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Synthesis of Recombinant Human Hemoglobin with NH_2-terminal Acetylation in *Escherichia coli*.

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ABSTRACT

The development of new technologies for the efficient expression of recombinant hemoglobin (rHb) is of interest for experimental studies of protein biochemistry and the development of cell-free blood substitutes in transfusion medicine. The expression of rHb in *E. coli* host cells has numerous advantages, but one disadvantage of using prokaryotic systems to express eukaryotic proteins is that they are incapable of performing post-translational modifications such as NH_2-terminal acetylation. One possible solution is to co-express additional enzymes that can perform the necessary modifications in the host cells. Here, we report a new method for synthesizing human rHb with proper NH_2-terminal acetylation. Mass spectrometry experiments involving native and recombinant human Hb confirmed the efficacy of the new technique in producing correctly acetylated globin chains. Finally, functional experiments provided insights into the effects of NH_2-terminal acetylation on O_2-binding properties.

***Basic Protocol 1*: Gene synthesis and cloning the cassette to the expression plasmid**

***Basic Protocol 2*: Selection of *E. coli* expression strains for co-expression**

***Basic Protocol 3*: Large scale rHb expression and purification**

***Support protocol 1*: Measuring O_2 equilibration curves**

KEYWORDS: blood substitute, recombinant hemoglobin, NH_2-terminal acetylation, post-translational modification.

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INTRODUCTION

Vertebrate hemoglobin (Hb) is a metalloprotein that is responsible for circulatory transport of O_2 and CO_2. The protein is a heterotetramer composed of two α-chain and two β-chain subunits, each of which contains a heme group that reversibly binds O_2 and other gaseous ligands. Hb is a model protein for the study of allosteric regulation (Kirschner, 1968; Perutz, 1989), and it has always held center stage in the fields of crystallography, biochemistry, and protein evolution (Storz, 2019).

Advances in recombinant DNA technology have led to the development of diverse approaches for synthesizing recombinant hemoglobin (rHb). The synthesis of rHb is of interest for advancing experimental approaches in protein engineering and for the development of rHb-based cell-free O_2-carriers for use as blood substitutes (Olson et al., 1997; Varnado et al., 2013). Heterologous expression of recombinant eukaryotic proteins in bacteria has always posed challenges concerning post-translational modifications. The first bacterial expression system for producing rHb involved expressing the β-globin chain subunit as a fusion protein (Nagai & Thogersen, 1984). One especially complicated step is the removal of the fusion tag, followed by the reconstitution of the protein with exogenous heme (Nagai, Perutz, & Poyart, 1985; Nagai & Thogersen, 1984, 1987). A second-generation prokaryotic system used a polycistronic design to express both globin chains under the control of a *tac* promoter (Hoffman et al., 1990). Hernan *et al*. optimized the codons for *E. coli* and expressed synthetic human α- and β-globin chains using a bacteriophage T7 promoter (Hernan et al., 1992). The above methods yield tetrameric Hb with correctly incorporated heme in *E. coli* host cells. However, neither of these methods yield rHb with the normal NH_2-terminal post-translational modifications that characterize vertebrate Hb *in vivo*. Both α- and β-globin chains retain the NH_2-terminal methionine, which interferes with allosteric binding of H^+, CO_2, Cl^- ions, and organic phosphates. One strategy is to mutate the first codon to valine, thereby replacing the initiator methionine. The second strategy is to co-express methionine aminopeptidase (MAP), an enzyme that cleaves the initiator methionines of the nascent peptides (Chang, McGary, & Chang, 1989; Looker et al., 1992; Shen et al., 1993).

In the polycistronic approach, the α- and β-globin chains were successfully co-expressed with the MAP enzyme under two separate *tac* promoters (Shen et al., 1997). The recombinant α-globin peptide solubility was increased in *E. coli* by co-expressing the α-hemoglobin stabilizing protein, a chaperone protein that binds specifically to free α-globin and prevents its precipitation (Gell, Kong, Eaton, Weiss, & Mackay, 2002; Kihm et al., 2002; Vasseur-Godbillon, Hamdane, Marden, & Baudin-Creuza, 2006). Efforts to develop rHb-based blood substitutes provided much of the motivation for improving expression yields (Olson et al., 1997). Based on earlier studies, we designed and optimized a bacterial plasmid system that can efficiently express authentic rHb in *E. coli* (Natarajan et al., 2011). This system has been used successfully to synthesize rHbs of diverse vertebrate species (Cheviron et al., 2014; Galen et al., 2015; Kumar et al., 2017; Natarajan et al., 2013; Natarajan et al., 2015; Natarajan et al., 2016; Natarajan et al., 2018; Projecto-Garcia et al.,

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2013; Signore & Storz, 2020; Signore et al., 2019; Tufts et al., 2015; Zhu et al., 2018).

NH_2-terminal acetylation is another post-translation modification that occurs in eukaryotic proteins and is of relevance to Hb function. N-α-acetyltransferase (Nat) is an enzyme complex consisting of a catalytic subunit and up to two auxiliary subunits that can add an acetyl group to the NH_2-terminus of proteins during translation. In mammals, at least six Nat (A-F) enzymes have been identified and characterized. By contrast, in the *E. coli* genome, only three acetyltransferases, *RimI*, *RimJ*, and *RimL*, are known to acetylate the NH_2-terminus of ribosomal proteins. In eukaryotes, Nat oligomeric complexes are unique and substrate-specific in adding the acetyl group to the proteins (Drazic et al., 2016). The NatA complex has a catalytic subunit *Naa10* that acetylates the nascent polypeptide, and *Naa15* is an auxiliary ribosomal anchoring subunit that helps to anchor the complex. The NatA complex binds to the ribosome and acetylates the smaller and polar amino acids (alanine, serine, threonine, cysteine, valine, and glycine) at the NH_2-terminus, and this process is performed simultaneously with the cleavage of the initiator methionine (Arnesen et al., 2005; Arnesen, Gromyko, et al., 2009; Arnesen et al., 2010; Arnesen, Van Damme, et al., 2009; Liszczak et al., 2013).

Here, we describe a new protocol for the expression and purification of human rHb with proper NH_2-terminal modification. In an earlier report, we optimized the expression conditions to produce functional rHb with proper cleavage of initiator methionines by co-expressing an additional MAP plasmid (Natarajan et al., 2011). In the current report, we extend our original protocol to produce human rHb with properly acetylated NH_2-termini. This was accomplished by co-expressing the pNatA complex in conjunction with the globin genes.

Methodology

Basic Protocol 1: Gene synthesis and cloning the cassette to the expression plasmid

This protocol describes the synthesis of the human globin gene cassette and subcloning the globin cassette to the pGM expression vector. The pGM expression plasmid was a T7 transcription-based system, and the expression is induced by T7 RNA polymerase in the *E. coli* host cells. The pGM system was customized by cloning the coding region of methionine aminopeptidase gene that removal of N-terminal methionine residues from nascent peptides. The pGM system was designed with dual promoters, where MAP was cloned tandemly upstream to the globin cassette with an independent T7 promoter. The globin cassette was controlled by the second T7 promoter downstream to the MAP cassette. The α- and β-globin genes were cloned by a unique pair of restriction enzyme sites (Fig. 1). This expression system was designed and developed specifically to express the vertebrate rHb in *E. coli* (Natarajan et al., 2011).

[*Place figure 1 near here.]

