Expression and Purification of Recombinant Hemoglobin in *Escherichia coli*

Chandrasekhar Natarajan  
*University of Nebraska-Lincoln*, chandrasekhar.natarajan@unl.edu

Xiaoben Jiang  
*University of Nebraska-Lincoln*

Angela Fago  
*Aarhus University*

Roy E. Weber  
*Aarhus University*

Hideaki Moriyama  
*University of Nebraska - Lincoln*, hmoriyama2@unl.edu

*See next page for additional authors*

Follow this and additional works at: [https://digitalcommons.unl.edu/bioscistorz](https://digitalcommons.unl.edu/bioscistorz)

Part of the Genetics and Genomics Commons

Natarajan, Chandrasekhar; Jiang, Xiaoben; Fago, Angela; Weber, Roy E.; Moriyama, Hideaki; and Storz, Jay F., "Expression and Purification of Recombinant Hemoglobin in *Escherichia coli*" (2011). *Jay F. Storz Publications*. 42.  
[https://digitalcommons.unl.edu/bioscistorz/42](https://digitalcommons.unl.edu/bioscistorz/42)

This Article is brought to you for free and open access by the Papers in the Biological Sciences at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Jay F. Storz Publications by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
Authors
Chandrasekhar Natarajan, Xiaoben Jiang, Angela Fago, Roy E. Weber, Hideaki Moriyama, and Jay F. Storz
Expression and Purification of Recombinant Hemoglobin in _Escherichia coli_

Chandrasekhar Natarajan¹, Xiaoben Jiang¹, Angela Fago², Roy E. Weber², Hideaki Moriyama¹, Jay F. Storz¹*

¹School of Biological Sciences, University of Nebraska, Lincoln, Nebraska, United States of America, ²Zoophysiology, Department of Biological Sciences, Aarhus University, Aarhus, Denmark

Abstract

**Background:** Recombinant DNA technologies have played a pivotal role in the elucidation of structure-function relationships in hemoglobin (Hb) and other globin proteins. Here we describe the development of a plasmid expression system to synthesize recombinant Hbs in _Escherichia coli_, and we describe a protocol for expressing Hbs with low intrinsic solubilities. Since the α- and β-chain Hbs of different species span a broad range of solubilities, experimental protocols that have been optimized for expressing recombinant human HbA may often prove unsuitable for the recombinant expression of wildtype and mutant Hbs of other species.

**Methodology/Principal Findings:** As a test case for our expression system, we produced recombinant Hbs of the deer mouse (_Peromyscus maniculatus_), a species that has been the subject of research on mechanisms of Hb adaptation to hypoxia. By experimentally assessing the combined effects of induction temperature, induction time and _E. coli_ expression strain on the solubility of recombinant deer mouse Hbs, we identified combinations of expression conditions that greatly enhanced the yield of recombinant protein and which also increased the efficiency of post-translational modifications.

**Conclusion/Significance:** Our protocol should prove useful for the experimental study of recombinant Hbs in many non-human animals. One of the chief advantages of our protocol is that we can express soluble recombinant Hb without co-expressing molecular chaperones, and without the need for additional reconstitution or heme-incorporation steps. Moreover, our plasmid construct contains a combination of unique restriction sites that allows us to produce recombinant Hbs with different α- and β-chain subunit combinations by means of cassette mutagenesis.

Citation: Natarajan C, Jiang X, Fago A, Weber RE, Moriyama H, et al. (2011) Expression and Purification of Recombinant Hemoglobin in Escherichia coli. PLoS ONE 6(5): e20176. doi:10.1371/journal.pone.0020176

Editor: Michael Hofreiter, University of York, United Kingdom

Received October 20, 2010; Accepted April 26, 2011; Published May 20, 2011

This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the Creative Commons CC0 public domain dedication.

