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Molecular Cloning and Characterization of a (Lys)₆-Tagged Sulfide-Reactive Hemoglobin I from *Lucina pectinata*

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Abstract

A poly-Lys tag was fused to the *Lucina pectinata* hemoglobin I (HbI) coding sequence and purified using an efficient and fast process. HbI is a hemeprotein that binds hydrogen sulfide (H₂S) with high affinity and it has been used to understand physiologically relevant reactions of this signaling molecule. The (Lys)₆-tagged rHbI construct was expressed in *E. coli* and purified by immobilization on a cation exchange matrix, followed by size-exclusion chromatography. The identity, structure, and function of the (Lys)₆-tagged rHbI were assessed by mass spectrometry, small and wide X-ray scattering, optical spectroscopy, and kinetic analysis. The scattering and spectroscopic results showed that the (Lys)₆-tagged rHbI is structurally and functionally analogous to the native protein as well as to the (His)₆-tagged rHbI. Kinetics studies with H₂S indicated that the association (k_{on}) and dissociation (k_{off}) rate constants were $1.4 \times 10^5/\text{M/s}$ and $0.1 \times 10^{-3}/\text{s}$, respectively. This results confirmed that the (Lys)₆-tagged rHbI binds H₂S with the same high affinity as its homologue.

Keywords

Hemeproteins; Fusion proteins; Cloning; Lysine tag; Recombinant hemoglobin I; Protein purification

Introduction

Many of the cloning and expression plasmids used to generate recombinants proteins allow the addition of polypeptide tags at either the *N*- or *C*-terminus of the proteins. These tagged proteins are used to facilitate expression [1, 2], detection [3], purification [1–4], and solubility [1, 2, 4]. The poly-histidine tail [5–8] (His-tag) is the most commonly used to purify and immobilize proteins on numerous surfaces [7] through a non-covalent, reversible immobilization [9]. On the other hand, tags of repeated polar amino acids such as aspartic

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acid, lysine [8, 10–13], and arginine [14–16] are suitable for covalent immobilization. In fact, we have employed the His-tag technology to study the interaction of hemoglobin I (HbI) from *Lucina pectinata* with the novel H₂S signaling molecule.

The monomeric sulfide-reactive HbI from the clam *L. pectinata* is one of the few known H₂S carriers and is considered the physiological receptor of H₂S. The affinity of ferric HbI for H₂S is very high and results from a fast association ($k_{\text{on}} = 2.3 \times 10^5/\text{M/s}$) and very slow dissociation ($k_{\text{off}} 0.22 \times 10^{-3}/\text{s}$) rate constants [17]. The orientation of the vinyl and the flexibility of the propionate peripheral groups of the heme, together with the heme pocket dynamic behavior facilitate H₂S binding in this hemeprotein [18]. Interaction of H₂S with nearby residues also contributes to the affinity of HbI for H₂S [18, 19]. This protein has a glutamine (Gln) residue at the distal E7 ligand-binding site instead of the typical histidine (His) found in many invertebrate and vertebrate organisms. In addition to Gln in the E7 position, HbI has phenylalanine residues near the heme, which generate what is known as the “Phe-cage” [20]. Results from site-directed mutagenesis, spectroscopic and theoretical approaches have shown that H₂S is stabilized by hydrogen bonding interactions with Gln and that the unusual amino acid composition of the HbI distal ligand-binding site is responsible for its high affinity for H₂S.

The expression of high concentrations of HbI and HbI mutants was obtained by cloning HbI cDNA into the pET28(a+) expression vector that incorporated a histidine tag at the N-terminal [21]. The recombinant HbI (rHbI) has comparable binding properties toward H₂S. The main difference of this particular rHbI mutant ((His)₆-tagged rHbI) is the histidine tag, which was used to purify the protein. Fusion proteins that have poly-oligo tags for purification purpose should take into consideration the simplicity of their purification methods, the purity of the recovered proteins, and the future applications where the tag are directly involved. The (His)₆-tagged rHbI has accomplished all these expectations; however, it has the limitation that it only interacts electrostatically with divalent metal ions (Ni²⁺, Cu²⁺, Zn²⁺) [22] restricting its use and versatility to further explore the chemistry of H₂S with hemeproteins. H₂S is a paradoxical gas, which has been extensively studied during the last decades since its dual effect in the human body depends on its levels [23]. Considering the specificity of HbI with H₂S, it represents an alternative to explore the detection of this gas, and the chemistry of hemeproteins with H₂S. However, to achieve these objectives, there is the need of a new modified rHbI containing a tag that facilitates its purification and at the same time also provides an alternative for a robust and covalent immobilization on surfaces without affecting the HbI reactivity with H₂S. This article is focused on the development of a new rHbI, now with a multipurpose lysine tag and without the previously used histidine tag. Although fused poly-lysine tags on the C-terminal of proteins have been previously reported [24, 25], in this work, it represents a new alternative to study this unique hemoglobin.