Materials

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Human globin cassette (see Fig.1; GeneArt® gene synthesis, Thermo Fisher Scientific)
pGM plasmid (Natarajan et al., 2011)
*Nco*I (New England Biolabs, cat. no. R0193)
*Sac*I (New England Biolabs, cat. no. R0156)
Invitrogen™ UltraPure™ Agarose (Invitrogen cat. no. 16500500)
T4 ligase (Invitrogen cat. no. 15224-017)
One Shot® TOP10 (Invitrogen cat. no. C404010)
GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, cat. no. K0503)
Zymoclean Gel DNA recovery kit (Zymo Research, cat. no. D4007)
Lauria Bertani (LB) medium (see Reagents and Solutions; Sigma, cat. no. L3022)
LB plates (see Reagents and Solutions)
Agar (Sigma, cat. no. A7002)
Ampicillin (100 mg ml-1; Sigma, cat. no. A9518)
Kanamycin (50 mg ml-1; Sigma, cat. no. 60615-25G)
Chloramphenicol (50 mg ml-1; Sigma, cat. no. C0378-25G)
Sequencing primer SDalpha: 5'- GAGATTAACTCAATCTAGAGGGTAT- 3'
Sequencing primer SDbeta: 5'- AATACCCTCTAGATTGAGTTAATCT - 3'

37°C water bath (Thermo Scientific)
42°C water bath/heating block (Thermo Scientific)
250 ml flat-bottom centrifuge bottles (Thermo Scientific)
50 ml Falcon conical centrifuge tubes (Thermo Scientific)
Vortex mixture (VWR)
Agarose gel electrophoresis system (Thermo Scientific)
Gel documentation system (BioRad)
Nanodrop (Thermo Fisher Scientific)

1) Synthesize the globin gene cassette with the coding region for the adult-expressed human α- (*HBA1*, UniProt accession: P69905) and β-globin genes (*HBB*, UniProt accession: P68871). Arrange the α and β-globin gene tandemly and interspace it with a Shine-Dalgarno region (SD). Flank the globin genes with a unique set of restriction enzyme sites (Fig. 1). Codon-optimize the whole cassette for *E. coli* and synthesize using GeneArt® gene synthesis (Thermo Fisher Scientific).
   The globin cassette is synthesized with unique restriction enzymes (NcoI and SacI) to help in a sticky end unidirectional cloning into the pGM custom vector.
2) Add 20 µl of RNase-free water to the lyophilized plasmid DNA and incubate for 1 hr at room temperature (25°C) to dissolve it.
3) Transfer ~1-2 µl of plasmid DNA to the One Shot™ TOP10 competent cell and do the transformation as per the manufacturer's instructions. Inoculate a single colony in LB media and grow the culture overnight at 37°C in a shaker incubator at 200 rpm (Sambrook, Fritsch, & Maniatis, 1989).
4) Isolate the plasmid using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) as per the manufacturer's protocol. Quantify the plasmid DNA using Nanodrop and store at -20°C for future use.
5) Subclone the globin cassette into the pGM vector using *Nco*I and *Sac*I restriction enzyme sites. Linearize the globin cassette and pGM plasmid by incubating for 1 hr at 37°C (see reaction recipe in Table 1).

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[*Place table 1 near here.]

6) Separate the digested products in 1.2% agarose gel, and purify the excised band by the Zymoclean Gel DNA recovery kit as per the manufacturer's protocol.
7) Set the ligation reactions, as shown in Table 2. The mixture is incubated for 25°C for 1 hr and transformed into the One Shot™ TOP10 cell.
If 1 hr ligation fails, please store the remaining ligation mix in the 4°C for overnight and repeat transformation. The success rate is higher for the overnight ligation.

[*Place table 2 near here.]

8) Isolate the plasmids from the positive colonies from the LB agar plate, and sequence the coding regions for the insert using the sequencing primers (SDalpha and SDbeta).
The invert sequencing primers were designed explicitly on the interspace (SD region) between the α- and β-globin genes for this purpose (Fig. 2).

[*Place figure 2 near here.]

***Basic Protocol 2*: Selection of *E. coli* expression strains for co-expression**

The expression of vertebrate rHb efficiently in bacteria is relatively challenging. This protocol describes the selection of appropriate *E. coli* host strains for the expression of rHb along with the additional post-translation modifying enzymes (MAP and NatA complex). Our current protocol is optimized to favor the expression of target and other additional proteins without affecting the yield of target rHb. The best experimental conditions and host strain for the rHb expression were reported in our earlier study (Natarajan et al., 2011). In this current protocol, along with human globin genes (α- and β-globin), and MAP, we also co-expressed the subunits needed for NatA complex.

Materials

pMAP (Natarajan et al., 2011)
pNATA plasmid (pACYCduet-naa10-naa15, Addgene plasmid # 72928; http://n2t.net/addgene:72928; RRID: Addgene_72928; Eastwood et al., 2017)
JM109 (DE3) (endA1, recA1, gyrA96, thi, hsdR17 (rk–, mk+), relA1, supE44, λ–, Δ(lac-proAB), [F–, traD36, proAB, lacIqZΔM15], lDE3; Promega, cat. no. P9801)
BL21 (DE3) pLysS [$F^–$, *omp*T, *hsd*$S_{B,}$ ($r_B^-m_B^-$)], *gal*, *dcm*, λ(DE3), pLysS, Cm^r; Promega, cat. no. P9811)
BL21 Star™ (DE3) [$F^–$, *omp*T *hsd*S_B ($r_B^-m_B^-$)] *gal, dcm, rne1*31 (DE3); Invitrogen, cat. no.C6010-03)
BLR (DE3) [$F^–$, *omp*T *hsd*S_B ($r_B^-m_B^-$)] *gal, lac, ile, dcm,* Δ(*srl-recA*) 306: Tn*10* (tet^R) (DE3); Novagen, cat. no.69053-3)
Origami™ (DE3) (Δara-leu 7697 ΔlacX74 ΔphoAPvull phoR araD139 galE galK rpsL F′ [lac+(lacIq) pro] gor522::Tn10(TcR) trxB::kan (DE3); Novagen, cat. no.70617-3)
Terrific Broth (TB) medium (RPI, cat. no. T15100, see Reagents and Solutions)
Glycerol (Sigma, cat. no. G5516)

Baffled Erlenmeyer flasks 1000 ml

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Shaker incubator (New Brunswick™ Excella® E25/E25R)
DU800 UV/Vis spectrophotometer (Beckman Coulter)
Sonicator (QSonica, Q500 model)
Refrigerated centrifuge (Sorvall Legend X1 Centrifuge)

1) The selection of *E. coli* host strain is a crucial process for prokaryotic protein expression. We used five commercially available expression strains for checking the rHb expression (JM109 (DE3), BL21 (DE3) pLysS, BL21Star™ (DE3), BLR (DE3), Origami™ (DE3)). We checked the quantity and quality of rHb protein among those different expression strains in our earlier study (Natarajan et al., 2011).
2) Based on our results from earlier experiments, JM109 (DE3) *E. coli* strain shows an optimal expression, significant NH_2-terminal methionine cleavage, and proper heme incorporation (Natarajan et al., 2011).
   The NH_2-terminal methionine cleavage was measured quantitatively among the E. coli strains and expression conditions in our earlier study. Among them, JM109 (DE3) has the best efficiency for cleaving the NH_2-terminal methionine of the globin peptides (greater than 95%).
   This protocol is designed strategically to co-express pGM, pMAP, and pNatA plasmids together in JM109 (DE3) host cells.
   The pNatA plasmid (pACYCduet-naa10-naa15) was a gift from Dan Mulvihill (Eastwood et al., 2017)*. The pGM plasmid has an ampicillin resistance gene. While pMAP and pNatA plasmids codes for kanamycin and chloramphenicol antibiotic resistance genes, respectively (Fig. 2).*
3) Add 100 ng of pGM, pMAP, and pNatA plasmids (equimolar ratio) to the JM109 (DE3) competent cells and incubated in ice before transformation (Sambrook, Fritsch, & Maniatis, 1989).
4) Select the co-transformed positive colonies by the triple antibiotics for the NH_2-terminal acetylated human rHb (pGM + pMAP + pNatA) and double antibiotics for unacetylated rHb (pGM + pMAP) on an LB agar plate (see Reagents and Solutions).

   Fewer colonies have appeared on the LB plates with triple antibiotic selection (2-10 colonies per LB agar plate).

5) Express and purify two versions of human rHbs. NH_2-terminal acetylated rHb (pGM + pMAP + pNatA) and the unacetylated rHb (pGM + pMAP).

***Basic Protocol 3*: Large scale rHb expression and purification**

This protocol is described in three major parts: expression, storage, and purification of rHbs. The expression of the human rHb was optimized cautiously, since the recombinant MAP, and NatA enzyme complex needs to be functional at the similar expression conditions (Fig. 3). A two-step ion-exchange chromatography was employed to purify the rHbs without any purification tags. We perform an anion-exchange followed by a cation-exchange chromatography to eliminate other bacterial heme proteins and overexpressed recombinant proteins (MAP and NatA complex proteins).

[*Place figure 3 near here.]

