Structural–functional characterization of the cathodic haemoglobin of the conger eel *Conger conger*: molecular modelling study of an additional phosphate-binding site

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The *Conger conger* (conger eel) haemoglobin (Hb) system is made of three components, one of which, the so-called cathodic Hb, representing approx. 20 % of the total pigment, has been purified and characterized from both a structural and functional point of view. Stripped Hb showed a reverse Bohr effect, high oxygen affinity and slightly low cooperativity in the absence of any effector. Addition of saturating GTP strongly influences the pH dependence of the oxygen affinity, since the reverse Bohr effect, observed under stripped conditions, is converted into a small normal Bohr effect. A further investigation of the GTP effect on oxygen affinity, carried out by fitting its titration curve, demonstrated the presence of two independent binding sites. Therefore, on the basis of the amino acid sequence of the α - and β -chains, which have been determined, a computer modelling study

has been performed. The data suggest that *C. conger* cathodic Hb may bind organic phosphates at two distinct binding sites located along the central cavity of the tetramer by hydrogen bonds and/or electrostatic interactions with amino acid residues of both chains, which have been identified. Among these residues, the two Lys- α (G6) (where the letter refers to the haemoglobin helix and the number to the amino acid position in the helix) appear to have a key role in the GTP movement from the external binding region to the internal central cavity of the tetrameric molecule.

Key words: Bohr effect, enthalpy, fish, haemoglobin, molecular modelling, phosphate binding.

INTRODUCTION

Among vertebrates, fish display the most extensive presence of multiple haemoglobin (Hb) components, which show considerable differences in amino acid sequence and functional properties [1–3]. Thus, in addition to Hbs that migrate anodically (the so-called anodic components) under normal electrophoretic conditions (pH $\approx$ 8.6) and are characterized by relatively low oxygen affinities, marked Bohr effect or Root effect (a dramatic reduction in oxygen-binding capacity upon acidification that induces oxygen unloading in the swim bladder and choroid rete and loss of subunit cooperativity at low pH), many fish species express cathodic Hbs (Hb C) that have high isoelectric points and lack significant pH effects. Although no unifying theory has been proposed for the biological significance of this multiplicity, cathodic Hbs have been suggested to safeguard oxygen transport to tissues under hypoxic and acidotic conditions [4,5] and also to play an important role during exercise stress, when the pH at the gills drops to values too low for efficient oxygen uptake by Root-effect Hbs [6].

Previous studies on Hb C from Anguilliformes have often shown the reverse Bohr effect, characterized by an increase in the oxygen affinity as the pH is brought towards acidic values. This peculiar pH dependence has been found in cathodic Hb

from the American and European eels *Anguilla rostrata* [7] and *Anguilla anguilla* [8] respectively, and the brown moray *Gymnothorax unicolor* [9]; in contrast, cathodic Hb (Hb I) from the Mediterranean moray *Muraena helena* has been shown to be fully insensitive to pH changes [10]. In a few other cases of air-breathing fish (the Amazonian catfish *Pterygoplichthys pardalis* and *Hoplosternum littorale*) and amphibians which still possess gills (the tadpole of the bullfrog *Rana catesbeiana* and the newt *Triturus cristatus carnifex*), the occurrence of reverse Bohr-effect Hbs has been related to the transition from water to air breathing, given that air breathing in fish is induced by water hypoxia and may occur in the case of internal acidosis [8].

In the present study, we report the functional characterization of Hb C from the conger eel *Conger conger* (order Anguilliformes) and the complete amino acid sequence of the α - and β -chains. A few years ago, a genetic study [11] suggested that there was a closer phylogenetic relationship of Anguillidae (*A. anguilla*) with Congridae (*C. conger*) rather than with Muraenidae (*G. unicolor* and *M. helena*) and, therefore, a comparative study of Hbs from these species could be useful to test this hypothesis.

The oxygen-binding features suggest that Hb C from *C. conger* contains an additional phosphate-binding site. The structural characterization of this site becomes very important in view of previous studies on other Hbs, e.g. human Hb A [12], dromedary

Abbreviations used: AUT-PAGE, PAGE in the presence of 5 % (v/v) acetic acid, 8 M urea and 2 % (v/v) Triton X-100; Hb, haemoglobin; IEF, isoelectric focusing; IHP, inositol hexakisphosphate; OPA, o-phthalaldehyde; P_{50} , partial pressure of oxygen required to saturate 50 % of the haems; ΔH , overall heat of oxygenation.

