Crystal structure of haemoglobin from donkey (Equus asinus) at 3Å resolution

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Abstract

Haemoglobin from donkey was purified and crystallized in space group C2. The present donkey haemoglobin model comprises of two subunits α and β. These α and β subunits comprise of 141 and 146 amino acid residues, respectively, and the haem groups. The donkey haemoglobin differs from horse only in two amino acids of α-chain (His20 to Asn and Tyr24 to Phe) and these substitutions do not significantly change the secondary structural features of donkey haemoglobin. The haem group region and subunit contacts are closely resemble with that of horse methaemoglobin.

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1. Introduction

Haemoglobin, the integral molecule for oxygen transport in erythrocytes, is found in nearly all mammals. Haemoglobin is composed of the protein globulin, which consists of four-polypeptide chains and four nitrogen-containing cyclic organic molecules called hemes. The protein of haemoglobin is composed of two identical alpha and beta polypeptide chains [1]. Each of the four-polypeptide chains binds to a heme group that is then bonded to a central iron ion. Each iron is capable of binding one oxygen atom, thus haemoglobin binds to a total of four oxygen atoms. Haemoglobin is capable of binding and releasing oxygen by changing conformations which attract or repel molecules of oxygen. Haemoglobin is in a “tense” state, commonly called the T-state, when oxygen is bound. In the absence of oxygen, haemoglobin conforms to a “relaxed” state, the R state. The T-state is characterized with high affinity for oxygen and the R state carries low affinity for oxygen (T and R states). Many papers have been reported on the elucidation of quaternary structure and its structure-function relationships of haemoglobin [2–8]. The oxygen affinity of haemoglobin is modulated by several non-heme ligands such as protons [9], chloride ions [10], and 2,3-diphosphoglycerate complex [11–13] which reduces the oxygen affinity by preferentially binding to haemoglobin in the T-state. The multiple sequence alignment of α-chain in donkey, zebra and horse haemoglobin was done using the programme CLUSTALX 1.82 [14] and is shown in Fig. 1. The sequence of α-chain in donkey haemoglobin is 98% identical to that of horse haemoglobin. The α-20 His and α-24 Tyr in horse are replaced by Asn and Phe, both in donkey and zebra (α-20 His → α-20 Asn and α-24 Tyr → α-24 Phe), respectively. Kitchen has reported the same substitutions in the α-chain as above and established the fact that the β-chain is conserved in all Equus species [15]. In order to understand the quaternary structure–function relationships and also to compare the three-dimensional structure of donkey haemoglobin with that of horse haemoglobin, a study on donkey haemoglobin was carried out. The sample was purified, crystallized and the structure determination was done.

2. Materials and methods

Purification, crystallization and data collection for donkey haemoglobin have been described elsewhere [16]. The haemoglobin crystals were obtained using hanging drop vapour diffusion method at room temperature from drops containing 10 μl
protein solution (80 mg/ml in water) and 4 μl reservoir solution, equilibrated against 1 ml of reservoir solution (2.8 M phosphate buffer, pH 6.8). The crystals were mounted in 0.5 mm Lindemann tubes and the data were collected using mar345 image plate detector system mounted on a Rigaku UltraX-18 rotating-anode X-ray generator operating at 40 kV and 80 mA with a copper anode. A dataset for 180° (1° oscillation frames, each exposed for 240 s) up to 3.0 Å resolution was collected with a crystal-to-detector distance of 100 mm at 293 K. All the data were indexed, integrated, merged, and scaled using DENZO and SCALEPACK from the HKL programme package [17]. Crystals of donkey haemoglobin are monoclinic with space group C2 and the data collection statistics are given in Table 1.

2.1. Structure solution

The structure solution was obtained by molecular replacement method using aquamet horse haemoglobin (PDB id: 2MHB) as starting model, using the programme AMoRe [18] implemented in CCP4 suite [19]. The solution resulted in a conventional crystallographic R-factor of 35.1% and correlation co-efficient of 72.1% with one molecule (αβ dimer) in the asymmetric unit.

