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Hemoglobin crystals immersed in liquid oxygen reveal diffusion channels

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ABSTRACT

Human hemoglobin (HbA) transports molecular oxygen (O_2) from the lung to tissues where the partial pressure of O_2 is lower. O_2 binds to HbA at the heme cofactor and is stabilized by a distal histidine (HisE7). HisE7 has been observed to occupy opened and closed conformations, and is postulated to act as a gate controlling the binding/release of O_2. However, it has been suggested that HbA also contains intraprotein oxygen channels for entrances/exits far from the heme. In this study, we developed a novel method of crystal immersion in liquid oxygen prior to X-ray data collection. In the crystals immersed in liquid oxygen, the heme center was oxidized to generate aquomethemoglobin. Increases of structural flexibility were also observed in regions that are synonymous with previously postulated oxygen channels. These regions also correspond to medically relevant mutations which affect O_2 affinity. The way HbA utilizes these O_2 channels could have a profound impact on understanding the relationship of HbA O_2 transport within these disease conditions. Finally, the liquid oxygen immersion technique can be utilized as a new tool to crystallographically examine proteins and protein complexes which utilize O_2 for enzyme catalysis or transport.

1. Introduction

Human hemoglobin (HbA) plays a vital role in transporting oxygen (O_2) from the lungs to the tissues [1]. HbA is a tetramer consisting of two α-subunits and two β-subunits [1]. In each HbA subunit, there is a prosthetic heme where an O_2 molecule can bind at the ferrous atom [2]. The tetrameric HbA is an allosteric protein in which each subunit can switch between the tense-state (low O_2 affinity) and the relaxed-state (high O_2 affinity) [3–5]. The subunits in the quaternary assembly show a positive cooperativity for O_2 binding [5]. The O_2 pressure in the lung is relatively high so that HbA in the lungs can bind O_2 molecules (oxy-HbA) [6]. When oxy-HbA is transported to other tissues though blood circulation, O_2 molecules are released where the pressure is lower. Acidic conditions also facilitate the release of O_2 molecules from oxy-HbA [7]. Therefore, there must be pathways for O_2 migration because the heme iron center is within the HbA protein. These pathways could allow HbA to sense the environmental O_2 pressure. O_2 molecules need to enter and exit HbA through these pathways.

In the O_2 binding pocket of HbA, there is a distal histidine residue (named HisE7; αH58, and βH63) that forms a hydrogen bond with the heme-bound O_2 molecule [8]. The hydrogen bond stabilizes the O_2 molecule bound in this pocket. In addition, HisE7 can be regarded as a gate to shield the heme-bound O_2 molecule from the solvent [9]. The sidechain of HisE7 can assume an open or closed conformation, which has been postulated to be a gate for O_2 to enter or exit the alleged E7 channel [10]. In a recent study by molecular dynamic simulation, however, it was shown that the mechanism of O_2 escape controlled by HisE7 may not be as what was previously postulated [11]. In fact, O_2 molecules can escape from the distal histidine pocket with the conformation of HisE7 sidechain open or closed. When the sidechain of HisE7 is protonated, it will adopt the open conformation, which allows water molecules to diffuse into the distal site, but hinders the escape of apolar gas molecules including O_2. The open conformation of HisE7 increases the volume of the distal pocket and may still increase the escape of O_2 molecules from this route, but not by a lot (around

Abbreviations: HbA, human hemoglobin; O_2, molecular oxygen; HisE7 or E7, referring to Histidine 58 and 63 on the α and β-subunits of human hemoglobin.

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12%). Mutations of HisE7 confirmed that the E7 channel is the main route of entering and escaping the heme-O_2 binding pocket [12].

However, it is possible that there are other pathways of O_2 migration in the HbA protein [13]. By structural determination of xenon docking sites in globins, it has been suggested that alternative pathways are possible [14,15]. These Xe docking sites are hydrophobic cavities, confirming the availability of space within HbA for alternative pathways. To verify potential pathways that include the Xe docking sites, photolysis of oxy-HbA was monitored and rebinding of O_2 molecules could be quantitated [16]. The rebinding kinetics were affected under 25atm of xenon gas, showing a decrease of escape from the α subunit. This clearly demonstrated that there are pathways for O_2 exit at least within the α subunit that go through Xe docking sites. Consistently, molecular dynamic simulations discovered multiple O_2 entrance pathways containing the Xe docking sites, in addition to the E7 channel [13]. In another simulation study, it was shown that multiple exit pathways can be found to include Xe docking sites [17]. The path of the diffusion is dependent on the allosteric state of the HbA quaternary structure. More interestingly, it was shown that O_2 molecules may escape through internal tunnels in significant portion in all allosteric states when the distal HisE7 is in the closed conformation, further confirming the presence of alternative pathways.

