

INTERACTION BETWEEN HAEMOGLOBIN AND SYNTHETIC PEPTIDES OF THE N-TERMINAL CYTOPLASMIC FRAGMENT OF TROUT BAND 3 (AE1) PROTEIN

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Accepted 13 July; published on WWW 10 September 1998

Summary

Two acidic peptides corresponding to the first 10 and 20 amino acid residues of the N-terminal, cytoplasmic fragment of rainbow trout band 3 (AE1) protein were synthesised in order to study their interaction with trout and human haemoglobin (Hb). The peptides did not influence the oxygen affinity of the main anodic trout Hb component (Hb IV) when tested at surplus peptide concentration ([peptide]/[Hb₄]=16), at high and low ionic strength and at pH values ranging from 6.5 to 7.6. With human Hb, however, the 20-mer peptide markedly decreased the oxygen affinity and increased the Bohr effect. These data suggest that the trout band 3 peptide binds

preferentially to the deoxy (T) conformation of human Hb, probably at the organic phosphate binding site in the central cavity between the β -chains, which is known to be the binding site for the acidic N terminus of human band 3. In trout Hb IV, the presence of negatively charged Asp at position NA2 of the β -chains (in contrast to positive or neutral residues in mammalian Hb) may weaken any interaction with the highly negatively charged peptides.

Key words: haemoglobin, band 3, anion exchange protein, allosteric interaction, rainbow trout, *Oncorhynchus mykiss*, human haemoglobin.

Introduction

Haemoglobin (Hb) is known to bind to internal membrane components of mammalian red blood cells (RBCs). The interaction is predominantly electrostatic in nature, and band 3 protein constitutes the main binding site (Low, 1986; Salhany, 1990). Band 3 (also known as AE1) is the most abundant membrane protein in human RBCs, being present at approximately 1 million copies per cell. The protein consists of a membrane domain and a cytoplasmic domain. The membrane part mediates anion exchange (e.g. the 1:1 exchange of HCO_3^- with Cl^-) that is central to blood CO_2 transport and pH equilibration across the RBC membrane. The cytoplasmic domain is anchored to the cytoskeleton and plays a structural role by connecting the cytoskeleton to the membrane. In addition, the N-terminal fragment of the cytoplasmic domain contains binding sites for glycolytic enzymes and haemoglobin (for reviews, see Low, 1986; Salhany, 1990).

Deoxygenated human Hb (T structure Hb) binds the N-terminal cytoplasmic fragment of band 3 more tightly than does oxygenated (R structure) Hb (Walder *et al.* 1984; Chétrite and Cassoly, 1985; Tsuneshige *et al.* 1987). The binding site in the Hb is the central cavity between the two β -chains, i.e. the same site as that to which the organic phosphate 2,3-diphosphoglycerate (2,3-DPG) binds (Walder *et al.* 1984). The N-terminal part of the human cytoplasmic band 3 domain contains several negatively charged amino acid residues that

seem to correspond stereochemically to the positive charges of the organic phosphate binding site of the Hb.

The interaction between Hb and membrane proteins, and in particular with band 3, has not been investigated in lower vertebrates, but is of interest in evaluating the generality of the interaction and in shedding light on its possible functional roles. Reversible binding of glycolytic enzymes, and competition between Hb and glycolytic enzymes for common binding sites, may be involved in the regulation of glycolysis (Walder *et al.* 1984; Salhany, 1990; Low *et al.* 1993). Furthermore, in fish RBCs, various ion transport pathways are highly sensitive to the degree of oxygenation and to the conformation of the Hb (Motais *et al.* 1987; Jensen, 1990). It has been hypothesised that these effects could be mediated by an oxygenation-dependent interaction between Hb and band 3 (Motais *et al.* 1987), but direct evidence for such an interaction is not yet available for fish. The nucleated RBCs of teleost fish possess band 3, and the number of copies and the anion transport capacity of this protein are close to those found in anucleated human RBCs (Romano and Passow, 1984; Jensen and Brahm, 1995). The primary structure of rainbow trout band 3 has recently been determined from the complete cDNA sequence (Hübner *et al.* 1992). The amino acid sequence reveals a cluster of negative charges in the N-terminal part of the cytoplasmic domain (Hübner *et al.* 1992), suggesting that

an interaction between Hb and the acidic N terminus of the cytoplasmic fragment of band 3 may be present in fish RBCs, as it is in human RBCs.

