Significantly Enhanced Heme Retention Ability of Myoglobin Engineered to Mimic the Third Covalent Linkage by Non-Axial Histidine to Heme (Vinyl) in *Synechocystis* Hemoglobin

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*Running Title: Ile107His prevents heme dissociation in myoglobin

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**Background:** Unprecedented stability of *SynHb* may be engineered in other globins.

**Results:** Myoglobin can mimic the covalent linkage between His117 and heme vinyl in *SynHb*, which dictates stability as expected.

**Conclusions:** Ile107His mutation in myoglobin significantly enhanced heme retention ability.

**Significance:** The additional covalent linkage engineered in myoglobin provides a novel evolutionary perspective and may help in the design of stable hemoglobin based blood substitute.

**Abstract**

Heme proteins, which reversibly bind oxygen and display a particular fold originally identified in myoglobin (Mb), characterize the “hemoglobin (Hb) superfamily”. The long known and widely investigated Hb superfamily, however, has been enriched by the discovery and investigation of new classes and members. Truncated Hbs typify such novel classes and exhibit a distinct 2-on-2 α–helical fold. The truncated Hb (*SynHb*) from the freshwater cyanobacterium *Synechocystis* exhibits hexacoordinate heme chemistry and bears an unusual covalent bond between the non-axial His 117 and a heme porphyrin 2-vinyl atom, which remains tightly associated with the globin unlike any other. It seems to be the most stable Hb known to date and His117 is the dominant force holding the heme. Mutations of amino acid residues in the vicinity did not influence this covalent linkage. Introduction of a non-axial “His” into sperm whale Mb at the topologically equivalent position and in close proximity to vinyl group significantly increased the heme stability of this prototype globin. Reversed phase chromatography, ESI-MS and MALDI TOF analyses confirmed the presence of covalent linkage in Mb Ile107His. The Mb mutant with the engineered covalent linkage was stable to denaturants and exhibited ligand binding and autooxidation rates similar to the wild type protein. This indeed is a novel finding and provides a new perspective to the evolution of Hbs. The successful attempt at engineering heme stability holds promise for the production of stable Hb based blood substitute.

**Introduction**

*Synechocystis* hemoglobin (*SynHb*) is a unique globin found in the non-nitrogen fixing unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (1-3). *SynHb* is “hexacoordinated” since His46 (distal) and His70 (proximal) function as internal
heme iron axial ligands (4) (Fig. 1A). This globin is unique among the Hbs discovered so far because of its several characteristic features not observed in any other Hb. It has ~120 amino acid residues in its polypeptide chain (truncated hemoglobin, trHb) as opposed to ~150 usually found in classical globins (5). It displays a 2-on-2 α-helical globin fold as opposed to the classical 3-on-3 α-helical fold. SynHb displays a unusual coordinate-covalent linkage between His117 and heme 2-vinyl group (6,7) (Fig. 1A). This post-translational modification provided by His117 is believed to confer stable association with heme, which remains tightly associated with the globin unlike in any other Hb (7). This is among the first hexacoordinate Hb (neuroglobin and cytoglobin being the other two) for which crystal structure is available in both ligand free and ligand bound states, with the latter showing unprecedented conformational changes and the former showing an absence of the generic “tunnel” in protein matrix (4,8). It has been almost 15 years since its discovery and in spite of several efforts, physiological function is yet to be assigned for this globin with certainty (9-11).

A large number of globins have now been discovered across all the life forms (12-14). Many of them displayed novel characteristics not seen in the classical globins like myoglobin (Mb). Literature search revealed that folding and stability of Hbs were not investigated for diverse globins (15,16). Most of the contributions relate to Mb and human Hb (17-23). Mb was extensively studied to identify the key residues which play an important role in stability and the knowledge may or may not be applicable to new globins (20,24,25), while SynHb is naturally a very stable Hb (7,26,27). It is known that the unusual third His (His117) in SynHb provides heme stability due to its covalent linkage to heme vinyl group, which is believed to be a post-translational modification (7). However, the influence of other residues in the heme pocket or elsewhere on such modification or heme stability has not been investigated in detail by mutational analysis. This prompted us to investigate SynHb, which could provide an excellent reference system for stable Hbs due to its novel properties.

Thus, we re-visited the post-translational modification to ascertain the extent of its role in the stability of SynHb. General knowledge about Hbs from investigations over last several decades as well as the existing studies on ligand binding kinetics of SynHb indicated some key residues in its heme pocket to be important. These residues were also targeted for the stability investigation (Fig. 1A). In addition, it was important to verify whether the unusual heme covalent linkage can be introduced in other globins, providing a novel insight as to why only this globin (and one from its related species Synechococcus (28)) displays such a unique bond. Engineering the covalent linkage to heme in other globins might also allow a new strategy to enhance heme stability. Such engineering of a covalent linkage has been attempted before but only in SynHb without the third linkage (His117Ala) for a different purpose at a different location and not in the classical globins (29).

Mb is invariably used as a prototype for the commercial development of extracellular Hb as a blood substitute (30,31). This monomeric, small, relatively stable globin is widely investigated and understood well to serve as a paradigm for investigation into the possibility of enhancement of heme and polypeptide stability using protein engineering approaches. Structure-based rational approaches and comparative mutagenesis was thus employed in the present investigation to enhance heme stability in sperm whale Mb. This investigation holds promise for engineering heme stability in recombinant hemoglobin based oxygen carriers (rHBOCs) for potential use as an artificial blood substitute.

**Experimental Procedures**

**Site directed mutagenesis**

Site directed mutagenesis was carried out by using the QuikChange Site-Directed mutagenesis kit (Agilent Technologies Inc., California, USA) and mutagenic primers as per the manufacturer’s protocol. The plasmids pET28a.synhb (5.7kbp) and pET28a.mb (5.9kbp) containing the genes encoding SynHb and sperm whale Mb (kind gifts from Prof. Mark Hargrove, Iowa State University, USA), were used as the template for mutagenesis,
respectively. The mutations were confirmed by gene sequencing.

**Expression of recombinant proteins**

*E. coli* BL21 (λDE3) cells (Invitrogen, California, USA) were transformed with plasmid vectors containing the genes for wild type or mutants under investigation and expressed in terrific broth (TB) media. For the *Syn*Hb wild type and mutant proteins, transformed competent cells were grown at 37 °C at 200 rpm in baffled flasks. Once O.D$_{600}$ nm reached 0.8, temperature was changed to 25 °C without IPTG addition and expression was continued for further 14 h. For the wild type Mb, expression was performed at 37 °C at 150 rpm for 14 h. MbI107H was expressed following the same procedure as *Syn*Hb but at 30 °C. Mb F138H, L72H and I111H, however, required a temperature of 25 °C and IPTG (0.5 mM) induction.