**Funding:** This work was funded by National Science Foundation Grant DEB-0614342 (http://www.nsf.gov/); and National Institutes of Health/National Heart, Lung, and Blood Institute Grant R01 HL087216 (http://www.nhlbi.nih.gov/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: jstorz@unl.edu

Introduction

Hemoglobin (Hb) and myoglobin (Mb) have long held center stage in studies of the relationship between protein structure and protein function. Hb and Mb were the first proteins whose structures were solved at high resolution by X-ray crystallography, and Hb has served as a highly profitable model system for establishing principles of allosteric regulation [1,2,3,4]. In the last few decades, our understanding of structure-function relationships has been greatly aided by the ability to introduce specific mutations into recombinant proteins by site-directed mutagenesis [5,6,7,8,9]. In the case of human embryonic Hbs, our understanding of structure-function relationships is solely based on experimental studies of recombinant proteins [10]. The expression and functional analysis of recombinant Hbs (rHbs) has also provided detailed insights into molecular mechanisms of pathophysiology and biochemical adaptation. For example, site-directed mutagenesis of human rHbs revealed the molecular mechanism responsible for elevated Hb-O2 affinity in birds that are capable of high-altitude flight [11,12]. Similarly, mutagenesis studies of human rHbs also aided the identification of sites responsible for the binding of bicarbonate ions as allosteric effectors in crocodile Hb [13]. By using ancient DNA as source material, this experimental approach has even provided insights into functional properties of Hb from extinct animals [14].

Recent advances in molecular biology have led to the development of increasingly refined and efficient methods for producing rHbs. Some of these methodological improvements have been motivated by the goal of developing cell-free, rHb-based blood substitutes [15]. Nagai and Thøgersen [16,17] developed the first bacterial expression system for producing rHbs in _E. coli_. These first efforts involved fusing α- or β-globin cDNAs to the coding region of a bacteriophage repressor gene. The insoluble fusion protein could then be digested to recover the intact globin chain [18]. One drawback of this system was that, once recovered, the globins had to be reconstituted _in vitro_ with heme. A second-generation expression system involved co-expressing the α- or β-globin genes as a polycistrionic transcript with tac promoter [19]. In this system, functional Hb tetramers were produced after the incorporation of exogenous heme in the _E. coli_ cytoplasm. One
Problem with this method was the lack of N-terminal processing, such that the N-terminal methionine residue was not cleaved from the α- and β-chain polypeptides. Two strategies have been adopted to solve this problem [19]. One strategy involves altering the first codon so that valine is substituted for the initiator methionine. The second strategy involves co-expressing the gene for methionine aminopeptidase (MAP), an enzyme that cleaves the N-terminal methionine from the nascent globin chains in *E. coli* [20,21]. In the polycistronic approach the α- and β-globin genes were successfully co-expressed with the MAP gene under the control of two separate *tac* promoters [22]. Expression of α-globin as a soluble fusion protein with exogenous heme has been attempted in bacteria, but expression and isolation proved to be more difficult than in the case of β-globin [23]. Vasseur-Godbillon et al. [24] were able to improve the yield of soluble α-globin in *E. coli* by co-expressing the gene for the α-Hb stabilizing protein (AHSP), an erythroid-specific chaperone protein that binds specifically to free α-globin and prevents its precipitation [25,26].

In the present report we describe modifications of expression conditions that increase the solubility of rHb and which also enhance the efficiency of post-translational modifications in *E. coli*. We identified optimal combinations of temperature, induction time, and expression strain for the efficient expression of soluble rHbs with proper cleaving of N-terminal methionine residues. The purified rHbs can be used for any number of downstream applications including structural and functional studies. Moreover, the protocol that we describe can be executed using resources of a standard molecular biology laboratory.