A recombinant HbI fused with six lysine (Lys) residues at the C-terminus [(Lys)₆-tagged rHbI] has the property of an ionic amino acid fusion system that is suitable for large-scale purification [12] and covalent immobilization [10] on different surfaces for other applications. In this work, a different cloning strategy than the one used for the (His)₆-tagged rHbI was employed. Our approach consisted in removing the nucleotides coding for

the Histag and the additional amino acids that are added by the vector, using restriction enzymes (*NcoI* and *XhoI*) that cut this segment in pET28(a+). To assure the ligation of the Lys tag, the insert had *NcoI* and *XhoI* sticky ends complementary to the digested pET28(a+) vector. The new construction avoided the addition of one or more amino acids inexistent in the native form of HbI, which typically occurs when using cloning and expressions systems to generate recombinant proteins as in the case of the (His)₆-tagged rHbI.

Here, we report the construction, expression, and purification of (Lys)₆-tagged rHbI together with its structural and functional properties. The results show that the (Lys)₆-tagged rHbI is structurally analogous to the native HbI and the (His)₆-tagged rHbI, and that it binds H₂S with the same high affinity. The lysine tag facilitates the purification of the new (Lys)₆-tagged rHbI construct by immobilizing the protein on a cation exchanger matrix. This cost-effective method is also suitable for large-scale purification. The fact that the lysine tail interacts strongly with the column matrix, suggests that the (Lys)₆ tag is surface-exposed, accessible, and available to be used also for direct immobilization on carbonyl-modified surfaces [22].

Experimental Section

Fusion of HbI with a Lys Tag: Synthesis and Cloning

Fusion of the Lys tag at the C-terminus was achieved by direct amplification of the HbI coding region via polymerase chain reaction (PCR). The PCR was conducted in a 50 µL reaction using the Advantage[®] cDNA PCR kit from Clontech. The pET28(a+) vector containing the coding sequence for HbI was used as the template for the PCR reaction [26]. The reverse primer was composed of 21 base pairs complementary to the 3' end of the HbI cDNA, the corresponding nucleotide sequences for 6 Lys residues, a termination codon, a Xho I restriction enzyme site, and six additional bases to ensure proper enzyme recognition of the restriction site. The forward primer contained an *NcoI* restriction site to facilitate subsequent directional cloning into the pET28 expression vector. The forward and reverse primer sequences were 5'-CCTCTACCATGGGTTCTCTCTCTGCTGCACAG-3' and 5'-CCGCGTCTCGAGTCACTTCTTCTTCTTCTTTCATGTTTGGTCTGATCATGCC-3', respectively, where the two restriction sites are underlined. Both primers were designed using the HbI cDNA sequence [27]. PCR amplification was performed as follows: 1 min at 95 °C for initial denaturation, then 30 cycles consisting of 30 s at 95 °C for denaturation, and 1 min at 68 °C that combine annealing/primer extension steps. The 473 bp-PCR product was purified using the QIAquick PCR Purification Kit and cloned into the pCR4[®]-TOPO cloning vector (Invitrogen) to introduce the sticky ends into the HbI cDNA fragment. The transformed clones were isolated, and the presence of the insert [(Lys)₆-tagged rHbI with sticky ends] was confirmed using restriction enzyme digestion (*NcoI*-HF and *XhoI*, New England BioLabs, Inc) and automated DNA sequencing (ABI-Prism 3130 automated DNA sequencer) with dye terminator chemistry (Applied Biosystems, Foster City, CA). The cloned insert with the correct reading frame was then ligated to the pET28(a+) vector, previously digested using the *NcoI* and *XhoI* restriction enzymes. This digestion process removed the His-tag from the pET28(a+) cloning site and generated the sticky ends complementary to the insert ends first cloned into the pCR4[®]-TOPO vector. Ligation of the

insert into the digested pET28(a+) vector was performed using a vector:insert molar ratio of 1:3 (14 fmol pET28a(+) and 43 fmol of (Lys)₆-tagged rHbI DNA insert) in the reaction mixture. The ligation reaction was done in 10 µL containing 1X Ligase Reaction Buffer with 1 mM ATP and T4 DNA ligase (3 Weiss units, New England Biolabs). The (Lys)₆-tagged rHbI construct was again confirmed by restriction enzyme digestion (*Nco*I-HF and *Xho*I) and automated DNA sequencing, as indicated above, to verify the correct orientation of the insert and for comparison to the reported HbI cDNA sequence, using the basic local alignment sequence tool, BLAST, available from the National Center for Biotechnology Information (NCBI).

Expression of Hemoglobin I Fused to a Lys Tag

The pET28(a+) + (Lys)₆-tagged rHbI plasmid, which contained the insert cloned in the correct reading frame, was transformed into BLi5 *E. coli* cells for protein expression [21]. To determine optimal conditions, the expression was first evaluated in 0.5 L Fernbach flasks following the protocol used for the His-tagged rHbI [21]. Large-scale expression was then conducted in a 5 L BioFlo 110 Modular Benchtop Fermentor (New Brunswick, Edison, NJ). Before the large-scale expression, 50 mL of Luria–Bertani Broth (LB broth) containing 30 µg/mL kanamycin and 25 µg/mL chloramphenicol was inoculated with a frozen stock of the BLi5 *E. coli* cells transformed with the pET28(a+) (Lys)₆-tagged rHbI plasmid, and incubated overnight on a shaker at 225 rpm at 37 °C. This overnight culture was grown in 1/10 volume of the total expression culture. For the large-scale expression, 4.5 L of fresh Terrific Broth (54.0 g tryptone, 108.0 g yeast extract, 35.0 mL glycerol) medium supplemented with the antibiotics indicated above were inoculated with the overnight culture. The new culture was grown and induced (when the OD₆₀₀ reached 0.75) with 1 mM IPTG, followed by supplementation with 1 % w/v glucose and 30 µg/mL hemin chloride. The temperature was set at 37 °C before induction and lowered to 30 °C after addition of IPTG. At this OD, the cells have reached an exponential growth. To increase cell density, the culture pH and the dO₂ content were monitored and regulated as previously described [28]. After induction with IPTG, cell growth was monitored by measuring the OD. When the culture reached the stationary phase (about 18–24 h after induction), the cells were harvested and centrifuged at 4000 rpm and 4 °C for 15 min. The cell pellets were stored at –20 °C until used for protein purification.

