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Engineering *Ascaris* hemoglobin oxygen affinity in sperm whale myoglobin: role of tyrosine B10

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Abstract The contribution to oxygen stabilization of a tyrosine residue in topological position (B10) has been studied in sperm whale myoglobin by simultaneous replacement of residues at positions (B10), (E7) and (E10) as suggested by analysis of the sequence of high oxygen affinity hemoglobins, such as that of the nematode *Ascaris suum*. Kinetic and equilibrium experiments with the gaseous ligands oxygen and carbon monoxide show that indeed the introduction of tyrosine (B10), together with replacement of the distal histidine (E7) with glutamine, is associated with a large decrease in the oxygen dissociation rate constant. Our results are consistent with the possible formation in the distal pocket of two hydrogen bonds with the iron-bound oxygen.

Key words: Myoglobin: Protein engineering; Oxygen affinity; Hydrogen bond

1. Introduction

Control of ligand binding in myoglobin (Mb) and hemoglobin (Hb) has been extensively investigated in the last few years by protein engineering. In order to assess the individual contributions of distal heme pocket residues to the thermodynamics and kinetics of ligand binding, several site-directed mutants of mammalian myoglobins have been expressed in *E. coli*. Successfull studies [1–5] have focused on the role of topological positions (E7) and (E11), clearly indicating that hydrogen bonding capability, polarity and steric hyndrance of the residues in these positions are of key importance in the stabilization and discrimination of gaseous ligands of the heme iron, such as CO and O_2. In most cases, these (highly conserved) distal residues have been replaced with others displaying alternative physico-chemical properties, leading to identification of a specific role mainly by its loss; introduction of a novel ligand stabilization mechanism prouved to be a much more difficult task [6–8].

A general guidance to implant some function specifically expressed in one Mb/Hb into another globin framework has been found [9] in the analysis of sequence alignments of globins from different species, along with structural information when available. Using this approach other distal residues over and above (E7) and (E11) may control ligand binding in Mb; among others, a residue which seems to play a crucial role in oxygen stabilization is that at position (B10), which is most frequently occupied by Leu [10], but in several globins displaying high oxygen affinity is either Phe or Tyr [11–14]. A striking example in this respect is the octameric hemoglobin from *Ascaris suum*, which has a very high oxygen affinity (P_{50} = 0.001–0.004 mmHg), due to an extremely slow oxygen dissociation rate constant (k = 0.004 s^{-1}) [15]; replacement of Tyr(B10) with either Leu or Phe in the cloned *Ascaris* Hb domain I was shown to lower the oxygen affinity by about 100-fold [16]. Protein engineering to mimic this extremely high oxygen affinity by substituting Tyr for Leu in the corresponding topological position of s.w. Mb failed, given that the single Mb mutant **YHT** (Leu(B10) → Tyr) displays a lowered oxygen affinity and a dissociation rate constant ($k \geq 500$ s^{-1}) much higher than the wild type s.w. Mb (k = 14 s^{-1}) [17]. On the contrary, it has been observed that replacement of Leu(B10) with Phe in s.w. Mb raises the oxygen affinity 10-fold [8] and, in the X-ray structure, the Phe benzene ring accomodates in the distal pocket and stabilizes the bound oxygen through a favourable contact.

We decided to further study a possible contribution of Tyr(B10) to oxygen stabilization in the s.w. Mb framework by simultaneously replacing residues at positions (B10), (E7) and (E10) in the distal pocket with those typically found in the very high oxygen affinity Hb's. Therefore we have produced and characterized two s.w. Mb mutants: a double mutant, His(E7) → Gln/Thr(E10) → Arg (**LQR**), and a triple mutant, Leu(B10) → Tyr/His(E7) → Gln/Thr(E10) → Arg (**YQR**). The results reported below show that substitution of Leu by Tyr starting from the double mutant **LQR** is associated to a large decrease of the oxygen dissociation rate constant (k = 1 s^{-1}) when compared to the values (k = 90–130 s^{-1}) observed for the single and double mutants having Gln at position (E7). In addition we observed that the presence of Tyr(B10) lowers the oxygen and carbon monoxide combination rate constants by approx. 10-fold compared to wild type. These results are not inconsistent with preliminary modeling of the distal site of the oxygenated triple mutant **YQR**, based on the coordinates of the oxygenated double mutant **LQR** obtained by X-ray crystallography [18].