2.2. Structure refinement

The structure refinement was done using REFMAC 5.0 implemented in CCP4 suite. About 5% of the reflections were used for the test set. The resultant model obtained from the structure solution was subjected to 20 cycles of rigid-body refinement, which led in a few cycles to an R-factor of 31.82% (R_free = 31.40%). 2Fo – Fc map was calculated and the model building was done using O and XtalView programmes [20, 21]. The refined model, which had the amino acid sequence of horse haemoglobin, was replaced to donkey haemoglobin using O programme, by positioning the donkey specific residues α-20 Asn and α-24 Phe in the electron density map. The position of the Fe atom was fixed in the 2Fo – Fc map contoured at 3.0σ cutoff. At this stage restrained refinement was carried out and the final R-factor converged to 18.6% (R_free = 28.5%). The refinement statistics for the final model is presented in Table 1. The coordinates are deposited in Protein Data Bank and the PDB id is 1S0H.

3. Results and discussion

The final model of donkey haemoglobin comprises of two subunits α and β. α and β subunits comprise of 141 and 146 amino acid residues, respectively, and the haem groups. The final model has an R-factor of 18.6% (R_free = 28.6%). The Ramachandran plot calculated for the final model with the programme PROCHECK [22] shows 85.9% of the residues in the most favoured regions and the remaining residues in the additionally allowed regions. It is interesting to see that almost

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### Table 1

Data collection and refinement statistics for donkey haemoglobin

<table>
<thead>
<tr>
<th>Data collection:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (Å)</td>
<td>30.0–3.0</td>
</tr>
<tr>
<td>Space group</td>
<td>C2</td>
</tr>
<tr>
<td>Unit cell dimensions (Å, °)</td>
<td>a = 107.664, b = 63.084, c = 54.042, β = 111.75</td>
</tr>
<tr>
<td>Asymmetric unit</td>
<td>αβ dimer</td>
</tr>
</tbody>
</table>

### Reflections

| Measured/unique | 14,220/5406 |
| Completeness (%) | 79 (34.3) |
| Mean <I>/σ(I) | 4.3 |
| Rmerge (%) | 16.3 (42.5) |

### Refinement:

| Resolution range (Å) | 21.18–3.0 |
| σ cut-off | 2.0 |
| Reflections used | 5133 |
| R-factor/R_free (%) | 18.6/28.5 |

### r.m.s deviations from ideal

| Bond lengths (Å) | 0.042 |
| Bond angles (°) | 1.512 |
| Chiral volume (Å³) | 0.131 |
| Mean B Values (Å²) | 33.32 |

### Ramachandran plot

| Most favourable regions (%) | 85.9 |
| Additional allowed regions (%) | 12.9 |
| Generously allowed regions (%) | 1.2 |

The numbers in parentheses refer to statistics for the higher resolution shell (3.00–3.08 Å).
all the residues in the structure are found in a helical conformation.

The α subunit contains eight helical segments whereas the β subunit contains nine helical segments. The secondary structural elements for donkey haemoglobin are shown in Fig. 2a. The secondary structure of the subunits are stabilized by hydrogen bonds formed by main chain NH and CO groups or with nearby polar side chains. In addition a few internal hydrogen bonds are linked with the various chain segments. The external polar side chains help to stabilize the tertiary structure by forming hydrogen bonds with each other. The overall tertiary structure is similar to that of horse haemoglobin with a root mean square (r.m.s.) displacement of 0.380 Å for main chain atoms. The Cα trace of the donkey haemoglobin and horse haemoglobin, superimposed for α and β subunits are shown in Fig. 2b.

The amino acid substitutions between the horse and donkey haemoglobins are: α-20 His (horse) → α-20 Asn (donkey) and α-24 Tyr (horse) → α-24 Phe (donkey). The substitutions have similar hydrogen bond patterns for donkey and horse haemoglobin but the hydrogen bond between side chain–side chain atoms (20 His Nδ1–23 Glu Oε1) is absent in the case of donkey haemoglobin. However, these amino acid substitutions have not affected the secondary structural features in donkey haemoglobin. The H-bonds were calculated using the HBPLUS programme [23]. The residues α-20 Asn and α-24 Phe in donkey haemoglobin have very clear electron densities.

