generally are highly sensitive to organic phosphates, pH and temperature, and cathodal ones with higher O₂ affinities and lower sensitivities to these factors (Binotti et al. 1971; Brunori, 1975; Weber, Wood & Lomholt, 1976; Weber, 1982). No information on the functional consequences of Hb multiplicity seems to be available for elasmobranchs, although they have highly variable patterns of Hb heterogeneity (Fyhn & Sullivan, 1975) which frequently also are polymorphic.

We studied the influences of potential erythrocytic modifiers of O₂ affinity (NTP and urea) on the composite and isolated Hbs, and the functional significance of Hb heterogeneity in the dogfish, Squalus acanthias, where the allosteric interactions are of additional interest in view of the almost complete dissociation of the tetrameric deoxygenated Hb molecules to dimeric halves upon oxygenation in vitro (Fyhn & Sullivan, 1975). We also report the dependence of blood pH on oxygenation at constant CO₂ tension, and the O₂ affinity in the Hb from juvenile specimens. The preceding paper (Wells & Weber, 1983) deals with the oxygenational properties of the whole blood and the regulation of O₂ affinity during in vitro incubation of the red cells.

**MATERIALS AND METHODS**

The study is based on seven specimens of the spiny dogfish, Squalus acanthias L., about 70–90 cm in length and 4–7 kg in weight. The sharks were caught by Esbjerg fishermen and kept at Esbjerg Marine Aquarium ('Fiskerimuseet') in sea water of about 35‰ salinity and at 10–15°C until use. We also used blood from a batch of 2-week-old, 30–40 g juveniles that were born in the aquarium.

Whole blood samples were obtained by acute venepuncture. This technique, together with those used for estimating haematocrit and for enzymic and chromatographic assay of the concentrations of ATP and guanosine triphosphate (GTP), are detailed elsewhere (Wells & Weber, 1983).
The relationship between CO₂ tension and pH in oxy- and deoxygenated blood was investigated by equilibrating 75 μl aliquots of blood in Radiometer BMS-II tonometers with gas mixtures containing varying CO₂ tensions, and O₂ tensions of either zero or 148–155 mm, balance N₂, for 20 min at 20°C. These gas mixtures were prepared with Wösthoff gas mixing pumps using 99-995% pure N₂. Millimolar bicarbonate concentrations were calculated from the Henderson-Hasselbalch equation as $10^{pH-pK'}$ $acO_2 PCO_2$ using a value for the CO₂ solubility coefficient ($acO_2$) of 0-0440 mmol l⁻¹ mmHg⁻¹, calculated from Pleschka & Wittenbrock's (1971) data for dogfish, and pK’ values interpolated from those given by Albers & Pleschka (1967) for elasmobranchs where pK’ at 20°C and pH 7-4 (corresponding to 77% CO₂ solubility in pure water) equals 6-042, and where pK’/°C = −0-008 and pK’/pH = −0-097.

Hb solutions were prepared by osmotic-shock and ultrasonification of red cells that had been washed 2–3 times in filtered sea water or 2% saline. The Hb was stripped of ionic cofactors by chromatography on Sephadex G25 Superfine, as preliminary attempts to strip samples with Amberlite MB 3 mixed ion exchange resin resulted in methaemoglobin formation. Other procedures for preparing haemolysates, administering cofactors and separating multiple Hbs were carried out as described earlier (Weber, Lykkeboe & Johansen, 1976; Weber & Lykkeboe, 1978) except that some O₂ equilibria were measured in Na-Hepes instead of Tris-Cl buffer (specified in legends).

A modified diffusion chamber technique (Weber, 1981) was used for measuring O₂ equilibria, evaluating cooperativity coefficients at half saturation ($n$) from the slopes of Hill plots (Hill, 1910). The effects of urea at varying ATP concentrations were investigated by preparing stock solutions of stripped Hb, ATP, urea, and Na-Hepes buffer, each containing 0-09 M of the buffer and 0-09 M-NaCl, then mixing these in the desired proportions. The apparent heat of oxygenation ($\Delta H_{app}$) was calculated from the derived van't Hoff equation (Wyman, 1948) as $R\Delta ln P_{50}/\Delta (1/T)$ where R is the gas constant (8-3143 J K⁻¹ mol⁻¹) and T the absolute temperature.

