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X-ray Analysis of Fine Structure of Heme in Normal and Thalassemic HbE/F Hemoglobin

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Thalassemic hemoglobin (Hb) differs from normal Hb in its structure and oxygen-binding capacity. To find out structural differences in their heme-iron sites, X-ray absorption fine structure (XAFS) was applied. Resulting data in the range of $0 \leq k \leq 16A^{-1}$ and $1 \leq k \leq 14$ A^{-1} obtained from frozen aqueous solutions (10 K) of normal and thalassemic HbE/F deoxy forms were refined using multiple-scattering (MS) analyses. To test the robustness of the refinements *R* starting models, constraints, restraints, and *k* ranges were varied; for normal relaxed R- and tense T-deoxy-Hb final XAFS *R* values were 13% and 17% and for thalassemic HbE/F relaxed R-deoxy-Hb *R* value was 19-21%. With normal R-deoxy-Hb, we obtained Fe-Np (pyrrole) and Fe-Nε (imidazole) distances of 2.05 and 2.14 , whereas in thalassemic HbE/F R-deoxy-Hb, these values were 2.11 and 1.9 , respectively. In the T-state of normal deoxy-Hb, Fe-Np distance (2.08A) was longer and Fe-Nε (1.96A) shorter than in the R-state; from thalassemic HbE/F T-deoxy-Hb we could not obtain sufficiently stable probes for XAFS measurement. In normal and HbE/F R-deoxy-Hb the Fe-His imidazole bond deviated from the z-axis by ~10^0, whereas it was on the z-axis in the T-state of normal Hb. Heme doming increased from normal R-deoxy via normal T-deoxy to thalassemic R-deoxy forms with simultaneously increasing dislocation of the central iron from the plane of the porphyrin. Oxygenation brings the iron back into the plane of the porphyrin ring.

Keywords: Beta-thalassemia; Heme fine Structure; Hemoglobin; Synchrotron; Thalassemic HbE/F; X-ray absorption fine structure (XAFS).

Heme proteins participate in a wide range of enzymatic redox processes, in electron transfer, and in the transportation and storage of dioxygen. Hemoglobin (Hb) of normal adults is 95% HbA with two alpha and two beta globin chains ($\alpha^2\beta^2$). Minor Hb species are HbA_2 consisting of two alpha and two delta globins ($\alpha^2\delta^2$), which amounts to 2-3.5% of total Hb and fetal Hb (HbF) made up of two alpha and two gamma globins ($\alpha^2\gamma^2$), which is less than 2%. At the third semester of pregnancy, HbF (up to 90% of Hb in maternal blood) synthesis starts to decline and is gradually replaced by adult HbA ($\alpha^2\beta^2$) over the first year after birth.[1-3]

Thalassemia is a hereditary Hb disease characterized by the absence or decreased production of normal globin chains which leads to varying degrees of anemia.[4-6] In Indonesia, a G to A shift in codon 26 causes the most common

(beta-26 Glu to Lys) mutation (HbE) frequently associated with beta-thalassemia.[7-11] Splice site mutation in exon 1 of the beta-globin chain makes HbE abnormal leading to microcytic anemia[11] and the clinical picture of thalassemia intermedia, especially when combined with further beta-thalassemic mutations or deletions. In thalassemia patients very often the production of fetal Hb does not drop off at birth but is maintained because it is still necessary for their survival.[2]

