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Research article

# Crystal structure of the ferric homotetrameric $\beta_4$ human hemoglobin

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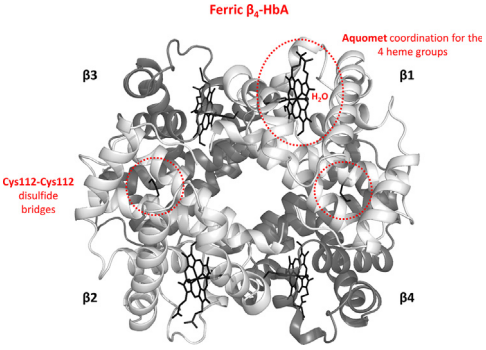
## HIGHLIGHTS

- Crystal structure of ferric $\beta_4$-HbA reveals no trace of endogenous bis-his coordination.
- Heme groups are in aquomet coordination in the crystal structure of ferric $\beta_4$-HbA.
- Ferric $\beta_4$-HbA presents a disulfide bridge between Cys112 of $\beta_1/\beta_4$ and $\beta_2/\beta_3$ chains.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Spectroscopic studies carried out in the early seventies have shown that the β-homotetramer of human hemoglobin ($\beta_4$-HbA) in the ferric state is a mixture of aquomet and bis-histidyl forms. Here we present the first crystal structure, solved at 2.10 Å resolution, of the oxidized form of $\beta_4$-HbA. The overall quaternary structure of the protein in the ferric state is virtually indistinguishable from that of the ferrous deoxygenated and carbomonoxy forms. The structure reveals that the four hemes are exclusively in an aquomet coordination, without any trace of bis-histidyl coordination. The oxidation of $\beta_4$-HbA is associated with the formation of a disulfide bridge between residues Cys112(G14) of $\beta_1/\beta_4$ and $\beta_2/\beta_3$ chains. The coordination state of $\beta_4$-HbA has been compared to that known for other organisms that exhibit bis-histidyl heme coordination in the $\beta_4$ state. This occurrence has been discussed in terms of different organism physiology.

## 1. Introduction

It is well known that adult human hemoglobin, HbA, is a $\alpha_2\beta_2$ heterotetramer. It is crucial to erythrocyte formation that the two globin chains are produced at balanced levels, since variations from the 1:1 α to β ratio have deleterious effects on red cell survival, a situation which is most clearly illustrated by thalassemia and related disorders [1–4]. Indeed, an overall deficit of one chain due to its reduced synthesis compared to the other chain led to the accumulation of non-canonical hemoglobins such as hemoglobin H ($\beta_4$ homotetramer) in α-thalassemia (reduced synthesis rate of the α-chain) [2].

Hb α- and β-chains have been found expressed in a plethora of non-erythroid cells [5]. Indeed, Hb functions are not exclusively restricted to the blood but this protein may play multiple roles in health and disease. In this last case, Hb is often related to precipitation of aggregates causing motor learning impairment, and ultimately cells' homeostasis [6]. Hb α- or

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β-chains have been found co-expressed in neurons, glial cells, alveolar cells, eye's lens, mesangial cells of the kidney, hepatocytes, and skeletal muscle [7–10]. Isolated α- or β-chains have been observed non-co-expressed in endothelial cells and macrophages [11,12].

Solubility, stability and aggregation propensity of the isolated α and β-chains are very different. Indeed, the $\beta_4$ homotetramer is highly soluble and stable in the ferrous state, whereas the isolated α-subunits do not form tetramers, tend to precipitate and *in vivo* are typically coordinated to the α-hemoglobin stabilizing protein (AHSP) [13].

The function and stability of the α-subunits of the $\beta_4$ tetramer are very different when compared to the $\alpha_2\beta_2$ heterotetramer. Particularly, isolated $\beta_4$ tetramers bind $O_2$ non-cooperatively and with a higher affinity than HbA. Notably, the peroxidase activity of both the separate subunits of ferric human hemoglobin is reduced when compared to the $\alpha_2\beta_2$ heterotetramer [14]. Notably, isolated human α-subunits complexed with the AHSP protein in a bis-hystidyl heme (hemichrome) coordination do not exhibit peroxidase activity [13]. This aspect is strictly related to the endogenous hexa-coordination in the ferric state. Indeed, spectroscopic studies performed on the ferric α- or β-subunits revealed that the α-chain preferentially adopts the bis-histidyl state (hampering the peroxidase activity). On the other hand, $\beta_4$ tetramer seems to be a mixture of aquomet and bis-His heme coordination forms [15]. It is worth noting that in other organisms, such as Antarctic [16–19] and sub-Antarctic [20,21] fishes (AF), the ferric $\beta_4$ tetramer is mostly in a bis-histidyl state. Furthermore, other mammalian globins, such as neuroglobins, cytoglobins, and myoglobin mutants, exhibit the ferric bis-histidyl coordination as well [22–26].

In the absence of any crystal structure of the ferric human $\beta_4$ tetramer, molecular dynamic (MD) studies were performed to predict the intrinsic tendency of the β-subunits to form endogenous hexacoordination. These investigations showed that the AF β-subunit is endowed with a higher tendency to adopt a bis-histidyl state when compared to the human counterpart [16].

