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Liganded and unliganded forms of Antarctic fish haemoglobins in polyethylene glycol: crystallization of an R-state haemichrome intermediate

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Liganded and unliganded forms of two Antarctic fish haemoglobins, from *Trematomus newnesi* and *T. bernacchii*, have been crystallized in low-salt media using polyethylene glycol as precipitant. In particular, crystals of air-exposed *T. newnesi* carbomonoxy haemoglobin were found to be isomorphous to the crystals grown in high-salt media. Preliminary X-ray analysis of the diffraction data revealed that the β-haem iron of this haemoglobin is in the haemichrome state, with both the proximal and distal histidyl residues linked to the iron. This is the first crystallization of a haemichrome intermediate of a vertebrate haemoglobin.

1. Introduction

Vertebrate haemoglobins (Hbs) have been thoroughly studied both functionally and structurally, since they are the first and most important molecular model for studying protein cooperativity (Perutz *et al.*, 1998). Over the years, many Hb structures from several vertebrate species have been reported with a variety of ligands bound to the iron of the haem. These studies have largely confirmed the general features of the liganded (R) and unliganded (T) states of Hbs (Perutz *et al.*, 1998). Nevertheless, some fundamental questions remain unanswered. In particular, two different quaternary structures of liganded Hbs (labelled as R and R2) have been identified and their role in solution is currently under debate (Mueser *et al.*, 2000; Tame, 1999). The preferential occurrence of R and R2 quaternary states in crystals grown in high- and low-salt media, respectively, has also been considered an indication that crystal packing could produce non-negligible effects on Hb structure (Tame, 1999).

While preserving a common general mechanism of action, Hbs extracted from different organisms have acquired specific functional properties in response to major evolutionary pressures. In Antarctic fishes, the evolutionary process of cold adaptation has produced unique haematological characteristics, which have been investigated in depth in the last two decades (di Prisco, 1998). Although many of these Hbs display the Root effect (di Prisco *et al.*, 1991), namely low oxygen affinity with loss of cooperativity at low physiological pH, the major Hb of *T. newnesi* (HbTn; D'Avino *et al.*, 1994) does not show this effect. Interestingly, this Hb exhibits very high sequence identity (95%) with Hb 1 of *T. bernacchii* (HbTb) which, conversely, is endowed with a strong Root effect. So far, the crystal structure of HbTb has been determined in both R (Camardella *et al.*, 1992) and T states (Ito *et al.*, 1995), whereas only the R-state structure of HbTn has been reported (Mazzarella *et al.*, 1999). It should be mentioned that the T-state structure of HbTb has been obtained from twinned crystals (Ito *et al.*, 1995). The analysis of these structures has not provided a conclusive answer to the long-standing problem of the molecular basis of the Root effect (Mazzarella *et al.*, 1999).

In this paper, we report the results of several crystallization experiments carried out on both HbTn and HbTb using polyethylene glycol (PEG) as precipitant, aimed towards better characterizing the T and R states of the two Hbs in low-salt media. In particular, we have grown crystals of HbTn in the deoxy state and of two oxidized forms of HbTb and HbTn from air-exposed solutions. Preliminary X-ray analysis of the oxidized form of HbTn has shown that the β-chain has the haemichrome structure with the distal histidyl residue acting as the sixth ligand of haem iron.

2. Materials and methods

2.1. Haemoglobin from *T. newnesi*

2.1.1. Crystallization of HbTn in the deoxy state. Crystallization trials of deoxy HbTn were performed in a glove box filled with argon using oxy HbTn, which was deoxygenated *in situ*. Single crystals of deoxy HbTn suitable for X-ray diffraction were grown in eight to ten weeks using MPEG 5000 as precipitating agent (Table 1). These crystals diffracted to 2.2 Å resolution at the Elettra Synchrotron. X-ray diffraction data were collected from one crystal frozen at liquid-nitrogen temperature (100 K) using glycerol as cryoprotectant. The presence

Table 1
Crystallization conditions.

	Technique	Protein concentration (mg ml^{-1})	Buffer and final pH	Precipitant concentration
HbTn R state	Free interface diffusion†‡	10	100 m*M* sodium acetate pH 7.6	10%(*w*/*v*) MPEG 5000
HbTb T state	Vapour diffusion†‡	13.5	100 m*M* potassium phosphate, 20 m*M* IHP§ pH 6.2	10%(*w*/*v*) PEG 6000
HbTn T state	Free interface diffusion†¶	10	100 m*M* sodium acetate, 2 m*M* dithionite pH 6.0	10%(*w*/*v*) MPEG 5000

† At 293 K. ‡ In air. § Inositol hexaphosphate. ¶ In argon.

of extra spots in several images suggested that the crystals were affected by a non-merohedral twinning, the twins being related by a 180° rotation around the *a* axis. Results and statistics of data collection and processing, not taking the twinning into account, are given in Table 2. Attempts to detwin the data are in progress.