On chromosome 16 are the genes which control α-globin production and on chromosome 11 the genes are located for β, γ, and δ globin synthesis. The β-globin chain which is made up of 146 amino acids binds heme covalently via the proximal histidine in position 92 (His-92) and alterations in the structure of the β-globin chain in hereditary Hb diseases mostly lead to an overproduction of HbF.[1] A distal His, in position 63, is not directly involved heme binding,[12] but may function as a ligand to the central Fe. Thus, the heme is fixed between helices E and F of the globin chain. Several amino acid residues form the hydrophobic heme pocket around the protoporphyrin and the two histidines. With distances less than 6A from the heme, Leu-28, Leu-31, Thr-38, Phe-41, Phe-42, Phe-45, Val-67, Leu-68, Ala-70, Phe-71, Phe-85, Leu-88, Leu-91, Leu-96, Val-98, Asn-102, Phe-103, Leu-106, Val-137, Leu-141 provide hydrophobic interaction, whereas the phenylalanines (Phe) may, in addition, interact via aromatic contact, and (besides the two 63 and 92 histidines) only two amino acid residues, Ser-44 and Lys-66, form hydrogen bonds within the heme pocket.[13]

Various spectroscopic methods, such as ultraviolet (UV) and visible spectroscopy, infrared, resonance Raman (RR), microwave spectroscopy and X-ray crystallography have been used to investigate heme proteins such as met-indoleamine 2,3-dioxygenase-2,[14] myoglobin,[15-17] and Hb.[2,11,18-20] Whereas structural changes in thalassemic globin chains have been widely reported, fine structure of their heme has not been known. Here we investigate the heme structure of a patient with 2/3 HbE and 1/3 HbF.

MATERIALS AND METHODS

To better understand the chemistry and dynamics of Hb, model compounds have been constructed, such as the "picket fence" complex[21] or *meso*-(2-methylimidazole)[tetraphenyl-porphyrinato(2-)]iron(II).[22] In both structures modeling the oxygenated heme complex, the bonds from iron to the porphyrin nitrogens are 0.09 Å shorter than in the deoxygenated complexes and the bond from the iron to the imidazole nitrogen deviates by 10.3° from the z-axis. Such models served as references for our study.

Sample Preparation

Blood from a 13-year-old non-transfused thalassemia intermedia patient with 1/3 HbF and 2/3 HbE was used for XAFS analysis. The reasons for this decision were: i) this non-transfused patient did not have mixtures of endogenous and exogenous Hb in his blood (as transfused patients do); ii) in Hb analysis, the patient had a clear relation of 67.5% HbE and 32.5% HbF; iii) frequently, patients show more complex Hb composition (including varying contents of thalassemic Hb and/or HbA_2), which complicates interpretation of XAFS data; iv) this patient had already been included in comprehensive thalassemia examinations on iron status, oxidative stress, and electron paramagnetic resonance (EPR) measurements of his isolated erythrocyte membrane.[23-25] Hence, the chosen sample may also serve as a model for the wide variety of thalassemic hemoglobins.

Blood samples were drawn from patients of the thalassemia ward in Cipto Mangunkusumo General Hospital in Jakarta after fully informed consent which conforms to the standards applied in Indonesia. The study was approved by the Ethical Clearance Committee of the Faculty of Medicine, University of Indonesia and hemolysate samples were transported on dry ice from Jakarta to Sydney, Australia (AQIS permit No 200012081). The Hb content was 0.5–2 mM, as measured by UV/vis spectroscopy (a Hewlett-Packard 8452-A diode array spectrophotometer or a CARY 5E spectrophotometer) at 406 nm (*met*-Hb) against a standard prepared from commercial *met*-Hb (Sigma Australia). The literature value of the molar extinction coefficients ε (406 nm) = 162 M^{-1} cm^{-1} was used to determine concentration.[26]

Control Hb A was purchased from Sigma Australia in the form of *met*-Hb. The molecular or formula weight (FW) was given as 64,000, equivalent to four heme moieties of FW 16,000, each (including the respective alpha- or beta-

peptide chains). For XAFS experiments, 250 µL of a solution containing 2 mM *met*-Hb (equivalent to 8 mM heme) was prepared with 60% bis-Tris (0.05 M) and 40% glycerol (v/v).[15,16] The glycerol was added to reduce random noise scattering from the sample surface and to prevent potential damage to the protein from ice crystals as well as interference with Bragg reflections from ice crystals in the XAFS spectra.