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The protein sequence data for the α - and β -chains have been deposited in the SWISS-PROT and TrEMBL protein knowledgebase under the accession numbers P83479 and P83478 respectively.

Hb [13] and Hbs from the Antarctic sea bird *Catharacta maccormicki* [14]; such studies have also indicated an additional site, whose molecular structure has been described. Among fish, the Hb models of the Arctic teleost *Anarhichas minor* revealed an unusual structure of the phosphate-binding region of Hb 3 [15]. The physiological ligand binds at a single site, and non-physiological inositol hexakisphosphate (IHP) migrates to a more internal partially overlapping region of the same domain [15]. Thus *C. conger* Hb C is the first example of a fish Hb having two binding sites for the physiological ligand, located in separate regions of the molecule. Molecular models of the additional site are reported in the present study. The possible physiological role of this site is also discussed.

EXPERIMENTAL

Materials

DEAE-cellulose (DE 52) was from Whatman, Sephadex G-25 was from Pharmacia, trypsin (E.C. 3.4.21.4), treated with L-1-tosylamido-2-phenylethylchloromethylketone, was from Worthington Biomedical, Tris, Mes, Hepes and Taps were from Sigma, and dithiothreitol was from Fluka. All sequanal-grade reagents were from Applied Biosystems, and HPLC-grade acetonitrile was from Lab-Scan Analytical. All other reagents were of the highest purity commercially available.

Hb purification

Adult *C. conger* ($n = 30$; 60–85 cm in length) were caught off the coast of Sardinia. Fish were kept in aquaria supplied with running sea water at a temperature of 10–13 °C. The specimens were anaesthetized by tricaine methane sulphonate and blood samples were drawn from the caudal vein by heparinized syringes. Immediately after sampling, erythrocytes were centrifuged at 1000 *g* for 5 min at 4 °C and washed twice through cycles of resuspension and centrifugation at 1000 *g* using an ice-cold isotonic NaCl solution. Lysis was achieved by adding 1 vol. of ice-cold hypotonic buffer [1.0 mM Tris/HCl (pH 8.0)]. Stroma were removed by centrifugation at 12000 *g* for 30 min at 4 °C. Isoelectric focusing (IEF) on thin-layer 5% (w/v) polyacrylamide slab gels (pH 3.5–10.0) was performed as described by Manca et al. [16] and Masala and Manca [17]. Proteins were stained with 0.1% (w/v) Bromophenol Blue.

The haemolysate was stripped by running through a Sephadex G-25 column (25 cm × 2.5 cm) equilibrated with 10 mM Tris/HCl (pH 8.0) containing 0.1 M NaCl. Hb C was isolated by ion-exchange chromatography on a DEAE-cellulose column (2.5 cm × 20 cm) in 10 mM Tris/HCl (pH 7.7).

Globin chains were analysed by PAGE in the presence of 5% (v/v) acetic acid, 8 M urea and 2% (v/v) Triton X-100 (AUT-PAGE). Gels were stained with 0.5% (w/v) Coomassie Brilliant Blue [18–20].

Globin purification

Globins were precipitated with 10 vol. of acetone containing 5 mM HCl at –20 °C. The α - and β -chains were separated by reverse-phase HPLC on a μ Bondapak C₁₈ column (0.39 cm × 30 cm; Waters). The eluents were 45% (v/v) acetonitrile containing 0.3% (v/v) trifluoroacetic acid (A) and 90% (v/v) acetonitrile (B) with a gradient of (B) in (A) at a flow rate of 1 ml/min [21].

Amino acid sequencing

Alkylation of the sulphydryl groups with 4-vinylpyridine, deacetylation of the α -chain N-terminus and tryptic digestions were carried out as described previously [21,22]. Tryptic peptides were purified by reverse-phase HPLC on a μ Bondapak-C₁₈ column (0.39 cm × 30 cm; Waters) as described previously [23]. Cleavage of Asp–Pro bonds was performed on polybrene-coated glass-fibre filters in 70% (v/v) formic acid for 24 h at 42 °C [24]. Asp–Pro-cleaved globins were treated with *o*-phthalaldehyde (OPA) before sequencing [25] in order to block the non-proline N-terminus and decrease the background. Sequencing was performed with an Applied Biosystems Procise 492 automatic sequencer equipped with on-line detection of phenylthiohydantoin amino acids.