Materials

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Tris-Base (Sigma, cat. no. T1503)
Tris-HCl (Sigma, cat. no. T3253)
Isopropyl β-D-1-thiogalactopyranoside (IPTG) (1M; Acros Organics, cat. no.302790250)
Dithiothreitol (DTT 1000 mM; Sigma, cat. no. D9760)
Lysozyme (200 mg ml^{-1}; Sigma, cat. no. L6876)
Polyethyleneimine (stock solution 1:1 v/v dilution in H_2O; Sigma, cat. no. P3143)
Glucose (Sigma, cat. no. G8270)
EDTA (Sigma, cat. no. E9884)
Hemin from bovine (50 µg $µl^{-1}$; see Reagents and Solutions; Sigma, cat. no.H9039)
Dimethyl sulfoxide (DMSO) (Sigma, cat. no. D8418)
Antifoam Y-30 (Sigma, cat. no. A6457)
Brilliant Blue G250 (Sigma, cat. no. B1131)
Orthophosphoric acid (Fisher Scientific, cat. no. A242-500)
Hydrochloride acid (Sigma, cat. no. 320331)
Ammonium sulfate (Sigma, cat. no. A4418-1KG)
β-Mercaptoethanol (Sigma, cat. no. M7522)
Ethyl alcohol (Sigma, cat. no. 459844)
10XTGS (BioRad, cat. no.161-0772)
PageRuler plus prestained protein ladder (Thermofisher Scientific, cat. no.26619)
Laemmli sample buffer (see Reagents and Solutions; BioRad, cat. no.161-0737)
Acrylamide (Fisher Scientific, cat. no. BP170)
Bis-Acrylamide (Sigma, cat. no. M7279)
Sodium dodecyl sulfate (SDS) (Sigma-Aldrich cat. no. L3771-1KG)
Ammonium persulphate (Sigma, cat. no. A3678)
Sodium dithionite (Sigma, cat.no.157953)
HEPES (Sigma, cat. no.83264)
Sodium chloride (Sigma, cat. no. S3014)
HiTrap Q-Sepharose Column (5 mL, GE Healthcare, cat. no.17-1153-01)
HiTrap SP-Sepharose Column (5 mL, GE Healthcare, cat. no.17-1152-01)
Amicon® Ultra-15 Centrifugal Filter Unit (30 kDa cutoff, Millipore Sigma, cat. no. UFC903024)
Amicon® Ultra-15 Centrifugal Filter Unit (50 kDa cutoff, Millipore Sigma, cat. no. UFC905024)

Tris lysis buffer (see Reagents and Solutions)
Tris Start buffer (see Reagents and Solutions)
Tris Elution buffer (see Reagents and Solutions)
HEPES Start buffer (see Reagents and Solutions)
HEPES Elution buffer (see Reagents and Solutions)
Staining solution (see Reagents and Solutions)
Shaker incubator (New Brunswick™ Excella® E25/E25R)
DU800 UV/Vis spectrophotometer (Beckman Coulter)
Sonicator (QSonica, Q500 model)
Refrigerated centrifuge (Sorvall Legend X1 Centrifuge)
Mini-gel for SDS-polyacrylamide gel electrophoresis (BioRad)
Fast Protein Liquid Chromatography (ÄKTA™ start, GE Health care)

Expression of rHb

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1) Pick a single colony and inoculated in a 100 ml LB medium (see Reagents and Solutions) overnight at 37ºC in a shaker incubator at 200 rpm.
Prepare the glycerol stock by adding 800 µl of culture with 200 µl of glycerol and store it in -80°C for future use.
2) Inoculate 10 ml of startup culture to a freshly autoclaved TB media for the large scale expression culture (300 ml x 6 Baffled Erlenmeyer flasks).

Allow the autoclaved TB medium to cool down before inoculation (See Reagents and Solution).

3) Add 50 ug/ml of antibiotics to final concentration to each flask containing the TB media.
We try to use a minimal amount of antibiotics for large-scale culture since double and triple antibiotics can suppress the growth of E. coli. Ampicillin, kanamycin, and chloramphenicol were added according to the plasmid combination.
4) Each batch of 1.8 L of TB medium is grown in a shaker incubator at 37ºC at 200 rpm for 4 - 5 hr until the absorbance reached 0.6-0.8 at 600 nm.
5) Induce the bacterial culture with a final concentration of 0.5 mM IPTG. All plasmids are induced by IPTG to produce the recombinant proteins.

We optimized the IPTG concentration to 0.2 mM in our previous study. Lowering the IPTG concentration can slow down the production of rHb and can have a beneficial tradeoff between the rHb production and NH_2-terminal methionine cleavage.

6) Supplement the culture with hemin (50 µg/ml), sodium hydrosulfite (50 mg/L), and glucose (20 g/L). See Table 3 for details.
Adding exogenous bovine heme at the time of induction keeps the induced globin peptides in the soluble fraction, and sodium hydrosulfite keeps the heme in reduced form $Fe2^+$. This process will avoid the methemoglobin formation to an extent.

[*Place table 3 near here.]

7) Induce the culture and incubate the sample for 16 hr at 28ºC in an orbital shaker at 200 rpm.
Lowering the temperature to 28ºC after induction helps to get the target protein in the soluble fraction, and it also helps to increase the MAP activity.

Storage and lysis

8) Saturate the overnight culture by bubbling with carbon monoxide (CO) for 15 min, and then pellet the cells and store at -80ºC.
Binding of CO helps to maintain the stability of rHb. Handle the CO gas inside a chemical hood with a proper exhaust. CO is a colorless and odorless gas, so it is essential to use proper protective gear and safety precautions. Cells are harvested and stored at -80ºC freezer. Freeze-thawing the cell pellets can help in proper lysis.
9) Resuspend the cell pellets with tris lysis buffer (5 ml/g of cell pellet, see Reagents and Solutions).
Lysosome and DDT are added just before the sonication.
10) Add the Lysozyme 1 mg/g of cells (200 mg/ml stock) to the sample for proper lysis.

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11) Add polyethyleneimine solution to the crude lysate to a final concentration of 0.5 to 1%.
It helps to precipitate the bacterial nucleic acids and other cell debris.
12) Sonicate the *E. coli* cells with 10 sec pulse on for 15 min with a 20 sec pause cycles at 3.0 output duty.

Place the samples carefully on the ice bath for the whole process.

13) Centrifuge the crude lysate for 15,000 x g for 45 min at 4ºC, and the clarified supernatants were dialyzed overnight against the Tris buffer (20 mM Tris with 0.5 mM EDTA, pH 8.8) for chromatography.

Cell debris and other excess heme impurities were precipitated during the overnight dialysis.

Anion-exchange chromatography

14) Perform a two-step ion-exchange chromatography using ÄKTA start protein purification system (GE Healthcare Life sciences).
To express and purify tagless rHb, we performed anion-exchange and cation-exchange chromatography in succession. This two-step procedure yields ultra-pure rHbs, and it also eliminates other bacterial heme-proteins.
Q-Sepharose column is a strong anion-exchange column (HiTrap QHP, 5 mL, 17-1153-01; GE Healthcare) equilibrated with Tris start buffer (20 mM Tris with 0.5 mM EDTA, pH 8.8) for purifying the rHbs.
15) The rHb binds to the column firmly and allows other bacterial proteins to elute in flow-through and washes (2 x 10 ml of Tris start buffer).
16) Elute the rHb against a linear gradient of 0-1.0 M NaCl with 20 mM Tris with 0.5 mM EDTA, pH 8.8.
The elution of rHb is visible in the Q-column since the rHb protein is bright red.
Monomeric and unbound heme (brownish) will get elute in 1-4% of elution buffer before the major tetramer rHb peak elutes.
17) Analyze the fractions by 15% SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis). Only the fractions with red color were pooled and concentrated using Amicon Ultra-15 Centrifugal Filter Unit (50 kDa cutoff)
18) Based on the SDS-PAGE results, pool the pure fractions and concentrate. Dialyze the fractions overnight against the HEPES start buffer for the second column.
The fractions were concentrated to a final volume of ~50 ml. Desalting and buffer exchange was carried out overnight in a dialysis tubing at 4°C.

Cation-exchange chromatography

19) Equilibrate a SP-Sepharose column (HiTrap SPHP, 5 mL, 17-1152-01; GE Healthcare) with (10X of column volume) HEPES buffer (20 mM HEPES with 0.5mM EDTA, pH 7.0).
20) Elute the rHb with a linear gradient of 0-1.0 M NaCl with the 20 mM HEPES with 0.5 mM EDTA, pH 7.0.
SP-column weakly binds to the rHb compared to Q-column. So all the fractions are evaluated by spectrophotometer and later by SDS-PAGE.

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21) Measure the eluted fractions for the absorbance using a spectrophotometer at 415 nm (Soret) and 280 nm wavelength (Fig.4).
This step can help in quantification and reduce the number of fractions to be analyzed by SDS-PAGE.

[*Place figure 4 near here.]

22) Analyze the purified rHb samples using SDS-PAGE (Fig.5).

[*Place figure 5 near here.]

23) Based on the SDS-PAGE analysis, the pure fractions containing the target proteins were pooled and desalted using the Amicon® Ultra 15 mL Centrifugal Filter Unit (30 kDa cutoff).
24) *In vitro* measurements of O_2-binding properties in the presence of allosteric cofactor are measured for rHbs and native Hb samples (see Support Protocol 1).