**Purification of recombinant proteins**

Purification of His tagged wild type and mutant proteins were performed using Ni-Sepharose (GE Healthcare, United Kingdom) affinity chromatography. Protein was further purified by DEAE-Sephadex anion exchange (GE Healthcare, United Kingdom) and S-200 Sephacryl (GE Healthcare, United Kingdom) based size exclusion chromatography. An absorbance ratio (A$_{Soret}$/A$_{280}$ nm) of 3.5 and higher indicated purity, which was also confirmed by SDS-PAGE. The purified recombinant protein was then subjected to reduction and oxidation by sodium dithionite and potassium ferricyanide, respectively, following desalting on a G-25 Sephadex (GE Healthcare, United Kingdom) column. Pure protein was stored at -80 °C till further use.

**Heme extraction**

Heme was extracted from the globins using Teale’s method (32). In brief, the pH of the sample protein was lowered to about 2.0 using ice-chilled 0.1 M HCl followed by mixing with an equal volume of cold butanone to separate the two phases. In general, heme partitions in the top organic phase and the resulting apoglobin in the bottom aqueous phase.

**Stability studies**

For pH-dependent stability studies, buffers were prepared in the pH range 2.0-11.0 as follows: 100 mM glycine-HCl for pH 2-3.5; 100 mM sodium acetate for pH 4.0 and 4.5; 100 mM potassium-phosphate for pH 5.0-8.0; 100 mM borate-boric acid or sodium hydroxide for pH 8.5-11.0. The relevant pure protein was diluted in a buffer of desired pH and incubated for 3-4 h at 25 °C prior to spectroscopic measurements. For GdmCl stability studies, pure protein was diluted in different concentrations of GdmCl (0-6.0 M) at pH 7.0 and incubated for 3-4 h. Thermal stability was also monitored using spectroscopic techniques in the range of 25-95 °C at pH 7.0 with 10 min incubation at each temperature. The protein concentration used for stability studies was ~0.15 mg/ml.

**UV-visible absorbance spectroscopy**

Absorbance measurements in the range from 260-700 nm were carried out using Cary Varian Bio100 UV-Vis spectrophotometer (Varian Inc., California, USA). Quartz cuvettes were used for the measurements.

**Circular dichroism spectroscopy**

CD spectra were recorded on a JASCO J-815 spectropolarimeter (JASCO Corporation, Tokyo, Japan) using a cylindrical quartz cell of path length 1 mm. Changes in the secondary structure of the protein were monitored in the far-UV region between 190 and 260 nm. Three consecutive spectral scans were averaged and corrected by subtracting corresponding blanks.

**Differential scanning calorimetry (DSC)**

For DSC experiments, the protein and the buffer solutions were degassed in a Thermovac degassing station for 15 min prior to loading into the DSC cells (Model VP-DSC from Microcal, LLC, Northampton, USA). The instrument was calibrated with the temperature standards provided by the company. The protein concentration used for DSC experiments was 0.12 mM at pH 7.0. A scan rate of 1 °C min$^{-1}$ was used, and the data were acquired using Origin™ software. The control buffer baselines were acquired and subtracted from the protein thermal denaturation data. The data were further analyzed using the
Ultra-performance liquid chromatography

Samples were analyzed by injecting 50µg of protein onto a 2.1 mm X 100 mm BEH C18 reversed phase column on a Waters Acquity UPLC instrument (Waters, Milford, MA). The protein was eluted using a linear gradient of solvent from 35 to 65% acetonitrile in water (containing 0.1% trifluoroacetic acid) over 25 min with detection at 280 and 409 nm. As a control, a stock solution of free heme was prepared by dissolving 5 mg in 100 µl of 0.1 M NaOH, followed by addition of 900 µl of 0.1 M potassium phosphate pH 7.0 and filtration with a 0.22 µm filter. Stock of free heme aliquot was diluted into 0.1% trifluoroacetic acid for analysis.

Mass spectrometry (MS)

Intact masses of Mb and MbI107H mutant were determined by an ESI based MS. Samples were desalted using C18 ZipTip and eluted with 50% acetonitrile in 0.1% trifluoroacetic acid. The positive-ion mass spectra were acquired on a Micromass LCT ESI-TOF mass spectrometer (+ESI-TOF MS, LCT, Micromass, UK) to obtain the final average mass of target protein. In-gel trypsin digestion of the proteins was performed following standard protocols (33) prior to peptide mass analysis using MALDI TOF/TOF. The peptide extract obtained from the gel spots were reconstituted in 10 µl of 50% acetonitrile and 0.1% trifluoroacetic acid. Reconstituted peptides were spotted onto 384 well MALDI plate after mixing with α-cyano-4-hydroxy cinnamic acid matrix in 1:1 ratio. The peptides were analyzed on AB Sciex 4800 Plus TOF/TOF analyzer in the reflector mode. The resulting spectra were analysed using Mascot (Matrix Science version 2.2) database search.

Electron paramagnetic resonance spectroscopy

EPR spectra were measured to determine the coordination state of Mb wild type and mutant proteins. The sample concentration used was 1.8 mM in 100 mM potassium phosphate buffer containing 100 mM NaCl, pH 7.0. The EPR spectra were recorded on a JEOL instrument, JES FA200 Spectrophotometer (Japan). The data were collected at 77 K in a liquid N2 finger Dewar. Power at the sample was 4 mW, the microwave frequency was 9.2 GHz and the center field was 220 mT. The EPR signal was observed at g = 6 (low field), indicating the presence of an axial high spin s = 5/2 species, as well as in the g = 2 region (high field) indicating the presence of a low spin s = ½ Fe (+3) centre.

Kinetic measurements

Kinetic measurements were performed using the stopped-flow and laser flash photolysis based spectroscopic methods as described by Olson and Quillin et al (34,35) and detailed by Kundu et al (36). The stopped-flow measurements for O2 dissociation rates and CO association rates of Mb and its mutant proteins were performed at 25 °C using SFM400 module equipped with four syringes in association with MPS70 syringe controller and MOSS0 spectrophotometer from BioLogic Science Instruments (Bio-Logic SAS, Claix, France). Laser flash photolysis was used for measurement of the O2 association rate constant for Mb and its mutants as described elsewhere (36-38). Oxygenated protein samples collected directly into gas tight syringes from desalting column were transferred to 3 ml quartz cuvette with 1 cm pathlength sealed with rubber septum. The O2-bound globin was subjected to flash photolysis using LKS.60 flash system (Applied Photophysics Ltd., Leatherhead, Surrey, United Kingdom) containing Nd-YAG pulsed laser at 1064 nm, frequency doubled to 532 nm of energy 10 or 20 Hz. White light from Xe lamp was used to probe the samples. The kinetic traces were followed by measuring changes in the Soret peak wavelength either for formation of oxy-bound globin or depletion of deoxy globin to obtain the O2 association rate constants.

Autooxidation measurements

Autooxidation rates were measured by monitoring the absorbance changes at 581 nm, as described by Hargrove et al (39). Briefly, 200 µl of 0.1 mM oxy-protein was diluted directly into about 1 ml of 100 mM potassium phosphate (pH 7.0) containing 1 mM EDTA and 3 mmole/mol of heme catalase and superoxide dismutase. The oxidation of the sample was followed by recording the entire visible spectra as well as monitoring the
decrease in absorbance at a single wavelength, usually at 581 nm, in scanning kinetics mode using Cary Varian 100 UV-vis spectrophotometer. Curve fitting to a single exponential equation was accomplished using the program Igor Pro (Wavemetrics Inc., USA) and the corresponding rates reported.