MS

The molecular mass of the *S*-pyridylethylated α - and β -chains and of peptides (less than 10 kDa) was measured by matrix-assisted laser-desorption ionization–time-of-flight ('MALDI–TOF') MS on a PerSeptive Biosystems Voyager-DE Biospectrometry Workstation. Analyses were performed on pre-mixed solutions prepared by diluting samples (final concentration, 5 pmol/ μ l) in 4 vol. of matrix [10 mg/ml sinapinic acid in 30% (v/v) acetonitrile containing 0.3% (v/v) trifluoroacetic acid (for globins), and 10 mg/ml α -cyano-4-hydroxycinnamic acid in 60% (v/v) acetonitrile containing 0.3% (v/v) trifluoroacetic acid (for peptides)].

Oxygen binding

Oxygen-dissociation curves were obtained spectrophotometrically [26] using a Varian spectrophotometer Cary 50. Experiments were performed in 0.1 M Mes/Hepes/Taps buffer (where Mes was used in the 6.7–6.9 pH range, Hepes for the pH 7.0–7.8 range and Taps for the pH 7.9–8.5 range) in the absence and presence of 0.1 M NaCl and/or 3 mM GTP or ATP at a protein concentration of 3–5 mg/ml. Curve-fitting as a function of effector concentration was carried out by using the least-squares fitting procedure according to a classical equation for two-site binding:

$$Y = (K_1 \cdot [\text{GTP}] + 2 \cdot K_1 \cdot K_2 \cdot [\text{GTP}]^2) / (2 \cdot (1 + K_1 \cdot [\text{GTP}] + K_1 \cdot K_2 \cdot [\text{GTP}]^2)) \quad (1)$$

where K_1 and K_2 are the overall affinity constants for the two GTP sites, and $Y = (P_{\text{obs}} - P_0) / (P_{\infty} - P_0)$, where P_0 is the partial pressure of oxygen required to saturate 50% of the haem molecules (P_{50}) in the absence of the effector and P_{obs} is the P_{50} measured at different GTP concentrations. The heat of oxygenation (ΔH ; J · mol^{–1}) was calculated from the integrated van't Hoff equation:

$$\Delta H = -2.303 R \Delta \log P_{50} [(T_1 \cdot T_2) / (T_1 - T_2)] \quad (2)$$

where T is the absolute temperature and $R = 8.31 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$.

Molecular modelling

Computer graphics, structural manipulations and energy-minimization calculations were carried out on a Silicon Graphics R12000 workstation using the Insight II software package (Biosym/MSI). The Discover-3 package (Biosym/MSI) was used for all energy-refinement procedures. Since the T-state structure of Hb has high affinity for phosphate ions, the X-ray structure of

Figure 1 IEF of the haemolysate and purified Hb C

IEF on thin-layer 5 % (w/v) polyacrylamide slab gels (pH 3.5–10.0) of the *C. conger* haemolysate (lane 1) and Hb C (lane 2), purified by DEAE-cellulose chromatography, was performed as described by Manca et al. [16] and Masala and Manca [17]. Proteins were stained using 0.1 % (w/v) Bromophenol Blue. The anode was at the bottom and migration was toward the anode.

deoxy Hb A [27] (Brookhaven Protein Data Bank accession number 1G9V) complexed with two effector molecules was used as template to build the molecular model of *C. conger* Hb C. Alignment of the amino acid sequences of the α - and β -chains of Hb C with Hb A showed 51 % and 52 % identity respectively (see Table 1).

The residues of Hb C identical with those of the template in the same sequence positions were assumed to maintain the same conformations, whereas replacements were manually modelled using the low-energy rotamer conformations [28]. Hydrogen atoms were added to the model at pH 7.0, partial charges were assigned to all atoms and refinement was performed by commonly used methods [29]. Minimization was carried out to eliminate bad contacts present in the X-ray structure of the template Hb. In order to ensure the stability of the model, a 5 Å (where 1 Å = 0.1 nm) water shell was added around *C. conger* Hb by using the Soak Assembly function of the Insight II package.

The molecular models of two GTP molecules were considered to each have a charge of -3.5 ; both molecules were added with the automated docking procedure. Automated docking was carried out with the Affinity package (Biosym/Molecular Simulation). This program finds the best binding structures of the ligand based on the energy of the ligand/receptor complex, with the ligand and the binding site of the receptor flexible during the search. The ligand/receptor system was partitioned into two subsets, i.e. bulk and movable atoms. Bulk atoms are defined as the atoms of the receptor that are not located in the binding site and were held rigid during the docking search. Movable atoms include those located in the binding region and can thus move freely, except for atoms of the binding site close to restrained bulk atoms.