Support protocol 1: Measuring O_2 equilibration curves

As oxygenated and deoxygenated Hb have different spectral properties (oxy peak = 415 nm, deoxy peak = 430 nm), the oxygenation state of the protein can be assessed by monitoring its spectral absorbance. By using a custom gas diffusion chamber with a built-in spectrophotometer, the Blood Oxygen Binding System (Loligo Systems), the Hb sample is exposed to sequentially increasing oxygen tensions (P_{O2}), and the corresponding changes in absorbance are recorded. The change in absorbance observed at each stepwise change in P_{O2} is used to determine the fraction of Hb saturated with oxygen. From these data, the P_{O2} at half-saturation (P_{50}), the standard metric by which Hbs oxygenation properties are compared, and can be calculated.

Materials

Potassium chloride (Sigma, cat. no. P9333)
Sodium Hydroxide (Sigma, cat. no. S8045)
2,3-Diphospho-D-glyceric acid pentasodium salt (Sigma, cat. no. D5764)
Disposable PD 10 Desalting Columns (GE Healthcare cat. no. GE17-0851-01)
Amicon® Ultra-4 Centrifugal Filter Unit (30 kDa cutoff, Millipore Sigma, cat. no. UFC803024)

Blood Oxygen Binding System (Loligo Systems)
pH meter (Orion Star A211 with an Orion™ PerpHecT™ ROSS™ Combination pH Micro Electrode)

1) The native Hb blood sample (100–200µl) was added to a 5x volume of 0.01 M HEPES/0.5 mM EDTA buffer (pH 7.4) and incubated on ice for 30 min to lyse the red blood cells.
2) Add NaCl to a final concentration of 0.2 M, and samples were centrifuged at 20,000 x g for 10 min to remove cell debris.

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3) Pass the clarified hemolysates, and purified recombinant hemoglobins through a PD-10 desalting column (GE Healthcare) equilibrated with 25 ml of 0.01 M HEPES/0.5mM EDTA (pH 7.4).
4) Concentrate the eluates using Amicon Ultra-4 Centrifugal Filter Units (Millipore).
5) O_2–equilibrium curves for Hb solutions (0.1 mM hemoglobin in 0.1 M HEPES/0.05 M EDTA buffer) were measured at 37°C using a Blood Oxygen Binding System (Loligo Systems), as detailed in steps 6 - 8.
6) Measure the O_2-equilibrium curves in the absence (stripped) and the presence of chloride ions (0.1 M KCl) and organic phosphates (0.2 mM 2,3-diphosphoglycerate (DPG) (Fig. 6). The stripped treatments provide a measure of intrinsic Hb-O_2 affinity. In contrast, the +KCl+DPG treatment provides a measure that is relevant to the *in vivo* conditions, as these ions are the principal allosteric modulators (i.e., non-oxygen ligands that alter Hb function) of Hb–O_2 affinity in mammalian red blood cells.

[*Place figure 6 near here.]

7) Equilibrate each Hb solution sequentially with an array of oxygen tensions (P_{O2}) between 3 and 21 mmHg. At the same time, monitor the sample absorbance continually at 430 nm (deoxy peak) and 421 nm (oxy/deoxy isobestic point) with the Blood Oxygen Binding System's built-in spectrophotometer. Each equilibration step was considered complete when the absorbance at 430 nm had stabilized (2 - 4 minutes). Only oxygen tensions yielding 30 - 70% Hb–O_2 saturation were used in subsequent analyses.
8) Construct Hill plots (log[fractional saturation/[1-fractional saturation]] vs. logP_{O2}) from these measurements and fit to a linear regression model. The resulting equation can be used to determine the P_{O2} at half-saturation (P_{50}) and the cooperativity coefficient (n_{50}), where the X-intercept and slope of the regression line represent the P_{50} and n_{50}, respectively.
9) Measure the P_{50} values at three different pH levels, where the pH of working solutions was adjusted with NaOH to as near 7.2, 7.4, or 7.6 as possible, then precisely measured with an Orion Star A211 pH Meter and Orion™ PerpHecT™ ROSS™ Combination pH Micro Electrode.
10) Fit a linear regression to plots of logP_{50} vs. pH, and use the resulting equation to estimate P_{50} values at pH 7.40 (± SE of the regression estimate).

Support protocol 2: Mass spectrometry to confirm the NH_2-terminal acetylation

This protocol describes the sample preparation and analysis of NH_2-terminal acetylation using MSMS. The human rHbs were separated on 4-20% SDS-PAGE and stained. The bands α- and β-globin chains were excised separately and digested with proteolysis enzyme. In-gel trypsin digestion was set up to analyze the desired post-translational modification for the globin samples. NH_2-terminal acetylation of the human α- and β-globin peptide can result in an increase of +42.01 Da mass compared to the unacetylated peptide fragment. MS/MS can detect this +42.01 Da mass shift in the digested peptides.

Materials

Acetonitrile (Sigma-Aldrich cat. no. 34851-1L)
Ammonium bicarbonate (Sigma-Aldrich cat. no. 09830-500G)

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UltraPure™ DNase/RNase-Free Distilled Water (ThermoFisher Scientific cat. no. 10977015)
Staining solution (see Reagents and Solutions)

Mini-gel for SDS-polyacrylamide gel electrophoresis (BioRad)
Vortex mixture (VWR)
Thermo Orbitrap Fusion Lumos Tribrid (Thermo Scientific™)
PEAKS X software

1) The NH_2-terminal acetylation of the α- and β-chains of recombinant human Hb were quantified with tandem mass spectrometry (MS/MS).
2) The purified rHbs were separated in a mini-protean precast 4-20% SDS PAGE gel (Bio-Rad, Hercules, CA) and then counterstained with Coomassie brilliant blue-G (see staining solution in Reagents and Solutions) for overnight.
The α-globin (~15 kDa) and β-globin (~15.9 kDa) are separated by 4-20% gel by longer run and stained. The bands were excised carefully and saved in a DNase/RNase-Free Distilled H_2O for MS/MS analysis.
3) The stained bands were excised and processed for in-gel digestion (Shevchenko et al., 2006). Add 200 µl of 100 mM ammonium bicarbonate: acetonitrile (1:1) and incubate it for 30 min with occasional vortexing. Add another 500 µl acetonitrile and incubate at room temperature until the coomassie stain washes off. Add 20 ng/µl trypsin in 100 mM ammonium bicarbonate (50-100 µl) to the gel and incubate in ice for 30 min. Add trypsin buffer accordingly to cover the gel piece. Incubate the gel for 60 min with trypsin and add 50 µl for ammonium bicarbonate and incubate overnight at 37°C. Add formic acid (final concentration 0.1%) to the tube and incubate for 15 min in a shaker incubator at 37°C. Centrifuge the samples at 12,000 g for 15 min and take 20 µl for MS/MS analysis.
4) The eluted peptides were then analyzed using a Thermo Orbitrap Fusion Lumos Tribrid (Thermo Scientific™) mass spectrometer in data-dependent acquisition mode.
5) Identify the peptides by searching MS/MS data against a customized reference database.
The human α- and β-chain peptide sequences are provided in fasta format for the software to do the analysis.
6) NH_2-terminal acetylation of α- and β-chain and the oxidation of methionines were included as variable modifications, and the carbamidomethylation of cysteines was set as a fixed modification.
7) The precursor mass tolerance threshold was set to 10 ppm, and the maximum fragment mass error was set for 0.02 Da.
8) Perform the qualitative analysis using PEAKS X software. The significance threshold of the ion score was calculated based on a false discovery rate of ≤ 1%.
The PEAKS DB is a workflow performing a protein database identification search. The software allows us to choose and customize the choice of proteolytic enzyme and the desired post-translational modifications.
9) We measured the relative percentages of acetylated and unacetylated NH_2-termini peptides for each globin subunits.

REAGENTS AND SOLUTIONS

LB medium

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Add 20 g of LB broth powder to 1 liter of distilled water. Dissolve the reagents by stirring and autoclave.

LB plates

Add 20 g of LB broth powder, 15 g of agar to 1 liter of distilled water. Dissolve the reagents by stirring and autoclave. Allow the medium to cool and then add ampicillin (100 µg ml^{-1}), kanamycin (50 µg ml^{-1}), and chloramphenicol (50 µg ml^{-1}) to final concentration, mix it gently by swirling and pour around 20 ml of LB agar to the Petri plates. Allow the agar to solidify, store the plates at 4ºC. Avoid air bubbles while pouring to Petri plates and store the plates in the cold room not longer than a month.