(Lys)₆-Tagged rHbI Purification Process

To isolate the soluble proteins, the wet cell pellets were weighted and resuspended in lysis buffer (580 mM Na₂HPO₄, 17 mM NaH₂PO₄, and 68 mM NaCl, pH 7.5, containing 1 mg/g of bacteria of lysozyme and protease inhibitor cocktail, Roche Applied Science) in a 1:3 cells/buffer (w/v) ratio. The suspension was incubated on ice for 30 min and homogenized by sonication for 1 min several times until a liquefied suspension was obtained. The cell debris was removed from the soluble proteins by centrifugation at 4 °C, 15,000 rpm for 1 h in a Beckman JA 20 rotor. The (Lys)₆-tagged rHbI was purified from the soluble fraction using cation exchange followed by size-exclusion chromatography with an automated AKTA FPLC System (Amersham Biosciences, Piscataway, NJ, USA). After both chromatographic processes, the collected fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The crude lysate was adjusted to

the composition of the starting buffer (50 mM MES pH 6.0) using an YM 10 (NMWL of 10 kDa) membrane on a Millipore stirred ultrafiltration cell. The sample was centrifuged at 10,000 rpm for 10 min or filtered through 0.45 μ m filters before loading it into the cation exchange Hi Prep SP_FF 16/10 column. The bound proteins were eluted from the column by continuously increasing the salt concentration of starting buffer from 0.0 to 1.0 M NaCl for 60 min at a flow rate of 2.0 mL/min. The pooled fraction containing the (Lys)₆-tagged rHbI was concentrated, and the excess of NaCl was eliminated using a Millipore ultrafiltration cell with a 50 mM NaH₂PO₄/0.5 M EDTA buffer, pH 7.5. The concentrated sample was loaded into a HiLoad Superdex 200 16/60 prep grade column, previously equilibrated with 50 mM NaH₂PO₄/0.5 M EDTA buffer (SEC buffer). The concentration of the purified (Lys)₆-tagged rHbI was determined by UV–Vis spectroscopy using the absorption coefficients reported for the ferric metaquo state (107/mM/cm at 406 nm) and ferrous oxy state (135/mM/cm at 416 nm) [17]. The identity of the purified protein was also determined by tandem mass spectrometry at the RCMi Translational Proteomics Center.

Small/Wide-Angle X-ray Scattering (SWAXS)

X-ray solution scattering in both the small-angle (SAXS) and wide-angle (WAXS) range (SWAXS) was used to determine the global three-dimensional structure of the (Lys)₆-tagged rHbI in solution. For comparison, the scattering profile of the H₂S-avid (His)₆-tagged rHbI was also determined. The measurements were carried out at the National Synchrotron Light Source (NSLS) beamline X9 using a PILATUS 300 k SAXS detector and a Photonic Science CCD WAXS detector. The scattering profiles of the proteins were measured at 2 mg/mL with a 50 mM phosphate buffer pH 7.0 used as a background. In general, 15 μ L of the proteins and buffer solutions were continuously flowed through a 1-mm diameter capillary and exposed to the X-ray beam for 30 s. The measurements were carried out in triplicate, and the data were processed using an automated Python-based package developed at the NSLS. The program converted the two-dimensional scattering patterns recorded on the SWAXS detectors into one-dimensional scattering profiles. SWAXS profiles were merged, averaged, and buffer subtracted to obtain relative scattering intensity (I) as a function of momentum transfer vector, q ($q = [4\pi\sin\theta]/\lambda$), where λ is the beam wavelength and θ is the scattering angle.

Data analysis was performed with the ATSAS software suite (<http://www.embl-hamburg.de/biosaxs/software.html>). Using Guinier analysis, the dimensions of the proteins were first estimated by the radius of gyration (R_g), the root mean square distance of an object from its center of mass. According to the Guinier approximation, for a monodisperse sample of globular protein, a plot of $\ln I(q)$ versus q^2 should be linear for $q < 1.3/R_g$ [29–31]. Deviations from linearity in the Guinier plots indicate aggregation or interparticle interactions, and were also evaluated to confirm that no aggregation was present. The shape and maximum dimensions (D_{max}) of the recombinant proteins were determined by the indirect Fourier transformation of their scattering data $I(q)$, using GNOM [32]. This process yields a pair distribution function $P(r)$, which is a real representation of the data and describes distances between pairs of elements within the entire volume of the scattering particle. The (Lys)₆-tagged rHbI SWAXS profile was also compared with the native HbI profile by calculating the SWAXS scattering curve of the HbI crystal structure (PDB 1FLP)

using CRY SOL. To evaluate the orientation of the (Lys)₆ tag, a theoretical three-dimensional model of the (Lys)₆-tagged rHbI protein was generated using the I-Tasser algorithm [29, 33–35]. CCP4MG [36] was used for graphical analysis.