**Methods**

**Study system**

As a test case for our expression system, we produced rHbs of the deer mouse, *Peromyscus maniculatus*. Deer mice have served as subjects for extensive research on mechanisms of Hb adaptation to high-altitude hypoxia [27,28,29,30,31]. Highland and lowland populations of deer mice possess structurally distinct Hbs due to allelic variation at two tandemly duplicated α-globin genes and two tandemly duplicated β-globin genes. We refer to the high- and low-altitude α-globin sequences as Hα and Lα, respectively, and we refer to the high- and low-altitude β-globin sequences as Hβ and Lβ, respectively. With regard to the α-chain subunits, the Hα and Lα sequences differ at eight amino acid positions: 50(CD8)Pro/His, 57(E6)Gly/Ala, 60(E9)Ala/Gly, 64(E13)Gly/Asp, 71(E20)Ser/Gly, 115(GH1)His/Leu, 115(GH3)Ala/Ser, and 116(GH4)Glu/Asp (the notation in parentheses indicates the sequential number of each residue in α-helices A-H, the interhelical segments, or terminal extensions). With regard to the β-chain subunits, the HB and Lβ sequences differ at four amino acid positions: 62(E6)Gly/Ala, 72(E16)Gly/Ser, 128(H16)Ala/Ser, and 135(H13)Ala/Ser. To measure the net contributions of the α- and β-globin mutations to variation in Hb-O₂ affinity, we synthesized the high- and low-altitude ‘wildtype’ isoHbs, HαHβ₂ and LαLβ₂, which we henceforth refer to as ‘HH’ and ‘LL’.

**Preparation of plasmid DNA**

We constructed two expression plasmids, pGM-HαHβ and pGM-LαLβ, which contain the α- and β-globin sequences that are characteristic of high- and low-altitude mice, respectively. The α/β-globin and MAP cassettes were synthesized by Genscript (Piscataway, NJ, USA). DNA sequences of the α- and β-globin genes were optimized with respect to *E. coli* codon preferences in order to maximize translational efficiency [23,32]. The α- and β-globin genes were tandemly cloned with a Shine-Dalgarno ribosomal binding site as an intergenic spacer [19]. In addition to expressing the α- and β-globin genes, the pGM plasmid also expresses the MAP gene under the control of a second independent T7 promoter (Figure 1A). The MAP enzyme is responsible for cleaving the N-terminal methionine residues from the nascent globin chains, a critical post-translational modification of tetrameric Hb. We also co-expressed a pCO-MAP plasmid that contained an additional copy of the MAP gene with kanamycin resistance to provide a means of antibiotic selection (Figure 1B). Alternative α-globin genes can be swapped by using two unique

---

**Figure 1. Design of the expression plasmids.** As shown in Panel A, the pGM-HαHβ plasmid system contains a cassette of tandemly arrayed α- and β-globin genes with a Shine-Dalgarno ribosomal binding site as a spacer DNA. The MAP cassette is located downstream of β-globin. As shown in panel B, the pCO-MAP plasmid contains a MAP cassette under the control of a T7 promoter along with a kanamycin resistance gene.

doi:10.1371/journal.pone.0020176.g001
restriction enzymes NcoI and HindIII, and alternative β-globin genes can be swapped by using a different pair of unique restriction enzymes, NdeI and SacI.

Optimization of expression conditions

Using the deer mouse pGM-HαHβ construct as a test case, we sought to increase the solubility of rHb by optimizing the expression conditions. Specifically, we conducted experiments to assess the combined effects of temperature, induction time, E. coli expression strain to enhance solubility and to improve the efficiencies of heme incorporation and post-translational modifications of deer mouse rHbs. The pGM-HαHβ plasmid was transformed into five commercially available expression strains: JM109 (DE3), BL21 (DE3) pLysS, BL21Star™ (DE3), BLR (DE3), and Origami™ (DE3). The E. coli cells were induced with 0.2 mM IPTG for 4 hr at 30°C in an orbital shaker at 200 rpm. The rHb samples were subsequently analyzed by 16% SDS-PAGE and yields were quantified by densitometric measures using the program Quantity One (BioRad, Hercules, CA, USA). We also evaluated four different combinations of temperature and induction time for the optimization of rHb expression: (1) 12°C for 24 hr, (2) 25°C for 16 hr, (3) 30°C for 4 hr, and (4) 37°C for 4 hr. As before, the samples were analyzed by 16% SDS-PAGE and yields were quantified by densitometric measures of band intensities. To assess whether the N-terminal methionine residues were properly cleaved, N-terminal peptide sequences of the α- and β-chain subunits were determined by means of Edman degradation. To prepare samples for peptide sequencing, the α- and β-globin monomers were separated by means of Acid-Urea-Triton (AUT) polyacrylamide gel electrophoresis [33], and were then transferred to PVDF membrane. The efficacy of the MAP enzyme in cleaving the N-terminal methionine residues was optimized by systematically altering the induction temperature, the induction time, and E. coli expression strain.