Control deoxy-Hb was prepared by reduction of *met*-Hb with sodium dithionite (6- to 10-fold molar concentration vs. heme in bis-Tris buffer 0.05 M, pH 6.5) using syringe techniques in an argon-filled glove bag.[15,16]

HbE/F solution (500 ìL, 0.5-2.0 mM) was oxidized with $K_3[Fe(CN)_6]$ (0.003 mg, 0.02 mM) and the solution was purified by passing it down a column of Sephadex G25-p10. The pooled red fractions were concentrated by dialysis against PEG400. The final *met*-Hb concentration for XAFS experiments was determined from UV/Vis spectroscopy to be 2.0-2.6 mM, equivalent to 8.0-10.4 mM heme, in NaP_i buffer (5 mM, pH 6.8). After hemolysis and oxidation, *met*-HbE/F was already dissolved in NaP_i buffer (5 mM, pH 6.8). Hence, reduction of HbE/F to deoxy-HbE/F was also carried out in this buffer (NaP_i, 5 mM, pH 6.8), but it needed a higher concentration of sodium dithionite (at least 10-fold over heme concentration) to reduce *met*-HbE/F.

All handling of proteins was undertaken using protein-clean glassware and every step in the Hb preparation was checked by UV/vis spectroscopy at wavelengths between 200 and 700 nm.[27]

X-ray Absorption Fine Structure (XAFS)

Numerous scans were collected and signal-averaged for a 1-2 mM Hb sample, equivalent to 4-8 mM heme. Solutions of Hb (6-8 mM heme) were syringed into a 140-µL Lucite cell with Mylar tape windows in an Ar-filled glove bag and frozen in liquid N_2 /*n*-hexane. A low temperature (10 K) was maintained using an Oxford Instruments continuous-flow liquid He CF 1208 Cryostat. The energy was calibrated using an iron (Fe foil) standard (first inflection point, 7111.2 eV). Iron K-edge X-ray absorption spectra (XAS) were recorded at the Stanford Synchrotron Radiation Laboratory (SSRL) on the beamlines 9-3 and 7-3 under dedicated ring conditions (3 GeV, 50-100 mA) using a Si (220) double-crystal monochromator.

We averaged the fluorescence XAS scans by rating the signal-to-noise ratio. The spectra obtained from each of the detector channels were checked and poor data due to noisy detector channels, low detector channel sensitivity, or those affected by step jumps, were removed from the averaged data because they resulted in a quality decrease of the averaged XAS and, hence, the accuracy of the XAFS fit. The background was corrected towards the pre-edge region and the fit was extrapolated into the XAFS region. Monochromator glitches were edited following calibration and subtraction of the background absorbance. For the removal of the underlying absorbance, a spline of three to five regions was fitted to the XAFS region and subtracted (Table 1). The resulting data were normalized to an edge jump of 1.0 and compensated for decreasing absorbance past the edge using a k^3-weighting, so that the final XAFS was relative to the edge (μ_0), and the amplitude enhanced at high values of the photoelectron wave vector k. After the background was subtracted, data were normalized, compensated for k^3, and plotted as a function of k.[28]

For the fits, already published heme models were used including that of *meso*-(2-methylimidazole)[tetraphenyl-porphyrinato(2-)] iron(II) with the imidazole ring of the histidine side chain as a reference. The starting bond lengths and angles were taken from the Protein Data Bank, ID: IGZX (T-oxy-Hb) and IC7B (R-deoxy-Hb). The model-fitting calculations were performed using the XFIT program,[28] which incorporates *ab initio* calculation of the XAFS using MS (version 6.01) of FEFF.

RESULTS

Photodecomposition Effects

To investigate any photodecomposition effects on the samples, the edge shifts from the first scan to the last scan were observed. Due to the negligible photodamage in the edge data of all Hb samples (the edge shifted less than 0.1 eV compared to the first scan), all scans were incorporated into the analyses. The edge positions of the T-forms were higher in energy than the R-forms.