In order to directly visualize the possible pathways of O_2 migration, we set-up a system to determine the crystal structure of HbA in liquid O_2. The idea is to trap O_2 molecules in HbA crystals that are frozen under cryo-conditions. The crystal structures of HbA were solved for different immersion times in liquid O_2. These structures are compared with previously reported HbA structures. In addition, we also observed autoxidation of the heme-iron to aquomethemoglobin when HbA crystals were immersed in liquid O_2 for a longer period of time. Accumulation of methemoglobin will cause methemoglobinemia that can be a severe disease leading to death [18–20]. Furthermore, we observed increased flexibility in areas previously postulated to be O_2 channels, giving the first direct evidence of O_2 channel entrances/exits with the endogenous ligand of HbA. These regions can also be correlated to medically relevant mutations which show decreased/increased O_2 affinity. Finally, the novel technique of liquid O_2 immersion can be used to crystallographically study enzymes which utilize O_2 in catalysis and examine the structure-function relationships of proteins and protein complexes which transport O_2.

2. Materials and methods

Crystallization and Protein Purification—Protein purification and generation of liquid oxygen were carried out as shown in the Supplemental Materials. The oxyhemoglobin was crystallized following the protocol of Perutz et al. [21]. 100 μL of toluene was added to each of 4 samples that contain the components described in Table S3. Crystals grown at 4 °C appeared after five to seven days and were allowed to grow for three weeks prior to experiments of immersing crystals in liquid oxygen (Figure S1). Hemoglobin crystals of approximately the same size were selected and transferred to a cryoprotectant solution comprised of 20% glycerol in crystallization mother liquor. Looped crystals were then immersed in the liquid oxygen for 20 s, or 1 min and rapidly transferred to a unipuck submerged in liquid nitrogen.

3. Results and discussion

Crystal Structure of HbA Immersed in Liquid O_2—When crystals of human HbA were immersed in liquid O_2, HbA was exposed to a high concentration of O_2 while the crystal was flash-frozen at cryogenic temperature (~90 K). HbA crystals were first immersed in liquid O_2 for 20 s, and 1 min, and then transferred to liquid nitrogen. X-ray diffraction data were collected under cryo temperatures and the structures were determined by molecular replacement (see Table S1 for statistics). All refined structures in this study are in the R state. Furthermore, in order to assure the utmost of accuracy in our study, we have collected and solved a control structure (see Table S2), taken from the same batch of crystals and frozen directly in liquid nitrogen—the standard method. However, due to higher resolution structures already deposited in the PDB, and our own control structure only being of modest resolution 2.28 Å, a conscious decision was made to utilize PDB code 2DN1 as the main structure used for comparison. For the purpose of this study, our control structure and 2DN1 are identical and the same conclusions can be drawn from both of the structures (Figure S3 and S4). The rationale behind choosing 2DN1 structure lies in the accuracy of the results provided by the higher resolution of the 2DN1 structure.

Under physiological conditions, the heme centers of HbA must exist in the ferrous state to bind molecular O_2 [2]. Once O_2 is bound, the HbA protein environment is highly tuned to stabilize a quasi-ferrous/ferric and O_2/superoxide resonance structure [22–24]. When O_2 is released, the active site is restored to its native ferrous state. However, in highly oxidative environments, it is possible for this process to become decoupled and HbA is left with a ferric center no longer capable of binding molecular O_2, which causes a plethora of medically relevant maladies [18–20]. Compared to a published structure cryo-cooled in liquid nitrogen (PDB CODE 2DN1, 1.25 Å resolution) [8] and our control structure (PDB CODE 6BB5), we noticed significant changes in the binding mode at the iron-O_2 heme centers. Crystals of HbA immersed in liquid O_2 for 20 s and 1 min showed significant oxidation at all of the heme centers (Fig. 1). In both the α/β-subunits of both crystals immersed for 20 s and 1 min a sizeable increase in the iron-O_2 bond length (2.1–2.2 Å) and a rounding of the electron density occurred. The longer bond in the new structures are similar to aquomethemoglobin with a bond length of 2.2 Å and an identical shape in electron density [25]. Thus, we have assigned these sites with a water ligand and believe that these heme centers have been oxidized to the ferric state, unable to bind molecular O_2. This indicates that, upon immersion, liquid O_2 can penetrate the crystal and access the heme sites to carry out oxidative inactivation of the protein.

Presence of O_2 Channels Indicated by Increased Structural Flexibility—Compared to 2DN1 and 6BB5, both structures immersed in liquid O_2 show only slight overall structural differences (Figure S3 and S5). This would imply that even under these O_2 saturated conditions, *in crystallo*, HbA does not undergo any large structural permutations deviating away from one of the many forms of R-state (R, R2, RR2, R3) [26].

Since HbA crystals were immersed in liquid O_2 prior to collection of X-ray diffraction data, it is expected that the channels through which the O_2 molecule is transported to and from the heme site in HbA would be occupied by O_2. Unfortunately, due to crystallographic averaging and O_2's propensity to look like a water molecule, we could not assign any new O_2 binding sites with certainty. Nonetheless, in order to investigate the possibility of intra-protein O_2 channels, the local flexibility in the structures was evaluated based on their B-factors. The rationale is that the penetration of molecular O_2 into the channels would induce flexible structural changes inside HbA. In this study, refinement of the 2DN1 structure was repeated at 1.54 Å resolution in order to compare all structures at the same resolution. B-factors and their differences were plotted by residue for structures from crystals immersed in liquid O_2 and 2DN1 (Fig. 2A and B). The major changes in flexibility are highlighted in the B-factor difference plots. Furthermore, each structure was visualized by the B-factor putty preset in PyMOL (Fig. 3 and S4). Based on these analyses, we found

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