A preferential binding of the N-terminal fragment of band 3 to deoxyhaemoglobin will be reflected in a lowering of the oxygen affinity of haemoglobin when the band 3 fragment is added to a Hb solution, as documented for human Hb (Walder *et al.* 1984). We have used this *modus operandi* to gain insight into the potential interaction between trout band 3 and Hb. Two peptides comprising (i) the first 10 and (ii) the first 20 amino acid residues of the N-terminal cytoplasmic fragment of trout band 3 were synthesised, and their influence on the oxygen equilibria of trout and human Hb was investigated.

Materials and methods

Peptide synthesis

The following peptides were synthesised, corresponding to the first 10 and 20 amino acid residues, respectively, of the N-terminal cytoplasmic fragment of trout band 3 (Hübner *et al.* 1992): 10-mer, H-Met-Glu-Asn-Asp-Leu-Ser-Phe-Gly-Glu-Asp-OH; 20-mer, H-Met-Glu-Asn-Asp-Leu-Ser-Phe-Gly-Glu-Asp-Val-Met-Ser-Tyr-Glu-Glu-Glu-Ser-Asp-Ser-OH.

Solid-phase peptide synthesis was carried out using standard continuous flow Fmoc-chemistry (Atherton and Sheppard, 1989) on a custom-made automatic synthesiser, using NovaSyn KA resins as the solid support and Fmoc-amino acid pentafluorophenyl esters or 3,4-dihydro-4-oxobenzotriazin-3-yl esters (Ser) in the coupling reactions. The Fmoc-amino acid NovaSyn KA resins and Fmoc-amino acid pentafluorophenyl and 3,4-dihydro-4-oxobenzotriazin-3-yl esters were purchased from Novabiochem (UK). The peptides were cleaved from the resins using tetrafluoroacetic acid:H₂O:thioanisole:phenol:ethane dithiol (82.5:5:5:5:2.5) and precipitated with ether. The crude peptides were dissolved in 1% ammonium acetate and isolated by freeze-drying. The peptides were further purified by size-exclusion chromatography (10-mer and 20-mer) followed by preparative high-performance liquid chromatography (HPLC) (10-mer). The peptides were analysed by reversed-phase HPLC and mass spectrometry (FAB⁺). The monoisotopic mass of the 10-mer was found to be 1156.5 Da (MH⁺), and the calculated value was 1156.4 Da; the mass of the 20-mer was found to be 2313.6 Da, and the calculated value was 2312.8 Da.

Preparation of haemoglobin and oxygen equilibrium measurements

Rainbow trout haemoglobin

Blood was sampled by cardiac puncture of three specimens of rainbow trout (*Oncorhynchus mykiss*; mass 200–390 g). The red blood cells from the combined samples were washed three times in 0.9% NaCl and lysed by adding 2 parts of distilled water and 0.2 parts of 1.0 mol l⁻¹ Tris buffer, pH 7.6. After rapid mixing, the lysate was kept on ice for 10 min and then centrifuged for 10 min at 14 000 revs min⁻¹. The supernatant

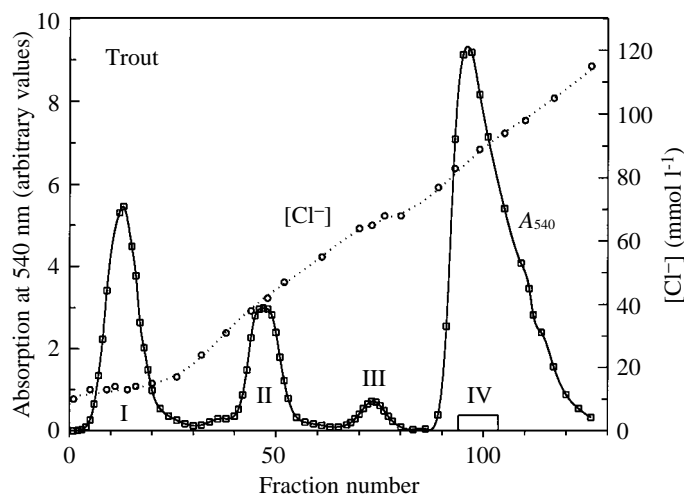


Fig. 1. Preparative separation of rainbow trout haemoglobin (Hb) components on a Sephacel DEAE anion-exchange column. Squares, absorption at 540 nm (A_{540}); circles, Cl^- concentration; fraction size 1.1 ml. The major cathodic and anodic components are Hb I and Hb IV, respectively. The horizontal bar marks the fractions containing Hb IV that were pooled for dialysis and O_2 equilibrium measurements.