TB medium

Add 47.6 g of terrific broth powder in 1 liter of distilled water. Dissolve the reagents by stirring and add 4 ml of glycerol. Autoclave the medium and set it to cool down before inoculation.

Hemin solution

Add 500 mg of bovine hemin dissolved in 10 ml of DMSO in a 15 ml tube (50 µg ml^{-1}).

Hemin dissolves slowly in the DMSO. Keep the tubes mixing overnight. Please make sure the heme is dissolved homogeneously before use.

Buffers

Sample loading buffer

The sample loading buffer for SDS page was prepared by adding 50 µl of β-Mercaptoethanol to 950 µl of 2X SDS sample dye (BioRad).

Lysis buffer

Dissolve 3.025 g of Tris base (50 mM) and 146 mg of EDTA (1 mM) in 400 ml distilled water. Adjust the pH to 8.0 with 10 N HCl and make up the volume to 500 ml. Add DTT (0.5 mM) and Lysozyme (1 mg per gram of cell pellet) to final concentration. DTT and Lysozyme were added just before use.

Tris Start buffer

Dissolve 3.152 g of Tris-HCl (20 mM), 146 mg of EDTA (0.5 mM) in 900 ml of distilled water. Adjust the pH to 8.8 and makeup to 1000 ml.

Tris Elution buffer

Dissolve 3.152 g of Tris-HCl (20 mM), 146 mg of EDTA (0.5 mM) 58.4 g of NaCl (1 M) in 900 ml of distilled water. Adjust the pH 8.8 and makeup to 1000 ml.

HEPES Start buffer

Dissolve 4.76 g of HEPES (20 mM), 146 mg of EDTA (0.5 mM) in 900 ml of water. Adjust the pH to 7.0 and makeup to 1000 ml.

HEPES Elution buffer

Dissolve 4.76 g of HEPES (20 mM), 146 mg of EDTA (0.5 mM) 58.4 g of NaCl (1 M) in 900 ml of distilled water. Adjust the pH to 7.0 and makeup to 1000 ml.

Make a stock solution for EDTA at pH 8.0 to avoid issues in dissolving EDTA

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Staining solution
Add 200 ml of ethanol (20%), 16 ml phosphoric acid (1.6%) with 0.08% Coomassie Brilliant Blue G-250 (solution A). Dissolve 80 g of ammonium sulfate (8%) in 600 ml of distilled water (solution B). Mix both the solutions and makeup to 1 liter; Use a magnetic stirrer to mix the solution overnight.
Dissolve the Coomassie Brilliant Blue G-250 (CBB) thoroughly in ethanol and add solution B later to avoid precipitation.

COMMENTARY

BACKGROUND INFORMATION:

Hemoglobin is a complex metalloprotein, and heme needs to be properly incorporated into each globin subunit to ensure correct tetrameric assembly. The bacterial cell membrane needs to be permeable to exogenous heme so that it can be transported across the bacterial membrane to assemble the functional rHbs upon induction by IPTG. The rHb is susceptible to heme autoxidation and denaturation throughout the expression and purification process. The purification buffers and all the solutions need to be bubbled with CO to prevent autoxidation of heme in rHb. Methemoglobin formation is also a concern, during expression and purification of rHb.

The α- and β-globin chains need to be expressed in equimolar quantities and should be present in the soluble fraction to ensure proper heme uptake. The selection of expression strain, IPTG induction concentration, and expression temperature are other crucial parameters that need careful optimization. Overexpressing multiple recombinant proteins (α- and β-globin chains, MAP, NatA complex) in *E. coli* host cells requires careful optimization (Fig. 3). NH_2-terminal modification can be achieved when there is a balance in the expression among the MAP, NatA complex, and globin chains. The MAP enzyme needs to be efficiently co-expressed to cleave the NH_2-terminal methionine residues from the nascent globin peptides (Natarajan et al., 2011). Likewise, the NatA complex also needs to be overexpressed at the right time to acetylate the NH_2-terminal.

Expressing recombinant proteins with purification tags is easier, but it will not resemble the native protein or its function. On the other hand, the tagless protein purification methods are complicated but can produce human rHb that possesses similar functional properties as native HbA. The crude bacterial cell lysate contains an abundance of heme-proteins, which complicates the purification of rHbs.

TROUBLESHOOTING:

Potential problems and solutions that may come up during this protocol are shown below in Table 4.

[*Place table 4 near here.]

UNDERSTANDING RESULTS:

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We developed a protocol to synthesize rHb with NH_2-terminal acetylation. Our spectroscopic analysis revealed the signature peaks (Soret and Q-band) for the rHbs showing the proper heme incorporation (Fig. 4). We successfully purified the unacetylated (pGM + pMAP) and acetylated versions (pGM + pMAP + pNatA) of human rHb (Fig. 5). Based on SDS-PAGE analysis, we pooled the pure fractions for downstream functional analyses. The O_2 binding properties were analyzed using the Blood Oxygen Binding System (Loligo Systems). O_2-equilibrium curves of purified Hb samples were measured in the presence and absence of allosteric cofactors (Fig. 6**,** Table 5). The data indicate that native and recombinant Hbs have highly similar O_2 binding properties. Our current system provides a means of assessing the role of NH_2-terminal acetylation in Hb function.

[*Place table 5 near here.]

We also analyzed the efficiency of NH_2-terminal acetylation using MS/MS for the co-expression of the pNatA plasmid system (Fig. 7). For purified samples of each rHb, we quantified the fraction of α- and β-chain peptides of NH_2-terminal acetylation using PEAK X software (Fago et al., 2020). We also measured the oxygenation properties of native human Hb and unacetylated rHb (expressed using the pGM + pMAP system) and acetylated rHb (expressed using the pGM + pMAP + pNatA system) (Fig. 8).

[*Place figure 7 near here.]

[*Place figure 8 near here.]

NH_2-terminal valine in both α- and β-chain forms a covalent bond with an acetyl group (CH_3CO), which can neutralize the charge on the NH_3 side chain. This reduces the net positive charge in the central cavity of Hb in the deoxy (T) conformation. This may not have major effects on the allosteric regulation of human Hb. However, in the Hbs of other vertebrates such as crocodilians, NH_2-terminal residues may play a more important role in the allosteric binding of organic phosphates and other cofactors so that acetylation can produce significant functional effects (Fago et al., 2020; Weber et al., 2013). Our new protocol for synthesizing rHb with NH_2-terminal acetylation provides a means of assessing how this post-translational modification affects the structural and functional properties of the protein. This protocol should yield about 15mg of purified rHb per 10g of wet cell pellet.

TIMING

Synthesis of cassette: 1-2 weeks
Ligation reaction: 1 hr - Overnight
Transformation of *E. coli* JM109 cells: 2-3 days
Preparation of glycerol stock: 15 min
Large-Scale expression of rHb: 3 days
Harvesting the cells: 45 min
Lysis of cells: 2 hr
SDS-PAGE analysis: 6-7 hr
Staining: 12 hr
Purification of rHbs: 4 days

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Acknowledgments

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Conflict of Interest

Authors have no conflict of interest to declare

Legends

Figure 1. Globin cassette sequence demarcated the coding region. Nucleotide and protein sequence for human α and β-globin showed along with Shine-Dalgarno region and restriction enzyme sites.

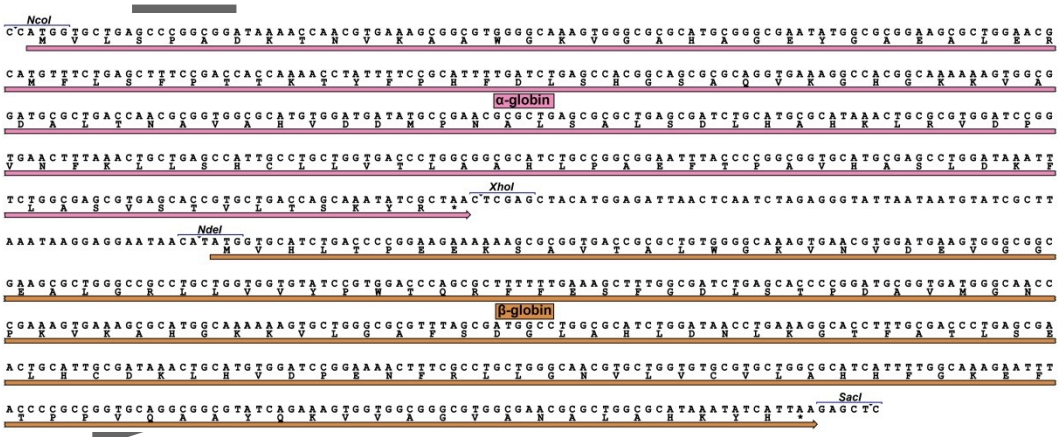

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Figure 2. The maps of expression plasmids. (A) pGM expression plasmid containing the ampicillin resistance gene and the Shine-Dalgarno (SD) sequence located between the α- and β- globin genes. The pNatA plasmid contains the genes that confer chloramphenicol resistance and the *naa10* and *naa15* genes of the NatA complex (Eastwood et al., 2017). The pMAP expression plasmid contains the kanamycin resistance gene and an additional copy of the MAP gene (Natarajan et al., 2011).