**Measurement of heme dissociation rates**

The heme dissociation rates were measured as described by Hargrove *et al* (40). Briefly, the transfer of hemin from 3 μM metmyoglobin and mutant proteins (holo) to 30 μM H64Y/V68F apomyoglobin was measured by following the entire visible spectra as well as monitoring the absorbance decrease at 410 nm as “green” H64Y/V68F holo-metmyoglobin was formed. The experiment was carried out in the presence of 0.15 M potassium phosphate buffer, pH 7.0 containing 0.45 M sucrose at 25 °C. Curve fitting to a single exponential equation was accomplished using the program Igor Pro and the rate constants were measured accordingly.

**Results**

**His117 is the sole determinant of heme retention ability of SynHb while other key residues do not influence the covalent linkage**

The functional and structural properties of the heme group are markedly affected by the amino acids in its vicinity. The influence of some key amino acid side chains in the heme pocket of SynHb on its stability and post-translational modification was thus investigated by mutational analysis (Fig. 1A). The most unique feature of this cyanobacterial Hb is the presence of an unusual third “His” (His117 or H16) which is covalently linked to the heme vinyl atom (6,7). This side chain, implicated in SynHb stability (7), was investigated here as well for reference. Prior to any attempt to engineer this covalent linkage in other globins, it was important to verify whether this post-translational modification is influenced by other amino acid residues in the vicinity of heme. SynHb displays “three His linkages” which includes distal His46 (E10), proximal His70 (F8), and the non-axial His117 (H16). Including these three, a total of six key residues surrounding the heme pocket and their combinations were selected for mutational studies (Fig. 1A). All these residues were known to be important for structure, ligand binding and other properties in SynHb (8,27,41-45). The bright red holo-proteins were successfully expressed and purified. The globins exhibited absorbance spectra typical of hexacoordinate Hbs, except H46L and H46LH117A, which were typical of pentacoordinate globins, indicating that H46L is indeed responsible for endogenous coordination to the heme iron (data not shown).

SynHb mutants were assessed for their ability to retain heme by utilizing a simple method of heme extraction by low pH and organic solvent (32). If heme dissociated from a globin, it partitioned to the upper organic layer providing a distinct red colour to it while the soluble apoprotein partitioned to the colourless aqueous layer (Fig. 1B). However, if heme was tightly held into the protein matrix, the upper organic layer was colourless, but the lower aqueous layer appeared red (Fig. 1B). Vertebrate Hb (bovine), used as a positive control, released heme in the red organic layer (top) and the apoprotein (without heme) remained in the colourless aqueous layer (bottom), as expected (vial 1, Fig. 1B). Same was true for Mb (vial 10, Fig. 1B) and several other Hbs tested in our laboratory (data not shown). As also shown previously (26), wild type SynHb was able to retain heme inside the protein matrix such that the top organic layer was colourless but the bottom aqueous layer was red (vial 2, Fig. 1B). This is a unique property of SynHb since all other globins known to date, release heme under such experimental conditions. The mutant proteins behaved similarly to wtSynHb, except two (vials 3-6, 8, Fig. 1B). The two exceptions, SynH117A and SynH46LH117A, behaved similarly to the control Hb and other globins and released heme readily in the top organic layer (vials 7 and 9, Fig. 1B). This clearly indicated that His117 is essential for heme retention in SynHb and the heme cannot be extracted from wtSynHb since it is tightly held by His117. Why SynHb specifically needs to retain heme so rigidly and why this is the only globin with such exceptional property, especially from an evolutionary perspective, is an open question. The data also showed that no other amino acids influence the covalent linkage by His117.
**His117 prevents denaturant induced heme dissociation in SynHb**

The above qualitative assay of the influence of heme pocket amino acid side chains on heme retention ability was confirmed further by spectroscopic and calorimetric investigations against denaturants (Fig. 1C-F). It is known that if a denaturant destabilizes a Hb, the characteristic Soret peak undergoes a blue shift in wavelength along with a significant concomitant decrease in absorbance intensity (46). Complete heme dissociation from a globin results in a broad visible absorbance peak typical of free heme with Soret wavelength maximum as low as ~350-370 nm and significant reduction in intensity (47). The changes in the Soret wavelength maxima of SynHb and its mutants were monitored with the change in pH (2.0-11.0). For wild type SynHb and all mutants except SynH117A and SynH46LH117A, it was observed that the Soret wavelength maxima (λ_soret) was independent of pH between 3.0 and 11.0, indicating that this cyanobacterial globin was highly stable between this pH range (Fig. 1C). Below pH 3.0, SynHb and mutants (except two) showed blue shift in λ_soret to ~395 nm suggesting that the heme was not dissociated from the protein matrix (Fig. 1C). The two mutants which were exceptions were less stable and at low pH their Soret wavelength was much more blue-shifted to about ~370 nm indicating heme dissociation. Absorbance spectra of SynH117A and SynH46LH117A at acidic pH (<3.0) were similar to the spectra of free heme, indicating complete heme dissociation (Fig. 1D). These findings were in agreement with those of the heme dissociation assay above indicating the importance of H117 in SynHb stability. The holo-mutants of SynHb displayed similar content of secondary structure as measured by far UV-CD (data not shown).

GdmCl, a strong denaturant, caused a similar effect as above at pH 7.0. Absorbance spectroscopic investigation showed that SynHb and all the other mutants, except SynH117A and SynH46LH117A, were stable upto 3.0 M GdmCl as evidenced by the absence of any major change in the Soret wavelength corresponding to 409 nm up to this concentration (Fig. 1E). At higher GdmCl concentrations, only the above two mutants showed significant blue shift in the Soret wavelength to about ~370 nm as seen for free heme or classical Hbs indicating heme dissociation (Fig. 1E). Thus, SynH117A and SynH46LH117A denatured completely releasing heme.

Differential scanning calorimetry (DSC) was performed to further validate the above results (Fig. 1F). It was observed that SynHb displayed apparent T_m corresponding to 96.5 °C (Table 1), not reported for any other Hb, and was significantly more stable than the classical Mb employed as a reference. The DSC data and apparent T_m's for other mutant proteins emphasized that His117 play significant role in SynHb thermal stability since the corresponding mutant substantially lowered the apparent T_m of SynHb (Fig. 1F, Table 1). All the above experiments suggested that H117 independently play substantial role in heme retention ability of SynHb.

**Four amino acid side chains in Mb might potentially mimic the role of His117 in SynHb**

Mb was used as the prototype for engineering a covalent linkage to heme vinyl group to mimic SynHb (H117) since it is a model protein and a common practice to test mutations on this paradigm globin before they are applied successfully to human Hb or other globins. It was evident that the amino acid side chain His117 was the predominant factor that dictated the stability of SynHb and a successful mimic in Mb also had the possibility of enhancing Mb stability.