The docking procedure was carried out starting from two different positions of the GTP molecules: (i) central Hb cavity, as suggested from X-ray structure of the complex of Hb A with two effector molecules [27]; and (ii) main (composed by residues of β -chains) and additional (formed by residues of α -chains) phosphate-binding sites found in bird Hbs [14].

Consistent valence force-field parameters were used in all calculations, with a distance-dependent dielectric constant $\epsilon = 1 \times r$ (where r is the distance) and a 12 Å cut-off distance for the treatment of the electrostatic and non-bonded interactions.

The quality of the minimized models of the complex of *C. conger* Hb C-(GTP)₂ was assessed by the analysis of their

Figure 2 AUT-PAGE

AUT-PAGE of dissociated globins from *C. conger* haemolysate (lane 3) and its anodic (lane 1) and cathodic (lane 2) components after separation by DEAE-cellulose chromatography. The gel was stained with 0.5 % (w/v) Coomassie Brilliant Blue.

Figure 3 Reverse-phase HPLC of the globins of Hb C

Reverse-phase HPLC was carried out on a μ Bondapak C₁₈ column (0.39 cm $\times$ 30 cm). The eluents were (A) 45 % (v/v) acetonitrile containing 0.3 % (v/v) trifluoroacetic acid and (B) 90 % (v/v) acetonitrile with a gradient of (B) in (A) at a flow rate of 1 ml/min.

stereochemical properties and by the use of the inverse protein folding approach [30] using the Profile_3D program.

RESULTS

The haemolysates from 30 specimens of *C. conger* were analysed by IEF. Two types of Hb components were found, namely one Hb C with $pI \approx 9.4$, the most basic Hb ever detected in teleosts [31], and two major anodic Hbs, with lower isoelectric point. The separation of Hb C, which accounted for 20 % of the total, was achieved by DEAE-cellulose chromatography (results not shown) and ascertained by IEF (Figure 1) Hb C runs unretarded through the column; two anodic Hbs are eluted, but not separately, by decreasing the pH of the Tris/HCl buffer to 7.0 and increasing the concentration of the buffer to 100 mM.

AUT-PAGE under dissociating conditions of the haemolysate and the two fractions showed that the α - and β -chains of purified Hb C differ from those of the anodic Hb fraction (Figure 2).

Purification of globins and amino acid sequence

The α - and β -chains of Hb C were isolated by reverse-phase chromatography (Figure 3). The calculated molecular masses of the α - and β -chains were 15 300 and 16 020 Da respectively.

The N-terminus of the α -chain was not available for Edman degradation. Matrix-assisted laser-desorption ionization–time-of-flight–MS analysis of the N-terminal tryptic peptide revealed that the blocking group was an acetyl group, similar to all teleost Hbs sequenced to date. An internal region became accessible in the α -chain after cleavage of an Asp–Pro bond. After treatment with OPA, sequencing proceeded from Pro⁹⁶ to Lys¹²⁸. In the β -chain, direct sequencing from the N-terminus proceeded for 40 residues. An internal region was sequenced in the β -chain after cleavage of an Asp–Pro bond. After treatment with OPA, sequencing proceeded from Pro¹⁰⁰ to Lys¹¹⁷.

The alignment of the tryptic peptides, purified by reverse-phase HPLC (results not shown), was established by overlaps and by similarity to other known fish Hb sequences. The complete amino acid sequences are shown in Figure 4.

The degree of sequence identity between the α - and β -chains of *C. conger* Hb C and those of human Hb A and the other known sequences of Hbs from Anguilliformes are shown in Table 1. It should be noted that the highest percentage of identity is that between the α - and β -chains of *C. conger* Hb C and the corresponding chains of *A. anguilla*, *G. unicolor* and *M. helena* cathodic Hb.

Oxygen-binding properties

The oxygen-binding parameters of freshly prepared Hb C were measured at 20 °C in the absence and presence of NaCl and/or ATP or GTP, i.e. the physiological effectors of fish Hbs. In the absence of chloride, Hb C showed a reverse Bohr effect throughout the pH range 6.5–8.0 ($\Delta\log P_{50}/\Delta\text{pH} = +0.28$), high oxygen affinity ($\log P_{50} = -0.12$ and 0.30 at pH 6.5 and 8.5 respectively) and low cooperativity ($h_{50} \approx 1.6$; where h_{50} is the Hill coefficient at 50 % saturation) (Figure 5). Addition of chloride ions only caused a small decrease in oxygen affinity ($\Delta\log P_{50} = 0.15 \pm 0.05$ in the pH range 6.5–8.0). Addition of saturating amounts of organic