RESULTS

O₂ affinity and its dependence on protons, organic phosphates, urea and temperature

The stripped Hb showed a high O₂ affinity, a weak Bohr effect (at 10°C and pH 7-85, $P_{50} = 2-3$ and $\phi = -0-21$) and very slight cooperativity ($n = 1-05$) which increased slightly with falling pH (Fig. 1). The O₂ affinity was much higher than that found in the whole blood ($P_{50} = 13$ mmHg – Wells & Weber, 1983).

The Bohr effect of the stripped Hb was slightly greater at 2°C than at 15°C ($\phi = 0-26$ and $-0-21$ respectively – Fig. 2). The overall heat of oxygenation ($\Delta H_{app}$, which includes the heat of solution of oxygen) was $-35$ and $-44$ KJ (mol O₂)⁻¹ at pH 7-0 and 7-9. This pH dependence of $\Delta H$ is compatible with greater proton binding at low pH. The haemolysate from juvenile specimens had a higher intrinsic O₂ affinity than the cofactor-free maternal Hb (Fig. 2), indicating that this difference contributes to the maternal-foetal O₂ transfer in S. acanthias.
Fig. 1. \( P_{S0} \) and \( n_{S0} \) values of the stripped haemolysate of adult specimens at 2, 10 and 15°C (Δ, □ and ○) compared with that of juvenile specimens at 15°C (●), measured in 0·1 M-Na-Hepes buffer. Haem concentrations, 0·12 mM.
GTP decreased O₂ affinity more than ATP at the same phosphate: Hb ratio, as previously established in teleost fish, where both these factors are found (cf. Weber et al. 1975). The phosphates exerted greater effect on $P_{50}$ at lower concentration ratios and raised $n$ from about 1 to 1.5 (Fig. 3).

The influence of urea on Hb oxygenation and the implications of its concurrence with ATP are illustrated in Figs 4, 5 and 6. Urea increased the O₂ affinity of *Squalus*
Hb and decreased its cooperativity (Fig. 4); the affinity effect was greater at pH 7.0 than pH 7.3. Urea also reduced the sensitivity of O₂ affinity to ATP (Fig. 5). Thus, in the absence of urea, ATP (at 1.5 molar excess over Hb tetramers) decreased O₂ affinity below pH 7.5; in the presence of 0.4 M urea this effect was evident only at pH below ~7.25 (Fig. 6). These observations suggest antagonism between oxygenation-linked binding of the two metabolites. Curiously, ATP increased O₂-affinity of the Hb at high pH (in the absence or presence of urea — Fig. 6).

**Influence of Hb oxygenation on blood bicarbonate and buffer capacity**

Fig. 7A, B shows the variations of CO₂ tension and bicarbonate concentration with pH for oxygenated and deoxygenated *S. acanthias* blood. The higher pH in the deoxygenated Hb at the same CO₂ tension (the Haldane effect) reflects binding of the Bohr protons to the Hb upon liberation of O₂ and a resultant increase in blood bicarbonate concentration ($\Delta$HCO₃⁻ = 1.4 mmol l⁻¹ at pH 7.9). The buffer capacity ($\Delta$HCO₃⁻/$\Delta$pH) decreased from about 10 and 11.8 mol (pH unit)⁻¹ for deoxy- and oxygenated blood at pH 7.85, to about 2.4 and 4.0 respectively, at pH 7.0.