*In silico* analysis was performed to identify the potential residues which could be mutated to His in sperm whale Mb to introduce the new covalent linkage. Sequence alignment of SynHb and Mb, using ClustalW server (48), revealed that Phe138 of Mb aligned with His117 of SynHb. Sequence equivalent position, Phe138, was thus targeted for substitution to His in Mb. The topologically equivalent position to His117 of SynHb in Mb was identified by structural analysis. It was seen in the crystal structure of SynHb that His117 was covalently linked to heme vinyl CAB atom. Using Swiss-PDB viewer (49), potential residues that are in the vicinity of heme 217CAB atom of Mb were identified at different distances (Fig. 2B). Upto 3 Å, no residues were observed in the vicinity of heme 217 CAB atom. At a distance
of 4 Å, only one residue was observed - Ile at 107th position. At a distance of 5 Å, four residues were seen: Val68, Leu104, Phe138, and Ile107. At a distance of 6 Å, seven residues were observed: Val68, Leu89, Leu104, Ile111, Ile107, Phe138 and Leu72. No attempts were made beyond 6 Å since the desired residues may not come in close contact with heme vinyl. Out of these residues, Val68, Leu89 and Leu104 were not mutated as they were shown to be involved in “water proofing” of the heme pocket (21). Mutations of these residues are expected to affect the hydrophobic environment that holds the heme inside the protein matrix resulting in rapid heme dissociation. Thus, the putative residues selected for mutation were Ile107, Leu72, Ile111, and Phe138. These four residues were mutated to His in silico using the crystal structure of sperm whale Mb (PDB ID: 5MBN) and the least distance of these residues from heme217 CAB atom was found to be lowest for MbI107H. Structure of SynHb and MbI107H were superposed (Fig. 2C) on each other using SuperPose server (50) to display the location of heme covalent linkage and it was found that the distance between His117 and heme vinyl atom in SynHb (2.06 Å) was comparable with the distance between His of MbI107H and heme vinyl atom (2.54 Å).

**Mb mutant proteins shared overall structural similarities to wild type globin**

Attempts were made to express in *E. coli* the four mutants targeted for further investigation. Only MbF138H and MbI107H were used further as they were soluble and could be purified easily. The other two mutants were insoluble and probably experienced stability and folding issues, characteristics not desirable for the current investigation. Appropriate absorbance spectra of the soluble mutants in presence and absence of ligands (CO, O2) indicated that they folded properly and contained a functional heme moiety (data not shown). Absorbance spectra of Mbwt, I107H and F138H mutant proteins showed the Soret peak at 409 nm, 409 nm and 408 nm, respectively (Fig. 3A). The Q bands for the three proteins were also similar. The identical Soret and charge-transfer spectra indicate that the introduction of His at specified positions in Mb did not alter the heme-polypeptide interaction or electronic configuration in the heme pocket. CD spectral profiles (Fig. 3B) showed that wtMb and mutant proteins were α-helical proteins with double negative minima at 208 nm and 222 nm. The mutants had similar mean residue ellipticities as wild type globin, indicating that all three proteins had similar fold and secondary structural arrangement.

**MbI107H displayed covalent linkage between His and heme vinyl group with enhanced heme retention ability**

Mb mutants were assessed for their heme retention abilities, in comparison to SynHb, using the heme extraction assay (32) described above. It was observed that wtMb and MbF138H readily lose heme in organic layer typical of globins whereas the mutant MbI107H was able to retain the heme just like SynHb (Fig. 3C). Heme remained within the protein matrix in bottom aqueous layer (red) for MbI107H reminiscent of SynHb (Fig. 3C), indicating that His at 107th position in Mb was able to form a covalent linkage with heme vinyl group leading to high heme affinity. This indeed is a very successful attempt at engineering heme stability and a novel finding for the field of hemoglobins in general.

The covalent attachment of heme moiety to the protein matrix was assessed by reversed phase UPLC experiments. Free heme eluted at ~8.2 min as probed by absorbance measurement of the Soret peak at 409 nm (Fig. 3D; black line). It was observed that almost the entire heme moiety from Mb wild type dissociated and eluted as free heme (Fig. 3D; red line; ~8.2 min), as also evidenced by lack of any protein peak when monitored at 409 nm. The resulting apomyoglobin eluted at ~21 min as indicated by the measurement at 280 nm (Fig. 3D; inset). Contrary to Mb wild type, for MbI107H mutant both the heme (measured at 409 nm; Fig. 3D; blue line) and the protein (measured at 280 nm; Fig. 3D; inset) co-eluted at ~18.4 min. Such co-elution was indicative of covalent heme attachment and the absence of free heme. It was, however, observed that a minor fraction (~10-15%) of MbI107H released heme under the experimental conditions, unlike in above experiment, whereas the major fraction remained associated covalently with the protein matrix.
Mass spectrometry (MS) provided further evidence that the heme prosthetic group in MbI107H was covalently attached to the polypeptide. ESI-MS spectrum of wtMb returned a molecular mass of 17552.76 ± 0.83 Da (Fig. 4A) which was the mass expected for apoMb (without heme). MbI107H mutant protein, on the contrary, revealed an average molecular mass of 18238.27 ± 0.97 Da (Fig. 4B) as expected for the covalently bound heme-protein. No traces of apoglobin were observed in the ESI-MS spectrum of the mutant indicating ~100% covalent linkage. The trypsin digested Mb and MbI107H mutant proteins unfortunately did not reveal masses that could be assigned to peptides including the 107th position with or without the heme moiety when analyzed by ESI-MS. Absence of few relevant peptides in MS experiments of tryptic digests have been reported for heme proteins before (51). Therefore, MALDI-TOF MS analysis of trypsin digested products of Mb wild type and mutant protein was performed. The relevant peptides, were however, very low in abundance and their masses needed to be minutely searched for. MALDI-TOF mass spectrum of holo-Mb showed a peak at m/z = 1927.118 (Fig. 4C; inset) corresponding to the expected mass of the peptide YLEFi107SEAIIHVLHSR. The MALDI-TOF mass spectrum of MbI107H (Fig. 4D) displayed a peak at m/z = 2560.109 (Fig. 4D; right inset) corresponding to the expected mass of peptide YLEFi107SEAIIHVLHSR covalently bound to the heme through a His-vinyl link. However, an additional peak was also observed at m/z = 1951.068 in the MALDI-TOF spectrum of MbI107H (Fig. 4D; left inset) which corresponds to the mass of the His(107) containing peptide without heme attachment. The heme-free peptide could arise either due to a fraction of MbI107H not undergoing covalent linkage or the heme attachment undergoing dissociation owing to the experimental conditions. Laser induced dissociation of heme from covalently linked globins and peptides have been reported previously (52). Nevertheless, it is evident that the major population of polypeptide (~90%) in MbI107H undoubtedly underwent covalent linkage with heme.