Optical Absorption Spectroscopy and Kinetics Analysis

The association rate (k_{on}) of H₂S with ferric (Fe³⁺) water bound (Lys)₆-tagged rHbI, also called metaquo, was measured using a π^* 180 stopped flow instrument from Applied Photophysics Inc. (Leatherhead, UK) equipped with a photodiode array detection, anaerobic accessory, and temperature control. Anaerobic reactions of 6.7 μ M metaquo (Lys)₆-tagged rHbI at pH 6 were mixed rapidly with solutions of sodium sulfide (0–2 mM). A stock solution of Na₂S (2 mM) was prepared in 100 mM succinic acid buffer at pH 6 and diluted into deoxygenated buffer to give the desired final concentrations (0–2 mM). Binding reactions were followed under pseudo-first-order conditions ($[H_2S] \gg [HbI]$) as reported by Pietri et al. [19]. The reaction kinetics were analyzed with ProK software from Applied Photophysics Inc, and the rate constant values (k_{obs}) at each concentration were determined using singular value decomposition (SVD). The slope of the plot between the observed rate constant and the H₂S concentration corresponds to the association rate constant (k_{on}). H₂S dissociation from the (Lys)₆-tagged rHbI was evaluated under anaerobic conditions by monitoring the decay of the (Lys)₆-tagged rHbI-H₂S absorption signal at 426 nm using UV–Vis spectroscopy. The (Lys)₆-tagged rHbI-H₂S was prepared by adding first potassium ferricyanide to the protein to obtain the metaquo derivative of the hemoglobin. A solution of Na₂S was then added to form the (Lys)₆-tagged rHbI-H₂S complex. Specifically, a 200 μ L sample of deoxygenated and degassed metaquo-(Lys)₆-tagged rHbI (140 μ M) was mixed with 0.84 μ L of a 100 mM sodium sulfide stock solution to yield a threefold molar excess of H₂S (minimum amount of H₂S to form 100 % HbI-H₂S complex) [19]. The integrity of the metaquo and sulfide (Lys)₆-tagged rHbI derivatives was monitored by their characteristic absorption spectra using a UV–Vis spectrometer (Agilent 8453). Dissociation of H₂S was then analyzed by mixing a small volume (125 μ L) of the (Lys)₆-tagged rHbI-H₂S complex with a large volume of deoxygenated buffer (1 mL, pH 6.5). The reaction kinetics at specific wavelength (426 nm) as well as spectra associated with the kinetics were acquired for 24 h.

Results and Discussion

Fusion of HbI with a New Lys Tag: Synthesis, Cloning, and Expression

To directionally clone the HbI cDNA containing the nucleotides that code for the Lys₆ tag into the pET28(a+), the *Nco*I and the *Xho*I restriction sites of the vector's polylinker region were used. The presence of the cloned insert was verified by restriction enzyme digestion with *Nco*I and *Xho*I, and the plasmids containing the cloned inserts were sequenced to confirm that the insert was in the correct reading frame for proper protein expression in *E. coli*. Figure 1a shows the sequence of HbI cDNA obtained in this work, including the nucleotides that code for the Lys tag. The cDNA-coding region contains 429 bp that code for the typical HbI amino acids (143), in addition to 18 bp that code for 6 additional Lys residues, a termination codon and 12 bp corresponding to the restriction sites for each one of the restriction enzymes. The derived amino acids sequence of the (Lys)₆-tagged rHbI is identical to the published sequences of the native HbI (95 %) and (His)₆-tagged rHbI except

for the addition of the Lys tail (Fig. 1b) [21, 27]. Comparison of the (Lys)₆-tagged rHbI with the (His)₆-tagged rHbI shows that not only the six Lys residues were added at C-terminus of the new construct but also that the six His residues at the N-terminus were effectively removed from the pET28(a+) vector, as desired. The (Lys)₆-tagged rHbI protein was successfully expressed in BLi5 *E. coli* cells, both in small and large quantities, using the protocols employed for the His₆-tagged protein with a slight modification. To obtain a better yield, the concentrations of kanamycin and chloroamphenicol were reduced from 70 to 30 µg/mL and from 30 to 25 µg/mL, respectively. The density profiles of the transformed BLi5 in both the micro (500 mL) and large (5 L) scale processes are shown in Figure S1. As expected, the density of the BLi5 cells increased ~24-fold (OD_{600 nm} = 1.558 vs. 36.998) in the large-scale protocol with a calculated wet biomass of 69.28 g/L. In both methods, the concentration of the purified (Lys)₆-tagged rHbI protein was nearly the same (3.7 and 3.1 g/L) and slightly higher than the one obtained for the His-tagged rHbI [21, 28], establishing the feasibility of expressing this new rHbI in large quantities.