Figure 4 Amino acid sequence of the α - (a) and β - (b) chains

The regions (marked by letters above the sequence) refer to the haemoglobin helices.

phosphate significantly increased cooperativity ($h_{50} = 2.1 \pm 0.2$) and strongly decreased oxygen affinity, with GTP exhibiting a larger effect with respect to ATP ($\log P_{50} = 1.27$ compared with 1.09 at pH 7.0). The larger effect of GTP is in agreement with the intracellular concentration of this ligand in the erythrocytes of *C. conger* and *A. anguilla*, which is 2–3 fold higher than that of ATP [32,33]. The large effect of GTP on the oxygen affinity displayed by *C. conger* Hb C ($\Delta\log P_{50}$ in the presence and absence of GTP at pH 7.0 is equal to 1.25) has the same extent as that reported for

Table 1 Sequence identity in α - (a) and β - (b) chains of human Hb A and Hbs from Anguilliformes

Sequences were obtained from the SWISS-PROT protein knowledgebase under the following accession numbers: *A. anguilla* cathodic Hb, P80727 and P80726; and *A. anguilla* anodic Hb, P80946 and P80945. *G. unicolor* sequences were from [9], and *M. helena* sequences were from [10].

(a) α -chain							
		Identity (%)					
		<i>C. conger</i>		<i>G. unicolor</i>		<i>A. anguilla</i>	
Hb	Hb C	anodic Hb	cathodic Hb	anodic Hb	cathodic Hb		
Human Hb A	51	46	52	43	48		
<i>A. anguilla</i> cathodic Hb	68	52	69	54			
<i>A. anguilla</i> anodic Hb	53	69	52				
<i>G. unicolor</i> cathodic Hb	65	51					
<i>G. unicolor</i> anodic Hb	58						
(b) β -chain							
		Identity (%)					
		<i>C. conger</i>		<i>M. helena</i>		<i>G. unicolor</i>	
Hb	Hb C	Hb III	Hb I	anodic Hb	cathodic Hb	anodic Hb	cathodic Hb
Human Hb A	52	45	52	47	51	45	52
<i>A. anguilla</i> cathodic Hb	81	54	76	55	76	56	
<i>A. anguilla</i> anodic Hb	56	78	54	82	56		
<i>G. unicolor</i> cathodic Hb	73	54	83	54			
<i>G. unicolor</i> anodic Hb	56	80	54				
<i>M. helena</i> Hb I (cathodic)	73	54					
<i>M. helena</i> Hb III (anodic)	57

Figure 5 Bohr effect and Hill coefficients

Effect of pH on the oxygen affinity (in terms of $\log P_{50}$) (b) and cooperativity (in terms of h_{50}) (a) of Hb C. Conditions, 0.1 M Mes/Hepes/Taps buffer at 20 °C and a protein concentration of 3–5 mg/ml; no effector (circles), plus 3 mM GTP (squares), plus 3 mM ATP (triangles) in the absence (open symbols) and presence (filled symbols) of 0.1 M NaCl. Oxygen pressure is expressed in torr units (1 torr = 0.133 kPa). Experiments were performed in triplicate.

Table 2 Oxygen-binding parameters of cathodic Hbs from four species of Anguilliformes

Values have been calculated from the equilibrium experiments in Figure 5. Values for *A. anguilla*, *G. unicolor* and *M. helena* have been taken from [8–10]. An average S.D. of $\pm 3\%$ for values of P_{50} [expressed in torr units (1 torr = 0.133 kPa)] was calculated.

Parameters	Species			
	<i>M. helena</i>	<i>G. unicolor</i>	<i>A. anguilla</i>	<i>C. conger</i>
Stripped Hb				
$\Delta \log P_{50} / \Delta \text{pH}$	0	+0.11	+0.28	+0.28
$\log P_{50}$ at pH 7.0	0.33	0.19	−0.08	0.02
h_{50}	1.1–1.2	1.1–1.2	1.4–1.9	1.5–1.7
Hb plus 3 mM GTP				
$\Delta \log P_{50} / \Delta \text{pH}$	−0.07	0	−0.1	−0.11
$\log P_{50}$ at pH 7.0	1.08	0.83	1.17	1.27
h_{50}	1.9–2.3	1.5–1.9	2.5–2.9	1.9–2.35
$\Delta \log P_{50}$ at pH 7.0	0.75	0.64	1.25	1.25

A. anguilla Hb C [8], and 30% stronger than that displayed by 2,3-diphosphoglycerate on human HbA. Table 2 summarizes the oxygen-binding feature of Anguilliformes.