2.1.2. Crystallization and preliminary X-ray analysis of the air-exposed HbTn. Spectrophotometric analysis of HbTn in PEG at pH 7.6 indicated that this Hb undergoes auto-oxidation when exposed to air (Riccio *et al.*, unpublished results). Fig. 1 shows spectral evidence of the evolution of the air-exposed carbomonoxy form of HbTn (HbTnCO) in a solution containing monomethyl polyethylene glycol (MPEG) 5000 [10%(*w*/*v*)] in 60 m*M* Tris–HCl at pH 7.6. The absorption spectra were recorded using a Jasco 560 spectrophotometer. The HbTnCO peaks at 569 and 540 nm gradually disappeared. After a few days, the peak at 535 nm and the shoulder at 565 nm indicated that most of the protein was in the form of the haemichrome derivative (Rifkind *et al.*, 1994), an intermediate along the denaturation pathway of the protein. In order to investigate the structural features of this intermediate, we set up several crystallization trials of HbTnCO in air using MPEG 5000 as precipitant. The free interface diffusion technique was used by pouring HbTnCO in 60 m*M* Tris–HCl pH 7.6 onto a solution containing 10%(*w*/*v*) MPEG 5000 in a capillary. Single crystals suitable for X-ray analysis were obtained in 2–3 d (Table 1). From these crystals X-ray diffraction data were collected to 2.8 Å resolution with a Nonius DIP2030b imaging plate mounted on a Nonius FR591 rotating-anode generator. Results and statistics of data processing are reported in Table 2.

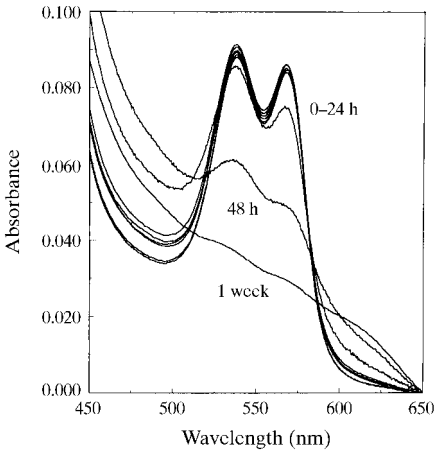

Figure 1
Spectral changes with time for the auto-oxidation reaction of HbTnCO in 60 m*M* Tris–HCl at pH 7.6. The evolution of the spectra indicates that a significant amount of HbTn is in the haemichrome state after 48 h.

The structure was solved using the carbomonoxy form of HbTn crystallized in ammonium sulfate pH 8.0 (PDB code 1t1n; Mazzarella *et al.*, 1999) as a starting model. The current model, refined using the program *X-PLOR* (Brünger, 1992), presents an *R* factor of 0.18 and an $R_{\rm free}$ of 0.23 using diffraction data extending to 2.8 Å resolution. The electron density in the haem regions shows that a CO molecule is bound to the haem iron in the α-chain, whereas the β-chain is in the haemichrome state with both the proximal and the distal histidyl residues directly bound to the iron (Fig. 2).

2.2. Haemoglobin from *T. bernacchii*

2.2.1. Crystallization of the oxidized HbTb in the T state. The presence of PEG has been reported to strongly favour the T state in Hbs (Liddington *et al.*, 1988). In HbTb the T state is additionally stabilized at low pH by the Root effect (Camardella *et al.*, 1992). Consequently, HbTb in PEG at pH 6.0 is forced into the quaternary T form even when the protein is exposed to air. The analysis of the HbTb visible spectra under these conditions reveals that HbTb also evolves toward the haemichrome state (data not shown). Crystals of air-exposed HbTb in the quaternary T state were obtained from a solution of HbTbCO, which was converted *in situ* to the T-deoxy state and then slowly shifted to the oxidized form. The precipitant and the buffer used in the crystallization trials (Table 1) were those reported by Ito *et al.* (1995). The crystals grew in 1 d and their dark brown colour was an indication that a significant amount of Hb was oxidized. Moreover, a change of colour from dark brown to purple was observed when sodium dithionite was added to the stabilizing solution containing the crystals. The colour change was fully reversible and no relevant crystal damage was observed.