3.1. Haem group region

Electron density map for haem group regions in α and β subunits are shown in Fig. 3a and b. The haem pockets of donkey haemoglobin are very similar to those of horse haemoglobin [4,24]. These regions of the molecule are well conserved and the equivalent residues overlap closely with slight deviation in α-subunit proximal histidine (His 87) and β-subunit distal histidine (His 63). Fig. 3c and d show the haem pockets of donkey haemoglobin (blue) and horse haemoglobin (red) least-squares fitted on the reference frame of 139 amino acid residues. Compared with horse haemoglobin, α-subunit distal histidine (His 58) and β-subunit proximal histidine (His 92) assume same symmetrical position on the haem axis. The distal histidine residue and haem group overlap closely in α-subunit.

Fig. 3e and f illustrate the detailed conformation of the two haem-linked helices E and F which are perfectly conserved between donkey and horse haemoglobin. The E helix of β-subunit is less regular than that of α-subunit. Helix E moves towards the haem in both subunits, but more in β than in α. In the
structures were superimposed by least-squares fitting the main chain atoms.

The term hinge to describe the rotating motion of residues 35–43 in the case of donkey haemoglobin was used to describe the switching action of residues 97β2–102β2 and the term switch for the hinge to describe the rotating motion of residues 35β2–43β2. Mueser et al. [26] had described the nature of α1β1 interface in horse carbonmonoxyhaemoglobin (liganded) and quaternary T-state deoxyhaemoglobin. The liganded horse carbonmonoxyhaemoglobin and human carbonmonoxyhaemoglobin have slightly different quaternary structures. Shibayama et al. [13] have reported that the quaternary structure of horse carbonmonoxyhaemoglobin–BZF (bezafibrate) complex remains the same as in horse carbonmonoxyhaemoglobin.

The exact number of non-covalent interactions at a protein–protein interface depends on the cut-off criteria used to define the direct polar and non-polar contacts. Ladner et al. [24] had used a cut-off ≤ 4.0 Å to identify the polar and non-polar contacts. The same cut-off value (≤ 4.0 Å) is used to define the polar and non-polar contacts at the subunit interfaces in donkey haemoglobin.

Perutz and Lehmann [27] had reported that the α1β1 contact comprised 34 residues whereas in α1β2 the number of contacts reduced to 19 in horse haemoglobin at 2.8 Å resolution. Later Ladner et al. [24] reported that the α1β1 contact comprised 35 residues and α1β2 contact had 18 residues in horse aquamethaemoglobin at 2.0 Å resolution and confirmed that the residues involved in contact are not the same ones.

In the present study of donkey haemoglobin, the α1β1 and α1β2 contacts comprises of 33 and 17 residues, respectively. In addition to the α1β1 and α1β2 interfaces, the dimer–dimer interface includes relatively weak α1α2 and β1β2 contacts.

The carboxyl group of α-Asp 94 is in van der Waal’s contact with the indole of β-Trp 37, and the guanidinium group of β-Arg 40 with the phenol of α-Tyr 42 as observed in horse methaemoglobin [24]. When the haemoglobin tetramer dissociates into dimer, these interactions of the aromatic rings in one subunit with the charged groups from a neighbouring subunit are lost and all the groups are exposed to water. The transition from the quaternary R to the T structure is accompanied by changes in the contacts made by the two aromatic side chains [28,29].

Chothia [30] has compared the α1β1 dimer in horse methaemoglobin with that of human deoxyhaemoglobin and found that despite the difference in amino acid sequence the α-carbon atoms in the region of contact superimpose as one rigid body. This means that the R⇆T transition causes no movement at the α1β1 contact.

Kilmartin and Bernardi [9] have found that the C-terminus of one α-chain was linked to the N-terminus of the other α-chain, and Perutz et al. [31] proposed the existence of a salt bridge between the carboxyl group of the C-terminal arginine (141α) and the positively charged α-amino group (Val 1α) of the opposite α-chain. The proximity of the negatively charged carboxyl group to α-amino group would stabilize the positive charge. The existence of the salt bridge between the α-chain C-terminal carboxyl group and the α-amino group of the opposite α-chain has been confirmed in deoxyhaemoglobin [32,33]. These residues are not seen in horse oxy-haemoglobin [34]. The salt bridge between the carboxyl group of the C-terminal arginine (141α) and the positively charged α-amino group (Val 1α) of the opposite α-chain exists in the present donkey haemoglobin structure. Also no salt bridges involving the β-chain...
α-amino group have been found in donkey haemoglobin as observed in the structures of oxy and deoxyhaemoglobin.

Acknowledgments

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References