To gain further insight into the effect of GTP and chloride ions on affinity, experiments were performed by adding increasing concentrations of organic phosphate (Figure 6). The curves in the absence and presence of a saturating concentration of chloride (0.5 M NaCl) can be fitted by using eqn. (1), assuming the presence of two independent binding sites. In the absence of chloride ions, the calculated overall affinity constants of these two sites differ by almost one order of magnitude (2.72×10^4 compared with $2.9 \times 10^3 \text{ M}^{-1}$). Saturating chloride significantly decreased the overall affinity constants of the two sites, but to a different extent. The overall affinity constant of the first and second site is indeed decreased by approx. 10 and 20 times respectively. In other words, in the presence of chloride the

Figure 6 Effect of GTP concentration on the oxygen affinity

Experiments were carried out in 0.1 M Hepes buffer (pH 7.0) at 20 °C in the absence (○) and presence (●) of saturating (0.5 M) NaCl. Oxygen pressure is expressed in torr units (1 torr = 0.133 kPa).

Figure 7 Overall oxygenation enthalpy as a function of pH

ΔH values (kJ $\cdot \text{mol}^{-1}$ oxygen) were calculated from oxygen-equilibrium experiments carried out at 20 °C (see the legend for Figure 5), and at 12 and 25 °C (results not shown), in Mes/Hepes/Taps buffer in the presence of 0.1 M NaCl alone (○) or of both 0.1 M NaCl and 3 mM GTP (●).

functional heterogeneity of the sites is increased. Moreover, in the presence of a saturating concentration of phosphate (3 mM), increasing concentrations of chloride caused no appreciable effect on oxygen affinity. Taken together, these results indicate that one of the two phosphate-binding sites is more competitive with respect to chloride than the other.

Thermodynamics of oxygen binding

The effect of temperature (within the range 12–25 °C) on the oxygen affinity was determined at various pH values in the presence of chloride alone and both chloride and GTP (Figure 7). In the absence of GTP, an increase in enthalpy of approx. 10 kJ $\cdot \text{mol}^{-1}$ was observed as pH decreased from 8.5 to 7.0; this behaviour reflects proton association upon oxygen binding, in accordance with the reverse Bohr effect displayed under these experimental conditions. The opposite pH-dependence of the overall heat of oxygen binding, observed in

Figure 8 Stereoview of the central cavity of *C. conger* Hb C along a direction parallel to the dyad axis of Hb

GTP molecules are in blue, Hb is in black, and only the side chains of residues, in yellow, in contact with GTP are drawn. The hydrogen bonds are in red.

the presence of GTP, is in agreement with the conversion of the reverse into a normal Bohr effect; the lower ΔH values reflect the endothermic contribution of phosphate binding.

Molecular modelling

The stereochemical parameters of the *C. conger* Hb C-(GTP)₂ complex suggest the quality of the minimized model. Similar to the X-ray structure of the complex of human Hb A with two molecules of effector [27] in the template structure, in the *C. conger* Hb C-(GTP)₂ complex the GTP molecules were located along the central cavity of Hb, interacting with residues of the α - and β -chains (Figure 8). As shown in Figure 8, the interactions between GTP and Hb C were symmetric with respect to the dyad axis of pseudosymmetry. The GTP molecules at the end of the docking procedure showed interactions with Hb very similar to those found in the X-ray structure of Hb A-(RSR-13)₂ complex {where RSR-13 is 2-[4-[(3,5-dimethylanilino)carbonyl]methyl]phenoxy]-2-methyl propionic acid} [27]. This result was independent of the starting GTP positions in the docking procedure (see the Experimental section). In fact, each GTP molecule displayed hydrogen bonds and/or salt-bridges with Tyr- α (C1), Lys- α (G6), Asp- α (H9), Arg- α (HC3) and Asp- β (G10) (where the letters refer to the haemoglobin helices and the numbers to the amino acid position in the helix). Interestingly, the two Lys- α (G6), unlike other residues engaged in the interactions with organic phosphate, undergo extensive side-chain movement during binding and, as suggested for avian Hbs [34], have a fundamental role in binding GTP molecules and in the ligand movement from the external binding region to the internal central cavity of Hb.