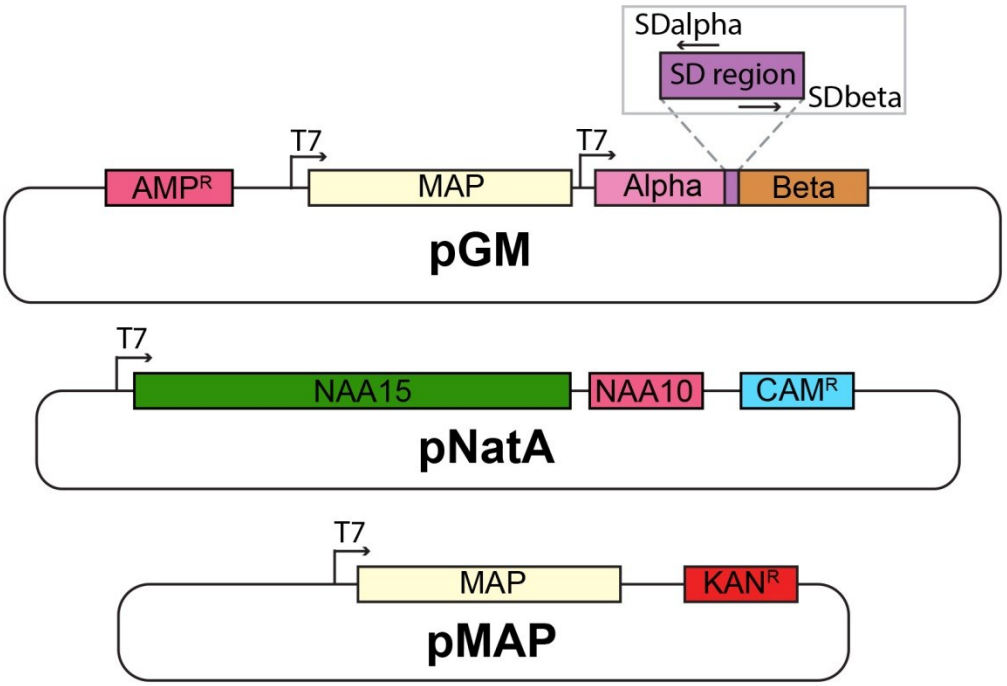

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Figure 3. Schematic diagram showing the post-translational modification in *E. coli.* Synthesis of rHb in the presence of pMAP and pNatA plasmids.

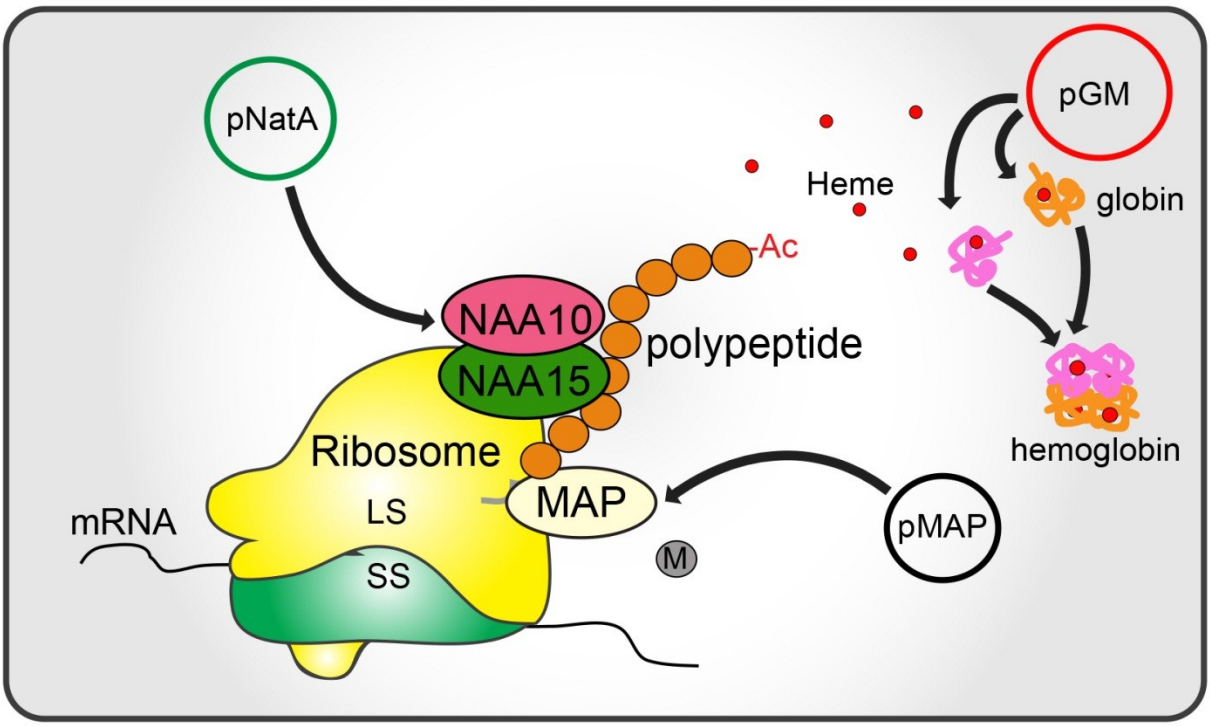

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Figure 4. Absorption spectra for human rHbs. The spectrum showing the characteristic of oxygenated soret peak at 415 nm and the Q-band centered at 541 nm and 575 nm for unacetylated and acetylated rHb.

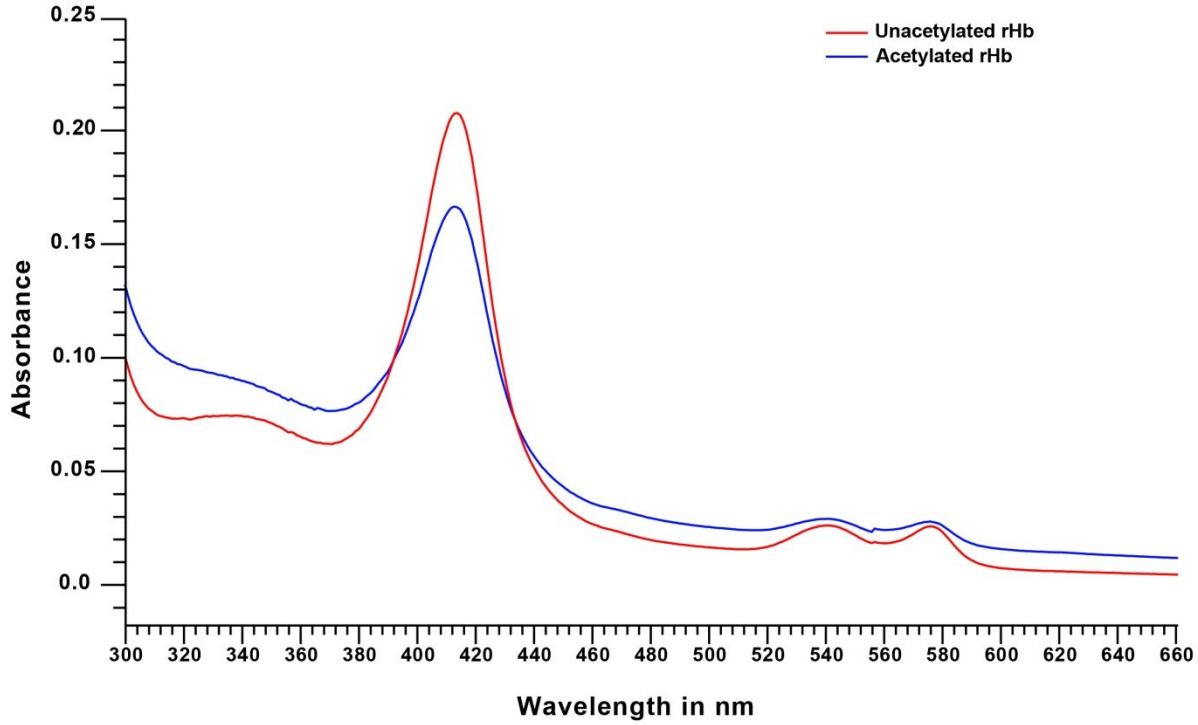

Figure 5. SDS-PAGE image showing the purified rHbs. (A) Elution profile for SP column, Lane M, prestained protein ladder (Thermo Fisher scientific); Lane1-8, flowthrough-1, wash-1, wash-2; 1%, 2%, 4%, and 8% of elution buffer respectively; Lane 9, purified unacetylated rHb; Lane 10, purified acetylated rHb. Asterisks denote the target rHb.