Several crystal structures of the HbA heterotetramer are available: the structure of the protein has been solved in both ferrous (R- and T-states [27], and R/T-intermediate quaternary state [28,29]) and ferric forms (including the R-state [30]). On the contrary, only few crystal structures are available for the isolated chains. The structure of the α-subunit is available only in complex with AHSP, both in the ferrous [31] and ferric [13] states, and the structure of the ferric state is biased by the presence of additives that strongly affects the heme coordination. Crystal structure of $\beta_4$ tetramer is available only in the ferrous states, in particular in the oxygenated [32] and deoxygenated forms [33].

We succeeded in crystallizing the ferric $\beta_4$ homotetramer for the first time. Here we report the X-ray structure of ferric-$\beta_4$-HbA, which shows that hemes are only in an exogenous hexacoordination (aquomet) with no trace of endogenous bis-histidyl coordination. The crystal structure of ferric human $\beta_4$ clearly reveals the presence of oxidation-induced disulfide bridges between Cys112(G14) residues of the two couples of β-chains ($\beta_1$-$\beta_4$, $\beta_2$-$\beta_3$) that make the overall structure more rigid, possibly hampering the bis-histydyl formation.

## 2. Materials and methods

### 2.1. Preparation of $\beta_4$ tetramers

$\beta_4$-HbA was obtained starting from separated β-chains. The separated oxy β globin chains from HbA ($\alpha_2\beta_2$) were obtained according to the method previously described by Geraci et al. [34] and Tsuruga et al. [35]. In solution β-chains readily associate into tightly assembled $\beta_4$ homo-tetramers [36].

### 2.2. Protein crystallization and structural refinement

Crystallization trials were performed at 293 K by using hanging-drop method [37]. After a preliminary screening of the crystallization conditions for carbomonoxy-$\beta_4$ [32] and deoxy-$\beta_4$-HbA [33], we were able to grow good quality crystals of ferric-$\beta_4$-HbA from the solution oxidized using potassium ferricyanide (4:1 M excess). We used stock solutions of ferric-$\beta_4$-HbA containing 20% glycerol to stabilize the tetramer, a protein-stabilizing ability common to many diols [38]. The crystals used in the diffraction experiments were obtained with a protein concentration of 8 mg ml$^{-1}$, at pH 7.0. The composition of the reservoir solution was 2.5 M ammonium sulphate and 0.26 M sodium phosphate buffer at pH 7.0. Diffraction data were collected in-house at 100 K using Cu Kα (1.54 Å) X-ray radiation from a Rigaku Micromax 007 HF generator equipped with a Saturn944 CCD detector. The structure was solved at 2.10 Å resolution. The crystals belong to the space group $P2_1$.

All the data were indexed, processed and scaled with HKL2000 program package [39]. Detailed statistics on data collection are reported in Table 1. Matthews coefficient [40] calculation suggested the presence of one tetramer in the asymmetric unit (Fig. 1).

**Table 1**
X-ray data collection statistics for ferric-$\beta_4$-HbA.

| Crystal data | |
|---|---|
| Space group | $P2_1$ |
| Unit-cell parameters | |
| a (Å) | 52.7 |
| b (Å) | 82.1 |
| c (Å) | 62.5 |
| α (°) | 90.0 |
| β (°) | 91.3 |
| γ (°) | 90.0 |
| Data processing | |
| Resolution limits (Å) | 62.5–2.1 |
| No. of reflections | 31,016 |
| Completeness (%) | 96.1 (94.1) |
| I/σ (I) | 20.6 (4.9) |
| Average multiplicity | 2.9 (2.8) |
| Rmerge (%) | 0.09 (0.40) |

$Rmerge = \Sigma_{hkl}\Sigma_i \ |I_i(hkl) - < I_i(hkl) > |/\Sigma hkl\Sigma_i \ I_i(hkl)$, where $I_i(hkl)$ is $i_{th}$ intensity measurement of the reflection hkl, including symmetry related reflections, and $< I_i(hkl) >$ is its average. Values in brackets are for the highest resolution shell (2.16–2.10 Å).

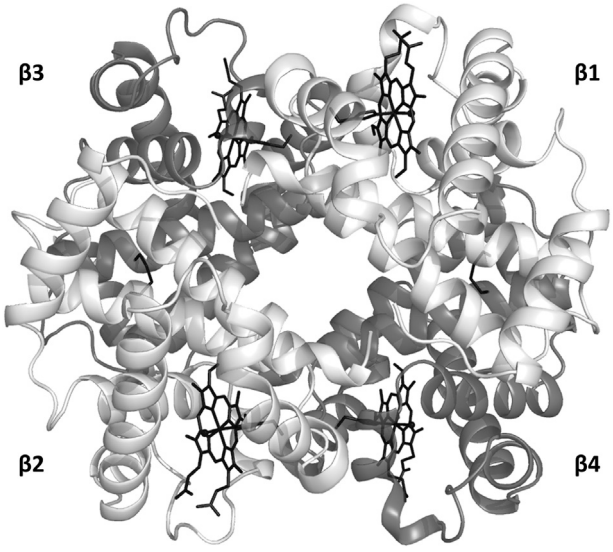


**Fig. 1.** Ribbon diagram of the overall structure of ferric-$\beta_4$-HbA. Disulfide bridges, heme groups, His92(F8) residues and water molecules involved in the iron coordination are shown as sticks.

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