DISCUSSION

The functional properties of *C. conger* Hb C match those of *A. anguilla* Hb C at a higher extent than those of the other Anguilliforme species characterized previously [8–10,35]. This finding, together with the high sequence identity between its α - and β -chains and the corresponding chains of *A. anguilla* cathodic Hb, is in agreement with the high identity found between Anguillidae (*A. anguilla*) and Congridae (*C. conger*) in a genetic

Table 3 Sequence alignment for selected residues in Hbs from human Hb A and some species of Anguilliformes

Sequences of *A. anguilla* cathodic Hb were obtained from the SWISS-PROT protein knowledgebase under the accession numbers P80727 and P80726; *M. helena* Hb I (cathodic) sequence were from [10], and *G. unicolor* cathodic Hb sequences were obtained from [9]. In the α - and β -chains, the letters refer to the haemoglobin helices and the numbers to the amino acid position in the helix. Ac, acetyl; nd, not determined.

Position	Species				
	Human Hb A	<i>C. conger</i> Hb C	<i>A. anguilla</i> cathodic C	<i>M. helena</i> Hb I (cathodic)	<i>G. unicolor</i> cathodic C
β-chain					
NA1	Val	Val	Val	Val	Val
NA2	His	Gln	Glu	Glu	Glu
D6	Met	Val	Gln	Ser	Ser
CD3	Ser	Lys	Lys	Gln	Asn
EF6	Lys	Lys	Lys	Lys	Lys
FG4	His	Asn	Asn	Asn	Asn
H21	His	Arg	Lys	Arg	Arg
HC3	His	Phe	Phe	Phe	Phe
G10	Asn	Asp	Asp	Asp	Asp
α-chain					
NA1	Val	Ac-Ser	Ac-Ser	Ac-Ser	Ac-Ser
C1	Phe	Tyr	Phe	nd	Tyr
C3	Thr	Gln	Ala	nd	Pro
C6	Thr	Thr	Val	nd	Ile
CD2	Pro	Ala	Ser	nd	Ser
G6	Lys	Lys	Lys	nd	Lys
H9	Asp	Asp	Asp	nd	Asp
HC3	Arg	Arg	Arg	nd	Arg

study [11] based on measurements of genome sizes and AT content in Anguilliformes.

The functional behaviour of *C. conger* Hb C may be discussed on the basis of the amino acid residues known to be critical for the allosteric properties of human Hb A, as well as of Hb C from other Anguilliformes (Table 3).

As far as pH-dependence of the oxygen affinity is concerned, the Bohr effect of human Hb A has mainly been ascribed to His¹⁴⁶ β (HC3), which forms a salt-bridge with Asp⁹⁴ β (FG1) in

the T-state. This bond is broken by the shift to the R-state, lowering the pK_a of the imidazole group and releasing protons under physiological conditions. No such interactions can take place in *C. conger* Hb C, since His¹⁴⁶ is replaced by phenylalanine. Hence this substitution may be considered largely responsible for the almost complete lack of the alkaline Bohr effect.

In Anguilliformes, cathodic Hb may be characterized either by almost complete insensitivity to pH changes, as in the Mediterranean moray *M. helenae* [10], or by a reverse Bohr effect, as in the American and European eels *A. rostrata* and *A. anguilla* [7,8] respectively, and in *G. unicolor* [9]. When present, the reverse Bohr effect indicates proton binding upon oxygenation, due to increase of the pK_a value of some still unidentified residues as a consequence of the conformational transition from the T- to the R-state of the molecule. However, several hypotheses have been suggested. For instance, in human Hb A, His¹⁴³β(H21) has been considered largely responsible for the reverse Bohr effect [36], because the adjacent positive charges of Lys⁸²β(EF6) and Lys¹⁴⁴β(HC1) could lower the pK_a value of deoxy-Hb His¹⁴³ with respect to that of oxy-Hb, giving rise to uptake of protons upon oxygenation. The reverse Bohr effect occurring in certain fish Hbs at higher pH values with respect to that of human Hb A might be due, at least in part, to an uncommonly high pK_a of His¹⁴³β, which is responsible for half of the acid Bohr effect in human Hb A [37]. Although the replacement His→Lys in eel and His→Arg in *G. unicolor* and *C. conger* Hb C at this position may be considered in keeping with this hypothesis, we cannot disregard the case of *M. helenae* cathodic Hb (Hb I), which has arginine in position β(H21) and does not display the reverse Bohr effect.