Hb was dialysed overnight against 500 ml of 20 mmol l⁻¹ Tris buffer (pH 8.49 at 5 °C). The main anodic Hb component (Hb IV) was isolated by anion-exchange (DEAE) chromatography (see Pelster and Weber, 1990) on a (27.6 cm × 2 cm) column, applying a 0 mol l⁻¹ to 0.3 mol l⁻¹ NaCl gradient in the 20 mmol l⁻¹ Tris buffer (Fig. 1).

Human haemoglobin

Human blood was taken by venopuncture from a healthy, non-smoking adult. The haemolysate was prepared and stripped of salts and organic phosphates as described previously (Weber, 1992).

The rainbow trout and human Hb samples were dialysed against three changes (500 ml) of 0.01 mol l⁻¹ Hepes buffer (pH 7.6) containing 0.5 mmol l⁻¹ EDTA and frozen at -80 °C in 150 µl samples that were thawed individually immediately before O_2 equilibrium curve determinations.

Oxygen equilibria were measured using a modified diffusion chamber (see Weber, 1981, 1992) and Wösthoff (Bochum, Germany) gas-mixing pumps to mix pure nitrogen (>99.998%) with oxygen or air. Chloride was administered as KCl and assayed using a Radiometer (Copenhagen, Denmark) CMT 10 coulometric titrator. ATP was used as its disodium salt, and its concentration was assayed using a Sigma (St Louis, MO, USA) test kit.

Hill plots of the individual O_2 equilibrium curves (O_2 saturations between 15 and 85%) were used to evaluate the oxygen affinity as P_{50} (the O_2 tension at half saturation) and the cooperativity of O_2 binding as n_{50} (the slope of the plot). The O_2 saturation achieved with 1 atm (101.3 kPa) pure O_2 was taken as 100% Hb O_2 -saturation. In fish Hbs demonstrating the Root effect, full saturation may not be achieved with 1 atm

O₂ at low pH values. This is, however, of no practical importance for the present investigation, since the main purpose was to detect differences in oxygen affinity in the absence and presence of band 3 peptides.

Results

The major trout Hb component, Hb IV, was isolated by anion-exchange (DEAE) chromatography (Fig. 1). In contrast with the cathodic component Hb I, which demonstrates a slight inverse Bohr effect and shows no interaction with organic phosphates, the anodic component Hb IV demonstrates a large Bohr effect and is greatly affected by organic phosphates (Weber *et al.* 1976). The large effect of phosphate on Hb IV implies that the phosphate binding site, and thus also the putative binding site for band 3, is present in this Hb.

The oxygen affinity of stripped trout Hb IV decreased strongly (P_{50} increased) with lowered pH, and cooperativity (n_{50}) decreased when pH fell below pH 6.8 (Fig. 2). These results confirm the large Bohr effect and the presence of a Root effect in trout Hb IV.

The two synthetic peptides correspond to the first 10 and 20 amino acid residues of the N-terminal cytoplasmic fragment of trout band 3. The peptides are very acidic. The 10-mer contains four negatively charged Glu or Asp residues and the 20-mer contains eight such residues. Addition of the 10-mer peptide to a concentration 16 times higher than the tetrameric Hb concentration ($[10\text{-mer}]/[\text{Hb}_4]=16$) resulted in a relationship between oxygen affinity and pH that could not be distinguished from that of stripped Hb (Fig. 2). The 10-mer had no influence on the oxygen affinity of trout Hb IV either at high ionic strength (in the presence of 0.1 mol l^{-1} KCl) or at low ionic

strength. Cooperativity was similarly not influenced by addition of the peptide (Fig. 2). The 20-mer contains more negative charges than the 10-mer and could be thought to be a more potent effector of oxygen affinity. However, even at a $[20\text{-mer}]/[\text{Hb}_4]$ ratio of 16, the peptide exerted no significant effect on the oxygen affinity of trout Hb IV (Fig. 2). The addition of ATP (at $[\text{ATP}]/[\text{Hb}_4]=16$), however, resulted in a large decrease in oxygen affinity (Fig. 2), illustrating the preferential binding of ATP to the low-affinity T structure of the Hb.