**Hb multiplicity**

Electrophoresis revealed the presence of three main anodal Hb bands in the adult as well as juvenile stages and showed no evidence for polymorphic variation in the material used. In preparative iso-electric focusing each of these bands resolved into two components; these six components accounted for at least 95% of total haem (Fig. 8). At 10°C the carboxy derivatives of the three pairs of components (I + II, III + IV and V + VI in Fig. 8) were isoelectric near pH values of 7.7, 7.4 and 6.9, respectively. O₂ equilibrium properties (incorporated into Fig. 1) showed that in the pH range of 7.0 to 7.9, which embraces physiological conditions, the most abundant fraction

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**Fig. 5.** Effects of urea on O₂ equilibria of the stripped haemolysate measured at 15°C and pH 7.26 in the absence (open symbols) and presence (closed symbols) of 0.42 M-urea, in 0.1 M-Na-Hepes buffer. Haem concentration, 0.41 mm; ATP/Hb (mol/mol) ratios, 0 (O, ), 0.97 (□, ), 2.90 (△, △) and 9.66 (▲, ▲).
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**Fig. 6.** Dependence of $P_{50}$ and $n_{50}$ of the stripped haemolysate on pH in the absence of added ligands (O) and in the presence of ATP (●), urea (□) and ATP + urea (■) measured at 15°C in 0·1 M-Na-Hepes buffer. Haem concentration, 0·30 mM; urea (where present), 0·46 M; ATP/Hb (mol/mol) ratio, 1·51.

**Fig. 7.** Influence of haemoglobin oxygenation on (A) the $P_{CO_2}$-pH relationship, (B) the bicarbonate-pH relationship and (C) the bicarbonate-$P_{CO_2}$ relationship, measured in a blood sample containing 0·93 mM-haem at 20°C. O, oxygenated Hb; ●, deoxygenated Hb.
Fig. 8. Isolation of component Hbs by isoelectric focusing in solutions of ampholines (LKB, Sweden) with pH ranges of 5 to 8 (0-42%) and 3-5 to 10 (0-28%). Open horizontal bars, fractions pooled for O₂ equilibrium determinations (given in Fig. 1).

(III + IV) had a similar O₂ affinity to the haemolysate but a slightly larger Bohr effect ($\phi = -0.35$), whereas fractions I + II and V + VI had smaller pH effects ($\phi$ near $-0.18$) and higher O₂ affinities. Again, ATP had a lesser effect on O₂ affinity of fractions I + II and V + VI than on those of the main fraction (III + IV) or the whole haemolysate. The closeness of the isoelectric points and the resultant difficulty in completely separating the main fraction (III + IV) from the smaller adjacent ones (cf. Fig. 8) suggest that the above differences will be more pronounced in pure, isolated components.

DISCUSSION

The high O₂ affinity ($P_{S0} = 2.3$ at pH 7.85 and 10 to 15°C), low cooperativity ($n = 1.05$) and small Bohr factor ($\phi = -0.20$) of stripped Squalus acanthias Hb conform broadly with corresponding earlier findings for other elasmobranchs (Manwell, 1963; Pennelly, Noble & Riggs, 1975; Mumm, Atha & Riggs, 1978; Martin et al. 1979). The available information thus suggests that homotropic and heterotropic intramolecular interactions will only play a modest role in adapting O₂ transport by haemoglobin in sharks, skates and rays to environmental and metabolic changes.

Unlike in teleost fish, where the alkaline Bohr effect disappears at high and low pH, that of Squalus acanthias is manifest over an extremely wide pH range (6.3 to 8.9), reflecting the implication of several acid groups with widely different pK values. The
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As also evident in stingray, Dasyatis, Hb (Mumm et al. 1978). The higher Bohr factor seen in the whole blood ($\phi = -0.28$, Wells & Weber, 1983) is consistent with the allosteric interaction of anionic erythrocytic phosphates, which raise $\phi$ by increased binding to Hb at low pH.