X-Ray Absorption Fine Structure (XAFS)

The parameters of interest: bond lengths between the iron and the four protoporphyrin nitrogens (Fe-N_p); the distance between the iron and the imidazole nitrogen of the proximal histidine (Fe-N^d_ε); whether the iron is located in the plane of the protoporphyrin ring or how far it is located out of the plane; changes in these parameters with changes from R- to T-states of the Hb; the distance between the iron center and the dioxygen in oxy-Hb; and the respective bond angles.

Multiple-Scattering Refinement of the Iron Site

The observed and calculated XAFS, $\chi(k) \times k^3$, the corresponding Fourier transforms, the residuals, $\Delta[\chi(k) \times k^3]$, and the window functions used in the Fourier filter for Normal R and T deoxy-Hb and thalassemic deoxy-Hb are shown in Figure 1. The MS analysis indicates differences in the Fe–N_ε of normal T-deoxy-Hb and of R-deoxy-Hb.

Distances of the Fe Atom from the Porphyrin Plane and the Imidazole Ring

The Fe–N_p distance of the normal R-deoxy-Hb is 2.05 Å. The imidazole of the normal R-deoxy-Hb is closer to the heme plane (0.29 Å) than that in the T-form, which is located further away (0.4 Å). These values are consistent with the predicted changes based on X-ray

Table 1. Spline and refinement parameters

Hemoglobin	Pre-edge Background (eV)	Spline Segments (eV)	S_0^2	R (%)
Normal-R-deoxy-	7094.7 – 7098.7	7130 – 7211.3		
		7211.3 – 7414.2	0.98	13
		7414.2 – 7823.8		
		7823.8 – 8257.4		
Normal-T-deoxy-	7072.35 – 7087.11	7134.2 – 7355.3		
		7355.3 – 7821.4	1.1	17
		7821.4 – 8244.3		
Thalassemic-Hb E/F	7075.2 –7108.8	7135.0 – 7310.3		
		7310.3 – 7381.9	1.15	19-21
		7381.9 – 7623.4		
		7623.4 – 7913.9		
		7913.9 – 8784.5		

Table 1. Legend: Data were collected from samples containing 6-8 mM Fe on the beamlines 9-3 and 7-3 under ring conditions, 3 GeV, 50-100 mA, using a Si (220) double-crystal monochromator and 13- and 30-element Ge array detectors. The XAFS (*k* space) window parameters were varied in the range 1-14 to 1-16 $Å^{-1}$. The (*r* space) window parameters were 0 5.0 and 0.75-5.0 Å for the multiple scattering analysis of normal deoxy-Hb and thalassemic Hb, respectively. The data were fitted according to previous work done with deoxy-leghemoglobin and deoxy-myoglobin at 10 K.[15,16,29]
Supplementary Material. Additional data can be provided on request.

Table 2. XAFS Structures – Fe-Ligand Bond Length

Hemoglobin	Fe-L bond length		Iron out of
	Fe-Np	Fe-N∈	porphyrin plane
Normal R-deoxy	2.05 Å	2.13 Å	0.29 Å
Normal T-deoxy	2.07 Å	1.95 Å	0.40 Å
Thalassemic R-deoxy	2.12 Å	1.87 Å	0.65 Å
	2.10 Å	1.92 Å	
Thalassemic R-oxy	2.01	2.0	-

Table 2. Legend: Distances: Fe-L, Fe-ligand; Fe-N_p, Fe-nitrogen(porphyrin); Fe-N_i, Fe-nitrogen(His-92 imidazole)

crystallography. The thalassemic Fe-N_p distance is longer (average 2.11 Å) than the distances for normal R- and T-deoxyhemoglobin (2.05 and 2.07 ú, respectively). The porphyrin ring in the thalassemic R-deoxy-Hb looks distorted. However, in the R-oxy-Hb oxygenation brings the central iron back into the plane of the porphyrin ring system.