In contrast with the lack of an effect on trout Hb IV, the 20-mer had a large effect on the oxygen affinity in human Hb. At a $[\text{peptide}]/[\text{Hb}_4]$ ratio of 15.8, it caused a large increase in P_{50} at both 15°C (the temperature used with trout Hb) and 37°C (Fig. 3). The effect was present throughout the pH range 6.7–7.4 (Fig. 3). The reduction in oxygen affinity was greater at low pH than at high pH, and the peptide therefore increased the numerical value of the Bohr factor ($\Delta\log P_{50}/\Delta\text{pH}$). Thus, binding of the peptide resulted in a decrease in oxygen affinity and in an increase in the Bohr effect. The influence of the 20-mer on $\Delta\log P_{50}$ at constant pH was larger at 15°C than at 37°C (Fig. 3). Cooperativity was not significantly affected by the peptide, but n_{50} tended to be higher at 37°C than at 15°C (Fig. 3).

Discussion

The two peptides corresponding to the first 10 and 20 amino acid residues of the N-terminal fragment of trout band 3 had no influence on the oxygen affinity of trout Hb IV (Fig. 2), but when the trout 20-mer peptide was added to human Hb, it produced a large decrease in oxygen affinity (Fig. 3). This

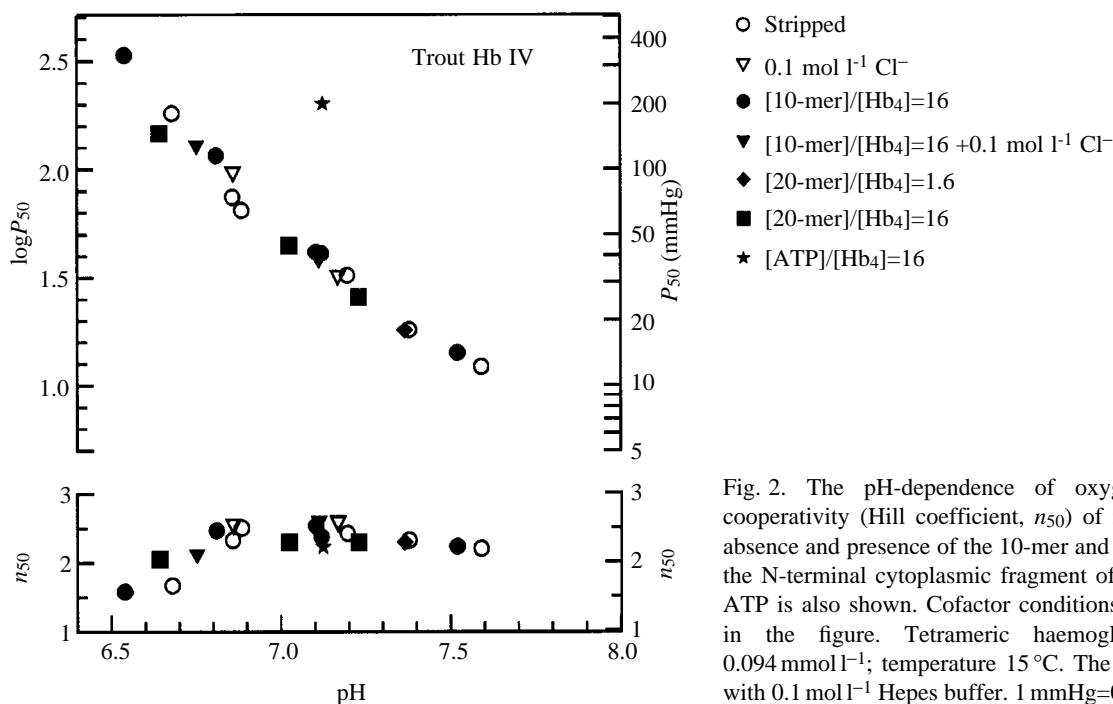


Fig. 2. The pH-dependence of oxygen affinity ($\log P_{50}$) and cooperativity (Hill coefficient, n_{50}) of rainbow trout Hb IV in the absence and presence of the 10-mer and 20-mer synthetic peptides of the N-terminal cytoplasmic fragment of trout band 3. The effect of ATP is also shown. Cofactor conditions and symbols are explained in the figure. Tetrameric haemoglobin (Hb_4) concentration 0.094 mmol l^{-1} ; temperature 15°C . The Hb solutions were buffered with 0.1 mol l^{-1} Hepes buffer. $1\text{ mmHg}=0.1333\text{ kPa}$.