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Abbreviations: s.w., sperm whale; Mb, myoglobin; Hb, hemoglobin. Each protein is identified by the one-letter code for the residues in positions B10, E7 and E10, respectively; these are capital letters for wild type residues (e.g. wild type Mb = Leu(B10)/His(E7)/Thr(E10) = LHT) and capital bold for the mutated residues (e.g. Leu(B10)/His(E7) → Gln/Thr(E10) = **LQT**).

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2. Materials and methods

Mutagenesis of the synthetic s.w. Mb gene [19] was performed using cassette mutagenesis in order to obtain the double mutant **LQR**; a set of oligonucleotides where the normal codon for His (CAT) and Thr (ACC) have been substituted with Gln (CAG) and Arg (AGG). The triple mutant **YQR** was then obtained on the double mutant template by oligonucleotide-directed mutagenesis, following the procedure of Kunkel et al. [20]. All DNA manipulations where as described in Sambrook et al. [21]; nucleotide sequences were determined with Sequenase Version 2.0 Reagents (United States Biochemicals, Cleveland, OH). Protein expression and purification was performed as described in Cutruzzolà et al. [6]. The double mutant **LQR** was easily purified in high yield in the oxygenated form; the triple mutant **YQR**, although obtained in low yield, was also purified as the oxygenated derivative.

Crystals of the **LQR** mutant Mb have been obtained following the procedure of Phillips et al. [22] and the 3D structure solved by X-ray crystallography [18]. Oxygen and carbon monoxide kinetics were measured at 20°C in 0.1 M Na-phosphate buffer pH 7.0 by means of an Applied Photophysics (Leatherhead, UK) DX.17MV stopped-flow apparatus. Oxygen affinities were determined under the same experimental conditions by tonometric measurements [23]. Autoxidation rate constants were assessed at 37°C in air in 0.1 M Na-phosphate pH 7.0, 1 mM EDTA, as described by Brantley et al. [24].

Molecular modeling has been performed using software programs from Biosym Technologies (San Diego, USA), running on an Indigo2 Silicon Graphics Workstation.

3. Results and discussion

The results of the equilibrium and kinetic experiments carried out on the two mutant Mb's **LQR** and **YQR** are compared in Table 1 with those of wild type Mb and other relevant mutants. Fig. 1 depicts the time course of oxygen dissociation as obtained by stopped-flow with four key proteins: LHT, **YHT**, **LQR** and **YQR**.

The double mutant **LQR** has an oxygen affinity ($K = 0.26 \times 10^6$ M^{-1}) which is similar to that of the single mutant His(E7) → Gln (LQT) (Table 1); both display a significant decrease in affinity if compared to the wild type s.w. Mb (LHT) mainly caused by a 10-fold increase in the oxygen dissociation rate constant. The time course for **LQR** (Fig. 1) was found to be biphasic: the slower phase accounts for about 80% of the overall reaction, with a dissociation rate constant of about 90 s^{-1}. This is only slightly different from that obtained for the single mutant **LQT** (Table 1 and [25]), which shows that in the double mutant **LQR** direct interaction of Arg at position (E10) with bound O_2 is either absent or ineffective. Indeed, preliminary inspection of the 3D structure of oxy**LQR** [18] shows that Gln(E7) is correctly located to form a H-bond with bound O_2, while Arg(E10) does not interact with the ligand but forms a H-bond with the carbonyl of Gln(E7).

The functional results obtained with the triple mutant **YQR** are somewhat unique among the Mb mutants. The presence of Tyr at position (B10) in **YQR** is associated to a large decrease in the oxygen dissociation rate constant (Table 1 and Fig. 1), which is 100-fold slower than that of the double mutant **LQR**, and more than 500-fold slower than that of the single mutant **YHT** [17]. In the latter case, unfavourable steric hindrance between the Tyr hydroxyl group and bound oxygen has been proposed [16] to account for the marked increase in oxygen dissociation rate constant, which was contrary to expectation; this proposal seem to be confirmed by our modeling analysis. Interestingly, kinetic analysis (J.S. Olson, personal communication) of oxygen binding by the double s.w. mutant **FQT** (with Phe(B10) and Gln(E7)) clearly shows that the presence of the Phe ring per se is insufficient to lower the oxygen dissociation rate constant anywhere near that of **YQR**(Table 1).