MbI107H exhibited stability similar to or slightly higher than Mb against denaturants

The structural and spectroscopic properties of the Mb mutants against denaturants were investigated in relation to SynHb and wtMb to verify the influence of the engineered covalent linkage on protein stability necessary for their biotechnological applications.

pH stability studies. The change in Soret peak wavelength was monitored in response to the change in pH and the comparative study clearly showed that MbI107H behaved similar to wtSynHb in that the blue shift in $\lambda_{soret}$ at low pH was only minimal, while both wtMb and MbF138H showed significant blue shift (Fig. 5A). This indicated higher heme stability for the MbI107H mutant protein. The complete spectra of the proteins at pH 7.0 and pH 2.0 revealed that wtMb at pH 2.0 displayed characteristic Soret peak (~373 nm) which is similar to free heme (Fig. 5B). MbI107H (Fig. 5C), on the other hand, displayed a characteristic Soret peak (395 nm) at pH 2 which is similar to SynHb (Fig. 5D) at the same pH, clearly indicating that there was retention of heme at lower pH for this mutant. Far UV-CD spectral changes revealed equivalent loss of secondary structure for both wtMb and MbI107H at lower pH (Fig. 5E) implying that although MbI107H has unique heme retention ability, the polypeptide for the mutant had stability similar to wtMb.

GdmCl stability studies. The absorbance measurements as above indicated that heme remained associated inside the protein matrix even at 6.0 M GdmCl for MbI107H similar to SynHb (Fig. 1E), as evident by the minimal blue shift in Soret peak wavelength (to about ~400 nm) (Fig. 6A). In contrast, MbF138H and wtMb showed significant blue shift in the Soret peak wavelength from 408 nm to ~373 nm indicating heme loss (Fig. 6A). CD spectroscopic investigation showed decrease in ellipticity at 222 nm with increase in GdmCl concentration for all three globins (Fig. 6B), indicative of loss of $\alpha$-helicity. The secondary structure of MbI107H had similar stability as Mb wild type while MbF138H was least stable. However, both the mutants showed denaturation over a broad range of GdmCl concentration, as opposed to steep denaturation for Mbwt,
indicating low cooperativity of unfolding in the mutants and a possible deviation from two-state denaturation.

**Thermal stability studies.** Thermal stability investigated by absorbance spectroscopy indicated results as above with similar stability for wtMb and MbI107H (data not shown). CD spectroscopic investigation also showed that MbI107H was similar to wtMb with slightly better stability at higher temperatures, while MbF138H was much less thermostable (Fig. 7A). However, it was interesting to note that the unfolding curves for MbI107H and MbF138H showed slow phase transitions at higher and lower temperature, respectively, indicating the presence of putative intermediates in the thermal unfolding (Fig. 7A). Moreover, even at 90 °C the globins had residual secondary structure (θ(222 nm)≈-7.9 mdeg) unlike that for GdmCl denaturation.

This finding was further confirmed by DSC measurements (Fig. 7B). Mb displayed much lower apparent T_m (~77.34 °C) compared to SynHbwt (~96.53 °C) (Fig. 7B, inset table). Both Mb and SynHb also exhibited sharp thermograms with single peaks corresponding to single apparent T_m. Surprisingly, however, MbI107H exhibited two peaks - one broad and the other sharp - in its DSC profile. The broad peak showed an apparent T_m about 8 °C lower than Mbw but the second sharp peak showed an equivalent higher apparent T_m than Mbwt (Fig. 7B). The reason for biphasic thermal denaturation of MbI107H needs further investigation, with potential to identify novel unfolding intermediates. However, it is safe to assume that overall MbI107H had stability at least similar or even higher than wtMb due to the engineered His at position 107.

**His107 substitution did not influence the high spin state of heme iron in Mb**

Introduction of “His” in Mb at a specific location increased the heme stability significantly and polypeptide stability mildly. It needed to be verified whether the extra “His” in Mb induced some conformational changes in the protein pulling down “distal His (E7)” close to the Fe atom of heme resulting in a hexacoordinated heme chemistry. Mb is pentacoordinate in both the ferrous and ferric oxidation states, also readily evident from its electronic absorbance spectra (Fig. 8A). The absorbance spectrum of MbI107H (Fig. 8B) showed characteristic visible region absorption bands that are weak and broad, with peaks near 507 nm and 630 nm for the ferric protein, and a single asymmetric absorbance band near 557 nm for ferrous protein, indicating that the heme iron was pentacoordinate in both the oxidation states similar to Mb wild type. On the other hand, His coordination to the sixth axial position converts the heme iron to the low spin electronic configuration in both the oxidation states giving rise to a stronger visible absorbance in the ferric state, and splitting of the ferrous visible absorbance band into two peaks near 557 nm and 528 nm as shown for a reference hexacoordinate Hb, rice Hb (Fig. 8C). These findings were confirmed by EPR spectroscopy since EPR is particularly sensitive of the spin state in ferric Hbs (53). Thus, MbI107H was pentacoordinate in nature since its EPR spectrum were typical of high spin electronic configuration with a strong axial signal at g=5.83 (Fig. 8E), similar to that observed for Mb wild type (Fig. 8D). Whereas, Hx rice Hb exhibited low spin electronic configuration with weak axial signals at g=2.40, 2.30 and 1.90 (Fig. 8F). Thus, heme coordination chemistry of MbI107H was clearly pentacoordinated.

**The covalent linkage in engineered Mb had insignificant influence on ligand binding rates**

The ability of Hbs to function as oxygen transport/ storage agents depends on the rates of association and dissociation of physiological ligands and the corresponding equilibrium rate constants. These rates determined for O_2 and CO as ligands are reported in Table 2 for the three Mb proteins for an estimation of the influence of the corresponding mutations on their kinetic properties. Mb showed k_0' and K_0 values similar to those reported earlier in literature (19,20) with an equilibrium association rate constant of 1.3 μM⁻¹ (Table 2). MbF138H had no influence on any of the oxygen rate constants which are similar to wtMb (Table 2). MbI107H, the stable mutant, also exhibited minimal differences in its rate constants. It had a lower k_0' and higher k_0 compared to wtMb protein (Table 2) of the order of ~2-fold. Consequently, the equilibrium association rate
constant or oxygen affinity ($K_{O2}$) of MbI107H was ~3-fold lower, which does not represent a significant change in the ligand affinity. A change of ligand affinity of the order of 10-fold is usually considered significant for Hbs (36,42). MbI107H also showed a similar change in $k'_{o2}$. The CO-off rate could not be determined due to unavailability of cheap and high quality NO gas in India. Lemon et al. (54) have shown that the chemical reaction with Hb does not limit O$_2$ transport as long as the rate constant for association ($k'_{o2}$) is $\geq ~1$ μM$^{-1}$s$^{-1}$ and that for dissociation ($k_{o2}$) is $\geq 10^{-15}$ s$^{-1}$, which MbI107H exhibited. It has been proposed that the key properties of an efficient and economical hemoglobin-based blood substitute are moderate O$_2$ affinity and large O$_2$ dissociation rate constants. Kinetics studies suggest that MbI107H fulfills these criteria and similar mutation may be attempted in artificial blood substitute to enhance stability.