Structural differences between R- and T-deoxy-Hb are minute and are more evident from the values in Table 2 than from the structural models in Figure 2.

The XAFS of thalassemic R-deoxy-HbE/F heme looks distorted with Fe located further out of the plane of the porphyrin ring system than in normal Hb reducing its oxygen affinity. Oxygenation in R-oxy-HbE/F brings the iron back to the plane of the porphyrin ring system (Figure 3).

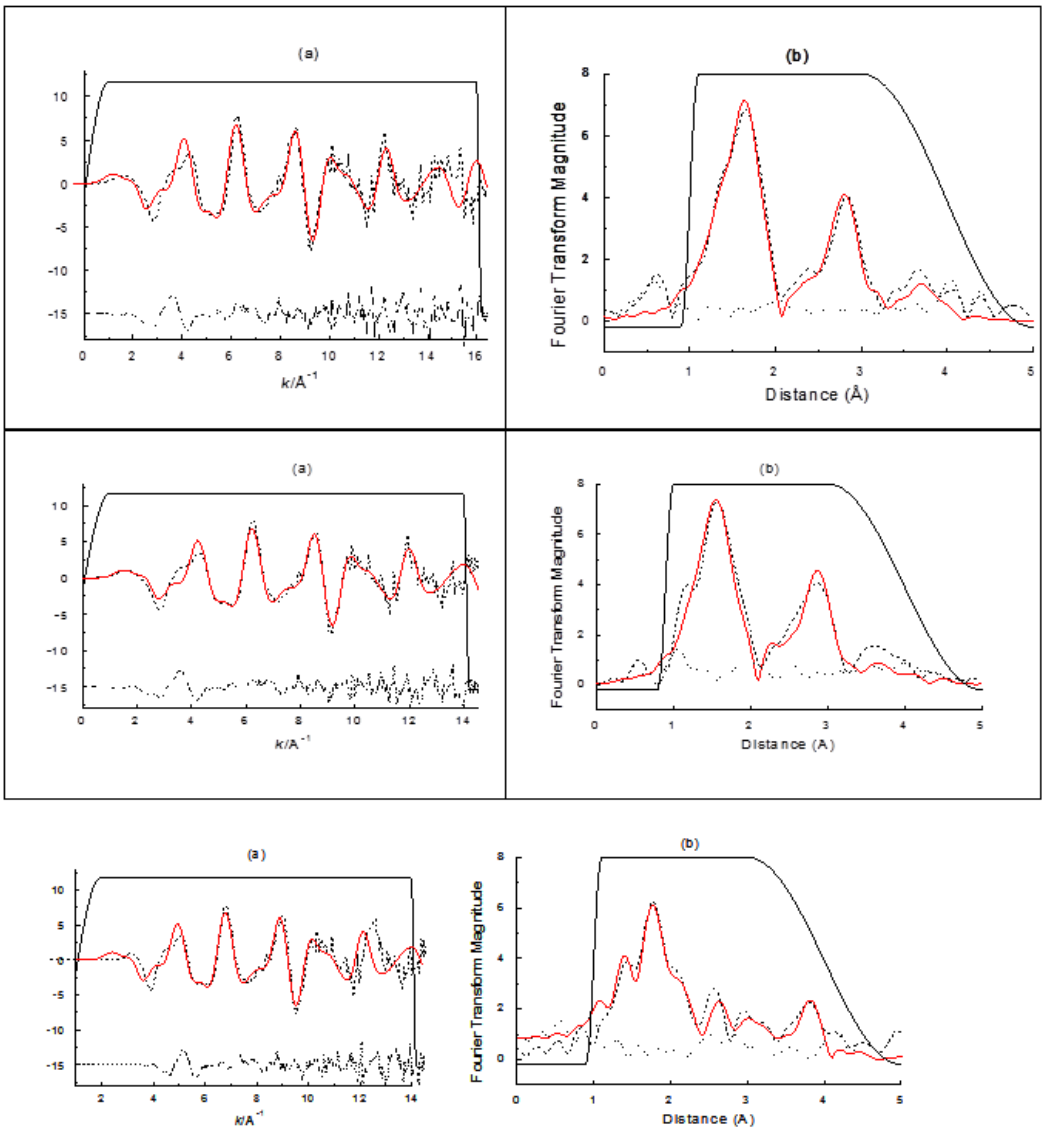

Fig. 1. (a) XAFS curves and (b) Fourier transform; observed (black-dash), calculated (red) and residual (black-dot); of normal R-deoxy-Hb (upper panel), T-deoxy-Hb (middle panel), R-deoxy-HbE/F (bottom)

DISCUSSION

All dioxygen-binding hemeproteins have in common the prosthetic group, iron protoporphyrin IX, the heme group, located in a hydrophobic pocket of the protein. The heme is connected to the globin chain on the proximal side by a covalent bond between the iron atom and an imidazole-nitrogen (N_ε) of the (proximal) histidine residue. Besides this covalent bond, the protoporphyrin is further fixed to the protein chain via hydrophobic