Large Scale rHb expression

The pGM-HαHβ and pGM-LαLβ plasmids were initially transformed into the E. coli strain JM109 [34]. Based on results of our optimization experiments (see Results), the plasmid vectors were subsequently transformed into the JM109 (DE3) cells for large-scale production of rHbs. The pCO-MAP expression plasmid was co-transformed with the pGM-HαHβ and pGM-LαLβ plasmids. The E. coli cells were grown overnight in 2xYT medium with supplemented ampicillin (100 μg/ml) and kanamycin (50 μg/ml) at 37°C in an orbital shaker at 200 rpm. A 5 ml inoculum of the overnight culture was added to 250 ml of fresh TB medium with a final volume of 50 μg/ml ampicillin and kanamycin in a 1000 ml conical flask. Production was scaled-up in batches of 4 to 6 flasks containing 1–1.5 liter of TB medium. The cells were grown at 37°C in an orbital shaker at 200 rpm until the absorbance reached 0.6–0.8 at 600 nm. The cells were induced with 0.2 mM IPTG and were then supplemented with hemin (50 μg/ml) and glucose (20 g/L). The cells were then subsequently grown at 28°C for 16 hr in an orbital shaker at 200 rpm.

Purification of rHb

The induced culture was saturated with CO for 15 min and the bacterial cells were harvested by centrifugation and stored frozen at −80°C. The cells were resuspended in lysis buffer (3 ml/g of cells), consisting of 50 mM Tris base, 1 mM EDTA, 0.5 mM

---

**Figure 2. SDS-PAGE (20%) image showing the purified rHb isoforms.** Lane BHb, Bovine Hb standard; Lane M, BioRad size standards; Lane 1, HH crude lysate; Lane 2, HH clarified fraction after dialysis; Lane 3, HH purified fraction; Lane 4, LL crude lysate; Lane 5, LL clarified fraction after dialysis; Lane 6, LL purified fraction; Lane NaHb, Native Hb from deer mouse blood.

doi:10.1371/journal.pone.0020176.g002
DTT, and 1 mM PMSF. Lysozyme (1 mg/g of cells) was added along with the lysis buffer prior to sonication. The *E. coli* cells were sonicated with 10 sec pulse and a 20 sec pause on ice bath with 3.0 output duty for 15 min. Nucleic acids were precipitated by adding polyethyleneimine solution to a final concentration of 0.5 to 1%. The crude lysates were centrifuged at 15,000 g for 45 min at 4°C, and the clarified supernatants were then dialyzed overnight against CO saturated Tris-TETA buffer (20 mM Tris-HCl and 0.1 mM triethylenetetraamine, pH 7.4). The dialysed samples were centrifuged at 15,000 g for 30 min at 4°C and the supernatants were used for further purification. Recombinant Hbs were purified by means of HPLC using Q-Sepharose (anion-exchange) followed by SP-Sepharose (cation-exchange) pre-packed columns. The Q-Sepharose column was equilibrated with 20 mM Tris-HCl and 0.1 mM triethylenetetraamine, pH 8.3, and the bound rHbs were eluted by a linear gradient of 0 to 160 mM NaCl in Tris-TETA buffer pH 8.3. The eluted fractions were dialyzed with 10 mM sodium phosphate buffer at pH 7.2 and were concentrated with YM-30 centrifuge filter. The concentrated fractions were passed through SP-Sepharose column equilibrated with 10 mM sodium phosphate buffer at pH 7.2. The bound rHbs were eluted with a linear gradient of equilibration buffer versus 10 mM sodium phosphate buffer at pH 8.0 and concentrated with ultra filtration and stored at 2–8°C in aliquots with 0.5–1 mM heme. The qualities of the purified rHb samples were analyzed by 20% SDS-PAGE (Figure 2).