(Lys)₆-Tagged rHbI Purification Protocol

Cation exchange chromatography (CEC) was employed to purify the Lys-tagged protein. The theoretical isoelectric point (pI) of (Lys)₆-tagged rHbI, determined with the *ExPASy Compute pI/Mw tool*, was found to be 9.68, indicating that the protein is positively charged at acidic and neutral pH and that it could be easily purified using a cation exchange matrix. Figure 2a depicts the purification profile obtained after loading the crude lysate containing the (Lys)₆-tagged rHbI protein into the cation exchange column. The profile was monitored at 280 nm, and the salt gradient was optimized to obtain the maximum amount of pure protein. In the profile, the first fraction (Fraction I) corresponds to unbound proteins that were washed out using the starting buffer. Three additional fractions were eluted from the column during the linear gradient. The second fraction (Fraction II) was eluted with 44–70 % NaCl, while the third and fourth (Fraction III and IV) fractions were removed with 72–86 % and 90–99 % NaCl, respectively. Fraction III was the only one that exhibited a translucent pale red color characteristic of the hemoglobin, indicating that this fraction corresponded to the (Lys)₆-tagged rHbI protein. This was corroborated by UV–Vis spectroscopy and SDS-PAGE. Figure 2b shows the UV–Vis spectrum of this eluent, which displays the characteristic Soret band at 416 nm, associated with the oxy form of the HbI heme (Fraction III). Lanes 4 and 5 in the SDS-PAGE gel (Fig. 2d) show that the molecular weight of Fraction III is about 17 kDa, which is very close to the (Lys)₆-tagged rHbI protein (~16 kDa) theoretical molecular weight. The SDS-PAGE of this fraction also showed a second band at lower molecular weight (lanes 4 and 5 in Fig. 2d), indicating contamination with another protein. This contamination was successfully removed by SEC (lanes 6 and 7 in Fig. 2d). Figure 2c shows the SEC elution profile of Fraction III containing the (Lys)₆-tagged rHbI after cation exchange. Two peaks were detected, and the pooled fractions were also evaluated by SDS-PAGE and UV–Vis. The first and major peak exhibited the translucent light red color of the hemoglobin and the SDS-PAGE gel (lanes 6 and 7 in Fig. 2d) showed a single defined band in the region corresponding to the molecular weight of the (Lys)₆-tagged rHbI protein (~17 kDa). The second small peak, which was eluted at longer times, was not observed in the SDS gel (lane 8) upon Coomassie Blue staining. Integration of the peaks in Fig. 2c suggests that the protein was more than 80 % pure. The spectral

properties of this purified (Lys)₆-tagged rHbI are shown in Fig. 3a. The spectra of the metaquo and H₂S (Lys)₆-tagged rHbI derivatives show an intense band at 407 nm and 425 nm, respectively. Small bands at 502 and 633 nm were also detected for the metaquo derivative, while the H₂S complex exhibited Q bands at 541 and 576 nm. As shown in the figure, these spectral features are identical to the ones reported for the native HbI and the (His)₆-tagged rHbI. This demonstrates that the heme configuration and the nearby environment are conserved in the (Lys)₆-tagged rHbI. Hemeproteins exhibit very characteristic UV–Vis absorption spectra, which are due to $\pi \rightarrow \pi^*$ electronic transitions of the porphyrin heme macrocycle. In particular, the spectra are characterized by an intense band near the ultraviolet region known as the Soret, and a pair of weak ones between 500 and 600 nm designated as the Q bands. These spectral bands are affected by the chemical nature of the heme (heme configuration) including coordination states of the heme iron as well as the protein environment surrounding the heme. Thus, the fact that the spectral properties of the (Lys)₆-tagged rHbI are the same as the native HbI and the (His)₆-tagged protein, indicate that the Lys tag did not affect the overall heme active site. In general, the results clearly showed that the new (Lys)₆-tagged rHbI construct can be immobilized on a cation exchange matrix for protein purification purposes and that the purity of the sample after SEC was close to 100 %. Moreover, the fact that high concentrations of NaCl were required to elute the protein from the cationic column indicates that the (Lys)₆ tag strongly interacts with the matrix.

To evaluate the disposition of all the lysine residues in the (Lys)₆-tagged rHbI, and to discern their availability for immobilization, a qualitative evaluation of the protein was performed using the CCP4MG [36] software. (Lys)₆-tagged rHbI has a total of eighteen lysine residues, six belonging to the lysine tag and twelve to the native HbI. The analysis showed that most of the native lysines fall within the interior of the protein, while only three are exposed to the surface (see Fig. 3b). To further identify the spatial location of the lysine residues, *in silico* solvent accessibility prediction was evaluated using the I-Tasser algorithm [33–35]. As shown in Figure S2, the predicted values ranged from 0 % for buried residues to 90 % for highly exposed residues. The results indicated that the lysine residues located in the tag are more solvent exposed (>60 %) than the three native lysine residues (<60 %). Thus, the poly-lysine tag is most likely responsible for the interaction between the (Lys)₆-tagged rHbI and the negatively charged media. Other amino acids such as arginine and histidine can be positively charged at pH 6; however, only Arg59 has an approximately 70 % accessibility to the solvent and could be available to interact electrostatically. Other arginine and histidine residues are buried within the protein and their accessibility is less than 40 %. Moreover, immobilization through the amine group of the *N*-terminus is less likely due to the charge density at the *C*-terminus versus the *N*-terminus. In summary, the theoretical three-dimensional model and the predicted solvent accessibility generated by I-Tasser demonstrate that the Lys tag residues are located at the surface of the protein and are most likely available to selectively react or anchor the rHbI on a suitable surface. These properties can be used to anchor electrostatically (Lys)₆-tagged rHbI on a charged surface, or even covalently on a carboxyl surface to generate a strong and robust peptide bond.