The rise in the Bohr effect as pH decreases from 8 to 7 (Fig. 1) correlates with the concomitant decrease in the temperature effect ($\Delta H = -44$ and $-36$ KJ mol$^{-1}$ at pH 8 and 7 – cf. Fig. 2), suggesting that the latter phenomenon results from endothermic protonation reactions that subtract from the exothermic oxygenation reaction proper. The overall heats of oxygenation fall within the range for Hbs from other vertebrate classes (Rossi-Fanelli, Antonini & Caputo, 1964) but not for the Hb from the porbeagle shark, Lamna nasus, where a $\Delta H$ value near zero reflects the occurrence of two types of functional subunits with different sensitivities within the same tetramers (Andersen, Olson & Gibson, 1973).

The pronounced sensitivity of the O$_2$ affinity of S. acanthias Hb to ATP and GTP contrasts sharply with ATP insensitivities observed in the stingrays, Dasyatis and Potamogon, and the torpedo ray, Torpedo nobiliana, (Mumm et al. 1978; Martin et al. 1979; Bonaventura, Bonaventura & Sullivan, 1974a, b), but accords with findings for the Japanese shark Triakis scyllia (Kono & Hashimoto, 1977). The available information thus suggests that ATP sensitivity may differ between the selachian (sharks) and batoidean (skates and rays) elasmobranchs.

The greater sensitivity of O$_2$ affinity of Squalus Hb to GTP than to ATP aligns the elasmobranchs with the teleost fish (cf. Weber et al. 1975; Petersen & Poluhowich, 1976). GTP may be formed by successive phosphate transfers from ATP to GMP, catalysed by guanosine monophosphokinase (GMPK) and nucleodiphosphokinase (NDPK) (cf. Weber, 1982). Both these enzymes occur in Squalus acanthias erythrocytes (0.05 and 25 units/ml cells, respectively, Parks et al. 1973). Such ATP to GTP conversion would increase the modulator effectiveness of the erythrocytic NTP, and may moreover account for the remarkable intraspecific variability in ATP/GTP ratio encountered in some species (e.g. the smooth dogfish, Mustelus canis, Borgese et al. 1978). Studies on the kinetic rates of ligand reactions in the Hb of the stingray Potamotrygon (Martin et al. 1979) show that ATP decreases O$_2$ affinity by raising the rate of O$_2$ dissociation without affecting that of ligand association, while proton binding (the Bohr effect) affects both rates. This picture is complicated by an observation of kinetic heterogeneity in Hb from the carpet shark, Cephaloscyllium, in the absence of phosphate cofactors. This appears to be a point of difference between elasmobranch and teleost haemoglobins (Brittain, Barber, Greenwood & Wells, 1982).

Urea markedly increases the O$_2$ affinity of Squalus acanthias Hb, as observed in Hbs of man (Rossi-Fanelli et al. 1964) and the toad, Xenopus laevis, where urea levels rise in response to salt-water acclimation and aestivation (Jokumsen & Weber, 1980). In contrast, Hb-O$_2$ affinity is virtually independent of urea concentration in the clearnose skate, Raja, the Amazonian stingray, Potamotrygon, (Bonaventura et al. 1974a, b; Martin et al. 1979) and in the African lungfish, Protopterus, which experiences high urea concentrations during aestivation (Weber, Johansen, Lykkeboe & Maloiy, 1977). Within elasmobranchs these data thus show a dichotomy which incides with the selachian-batoidean differentiation.
Kinetic carbon monoxide binding studies with *Raja* Hb indicate that urea insensitivity is dependent on the integrity of the tetrameric molecular structure of the Hb, whereas the urea-sensitive, human Hb dimerizes extensively in the presence of urea (Bonaventura *et al.* 1974a, b). In this light the large urea sensitivity of *Squalus* Hb correlates with evidence that 94% of non-oxidized pigment in solution consists of dimers, which aggregate to tetramers upon deoxygenation (Fyhn & Sullivan, 1975). That molecular dissociation alone will not account for the urea-induced increase in O₂-affinity, however, follows from the persistence of the ATP effect in the presence of urea, since ATP modulation, in analogy with mammalian Hb and DPG, is dependent upon the integrity of the tetrameric structure.