forces. Only two hydrophilic residues are present on the heme moiety, i.e., propionates, which possibly interact electrostatically with external peptide residues, in an analogous way to the heme interactions in myoglobin.[30] In all hemoglobins, the four nitrogens of the porphyrin (N_p) and N_i of the proximal His coordinate the central iron: in deoxy-Hb, Fe is five-coordinate and paramagnetic with a spin of $S = 2$.[22,31,32]

In our measurements, the Fe–N_p distance (2.05 Å) of the normal R-deoxy-Hb is 0.03 Å shorter compared to that of the deoxy-Hb investigated by Brucker,[33] but similar to the Fe–N_p distance (2.06 Å) in the myoglobin studied by Rich.[15] The imidazole of the normal R-deoxy-Hb is closer to the heme plane (0.29 Å) than that in the T-form, which is located further away (0.4 Å). These values are consistent with the predicted changes based on X-ray crystallography. The thalassemic Fe-N_p distance is much longer (average

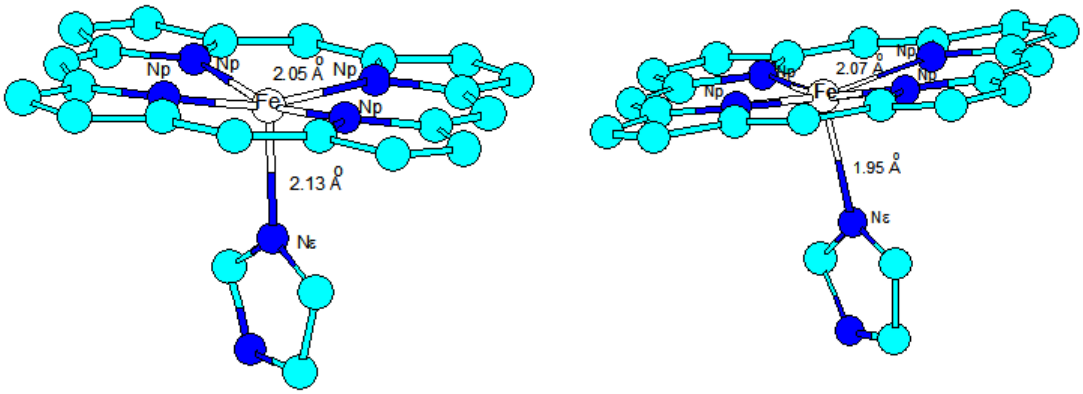

Fig. 2. XAFS-derived molecular structures of the active site of R-deoxyhemoglobin (left) and T-deoxyhemoglobin (right)