Identification of (Lys)₆-Tagged rHbI by Mass Spectrometry

Tandem mass spectrometry (MS/MS) was used to characterize the purified (Lys)₆-tagged rHbI. After SEC isolation, the (Lys)₆-tagged rHbI was enzymatically digested with trypsin, and the generated peptides were identified by LC–MS/MS [37]. The analysis was performed in duplicate. The obtained MS/MS spectra for each of the fragmented peptides were compared against the Swiss Prot protein sequence database using the MASCOT computer algorithm to establish the identity of the protein in both samples. Based on the criteria established by Ommen et al. [38], proteins with four or more matched peptides are predicted to be correct with better than 0.99 confidence. Table 1 summarizes the results obtained for the two samples using MASCOT, one of them identified as sample 1 and another one as sample 2. Seven unique peptides were identified for sample 1, while six peptides were detected in sample 2. For sample 1, the probability that the peptides were associated with the sequence of native HbI was more than 80 %, while for sample 2 all had a probability of 99 %, keeping in mind that 80 % or more is considered an excellent probability for identification. The additional peptide in sample 1 (TFAANHKAR) is found at rather low probability (80 %) and it is most likely that it was a low relative abundance peptide selected for MS/MS in one analysis but missed in the other. Variance between duplicates is very common in LC–MS/MS, since the technique works by selecting peptides ranked by order of abundance, from high to low and the MS/MS is typically not able to sequence all the peptides eluting from the HPLC in time leading to some variability in the analyses. Despite this variance, which is common in these procedures, the data are consistent as explained below. The HbI amino acid sequence (GeneBank no. P41260) used by the MASCOT algorithm to match the peptides obtained in the sample 1 is presented in Figure S3. The peptides are highlighted in gray and they represent 64 % (or 92 residues) of the total amino acid sequence. For sample 2, the peptides represented 57 % (82 residues) of the full HbI sequence. The intact (Lys)₆ tag is not present in the identified peptides due to the nature of the digestion process with trypsin. The procedure cleaves peptide bonds on the carboxyl side of Arg and Lys, and will in principle break the Lys tag at different positions in the tail. By Ommen's criteria [38], the data suggest, with high confidence, that the peptides in both samples are true positives and that they belong to the sequence of the native HbI protein. In both analyses, the same unique peptides were identified, validating the identity of the (Lys)₆-tagged rHbI protein.

Structural Analysis of (Lys)₆-Tagged rHbI by SWAXS

SWAXS offers information about the global three-dimensional shape of biological macromolecules in solution and was used here to evaluate the overall shape and dimension of (Lys)₆-tagged rHbI. The SWAXS profile of the (His)₆-tagged rHbI was also determined and compared with that of the (Lys)₆-tagged rHbI protein. Figure 4a shows the experimental scattering curves of the recombinant proteins. The scattering patterns were quite similar, reflecting analogous topologies and global shapes for both proteins. The scattering curves of the proteins and the linearity of the Guinier plots indicated no detectable protein aggregation. The Guinier analysis allowed the determination of the proteins radius of gyration (R_g), which offers information related to the size of the proteins. The (Lys)₆-tagged rHbI had a slightly larger dimension with an R_g of 21 Å, while for His-tagged rHbI was about 19 Å. As shown in Fig. 4b, the *P(r)* functions of the proteins were asymmetric bell-

shaped, indicating that they have globular elongated structures. A maximal dimension ($D_{\max}$) of 67 Å was obtained for the (Lys)₆-tagged protein, whereas the (His)₆-tagged rHbI had a $D_{\max}$ of 64 Å. This small difference can be attributed to the amino acid tags (Lys vs. His).

Figure 4c depicts the experimental scattering curve of the (Lys)₆-tagged rHbI and the one calculated for the untagged native HbI using the atomic crystal structure of the protein. The overall profiles are very similar, indicating that the (Lys)₆-tagged rHbI retains the global structural shape of the native HbI. The small discrepancies at small angles ($q \sim 0.2$) might reflect slightly different global dimensions due to the introduction of the (Lys)₆-tag on the (Lys)₆-tagged rHbI as opposed to the native HbI. In addition, the lattice of the native HbI crystal structure may hamper the dynamic behavior of the protein in solution. Overall, the SWAXS data indicated that the global three-dimensional structure of the (Lys)₆-tagged rHbI is comparable to the (His)₆-tagged rHbI as well as to the native HbI.