Functional analysis of rHbs

Using the purified rHb samples, absorbance spectra of oxy, deoxy (obtained by adding a small amount of solid dithionite), and CO derivatives were measured at 450–600 nm to confirm that the absorbance maxima corresponded to those of native Hb. O₂-binding equilibria of rHb solutions (0.2 mM heme) were measured at 37°C and in 0.1 M Hepes, pH 7.4, in the absence (stripped) and presence of added cofactors (0.1 M KCl, and/or 2,3-diphosphoglycerate [DPG] at 2.0-fold molar excess over rHb tetramers [29]) as previously described [35,36]. The measurements were conducted by using a modified diffusion chamber where changes in absorbance are recorded during stepwise changes in the O₂ tension of equilibration gases. Experimental measures of P₅₀ (O₂ tension at half-saturation) and of the cooperativity coefficient n₅₀ were obtained from the zero-intercept and the slope of Hill plots (log(Y/(1−Y)) vs. logPO₂, respectively, where Y is the fractional O₂ saturation.

Results and Discussion

We initially tried to optimize the deer mouse rHbs using the protocol of Shen et al. [22]. In contrast to results obtained with human Hb (HbA), the deer mouse Hbs were expressed mostly in the insoluble fraction. Tetramers that incorporated the Hₐ subunit were characterized by an especially low solubility. The observed solubilities of the two deer mouse rHbs were consistent with *in silico* predictions using the method of Wilkinson and Harrison [37]. Using this same *in silico* method, we found that α- and β-chain Hbs from a diverse array of vertebrate species span a broad range of predicted solubilities (Table S1). These results suggest that obtaining adequate yields of soluble rHb may represent a chief obstacle to the experimental study of rHbs from many non-human animals.

Our experimental results indicate that the yield of soluble rHb and the efficiency of heme incorporation vary among different *E.
*E. coli* strains. The selection of the right expression strain can greatly enhance the quality and quantity of rHb production. Of the five *E. coli* expression strains that we tested, BL21Star™ (DE3) produced the highest yield of soluble rHb ([Figure S1 a and S1 b]). Yields can be further enhanced by using the right combination of induction temperature and induction time. Using the pGM-Hb expression plasmid in the BL21Star™ (DE3) expression strain, we found that the highest yield of soluble rHb – relative to the insoluble fraction – was produced at low temperature with an extended induction time (12 °C for 24 hr; [Figure S1 c and S1 d]). When supplemented with a rich medium, *E. coli* can continue to maintain a balanced growth in temperatures ranging from 10 to 49 °C [38]. At low temperature, cold shock proteins such as CsdA, RbfA and CspA will be induced and they play important roles in protein synthesis [39–41]. Our preliminary results suggest that induction of the BL21Star™ (DE3) expression strain at 12 °C for 24 hr yields good quantity of rHbs in the soluble fraction. We also assessed whether the N-terminal methionine residues were properly cleaved from the α- and β-chain subunits of the rHbs. To do this, the recombinant globin chains were separated by means of AUT gel electrophoresis and were then subjected to N-terminal peptide sequencing. We established an optimal culture condition to enhance the MAP activity without affecting the solubility of recombinant globin chains. We compared three combinations of induction temperature, induction time and expression strains: A) BL21Star™ (DE3) at 12 °C for 24 hr, B) BL21Star™ (DE3) at 30 °C for 4 hr with co-expression of pCO-MAP expression plasmid, C) JM109 (DE3) at 28 °C for 16 hr with co-expression of pCO-MAP expression plasmid. The N-terminal peptide sequencing revealed that the rHbs expressed in JM109 (DE3) at 28 °C for 16 hr with an additional copy of the MAP gene had the lowest fraction of intact N-terminal methionine residues in both the α- and β-chains (i.e., the post-translational processing was the most efficient; [Figure 3]). Our results also revealed that the JM109 (DE3) strain generally produces rHb with a higher efficiency of heme incorporation compared to the BL21Star™ (DE3) strain. In general, an increased induction temperature enhances MAP enzyme activity but decreases the solubility of recombinant globin chains. Thus, the optimal expression conditions strike a balance between protein yield and proper post-translational processing.