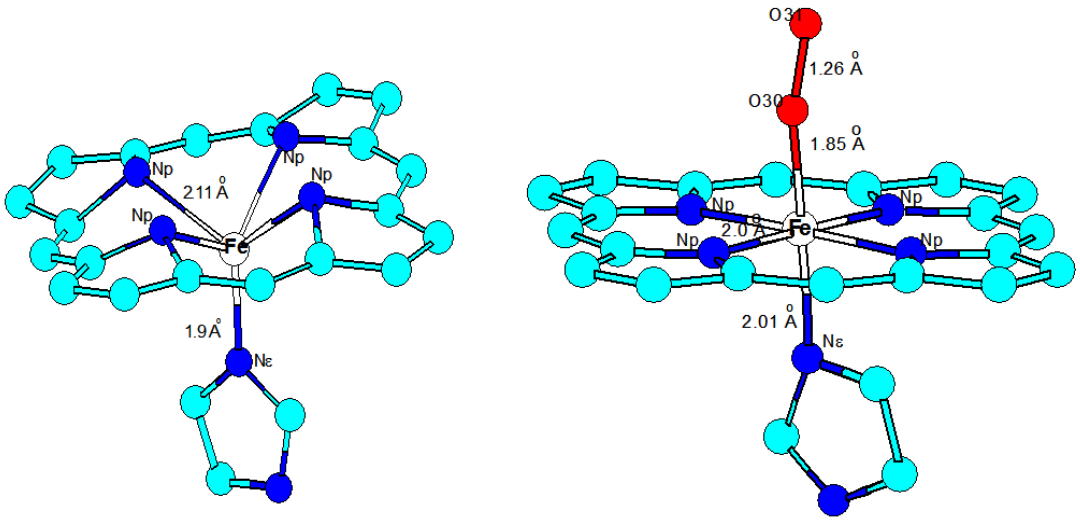

Fig. 3. Molecular structures of thalassemic R-deoxy-HbE/F (left) and thalassemic R-oxy-HbE/F (right)

2.11 Å) than those for normal R- and T-deoxy-Hb (2.05 and 2.07 Å, respectively). The porphyrin ring in the thalassemic deoxy-Hb looks distorted. The extent of the distortion produced in the fit may be overrepresented which may, in part, be due to the mixture of HbF and HbE and the variation of the Fe-N_p distance between 2.10 Å and 2.12 Å.

Normal R- and T-deoxy-Hb

The fact that the iron is out of the heme plane in the normal R-deoxy-Hb is consistent with the theory that the diameter of a high-spin $t_{2g}^4 e_g^2$ Fe(II) ion is larger than that of the central hole in the porphyrin ring.[32] In the oxygenated form, the complex converts to low spin (corresponding to the t_{2g}^4 configuration of Fe(II)), the Fe ion shrinks a little, and moves into plane.[34]

We do not want to go into the controversial views about the nature of R- and T-states of deoxy-Hb as discussed by Park and colleagues,[35] however, we refer to their values of Fe deviation from the heme plane in 1.25 Å resolution crystal structures. The calculations of the authors differentiate between all 24 porphyrin atoms where the iron deviates from the plane by 0.2-0.3 Å and only the four porphyrin nitrogens, where the iron displacement from the plane is 0.3-0.4 Å. These values correlate well with our measurements of 0.29 Å (R-form) and 0.4 Å (T-form). In the T-form of normal deoxy-Hb, the iron is pulled out of the heme plane 0.11 Å further than in the R-form (0.29 Å) towards the proximal His, concomitantly it has lower affinity for dioxygen.[36] The shift from R- to T-quaternary structure is consistent with the bond lengths of Fe-N_p and Fe-N_ε and the $\alpha_1\beta_2$ contacts in crystal structures.[37]