Functional Properties of (Lys)₆-Tagged rHbI by UV–Vis and Kinetics Analysis

UV–Vis spectroscopy was used to determine oxidation and binding properties of (Lys)₆-tagged rHbI with different ligands, including H₂S. The spectrum of the purified (Lys)₆-tagged rHbI had a strong Soret band at 413 nm with corresponding Q bands at 538 and 574 nm characteristic of the oxy complex, as shown in Fig. 5a. The fact that the protein is purified in the oxy form indicates similarity to native HbI, where O₂ binding is stable in the absence of H₂S. Upon reaction with ferricyanide, the Soret band shifted toward 407 nm, indicating changes in the oxidation state of the heme iron from ferrous Fe^{II} to ferric Fe^{III}, as presented in Fig. 5a. When ferric (Lys)₆-tagged rHbI sample was exposed to H₂S, spectra similar to native HbI–H₂S and (His)₆-tagged rHbI–H₂S complexes were obtained with a Soret band at 426 nm and Q bands at 541 and 576 nm. These results demonstrate that (Lys)₆-tagged rHbI can be readily oxidized and it can form a stable complex with H₂S. This new recombinant protein retains the heme group functional features of the native and (His)₆-tagged rHbI counterparts. Association kinetics studies between (Lys)₆-tagged rHbI and H₂S (Fig. 5b), show a rapid association rate constant, k_{on} , of $1.4 \times 10^5/\text{M/s}$, which is very similar to the k_{on} for (His)₆-tagged rHbI, $1.9 \times 10^5/\text{M/s}$. Both recombinant HbIs showed an increased linearity rate constant with respect to H₂S concentration, obtaining an R^2 equal to 0.990 and 0.994, for the (Lys)₆-tagged rHbI and (His)₆-tagged rHbI, respectively. These association rate constants are also comparable with those reported for the wt-HbI, k_{on} of $2.3 \times 10^5/\text{M/s}$ [17]. As previously pointed out by Fernández-Alberti, et al. [39], the fast sulfide association rate constant is highly affected by changes in the HbI distal pocket and its periphery. Hence, the association rate constant reported here demonstrates that the distal area as well as the entrance of H₂S to the heme pocket was not affected by the fusion of the Lysine tag at the C-terminus of the protein.

To further define the reactivity of (Lys)₆-tagged rHbI with H₂S, the dissociation kinetics of the ligand were evaluated and results are shown in the inset of Fig. 5c. The displacement of the Soret band as a function of time from 426 to 407 nm demonstrates that H₂S slowly dissociates leading to the formation of the final ferric metaquo derivative. Fitting the kinetic trace associated with H₂S release with a single exponential function yielded a dissociation

rate constant, k_{off} , of $0.1 \times 10^{-3}/\text{s}$, which differs by a factor of 2 from the value ($0.22 \times 10^{-3}/\text{s}$) reported for the native HbI protein. The discrepancy in the estimated k_{off} is attributed to the complex behavior of H_2S dissociation. Pietri, et al., reported that the extraordinarily slow dissociation of H_2S from ferric HbI is restricted by two competing reactions: (1) simple H_2S dissociation from the ferric adducts and (2) reduction of the heme iron center to the ferrous state followed by H_2S dissociation [19]. These competing processes are reflected in the H_2S dissociation kinetics and therefore the rate constant. Despite the small discrepancy in the k_{off} , the results showed that, similar to the native protein, H_2S release is very slow. Overall the k_{on} and k_{off} data strongly suggest that the heme pocket configuration as well as the orientation of residues in the heme distal site is not significantly altered in the new (Lys)₆-tagged rHbI. The high H_2S affinity for the (Lys)₆-tagged rHbI is supported by its fast association constant ($k_{\text{on}} = 1.4 \times 10^5/\text{M}/\text{s}$) and its very slow dissociation process ($k_{\text{off}} = 0.1 \times 10^{-3}/\text{s}$).

Conclusions

In this work, we presented a new recombinant HbI fused with a poly-Lys tag without adding other amino acids that are not present in the native sequence, or affecting the protein function. In our approach, the poly-Lys tag was fused by directly modifying the cDNA of HbI and inserting the segment in an expression vector. Incorporation of the tag into the HbI cDNA was confirmed by DNA sequencing. The protein was successfully expressed in large quantities and purified to ~100 % using a two-step chromatographic protocol. The identity of the purified (Lys)₆-tagged rHbI was validated by MS/MS, and the functional and structural properties of the protein were determined by UV-Vis, ligand-binding kinetics, and SWAXS. All together, the results showed that (i) the (Lys)₆-tagged rHbI is structurally and functionally analogous to the native and (His)₆-tagged rHbI proteins; (ii) the Lys tag did not affect the integrity of the heme environment, and (iii) the new recombinant protein binds H_2S with high affinity.

More importantly, this work shows that the (Lys)₆-tagged rHbI can be purified effectively by immobilizing the protein on a cation exchange matrix without the use of expensive immobilized metal-affinity resins and chemicals (such as heavy metal ions and imidazole). The results also demonstrated that the Lys tag in the (Lys)₆-tagged rHbI moiety interacts strongly with the cation exchange chromatography matrix, suggesting that the location of the poly-Lys tag can facilitate site-selective electrostatically immobilization and it also represent an alternative for covalent immobilization.

This poly-Lys tag does not affect the high expression of the protein and it provides a simple straightforward purification strategy available for overproduction of other proteins. In addition, it offers an alternative for oriented immobilization of proteins without the addition of a second tag [8, 10, 40]. This new HbI variant will allow determining the chemistry of H_2S with heme proteins.