Urea decreases the effect of ATP on O₂ affinity (Figs 5, 6). This suggests that *in vitro* oxygenation studies conducted in the absence of urea overestimate the modulator role of the phosphate in elasmobranchs. Urea, however, does not obliterate NTP sensitivity completely (see Fig. 5). Hypoxic incubation of intact *S. acanthias* erythrocytes decreases cellular NTP and increases O₂-affinity (Wells & Weber, 1983).

The effect of urea on the oxygen equilibria may be due to oxygenation linked binding of this cationic compound to the negatively charged carboxyl termini of the polypeptide chains of Hb. Binding to Hb and other intracellular proteins at these sites is consistent with the greater effect of urea on O₂ affinity at high pH (Fig. 4), where the carboxyl groups will show greater ionization. Such binding may explain the higher urea concentrations in erythrocytes than in plasma (Krichevskaya *et al.* 1973; Browning, 1978) since urea diffuses freely through the erythrocyte membrane (Hunter, 1976). Urea binding at the carboxyl groups might thus increase affinity by hindering the oxygenation-linked binding at these sites of the Bohr protons which have a negative effect on O₂-affinity (removal of the C-terminal histidines of the β chains of human Hb decreases its Bohr effect by half – Kilmartin & Wootton, 1970). Urea may also affect O₂ affinity of Hbs due to its spontaneous conversion to cyanate, resulting in the slow, irreversible carbamylation of the NH₂-terminal valine residues of the Hb protein chains (Cerami *et al.* 1973; Bonaventura *et al.* 1974a, b). Carbamylation of these α-amino N-termini would explain the urea induced reduction of the ATP-effect (since those of the β-chains contribute two of the seven positively charged sites where DPG interacts in human Hb – cf. Arnone, 1972). The irreversibility of the carbamylation reaction and the fact that the amino terminal residue of stingray Hb is ‘uncarbamylated’ valine (Mumm *et al.* 1978), however, appear to be evidence against the implication of this reaction in the urea effect.

In whole blood, the Hb of *Squalus acanthias* shows a distinct Haldane effect. Curiously, Lenfant & Johansen (1966) found no such effect in the Pacific *S. suckleyi* (which now is considered to be *S. acanthias*). A Haldane effect is also lacking in the dogfish, *Scyliorhinus stellaris* and *Mustelus mustelus*, and in the electric ray, *Torpedo ocellata* (Albers & Pleschka, 1967). These data predict the absence of a Bohr effect in the latter three species (as recorded for *S. suckleyi*, Lenfant & Johansen, 1966). Since the Haldane effect is a thermodynamic consequence of the Bohr effect the presence of both effects in the Hb of *S. acanthias* appears to be internally consistent. The steepness of the (HCO₃⁻)/PᵣCO₂ curve at low, physiological CO₂ tensions suggests transport of significant blood CO₂ as bicarbonate. Our values for the log PᵣCO₂/pH relationship...
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-1.9 to -2.1) and buffering capacity (10–12 mM-HCO3-/pH unit at pH 7.85 – Fig. 6A, B) agree well with those in other elasmobranchs (Albers & Pleschka, 1967).

We find at least six Hb components in Squalus acanthias compared to previous electrophoretic studies showing one and two components (Buhler & Shank9, 1959; Manwell, 1963). Although the shark haemolysate does not display such striking functional heterogeneity as teleosts with cathodal Hbs, there is evidence for some degree of molecular ‘division of labour’ – the main components showing higher heterotropic interactions (associated with proton and ATP binding) than the other components, with the composite haemolysate having intermediate properties (reflecting the absence of hybridization). The absence of radical functional heterogeneity and of large heterotropic interactions (see Results) in elasmobranch Hb may contribute to the low tolerance of elasmobranchs to variation in water O2 tension, and to their low capacity for maintaining constant O2 uptake rates as O2 tension falls (cf. Piiper, Baumgarten & Meyer, 1970).

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