The covalent linkage in Mb did not alter the rate of autooxidation and prevented hemin dissociation

Influence of the introduced mutations on autooxidation and heme dissociation rates of Mb were investigated since a stable globin suitable for use as a blood substitute must have moderate or low rates of these kinetic events to maintain the heme-iron in a reduced (Fe$^{2+}$) physiologically active state. The rate of conversion of oxygenated Mb into the ferric form was measured by absorbance spectroscopy for an estimate of the autooxidation rate (20,55). The peaks typical of the oxygenated form of Mb were observed in the samples with absorbance maxima at 416 nm, 536 nm, and 581 nm. The experiment was designed to mainly measure, as a function of time, the absorbance decrease at 581 nm ($\alpha$-form of Q-band), since this peak is associated primarily with the oxygenated form (20,55). It was found that both MbI107H (0.181 h$^{-1}$) and MbF138H (0.173 h$^{-1}$) had almost similar autooxidation rates as compared to wtMb (Fig. 9A). Thus, the engineered mutants did not influence the autooxidation rates of the globin. The rate of autooxidation of wtMb and mutant proteins were, however, low in comparison to the cyanobacterial Hb (SynHb). The autooxidation rate of SynHb was found to be 1.01 h$^{-1}$ compared to 0.141 h$^{-1}$ (reported value: 0.1 h$^{-1}$) observed for Mb wild type (Fig. 9A).

The effect of mutations on heme loss was quantitated and the results are represented in Fig. 9B. Mbwt showed a profile similar to that observed in literature (55). MbF138H showed a rapid loss of heme compared to Mb wild type. MbI107H showed no dissociation of heme at pH 7.0 similar to SynHb. Thus, introduction of “His” at 107th position in Mb was found to be successful in preventing hemin dissociation because a covalent bond was introduced between heme and His. Hence, in summary, it is evident that SynHb based protein engineering in Mb was outright successful in enhancing its heme retention ability to the same order as SynHb itself.

**Discussion**

SynHb is a hexacoordinated and truncated bacterial Hb that displays 2-on-2 alpha helical globin fold and unique features not observed for any other Hb (4,56,57). The present investigation, partly an analysis of the influence of key heme pocket residues on SynHb stability, validated some earlier propositions and provided additional extended insight (7,26). The extraordinary thermal stability of SynHb as highlighted by DSC (Fig. 1F) is unprecedented in the globin family. SynHb also displayed unique stability to pH (Fig. 1C) and the chemical denaturant GdmCl (Fig. 1E). It is also evident that the other residues in the vicinity do not influence the post-translational modification of heme vinyl by His117. The presence of the third covalent linkage in which heme 2-vinyl group is covalently linked to His117. The presence of the third covalent linkage in which heme 2-vinyl group is covalently linked to His117, was thus found to be the most important structural parameter for stability in SynHb as predicted before (26). Mutational studies reiterated and validated that this covalent linkage is the major force holding the heme and is thus suitable for introduction into other globins for enhancing their stability.

The evolutionary significance of His117 (H16) and the unique covalent linkage it undergoes, for one, is a pertinent question in hemoglobin biology. Obviously, it is intriguing to know whether a similar covalent linkage can be mimicked in other globin architecture, especially in classical Hbs (like Mb), or whether it is specific to SynHb and its related members. Based on comparative
mutagenesis (Fig. 2), we were indeed able to engineer a covalent linkage between His and heme vinyl moiety in the paradigm globin, Mb. The “His” (to mimic His117) was introduced into Mb at the topologically equivalent position and in close proximity to the vinyl group. This mutant, MbI107H, was found to have high heme affinity comparable to SynHb (Fig. 3C). The fact that such covalent linkage could be introduced into classical globin like Mb suggests that the ancestral globins in nature might have harboured such post-translational modification prior to their evolution into modern globins lacking the covalent linkage, since it ceased to be necessary for their function. The ancient globins might have had such strong linkage to avoid loss of heme and concomitant loss of ligand, like O₂, which was limited in early evolution (58). However, as O₂ concentration increased to favourable limits, globins evolved without the additional covalent linkage, since it was no longer necessary. Such stable globins could have also been difficult to degrade and recycle in cells. Synechocystis Hb (and Hb from related species Synechococcus sp.) may have retained such novel structural feature as a “fossil” for a very specific, yet unknown function (59,60).

The third “His” of SynHb, successfully engineered in Mb, enhanced the stability of the classical globin probably due to a covalent linkage to proximal heme vinyl group, as expected by design (Fig. 2). While a crystal structure awaits the final confirmation, a combination of reversed phase chromatography, ESI-MS and MALDI-TOF analyses, clearly indicated a linkage between His107 and heme vinyl group (Fig. 3D and 4). The corresponding mutant, MbI107H, behaved like SynHb and did not dissociate heme under most experimental conditions (Fig. 3C, 4A) and had absorbance spectral properties remarkably similar to SynHb (Fig. 5B, 5C and 5D). Heme dissociation from this stable mutant, if any, was minor (~10-15%), as observed under some experimental conditions (Fig 3D, 4D). “His” introduced at other sequence and topologically equivalent positions either resulted in insoluble protein or did not improve heme retention at all, but on the contrary reduced heme stability (Fig. 5A; Fig. 6; Fig. 7A). MbI107H, the successfully engineered mutant, was investigated for protein (polypeptide) stability as well as other properties. Biochemical characterization at different pH, temperature and different concentrations of GdmCl showed that MbI107H as a protein was either marginally more stable than Mbwt or is atleast similar to Mb (Fig. 5; Fig. 6; Fig. 7). However, MbI107H showed multiphasic thermal denaturation profiles in DSC experiment (Fig. 7B) and non-cooperative unfolding in the other denaturing conditions (Fig. 6B, 7A). The possibility existed for the presence of intermediates in the unfolding process of MbI107H which was also seen for SynHb (data not shown). It is surprising for a single globular domain, MbI107H, to have a slow phase transition indicating a distinct intermediate formation at higher temperature as implied by DSC. The formation of intermediates during protein unfolding is a characteristic of multidomain proteins (61), where separate unfolding transitions can be observed with one domain unfolding faster compared to the other. This aspect for MbI107H will need further investigation.

EPR data showed that introduction of “His” did not affect the coordination state of the protein (Fig. 8). MbI107H was still pentacoordinated and bound exogenous ligands in a manner similar to Mb wild type (Fig. 8A and 8B). The affinity of oxygen binding for the engineered mutant was mildly lower than wild type Mb (Table 2); however, MbI107H might be still good enough to work as an oxygen transport protein since its kinetic parameters lies within allowed range required for oxygen transport/ storage (54). Autooxidation results showed that the rate of autooxidation of MbI107H was similar to Mb wild type (Fig. 9A). Quantitative hemin dissociation experiment clearly demonstrated that MbI107H had extremely high hemin affinity compared to Mb wild type (Fig. 9B).