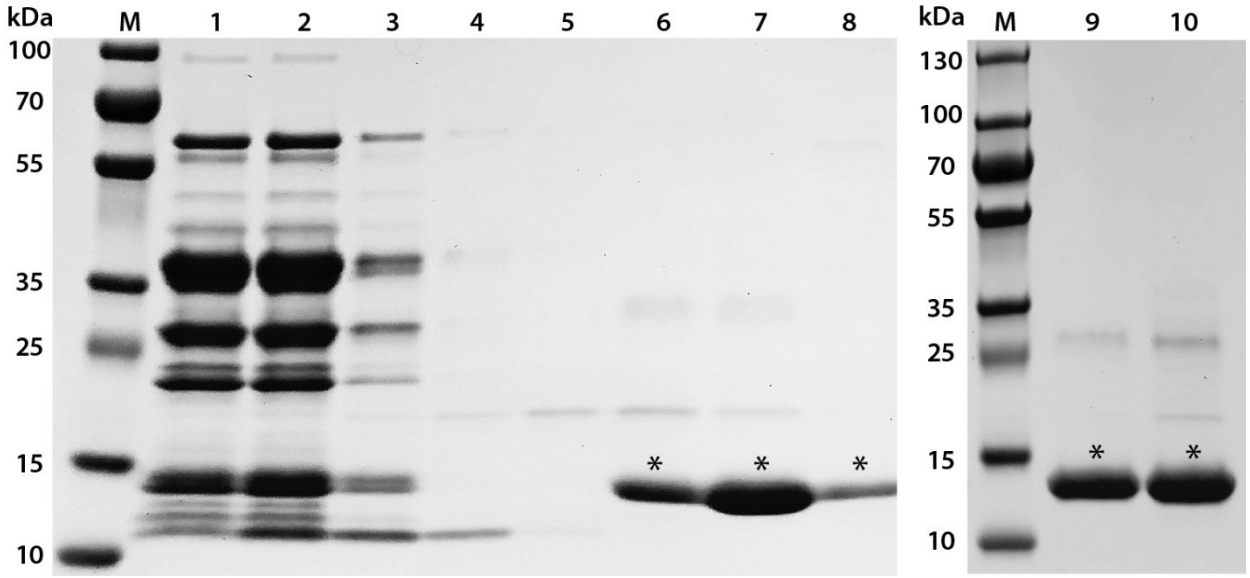

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Figure 6. O_2 binding properties of native human HbA and rHbs. Data showing the $LogP_{50}$ for stripped and other treatments (Cl^- ions, DPG, DPG+Cl^-) for native Hb, unacetylated rHb (produced using the pGM+pMAP plasmid), and acetylated rHb (produced using the pGM+pMAP+pNatA plasmid).

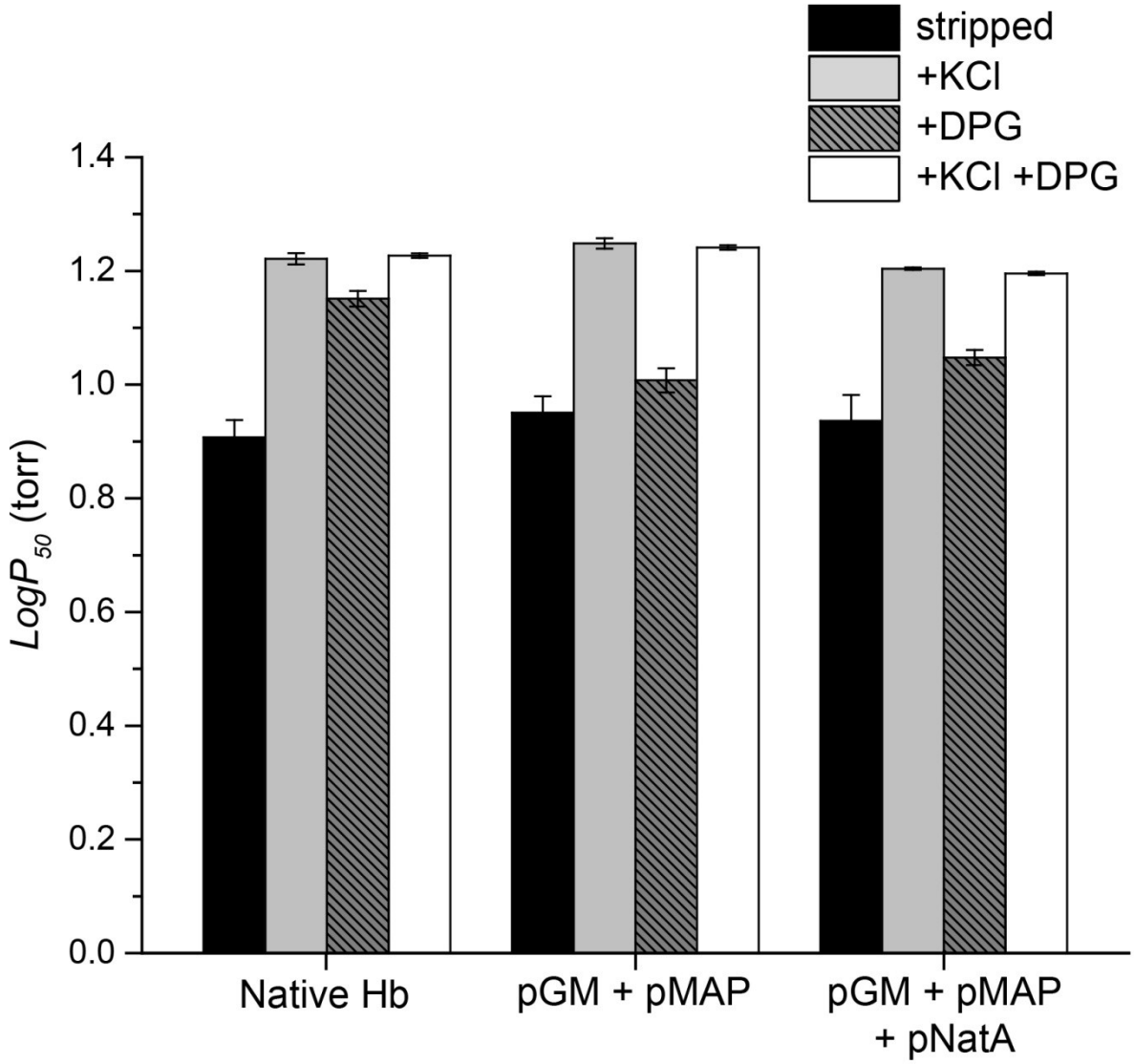

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Figure 7. NH_2-terminal acetylation profile for recombinant Hbs produced using different plasmid systems. Bar graph depicting the efficiency of N-terminal acetylation on α- and β-chain subunits for the pGM+pMAP and pGM+pMAP+pNatA plasmids.