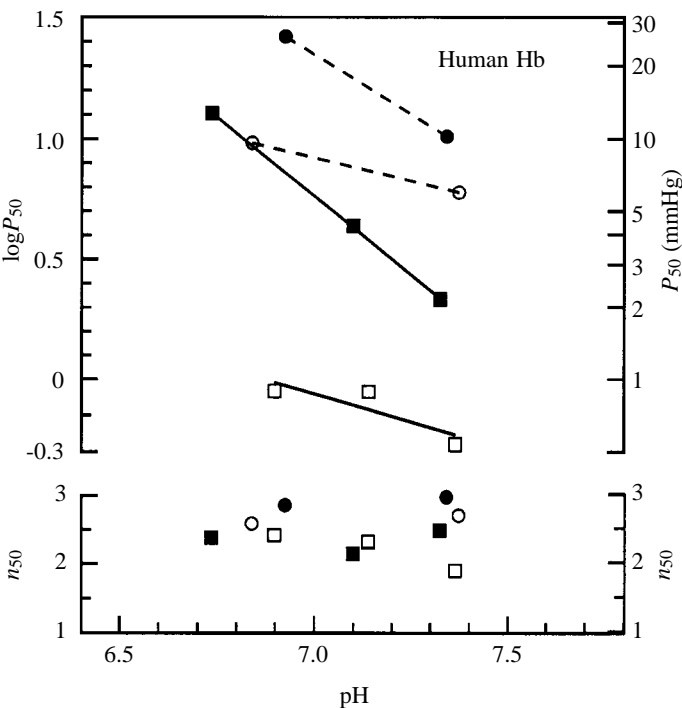


Fig. 3. The pH-dependence of oxygen affinity ($\log P_{50}$) and cooperativity (Hill coefficient, n_{50}) of human haemoglobin (Hb) in the absence (open symbols) and presence (filled symbols) of the 20-mer synthetic trout band 3 peptide at 15 °C (squares, solid lines) and 37 °C (circles, dashed lines). $[20\text{-mer}]/[\text{Hb}_4]=15.8$. Tetrameric Hb concentration 0.157 mmol l^{-1} . Hepes buffer, 0.1 mol l^{-1} . $1\text{ mmHg}=0.1333\text{ kPa}$.

indicates that the trout 20-mer peptide binds preferentially to the deoxy (T) conformation of human Hb, whereas this is not the case with trout Hb IV.

Interaction between peptide and human Hb

The magnitude of the effect of the trout 20-mer peptide on P_{50} of human Hb is similar to that of the human 11-mer peptide tested by Walder *et al.* (1984) at pH 7.2. This 11-mer human band 3 peptide contained six negatively charged Asp or Glu

residues, and X-ray crystallographic studies showed that the binding site for the peptide extended deep into the central cavity between the β -chains of the Hb (Walder *et al.* 1984). The conformation of the peptide appeared to be extended, with only 5–7 residues in direct contact with the Hb (Walder *et al.* 1984). It is likely that parts of the 20-mer trout peptide (which contains eight negatively charged Asp or Glu residues) interact with human Hb in a manner very similar to the human peptide.

The human 11-mer used by Walder *et al.* (1984) is compared with the two trout peptides in Fig. 4. Direct comparison reveals that negative charges are shared in three positions (i.e. positions 2, 9 and 10; see Fig. 4). The tyrosine in position 8 of human band 3 is important for its function, and it may be positioned close to residues involved in binding. Phosphorylation of the Tyr residue inhibits both the binding of Hb and the binding of glycolytic enzymes (Low *et al.* 1987). If the human peptide is aligned to the trout 20-mer according to the position of Tyr, negative charges are found to be shared in four positions (Fig. 4), which is the the best charge alignment obtainable between the two peptides. A similar charge agreement is obtained if the human peptide is displaced farther to the right by one and two positions. However, assuming a central position for the Tyr residue, then residues 9, 15, 16 and 17 in the trout 20-mer and residues 3, 9, 10 and 11 in the human peptide could be the important residues in the binding to Hb. In each peptide, the four residues will come to lie close to each other if the peptide assumes an α -helix. An involvement of these residues would explain the similarity in the effects of the trout 20-mer and the human peptide on the O_2 affinity of human Hb. A firm conclusion regarding the amino acid residues involved in the interaction with Hb must, however, await further study.

In addition to decreasing O_2 affinity, the trout 20-mer increased the Bohr effect of human Hb (Fig. 3). A similar augmentation of the Bohr effect is observed for 2,3-DPG binding to the organic phosphate binding site of the Hb. The effect of the peptide on $\Delta\log P_{50}$ was larger at 15 °C than at 37 °C (Fig. 3). This temperature-dependency also corresponds to that observed with organic phosphates (Jensen and Weber, 1987).