Successful introduction of a stability enhancing covalent linkage in Mb presents a new strategy to enhance heme stability in globins in general and recombinant hemoglobin based oxygen carrier (rHBOC) in particular. To avoid the problems associated with blood transfusions and to meet the need for pre-hospital emergency resuscitation fluids, acellular hemoglobin solutions are being developed as one kind of clinical blood substitutes among others (62). Extensive efforts
over the years have successfully provided rHBOCs many of the desired properties like suitable $O_2$ binding kinetics, reduced NO binding and associated hypertension and others (31). A key problem that limits wide use of rHBOCs as of today is their limited stability, poor expression and frequent loss of heme which results in cytotoxicity (63). The present investigation might provide a solution to this persistent problem. Mb, a prototype for rHBOC, has been engineered here to enhance its heme retention ability and general polypeptide stability. The heme retention advantage is also coupled to the fact that MbI107H did not show any major influence in its structural or biophysical properties or protein folding or heme coordination. It enhanced heme retention ability with minimal influence on the other properties of the globin. Other important requirements for blood substitutes such as suitable rates of ligand binding, autooxidation and hemin dissociation were also preserved in the novel mutant. The present investigation thus provides a first step in the novel design of a stable rHBOC.

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FOOTNOTES

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4The abbreviations used are: SynHb: Synechocystis Hb; Mb: myoglobin; Hb: hemoglobin; trHbs: truncated hemoglobins; hxHbs: hexacoordinate hemoglobins; GdmCl: guanidine hydrochloride; CD: circular dichroism; wt: wild-type; DSC: differential scanning calorimetry. MS: mass spectrometry, ESI: electrospray ionisation, MALDI: matrix associated laser desorption ionisation, TOF: time of flight, UPLC: ultra performance liquid chromatography; EPR: electron paramagnetic resonance; MRE: mean residue ellipticity.

FIGURE LEGENDS

FIGURE 1. Side chain amino acids mutated in Synechocystis hemoglobin and their influence on the heme retention ability and protein stability. (A) Structural representation of Synechocystis hemoglobin (PDB ID: 1RTX). The truncated helical fold (2-on-2) is represented in grey and the heme prosthetic group in black. Various key residues (B10, E7, E10, F7, F8 and H16) decorating the heme pocket of SynHb are displayed in color. The three histidines that are covalently associated with heme, and are unique to SynHb, are shown in red. His46 and His70 directly coordinate to heme iron and constitute “hexacoordination”. The third His (His117) is covalently associated to heme vinyl group. (B) Comparison of the heme extraction results for Synechocystis wild type and mutant proteins. Bovine hemoglobin was used as a positive control and it released heme in the organic (top) layer (1). The purified proteins used for heme extraction were as follows: 2) SynHbWT; 3) SynQ43V; 4) SynH46L; 5) SynY22L; 6) SynH70G; 7) SynH117A; 8) SynA69S; 9) SynH46LH117A; 10) Myoglobin. (C) Comparison of pH titration profiles of SynHb wild type and mutant proteins monitored by change in the Soret wavelength against pH (2.0-11.0). SynH117A and H46LH117A mutant proteins showed significant blue shift in the Soret wavelength at acidic pH. (D) Absorbance spectra of SynH117A and SynH46LH117A at acidic pH were typical of free heme (E) Changes in the Soret peak wavelength were monitored with the increase in GdmCl concentration using absorbance spectroscopy. SynH117A and H46LH117A were significantly less stable than wtSynHb. SynH46L was also seen to be less stable. (F) DSC thermograms of SynHb wild type and
mutant proteins. Mb wild type protein was used as a control. The concentration of protein used was 0.12 mM in 100 mM potassium phosphate buffer, pH 7.0.

FIGURE 2. Selection of amino acid side chains and the strategy to introduce an additional covalent linkage in myoglobin. (A) Sequence alignment of Mb and SynHb generated using the ClustalW software. His117 (SynHb) aligned with Phe138 (Mb) as shown in red colour, indicating Phe138 to be a putative amino acid side chain for substitution. (B) Identification of amino acid side chain in Mb that is within 3 Å, 4 Å, 5 Å and 6 Å radius of heme: CAB atom. These are the side chains that are most likely to form covalent linkage to vinyl heme upon substitution to His. (C) Structural alignment of Mb (red) and SynHb (blue) generated by using the SUPERPOSE software. Ile107 in Mb was mutated to His \textit{in silico} and the distance between heme: CAB and HisNε2 was calculated to be 2.54 Å similar to that between His117Nε2 and heme of SynHb. PDB ID used: 1RTX (SynHb) and 5MBN (Mb).

FIGURE 3. Spectral properties, heme extraction assay and assessment of extent of covalent linkage for myoglobin and its mutant proteins. (A) Absorbance spectra (B) CD spectra of Mb mutants in comparison to wild type. (C) The peptides used for heme extraction were as follows: a) Mb wild type b) MbF138H; c) MbI107H d) SynHbWT. Myoglobin (a) released heme in the organic (top) layer. Similar result was observed for MbF138H. MbI107H did not release heme in the organic layer as also observed in SynHbWT. (D) Reversed phase UPLC analysis of purified Mb wild type and I107H mutant protein in BEH C18 column. The elution of the heme moiety was determined at 409 nm, whereas the protein (inset) was detected at 280 nm. An acetonitrile gradient was used to determine the elution position of free heme, Mb wild type and MbI107H protein.

FIGURE 4. Mass spectrometric analysis of covalently bound heme in MbI107H mutant protein compared to myoglobin wild type. (A) ESI-MS spectrum of intact myoglobin protein with an average molecular mass corresponding to ~17552.87 Da similar to the mass expected for apo-myoglobin (without heme). (B) ESI-MS spectrum of intact myoglobin I107H mutant protein with an average molecular mass corresponding to ~18238.27 Da similar to the mass expected for the MbI107H mutant protein covalently bound with heme. (C) MALDI-TOF mass spectrum of trypsin digested myoglobin in the region of m/z = 899 - 3010. Inset shows the expanded view (m/z = 1922-1943 region) of the MALDI-TOF mass spectrum of the peptide fragment containing Ile at 107th position at m/z = 1927.118. (D) MALDI-TOF mass spectrum of trypsin digested myoglobin Ile107His mutant protein in the region of m/z = 899 - 3010. Left inset shows the expanded view (m/z = 1943-1954 region) of the MALDI-TOF mass spectrum of the peptide fragment containing His at 107th position at m/z = 1951.068 and right inset shows the expanded view (m/z = 2552-2576 region) of the MALDI-TOF mass spectrum of the heme-peptide fragment at m/z = 2560.109.

FIGURE 5. pH stability studies of myoglobin wild type and mutant proteins. (A) Comparison of pH titration profile of myoglobin mutant proteins (MbF138H and MbI107H) with Mb wild type protein measured by monitoring Soret peak wavelength maxima. pH titration profile of MbI107H was found to be similar to SynHb WT (inset). Absorbance spectral profiles of Mb wild type (B), I107H (C) and SynHb WT (D) at pH 2.0 (red) and pH 7.0 (black). (E) The stability of secondary structure at different pH were investigated using far UV-CD for Mb wild type and mutant proteins. CD_{222 nm} (mdeg) were plotted as a function of pH from 2.0-11.0.

FIGURE 6. GdmCl stability studies of myoglobin wild type and mutant proteins. (A) Changes in the Soret peak wavelength were monitored with increase in GdmCl concentration. (B) Changes in the secondary structure were monitored with increase in the GdmCl concentration using far UV-CD signal at 222 nm.