Our results also show that the oxygen combination rate constant for **YQR** ($k' = 1\text{–}2 \times 10^6$ $M^{-1} \cdot s^{-1}$) is 10-fold smaller than that typical of other Mb's, with the exception of (i) the slower process observed for **YHT**, and (ii) *Ascaris* Hb ($k' = 1.2 \times 10^6$ $M^{-1} \cdot s^{-1}$) which is also slow and very similar to **YQR**. We have also determined for the triple mutant **YQR** the combination rate constant for CO ($k' = 4.7 \times 10^4$ $M^{-1} \cdot s^{-1}$), which is also 10-fold slower than that typically observed for other Mb's, including wild type and **LQR** ($k' = 9.8 \times 10^5$ $M^{-1} \cdot s^{-1}$). This considerable decrease of the bimolecular rate constant for the binding of both gaseous ligands to **YQR** would be difficult to explain on the basis of the increased steric hindrance of Tyr at position (B10) as compared to Leu, because **FHT** and **FQT** (both with the bulky Phe in B10) display only a slight decrease in the CO combination rate constant (Table 1). More likely (but yet speculative) is the possibility that the

Table 1
Oxygen and carbon monoxide binding parameters of wild type and mutant sperm whale Mb at pH 7.0, 20°C

Positions B10–E7–E10	k_{O_2} (s^{-1})	k'_{O_2} ($M^{-1} \cdot s^{-1}$)	K_{O_2} (M^{-1})	k_{CO} (s^{-1})	k'_{CO} ($M^{-1} \cdot s^{-1}$)	$k_{autox.}$ (h^{-1})
L H T[a] wild type	14	1.5×10^7	0.9×10^6	0.019	5.5×10^5	0.05[#]
Y H T[a]	500	3×10^6	–	0.004	5×10^3	–
	800	2.6×10^7		0.14	3.5×10^5	
F H T[b]	1.4	2.1×10^7	1.5×10^7	0.006	2.2×10^5	0.005
L **Q T**[c]	130	2.4×10^7	0.18×10^6	0.012	9.8×10^5	0.21[#]
F Q T[d]	54	3.0×10^7	5.5×10^7	0.006	6.0×10^5	0.072
L Q R	90	2.3×10^7*	0.26×10^6	0.014	9.8×10^5	0.26
	400					1.7
Y Q R	1	1.9×10^6+	1.2×10^6	0.014	4.7×10^4	0.027
		1.2×10^6*				

[a] From Gibson et al. [17].
[b] From Carver et al. [8].
[c] From Rohlfs et al. [25].
[d] From J.S. Olson, personal communication.
[#] From Brantley et al. [24].
*Calculated from equilibrium constant and dissociation rate constant.
+ By stopped flow.

polarity of Tyr(B10) may stabilize a water molecule in the active site, possibly leading to an increased kinetic barrier in the binding of O_2 and CO [26].

To further characterize the oxygen reactivity of our mutants, we have measured their autoxidation rate constant. The results (Table 1) indicate that in the case of **YQR** autoxidation proceeds with a much slower rate compared to that of LQT and LQR; this evidence further supports the involvement of Tyr(B10) in the stabilization of the oxygenated complex and hence in the decrease of rate of metMb formation. Indeed Brantley et al. [24] have recently proposed that the O_2 affinity is inversely related to the autoxidation rate in mammalian Mb's.

The *Ascaris* site-directed mutagenesis study of De Baere et al. [16] shows that the single Tyr(B10) → Leu mutant in domain I of this protein displays a 100-fold reduction of the oxygen affinity and an increase in the dissociation rate constant with respect to the wild-type. On this basis they have proposed that, in addition to that formed with the distal Gln(E7), a second H-bond between bound oxygen and the hydroxyl group of Tyr(B10) is present in *Ascaris* Hb.