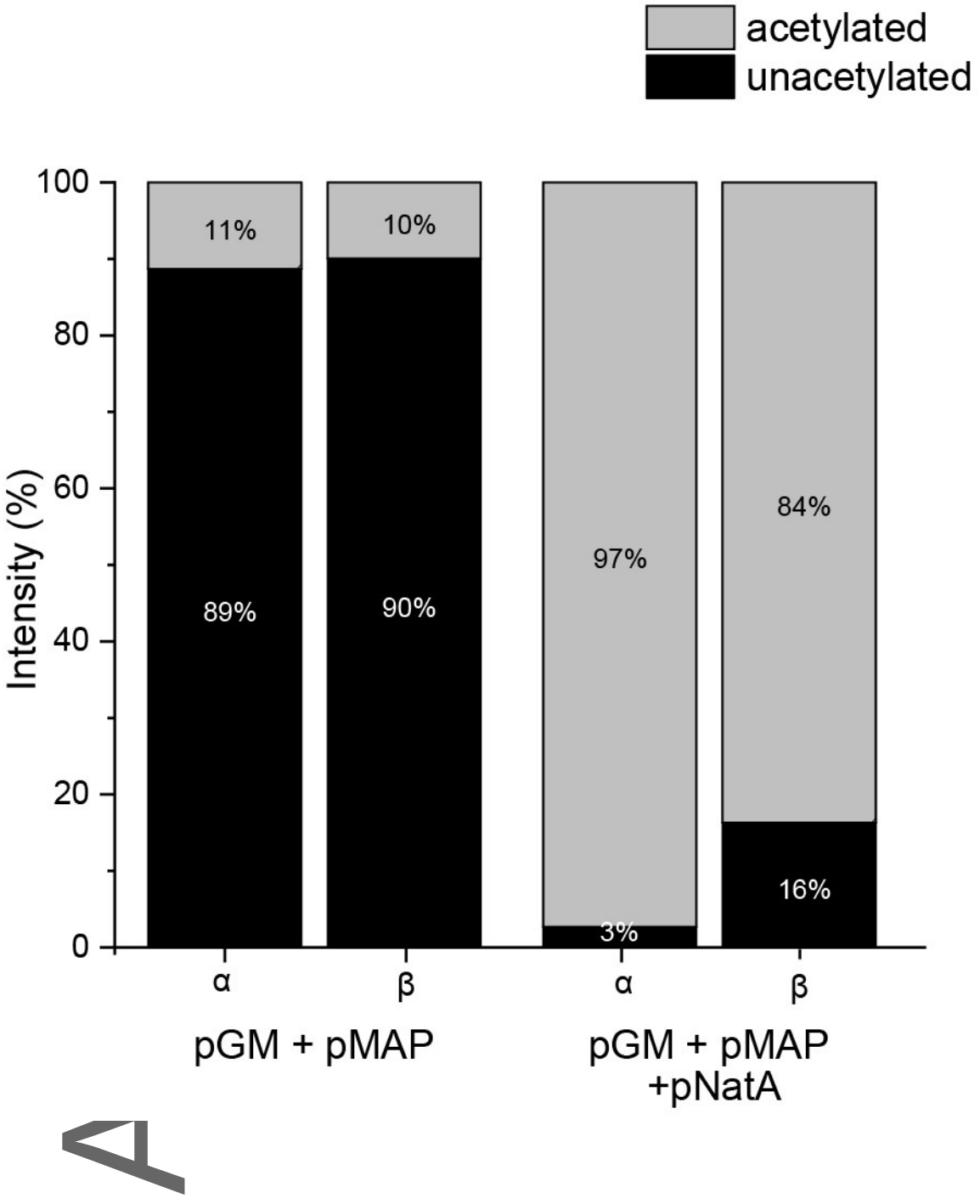

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Figure 8. Effect of NH_2-terminal acetylation on oxygenation properties of Hb. Plots showing the pH sensitivity and cooperativity for the Native human HbA (A), unacetylated rHb (B), and acetylated rHb (C).

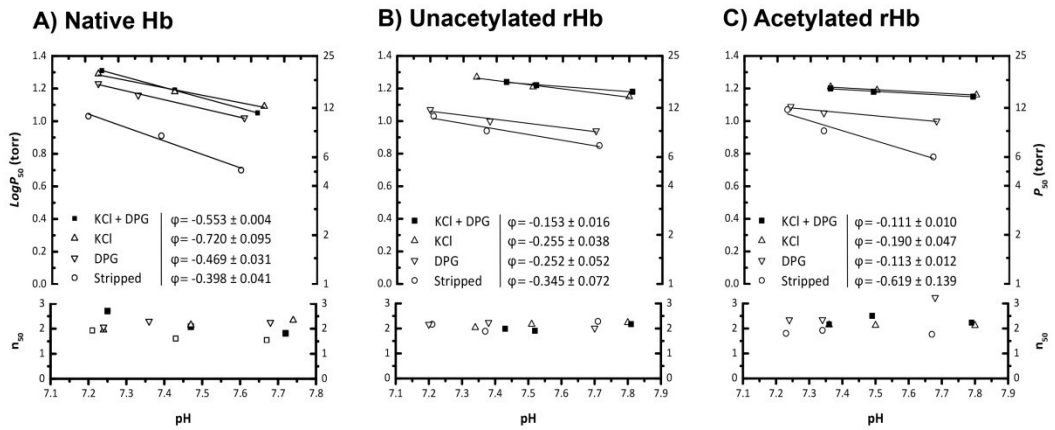

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Table 1 Double-restriction digestion recipe.

Reagent	Volume
Plasmid (2-3 ug)	15.0 µl
10X NEBuffer-1	3.0 µl
BSA (100X)	0.2 µl
*Nco*I	1.0 µl
*Sac*I	1.0 µl
Nuclease-free water	9.8 µl
Total volume	30 µl

Table 2 Pipetting Scheme for setting the ligation reaction.

Reagent	Volume
pGM vector DNA (40-80 fmol)	2.0 µl
Globin cassette insert DNA (120-200 fmol)	6.0 µl
T4 ligase	1.0 µl
T4 ligase buffer (10X)	1.2 µl
Nuclease-free water	1.8 µl
Total volume	12 µl

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Table 3 List of reagents added for protein induction

Reagent	volume/weight
Glucose	6.0 gm
IPTG*	150.0 µl
Sodium hydrosulfite	15 mg
Hemin*	300.0 µl
To be added to 300 ml of TB media in 1000 ml flask	

*see reagents and solutions for preparation of stock

Table 4. Troubleshooting guide for the purification of rHbs.

Step	Problems	Reasons and Solutions
Restriction Digestion	Self-ligation pGM plasmid	The *Nco*I restriction enzyme needs more time to digest the plasmid relative to *Sac*I. Incomplete digestion resulted in self-ligation. Increasing the amount of enzymes and the digestion time enhances the complete digestion.
Ligation	Ligation failure	Ligation can fail due to nicks caused to the vector or insert DNA during gel excision. Use the low setting for UV-lamp in trans-illuminator to excise the DNA band from the agarose gel.
Ligation	Ligation failure	When the digested DNA amount is less. Low DNA concentration can cause a failure in 1 hr ligation. Overnight ligation at 4°C can provide success.
Cloning	Transformation failure	Sub cloning three plasmids into JM109 (DE3) was challenging. Only cells that received the three plasmids can survive in the LB plate with antibiotics. Prolonging the recovery time after transformation can help to get more positive clones.
Large-scale Expression	Expression strain growth	The delay in the growth of the JM109 (DE3) cells after inoculation may be

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		due to multiple antibiotic selections. Antibiotics concentration can be reduced to avoid delay in culture growth.
Large-scale Expression	Insolubility of hemin in the culture medium	Hemin is dissolved in DMSO and added to the culture media at the desired concentration. DMSO is known for its cell permeability
SDS-PAGE	Smearing of lanes, Interference of bacterial heme-proteins and free heme	Bacterial heme-proteins interfere with the sample separation and migration in SDS-PAGE. Bacterial heme-proteins and the nucleic acids are precipitated by adding polyethyleneimine solution to a final concentration of 0.5 to 1%. Centrifuge crude lysates at 15,000 g for 45 min at 4ºC and analyze the supernatants using SDS-PAGE.
Purification	Autoxidation of heme or methemoglobin formation	Equilibrate all buffers with CO, keep on ice between purification steps. Adding Sodium dithionite at the time of induction during expression can also help to keep the heme in reduced form. Add it in the later stage can affect rHb stability.
Purification	The protein not binding to the anion-exchange column	If the dialysis step was too short, or the buffer needs to be exchanged. Use at least a 1:250 volume ratio sample: dialysis buffer. Alternatively, the column was poorly equilibrated, and still contains some salt from the previous purification. Increase the volume of equilibration buffer A before sample loading.
Storage	Protein degradation and precipitation	Some of the recombinant hemoglobin falls apart quickly due to buffer pH or buffer composition. It is always better to concentrate the protein and desalt it before any storage. For long term storage at -80°C, flash freezing by dry ice is the best method.

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Table 5. Oxygen binding properties of the human native hb and rHbs.

Human	Stripped		+KCl		+DPG		KCl + DPG	
	P_{50}	n_{50}	P_{50}	n_{50}	P_{50}	n_{50}	P_{50}	n_{50}
Native Hb	8.43±0.09	1.69±0.10	16.90±0.70	2.15±0.09	14.42±0.72	2.21±0.09	17.25±0.69	2.20±0.22
Unacetylated rHb	9.05±0.74	2.11±0.10	17.74±0.46	2.15±0.05	10.25±0.58	2.14±0.05	17.40±0.18	2.02±0.06
Acetylated rHb	8.95±1.27	1.83±0.04	16.00±0.06	2.13±0.01	11.20±0.38	2.79±0.26	15.69±0.12	2.30±0.08

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