Crystals of air-exposed HbTb diffract to 2.2 Å with a conventional X-ray radiation source and to 1.8 Å at the Elettra synchrotron. Synchrotron data were collected from one crystal frozen at 100 K using glycerol as cryoprotectant. Statistics of data processing are reported in Table 2. These crystals are strictly isomorphous to the deoxy HbTb crystals (Ito *et al.*, 1995) and display the same type of merohedral twinning. Refinement of the structure using the twinning handling implemented in the program *SHELXL* (Sheldrick & Schneider, 1997) is in progress.

3. Results and discussion

Recent literature data have suggested that the crystallization medium may have a significant effect on the structure of liganded Hbs (Mueser *et al.*, 2000; Tame, 1999). In particular, low-salt solutions (containing

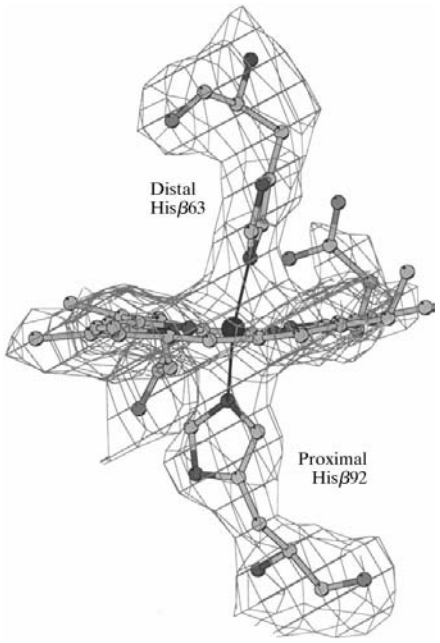

Figure 2
$F_o - F_c$ omit electron-density map (contoured at 2.5σ) of the β-haem region of HbTn.

Table 2
Crystal data and processing statistics.

	HbTn R state	HbTb T state	HbTn T state
Space group	*C*2	$P2_1$	*C*2
Unit-cell parameters (Å, °)	a = 89.8, b = 88.19, c = 56.2, β = 97.8	a = 62.6. b = 96.5, c = 62.6, β = 90.3	a = 90.0, b = 84.4, c = 89.4, β = 101.5
Resolution limits (Å)	12.0–2.8	15.0–1.8	15.0–2.2
Asymmetric unit	$\alpha\beta$ dimer	$\alpha_2\beta_2$ tetramer	$\alpha_2\beta_2$ tetramer
No. of reflections	25439	297663	116518
No. of unique reflections	9764	65547	32744
Completeness (%)	92.2	97.5	98.6
$R_{\rm merge}(I)$† (%)	6.0	3.3	4.8‡

† $R_{\rm merge}(I) = \sum_{hkl}\sum_i |I_i - \langle I\rangle|/I_i$. ‡ This value was calculated without data detwinning.

PEG) may favour the R2 state (Mueser *et al.*, 2000; Silva *et al.*, 1992), whereas high-salt media lead to the R state (Ladner *et al.*, 1977; Vásquez *et al.*, 1998). Conflicting data refer to the physiological role played by these two forms (Mueser *et al.*, 2000; Srinivasan & Rose, 1994; Tame, 1999). Here, we report preliminary results on the structure of HbTn from crystals grown in air using MPEG 5000 as precipitant. The quaternary structure of HbTn is very similar to that of the carbomonoxy form crystallized from ammonium sulfate (Mazzarella *et al.*, 1999), showing that the R state is stable under a wide range of conditions. However, despite the close similarity between the two crystal forms, the electron-density map corresponding to the crystals grown in MPEG (Fig. 2) reveals that the β iron is axially linked to both proximal and distal histidyl residues, whereas a CO molecule appears to be linked to the α iron. So far, the haemichrome state has only been reported in the monomeric Hb extracted from sea cucumber *Caudina arenicola* (Mitchell *et al.*, 1995) and in the dimeric non-symbiotic rice Hb (Hargrove *et al.*, 2000). Therefore, air-exposed HbTn is the first example of a vertebrate Hb crystallized in the haemichrome state. The refinement of this structure will provide new interesting insights into the molecular bases of the Hb-degradation pathway. This process is of great physiological importance because many abnormal Hbs are unstable and often form haemichromes (Rifkind *et al.*, 1994). Because of the unforeseen interest of this form, we are now trying to optimize its crystal quality, with the aim to obtain a more detailed structure.

In addition to the crystals of HbTn in the haemichrome state, we have also obtained crystals of deoxy HbTn and of air-exposed HbTb in the T state. The elucidation of these structures may provide clues to the structural bases of the Root effect in fish Hbs. The development of refinement procedures, taking twinning in both crystal forms into proper account, is now in progress.

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