

Comments on *Structural studies of haemoglobin from pisces species shortfin mako shark (Isurus oxyrinchus) at 1.9 Å resolution* by P. Ramesh *et al.* (2013). *J. Synchrotron Rad.* **20**, 843–847

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The recent paper by Ramesh *et al.* (2013) reports a crystallographic study on the tetrameric haemoglobin (Hb) isolated from the shortfin mako shark (*Isurus oxyrinchus*) (SMS). From the analysis of the overall and local features of the model the authors conclude that the structure corresponds to an unliganded deoxy T state conformation.

Among the several distinct forms that tetrameric Hbs may assume, the deoxy state is referred to Fe²⁺ not binding any exogenous ligand, usually in a penta-coordination state. In this context, the interpretation of the binding state of SMS Hb in the structure reported by Ramesh *et al.* is questionable. Indeed, the analysis of Fig. 4(b) of the paper, along with an independent inspection of the electron density that we calculated by retrieving the structure factors from the Protein Data Bank (code 3mkb), clearly indicate that the iron of the β -heme is exogenously hexa-coordinated. The authors reasonably modelled a water molecule at 2.20 Å from the iron. This hexa-coordination of the β -iron, with an exogenous ligand containing a single non-hydrogen atom, strongly resembles a met-haemoglobin state, in which the iron is oxidized to Fe³⁺ and the sixth coordination position is occupied by either a water molecule or a hydroxyl ion. This coordination exhibits an average Fe–O distance of 2.08 Å (Vergara *et al.*, 2009). The oxidation of the iron would not be a surprising event since, according to the protocols described in the experimental section, no particular care, such as the use of a reducing agent or of a controlled inert atmosphere, was employed to prevent oxidation.

The occurrence of SMS Hb oxidation is corroborated by the analysis of the α -heme region, which exhibits an atypical iron coordination. As shown by the electron density [also reported in Fig. 4(a) of the paper], the iron is likely coordinated by both the proximal (His87) and distal (His58) histidyl residues. Although the coordination of the iron is slightly distorted, it can be confidently assumed that the metal is endogenously hexa-coordinated with the two histidyl residues acting as axial ligands. The binding of the distal His to the iron induces a local compression of the EF corner of the protein, as observed in other tetrameric Hbs (Merlino *et al.*, 2011). Indeed the distances between the C α atoms of the proximal and distal histidyl residues, which are 14.0 and 14.2 Å in the deoxy (PDB code 1gev) and carbonmonoxy (1gcw) forms of the closely related *Mustelus griseus* shark Hb (Naoi *et al.*, 2001), decreases to 12.9 Å. Although bis-histidyl adducts may be formed by both Fe²⁺ and Fe³⁺ (hemochrome or hemichrome), in tetrameric Hbs this peculiar coordination has been reported, although very occasionally, only for oxidized proteins (Vergara *et al.*, 2008). On the basis of these considerations, we believe that the structure solved by Ramesh *et al.* likely corresponds to a ferric Hb of the type α (hemichrome) β (aquo/hydroxymet).

The finding that SMS Hb is able to form a hemichrome with a well defined three-dimensional structure holds interesting implications

that may be the subject of further investigations. It was initially believed that hemichrome formation, which requires the simultaneous binding of the iron by the proximal and distal His, was not compatible with a properly folded state of the complex architecture of tetrameric Hbs (Rifkind *et al.*, 1994). The first crystallographic characterization of tetrameric Hb in a hemichrome state was reported for the major Hb component of the Antarctic fish *Trematomus newnesi* (Ricchio *et al.*, 2002). In this structure hemichrome formation occurred at the β -iron. Subsequent studies have described hemichrome structures of horse haemoglobin and of the Hb isolated from another Antarctic fish *Trematomus bernacchii* (Vitagliano *et al.*, 2004). Interestingly, in horse haemoglobin hemichrome was detected at the α -iron (Robinson *et al.*, 2003). In all cases a single Hb chain underwent hemichrome formation. This trend is confirmed by the crystallographic data collected on SMS Hb. Altogether these results indicate that the formation of a complete hemichrome likely represents a too strong perturbation of the structures of tetrameric Hbs that prevent their crystallization. It is important to note that hemichrome formation has different effects on the overall structure of these Hbs. For Antarctic fish the mixed hemichrome state assumes a structure that falls in the R–T transition pathway (Vitagliano *et al.*, 2008). On the other hand, SMS Hb mixed state has a quaternary structure that is very close to the T state. Therefore, the propagation of the perturbation induced by the endogenous hexa-coordination depends on the chain involved and/or on the intrinsic plasticity of the specific Hbs (Balsamo *et al.*, 2012).

In conclusion, we believe that detailed structural data, such as those obtained by Ramesh *et al.*, are fundamental in order to reach a molecular-level understanding of the structure–function relationship of Hbs. As for Antarctic bony fish Hbs, the detection of a peculiar state along the oxidation process of the cartilaginous SMS Hbs represents a stimulating finding for a complete characterization of the process that may disclose other unexpected features. Moreover, the observation that Hbs isolated from both cartilaginous and bony fish also holds interesting implications (Komiya *et al.*, 1991) that certainly deserve future investigations.

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