The cooperativity of oxygen binding displayed by cathodic Hbs of Anguilliformes (lower than that of human Hb A) is decreased to different extents in the four species examined, and it is also differently enhanced by organic phosphates. This finding could be related to the amino acid substitutions at the α₁β₂ 'dovetailed' switch region [i.e. Thr³⁸α(C3), Thr⁴¹α(C6), Pro⁴⁴α(CD2), His⁹⁷β(FG4) in human Hb A], which has a primary role in the cooperative, quaternary T→R transition of most vertebrate Hbs (see Table 1 in [8]). If the residues present in α(CD2) and β(FG4) are considered, no relationship with cooperativity can be found; moreover, although threonine is still present in α(C6) in *C. conger*, it is replaced by valine and isoleucine in *A. anguilla* and *G. unicolor* respectively, despite their very different cooperativity. Therefore we suggest that the α(C3) residue replaced by alanine and glutamine in *A. anguilla* and *C. conger* respectively, and by proline in *G. unicolor*, is likely to have a crucial role in modifying the ability of the tetramer to switch from T to R, since the peptide link involving both alanine and glutamine is more flexible than that involving proline.

The high oxygen affinity of *C. conger* Hb C may be explained, at least in part, on the basis of an amino acid substitution in the β-subunits. Hence, as in *A. anguilla*, Ser(CD3), close to distal His(E7) in human Hb A, is replaced by lysine in *C. conger* Hb C. This residue may allow the formation of a hydrogen bond or a salt-bridge with one of the propionic groups that may alter the orientation of the haem plane and the β-subunit affinity, whereas in human Hb A the side chain of Ser(CD3) is too short to allow such a bond [8,38]. Other potentially important replacements with respect to human Hb A are displayed by *C. conger* Hb C; for instance, in β-chains, Lys(E3) and Lys(E10) are replaced by asparagine and threonine respectively, producing large electrostatic variations in the distal portion of the haem pocket.

As shown by the oxygen-binding findings in the absence of organic phosphates, *C. conger* Hb C displays a reverse Bohr effect, whereby oxygenation is associated with uptake of protons.

However, the negative Bohr effect observed under stripped conditions is reversed to a small positive Bohr effect in the presence of saturating organic phosphates. The ability to convert the reverse Bohr effect may be physiologically very important considering that organic phosphates cause not only a drop in the oxygen affinity, but also a large change in the Bohr effect, whose amplitude and sign is under their control.

The finding that GTP is the most abundant phosphate in *C. conger* erythrocytes [32], similar to *A. anguilla* [39] and *A. rostrata* [40], and that its concentration decreases more significantly than that of ATP during hypoxia [35], indicates this triphosphate as a key factor in the adaptation of conger eel blood to oxygen levels in the environment. In the presence of low GTP levels, as in hypoxia, the functional heterogeneity between *C. conger* Hb components (anodic Hbs have low oxygen affinity) is thus increased, enabling the whole blood to meet its physiological role under a wider range of oxygen pressure.

Molecular modelling confirmed the existence of two GTP-binding sites centred along the central cavity of Hb C, as suggested by the oxygen equilibria. *C. conger* Hb C is the first example of a fish Hb with a second phosphate-binding site. So far, a double site has been found in mammalian Hbs [12,13,27] and in the two Hbs of the Antarctic bird *Ca. maccormicki* [14,34]; however, in such cases, the molecular structures are totally different from those of *C. conger*.

The GTP molecules bind to Hb, establishing interactions with residues belonging to α- and β-chains. This finding is in agreement with the X-ray structure of Hb A complexed with two effector molecules [27], namely our starting molecular model.

A double site for GTP should stabilize the low-affinity T structure; this finding is in keeping with the strong decrease in affinity of Hb C in the presence of GTP. In particular, an internal site might be more efficient in stabilizing the T structure than a site localized at the surface of the molecule. A similar hypothesis has been suggested for the Arctic fish *An. minor*, whose three Hbs show differential modulation of oxygen affinity in the presence of IHP [15]. In *An. minor* Hb 3, the oxygen affinity was decreased much more strongly by IHP than in the other two Hbs. In fact, in Hb 3, a docking procedure indicated a more internal IHP site than that of the other two Hbs, where IHP was bound at the surface. The GTP-binding region in *C. conger* Hb C was also different from the additional IHP sites of the two Hbs of the Antarctic bird *Ca. maccormicki* and of Hb A. This result may be ascribed to the different molecular structure of GTP and IHP. IHP has an essentially spherical shape, whereas GTP has a linear structure; thus, two IHP molecules would be unable to bind at the same time along the Hb central cavity, because there would not be space.

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