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|--------|-----|-----|-----|-----|-----|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--------------|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | |
| Ac-Met | Glu | Glu | Leu | Gln | Asp | Asp | Tyr | Glu | Asp | Glu | | | | | | | | | | Human 11-mer |
| Met | Glu | Asn | Asp | Leu | Ser | Phe | Gly | Glu | Asp | | | | | | | | | | | Trout 10-mer |
| Met | Glu | Asn | Asp | Leu | Ser | Phe | Gly | Glu | Asp | Val | Met | Ser | Tyr | Glu | Glu | Glu | Ser | Asp | Ser | Trout 20-mer |
| | | | | | | Ac-Met | Glu | Glu | Leu | Gln | Asp | Asp | Tyr | Glu | Asp | Glu | | | | Human 11-mer |

Fig. 4. A comparison between the human 11-mer N-terminal band 3 peptide and the trout 10-mer and 20-mer N-terminal band 3 peptides. Negatively charged residues are highlighted. The bottom row aligns the human peptide with the trout 20-mer on the basis of the position of the tyrosine residue. See text for further explanation.

Absence of a peptide effect in trout Hb IV

On the basis of the large effect of the trout 20-mer peptide on P_{50} in human Hb, it is surprising that the trout band 3 peptides had no effect on the oxygen affinity of trout Hb IV. The experimental conditions were chosen to maximise an effect, if one were present. Oxygen equilibria were studied mainly at low ionic strength, where the effect of the peptides on P_{50} should be greater than at high ionic strength (Walder *et al.* 1984). The peptide-Hb interaction was studied at [peptide]/[Hb₄] ratios which, by analogy with the human case, can be expected to be close to saturation binding of the peptide and compatible with a maximal change in O₂ affinity (Walder *et al.* 1984; Tsuneshige *et al.* 1987). In addition, different pH values were tested, including low pH values, where the Hb-peptide binding constant should be larger than at high pH. The possibility that the absence of a peptide effect in trout Hb IV was caused by either the Hb or the peptides being non-functional can be ruled out. The presence of a large effect of ATP on O₂ affinity (Fig. 2) confirms that the Hb was intact and functional with respect to binding of ligand at the central cavity between the two β -chains, and the functionality of the peptide was verified by its large effect on human Hb (Fig. 3). The lack of an effect of the peptides on oxygen affinity in trout Hb IV suggests that an oxygenation-linked binding of band 3 peptides to the Hb does not occur and a molecular explanation must be sought for this.

The binding site for organic phosphates in fish Hb is the same as in mammalian Hb, but a few amino acid substitutions have changed the site from one that is stereochemically complementary to 2,3-DPG (mammalian Hb) to one that fits ATP and GTP, the organic phosphates found in the red blood cells of fish (Perutz and Brunori, 1982). Mammalian Hbs have His, Gln or Asn in position NA2 of the two β -chains, whereas teleost fish Hbs commonly have either Glu or Asp (the latter applying to trout IV); in position H21 of the β -chains, mammalian Hbs have His, whereas teleost Hbs have Arg. In fish Hbs, the substitution to Glu or Asp at NA2 β allows a hydrogen bond to be made with the adenine group of ATP (Perutz and Brunori, 1982). The negative charges on the phosphates in ATP are neutralised by positive charges on Val NA1 β , Arg H21 β , Lys EF β 1 and Lys EF β 2 (Perutz and Brunori, 1982). It is possible that the presence of negatively charged Asp at NA2 β in trout Hb IV (in contrast to positive or neutral residues in mammalian Hbs) introduces electronic repulsion to the binding of the highly negatively charged peptides. This hypothesis would explain the weaker (or perhaps absence of) peptide binding to trout Hb IV and the stronger binding to human Hb.

Although an interaction between band 3 peptides and trout Hb could not be established *in vitro*, this does not exclude an interaction between Hb and band 3 in intact fish red blood cells. The cellular scenery is more complex than the system analysed here, and the structure of the peptides in solution may deviate from their structure when attached to the remaining portion of band 3. An alternative conformation of the N-terminal fragment of band 3 in intact erythrocytes may be compatible with an interaction with Hb. Thus, further studies are needed

at the cellular level to describe fully the interaction between Hb and membrane proteins in fish red blood cells.

We thank Anny Bang for skilled technical assistance. The work was supported by the Danish Natural Science Research Council (Centre for Respiratory Adaptation).

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