Supporting Information available: [(1) Expression profiles of the BLi5 *E. coli* cells transformed with the pET28(a+) + (Lys)₆-tagged HbI plasmid, (2) Predicted solvent accessibility generated by I-Tasser server, and (3) peptide sequences identified by MS/MS].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

a Partial nucleotide sequence of the cloned HbI cDNA, including the nucleotides for the Lys tag (AAG sequence), and the derived amino acids sequence. Important sequences are *highlighted*: initial methionine and its corresponding codon (*bold and underline*), the stop codon (*italic*), and Lys sequences (*bold italic*). **b** Multiple sequence alignment of the native HbI, the (His)₆-tagged rHbI and (Lys)₆-tagged rHbI. The *asterisks* indicate the common amino acids in the sequences. Sequence alignment was performed with the Clustal 2.1 multiple sequence alignment program, available at <http://www.ebi.ac.uk/Tools/msa/clustalw2/>

Fig. 2.

Purification profile of (Lys)₆-tagged rHbI after cation exchange. **b** UV-Vis spectra of the CEC fractions. Fraction III, which corresponds to the (Lys)₆-tagged rHbI, clearly shows the typical spectra of hemoglobins in the metaquo state with a Soret band at 407 nm. **c** Size-exclusion chromatography profile of (Lys)₆-tagged rHbI (Fraction III from CEC) and **d** SDS-PAGE analysis of the fractions obtained in each purification step. *Lane 1* shows the EZ RUN Pre-Stained Rec Protein Ladder, and *lanes 2* and *3* correspond to the crude lysate. Fraction III from CEC is shown in *lanes 4* and *5* and Fraction A and B from SEC are shown in *lanes 6* and *7* and *8*, respectively. The molecular weights of interest are highlighted in the red square (Color figure online)

Fig. 3. UV-Vis spectra of **a** (Lys)₆-tagged rHbI, native HbI and (His)₆-tagged rHbI in its metaquo and sulfide forms. The *inset* shows the Q bands associated with the complexes described above. The (Lys)₆-tagged rHbI spectrum shows the characteristic absorption bands for porphyrins as explained by M. Gourteman and his four-orbital model, confirming that the heme configuration was unaltered. **b** Theoretical three-dimensional model of the (Lys)₆-tagged rHbI protein generated by I-Tasser. CCP4MG was used for graphical analysis. The orientation of the Lys tag (*red circle below*), other possible Lysine or positively charged amino acids at the surface, as well as the *N*-terminus in the (Lys)₆-tagged rHbI are indicated (Color figure online)

Fig. 4. SWAXS scattering profiles of **a** the (Lys)₆-tagged rHbI (*dashed line*) and the (His)₆-tagged rHbI (*solid line*). The Guinier plot of the (Lys)₆-tagged rHbI is shown in the *inset*. **b** Distance distribution function of the (Lys)₆-tagged rHbI (*dashed line*) and (His)₆-tagged rHbI (*solid line*). **c** Experimental scattering profile of the (Lys)₆-tagged rHbI (*dashed line*) and the theoretical profile of the native HbI (*solid line*) calculated using Crysol

Fig. 5.

a UV-Vis spectra of the (Lys)₆-tagged rHbI in the oxy form (*dashed line*), the ferric metaquo derivative (*solid line*) and coordinated with H₂S (*dots*). The *inset* shows the Q bands associated with the complexes described above. **b** Plot of the observed association rate constants as function of H₂S concentration. The *inset* displays the exponential kinetics trace for (Lys)₆-tagged rHbI (*black squares*) and (His)₆-tagged rHbI (*gray circles*) at 426 for a ratio of 1:10 [rHbI:H₂S] in both case. **c** UV-Vis spectra associated with H₂S dissociation kinetics under anaerobic conditions at different times: 18 h. (*solid*), 15 h. (*dash*), 12.5 h. ks (*dot*), 8 h. (*dash dot*), 0 h. (*short dash*). The *inset* shows the H₂S dissociation kinetic curve monitored at 426 nm

Table 1

Summary of identified peptide sequences

Sample	Sequence Previous and next amino acids (aa)	Percentage sequence coverage	Exclusive unique peptide count	Best peptide identification probability
1	(K)ASAAWGTAGPEFFMALFDAHDDVFAK(F)	63.60	7	79.90
	(R)GISAGQLEAAFK(V)			99.70
	(K)NTPEMAAQAQSF(K)			99.70
	(K)SYGGDEGAWTAVAGALMG(MIR(P)			99.70
	(K)SYGGDEGAWTAVAGALMG(MIRPNM(–)			99.70
	(K)TFAANHKAR(G)			80.30
	(K)VLAGFMK(S)			87.40
2	(K)ASAAWGTAGPEFFMALFDAHDDVFAK(F)	57.30	6	99.70
	(R)GISAGQLEAAFK(V)			99.70
	(K)NTPEMAAQAQSF(K)			99.70
	(K)SYGGDEGAWTAVAGALMG(MIR(P)			99.70
	(K)SYGGDEGAWTAVAGALMG(MIRPNM(–)			99.70
	(K)VLAGFMK(S)			99.70