Our results with the triple mutant **YQR** are, to a first approximation, consistent with the proposal of De Baere et al. [16]. We have carried out some modeling of the structure of **YQR**, starting from the coordinates of oxyLQR as determined in [18]. A picture of the active site obtained after energy minimization (illustrated in Fig. 2) indicates that Tyr(B10) may fit in the distal pocket, and is located at a distance consistent with formation of a second H-bond with bound O_2. Modeling of **YQR** suggests that replacement of His(E7) with Gln is crucial to allow a favourable stereochemistry of Tyr(B10), and is consistent with the very large decrease in the oxygen dissociation rate constant with respect to YHT, where the bulkier His at position (E7) appears to clash with Tyr(B10). The failure of **YQR** to display the extremely low oxygen dissociation rates characteristic of *Ascaris* Hb ($k = 0.004$ s^{-1}), in spite of the presence of two putative H-bonds (Fig. 2) may well be due to differences in helices topologies in the two different tertiary frameworks. As pointed out by De Baere et al. [16], the relative position of the helices in the distal pocket is a crucial factor to achieve optimal contact distance between the ligand and the side-chains involved in its stabilization. Naturally, further evidence to substantiate the H-bonding network leading to oxygen overstabilization will come from determination of the 3D structure of both *Ascaris* Hb and **YQR** s.w. Mb mutant by crystallography.

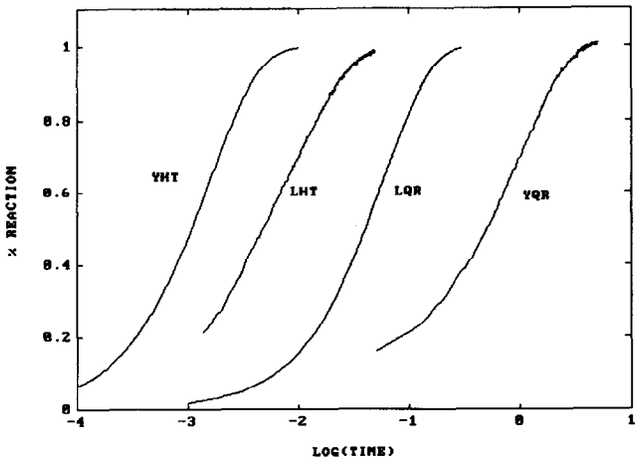

Fig. 1. Time courses of oxygen dissociation for the wild-type Mb (LHT) and 3 mutant proteins (YHT, LQR, YQR) as measured either by stopped flow using the dithionite method [23]. Experimental conditions were 0.1 M Na-phosphate buffer pH 7.0; 20°C; 2–4 μM protein concentration. Time in seconds.

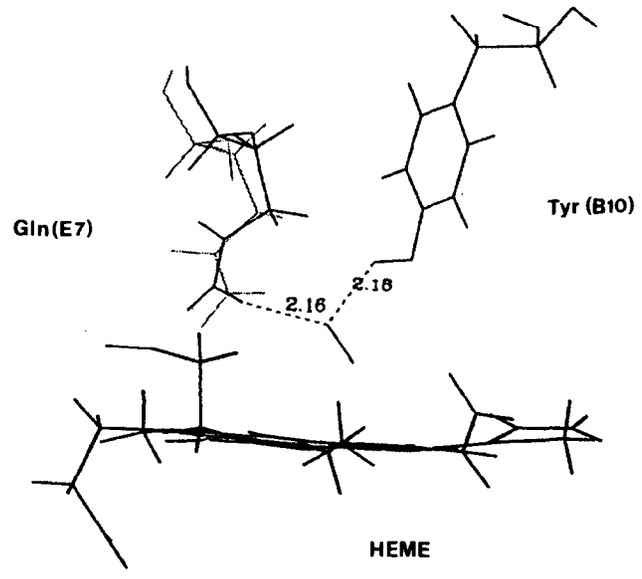

Fig. 2. Stereochemistry of the active site of **YQR** mutant Mb in the oxygenated state. Thin line indicates Gln(E7) before minimization; numbers indicate distances in Å to the distal atom of iron-bound dioxygen. The starting coordinates used for the minimization were those of oxyLQR Mb; the dioxygen molecule orientation is that obtained from refinement of this mutant [18].

Characterization of other s.w. Mb mutants carrying additional substitutions could be also important in order to achieve the high oxygen affinity observed in the nematode Hb. First of all, it would be interesting to study the effect of Tyr(B10) in the absence of a H-bond donor side-chain at position (E7). Second, comparison of aligned sequences of s.w. Mb and *Lucina pectinata* Hb II, as well as molecular modeling [14], suggest that Tyr(B10) is more easily accomodated in the distal pocket of *Lucina* Hb II because at topological position (CD4) Leu substitutes for the conserved bulky Phe; interestingly *Ascaris* and *Pseudoterranova* Hb's both lack Phe(CD4), having an Arg at this position.

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