FIGURE 7. Thermal stability studies of myoglobin wild type and mutant proteins. (A) Changes in the secondary structure of proteins were monitored with the increase in temperature using CD spectroscopy. CD_{222 nm} values were plotted against temperature. (B) DSC thermogram of myoglobin and
mutant proteins. MbI107H shows biphasic denaturation profile compared to Mb and SynHb wild type proteins, with the second peak having higher apparent \( T_m \) than Mb wild type protein. The concentration of protein used was 0.12 mM in 100 mM potassium phosphate buffer, pH 7.0. (Inset table) Melting temperature (apparent \( T_m \)) of Mb wild type, MbI107H, and SynHb WT obtained from differential scanning calorimetry.

**FIGURE 8. Electronic and paramagnetic spectral characteristics of MbI107H.** (A) and (B) Absorbance spectra of Mb and Mb I107H in both ferric and ferrous forms. MbI107H (B) demonstrates characteristics of high spin pentacoordinate Hb like wild type Mb (A). (C) Absorbance spectra of ferric and ferrous hexacoordinate Hb (Rice Hb) demonstrate characteristics typical of low spin hexacoordinate Hb. The EPR spectrum of ferric Mb (D) and MbI107H (E) shows the axial high spin signal. (F) The EPR spectrum of ferric HxHb (Rice Hb) shows axial low spin signals.

**FIGURE 9. Influence of mutations on autooxidation and hemin loss in myoglobin.** (A) Autooxidation kinetics of myoglobin wild type and mutant proteins in comparison to wild type SynHb. Time courses showing the normalized changes of the ratio \( A_{581\text{ nm}}/A_{630\text{ nm}} \) for Mb wild type, F138H and I107H indicated similar autooxidation rates. SynHb displays a much faster rate of autooxidation (B) Time courses for hemin dissociation at pH 7.0 for SynHb, Mb and its mutants. Normalized changes of the ratio, \( A_{409\text{ nm}}/A_{630\text{ nm}} \), of the globins were plotted. SynHb and MbI107H were resistant to heme dissociation while MbF138H had a very high rate of heme dissociation.

**TABLES**

**Table 1.** Comparison of melting temperature (apparent \( T_m \)) of SynHb wild type and mutant proteins measured using differential scanning calorimetry.

**Table 2.** Kinetic parameters of ligand binding for Mb wild type, F138H and I107H mutant proteins.
Table 1

| Protein                  | Apparent T<sub>m</sub> (°C) |
|--------------------------|-----------------------------|
| 1. SynHb WT              | 96.53 ± 0.017               |
| 2. SynA69S (F7)          | 95.83 ± 0.033               |
| 3. SynH70G (F8)          | 98.59 ± 0.044               |
| 4. SynH117A (H16)        | 79.50 ± 0.028               |
| 5. Mb WT                 | 77.34 ± 0.024               |

Table 2

| Protein      | k'<sub>O<sub>2</sub></sub> (μM<sup>-1</sup>s<sup>-1</sup>) | k<sub>O<sub>2</sub></sub> (s<sup>-1</sup>) | K<sub>O<sub>2</sub></sub> (μM<sup>-1</sup>) | k'<sub>CO</sub> (μM<sup>-1</sup>s<sup>-1</sup>) |
|--------------|---------------------------------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
| MbWT         | 16                                                             | 12                                    | 1.3                                    | 0.58                                    |
| MbF138H      | 15                                                             | 10                                    | 1.5                                    | 0.8                                     |
| MbI107H      | 10                                                             | 20                                    | 0.5                                    | 0.15                                    |

Note: k'<sub>O<sub>2</sub></sub> = Association rate constant for O<sub>2</sub>; k<sub>O<sub>2</sub></sub> = Dissociation rate constant for O<sub>2</sub>; K<sub>O<sub>2</sub></sub> = Equilibrium affinity constant for O<sub>2</sub>; k'<sub>CO</sub> = Association rate constant for CO.
Figure 1

(A)  
Ty22 (B10)  
Gln43 (E7)  
His46 (E10)  
His70 (F8)  
Ala69 (F7)  

(B)  
1 2 3 4 5 6 7 8 9 10

(C)  
Soret Peak Wavelength (nm)  

(D)  
Absorbance  

(E)  
Soret Peak Wavelength (nm)  

(F)  
Heat capacity (cal/mole·°C)  

(A) and (B) illustrate the protein structure and samples, respectively. (C) shows the Soret peak wavelength vs. pH for different Hb mutants. (D) presents the absorbance spectra for free heme and synthetic Hb mutants. (E) displays the Soret peak wavelength vs. GdnCl concentration for various Hb mutants. (F) presents the heat capacity vs. temperature for different synthetic Hb mutants.
Figure 2

(A) SynHb: ---------------STLYEKLGGTTA---VDLAVDKFY---ERVLQDDRIKHFFADVDMAKQRA 44
Mb: VLSEGEWQLVHLHWAKVEADVAGHQDILIRLFKSHPETLEKFDKFKHLKTEAEMKASED 60

(B) SynHb: HQKAF---FLTYFGGTDDKYDGFMREA\-----KELVENHGLNGHFDVDAEDLLATLKEMGV 99
Mb: LKKGTVTLAIGLKKGHEALKPLAQSHATKHIIPIKYLEFISEAIHVLHSR-H 119

(C) SynHb: PDELIAEVAAG---APAHKRDVLNQ-------- 123
Mb: PGDFGADAQGAMNKAELFRKDIAAKYKELGYQG 153
Figure 3

(A) Absorbance vs. Wavelength (nm) for MbWT, Mb107H, MbF138H (439 nm) and Mb107H (416 nm).

(B) MRE (deg•cm²•dmol⁻¹) vs. Wavelength (nm) for MbWT, MbF138H, and Mb107H.

(C) Images of MbWT, MbF138H, Mb1107H, and Mb107H.

(D) AU vs. Minutes graph for MbWT, Mb107H, MbF138H, and Free Heme at 280 nm and 409 nm.
Figure 4

(A) 

(B) 

(C) 

(D)
Figure 5

(A) Soret Peak Wavelength (nm) vs pH

(B) Absorbance vs Wavelength (nm)

(C) Absorbance vs Wavelength (nm) for Mb1107H

(D) Absorbance vs Wavelength (nm) for SynHb WT

(E) CD (mdeg) vs pH for MbWT and Mb1107H
Figure 6

(A) Soret Peak Wavelength (nm) vs. GdmCl (M)

(B) CD_{222nm} (mdeg) vs. GdmCl (M)

(A) (B)

MbWT
MbF138H
MbI107H

MbWT
MbI107H
MbF138H
Figure 7

(A) Protein Apparent T_m (°C)

| Protein    | Apparent T_m (°C) |
|------------|-------------------|
| MbWT       | 77.34 ± 0.024     |
| MbI107H    | 69.31 ± 0.013     |
| SynHb WT   | 96.53 ± 0.017     |

(B)
Figure 8

(A) Mb

(B) MbI107H

(C) HxHb (Rice Hb)

(D) Mb

(E) MbI107H

(F) HxHb (Rice Hb)
Figure 9

(A) 

(B)