Thalassemic deoxy-Hb and oxy-Hb

The iron in thalassemic R-deoxy-Hb moves even further towards the proximal histidine (0.65 Å) than in the T-form of normal deoxy-Hb. The T-form has lower oxygen affinity than the R-form,[36] accordingly, oxygen affinity of thalassemic R-deoxy-Hb is even much lower. In analogy to normal deoxy-Hb, the iron in the thalassemic T-form should be displaced even further from the plane of the porphyrin. Apparently, the thalassemic Hb does not only exert lower oxygen affinity but also lower molecular stability than normal deoxy-Hb. We managed to produce sufficiently stable probes for XAFS measurements from normal deoxy-Hb both in the R- and T-forms, but only from the R-form of thalassemic deoxy-Hb. X-ray crystallography with normal Hb and HbS indicated that hydrophobic amino acids Phe-β85 and Leu-β88 stabilize the globin structure, Hb assembly, and heme binding.[38] HbS variants containing Glu in one of these positions, or in both, destabilize the pocket structure, but it is uncertain as how this relates to the thalassemic mutants. Besides genetically caused structural destabilization[39] oxidative stress by iron overload[25] induces radical formation and additional destabilization of thalassemic hemoglobins.[40]

In their study, Rees and colleagues[41] suggest that HbE itself is not unstable at normal body temperature, but may become unstable under oxidative stress at increased, febrile temperatures. Oxidative stress leads to the formation of free radicals within the globin chains including higher oxidative iron states and initiates a pathophysiological cascade of hemoglobin destabilization and damage of the erythrocyte membrane.[42,43] HbE itself presents a rather mild clinical picture but the combination HbE/ß-thal can cause severe problems.[44] In HbE/ß-thalassemia two thalassemia alleles interact in varying degree of globin chain imbalance, from which the marked variability in severity between different individuals with HbE/ß-thalassemia may principally result. The thalassemic Hb in our study contains 33% of HbF. Hirsch and colleagues[42] stated that high levels of HbF are associated with more moderate clinical symptoms. In Hb E/ß-thal HbF levels have been reported to vary from 10% to 80%.[45]

Strader and his working group[46] conclude that the occurrence of free α-subunits increases the oxidative instability of HbE. Samuel and colleagues[47] investigated the mechanism and intermediates of the disassembly and unfolding of hemoglobins which pathway may further contribute to instability.

CONCLUSION

On the one hand, we managed to show the X-ray absorption fine structures of thalassemic R-deoxy-HbE/F and R-oxy-HbE/F. On the other hand, our probes from T-deoxy-HbE/F were not sufficiently stable for XAFS measurements. We used one form of pathological Hb with a defined

composition of 2/3 HbE and 1/3 HbF, exemplary and representative of thalassemic hemoglobins. However, there are several hundreds of mutations and deletions both in α- or β-thalassemias. Each of these mutations is likely to have its unique structural modification of the heme pocket of their respective Hb. There are also various combinations with HbE, for example, a rare case of Hb Constant Spring and HbE was recently reported from China.[48] Hence, our study is a start and further investigations should be done with other forms of thalassemic Hb for comparison and to obtain a more comprehensive knowledge of thalassemic heme fine structure.

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Conflict of Interest

The author(s) do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

The study was approved by the Ethical Clearance Committee of the Faculty of Medicine, University of Indonesia and hemolysate samples were transported on dry ice from Jakarta to Sydney, Australia (AQIS permit No 200012081).

Informed Consent Statement

Informed Consent as stated in Methods and Materials – Sample Preparation.

Clinical Trial Registration

This research does not involve any clinical trials

Author Contributions

SKUF: experimental laboratory work, data collection and analysis, visualization, writing-original draft, approval of the final version; SKUF conducted this study as part of her dissertation at the University of Sydney; HJF: conceptualization, methodology, blood sampling in Indonesia and transport to Australia, writing-review, approval of the final version, supervision, project administration; HJF designed the study concept for the collaboration between the Study Program Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, and the Chemistry Department, University of Sydney.

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