Relative to the LL rHb, the O₂-binding experiments revealed that the HH rHb has a higher intrinsic O₂ affinity (i.e., lower P₅₀ stripped) under the buffer conditions investigated ([Figure 4] but similar affinity in the presence of anionic cofactors. The measured difference in intrinsic O₂-affinity between the HH and LL rHbs is slightly larger than the measured O₂-affinity differences between stripped red cell lysates from high- and low-altitude deer mice [30,31]. The stripped HH and LL rHbs exhibited P₅₀ values of 4.5 and 5.7 Torr and ν₂₅ values of 1.8 and 2.1, respectively (37 °C; 0.1 M Hepes buffer, pH 7.4, heme concentration = 0.2 mM). Under identical experimental conditions, the stripped red cell lysates of high- and low-altitude mice exhibited mean P₅₀ values of 7.4 and 7.9 Torr and mean ν₂₅ values of 2.1 and 2.3, respectively [31]. Individual deer mice generally express at least 3–4 distinct Hb components that contain different combinations of amino acid mutations in both the α- and β-chain subunits [30,31], but the most common native Hb types in mice from high- and low-altitude have α- and β-chain primary structures that are similar (or identical) to those of the HH and LL rHbs, respectively. Although the red cells of high-altitude deer mice contain a preponderance of Hb components that are highly similar to the HH rHb, such components are typically co-expressed with other α- or β-chain isoforms that bear closer similarities to the LL rHb. Given the heterogeneous mix of distinct Hb types in the red cells of deer mice, we would not expect an exact match between P₅₀ values for the composite hemolysates and those of isolated Hb components. Nonetheless, as expected, we do see a clear correspondence in the direction and magnitude of O₂ affinity differences in the comparison between HH and LL rHbs and in the comparison between red cell lysates from high- and low-altitude mice. Because of the high level of Hb heterogeneity in the red cells of deer mice,

![Graph](image-url)

**Figure 4.** O₂-binding properties of purified deer mouse rHbs. Panels A and B show O₂ equilibrium curves for purified rHbs HH and LL, respectively. The rHbs HH and LL were expressed in the JM109 (DE3) *E. coli* strain at 28 °C for 16 hr with co-expression of the pCO-MAP plasmid. O₂-binding measurements were conducted at pH 7.4 at 37 °C in presence and absence of allosteric cofactors (0.1 M Cl⁻, 0.1 M Hepes, 2.0-fold ratio DPG / rHb tetramers and 0.2 mM heme). Horizontal lines denote the half-saturation value for each rHb.

DOI:10.1371/journal.pone.0020176.g004
it is clear that site-directed mutagenesis experiments involving recombinantly expressed Hbs will provide the most powerful means of identifying the specific amino acid mutations that are responsible for the observed differences in intrinsic O₂ affinity between the high- and low-altitude Hb variants.

In summary, we identified combinations of expression conditions that can be expected to improve the qualitative yield of rHbs with low intrinsic solubilities in E. coli. This protocol should prove useful for the experimental study of rHbs in many non-human animals. Previous methods for expressing rHbs in E. coli involved the independent production of α- and β-chain subunits, followed by a second step to ensure proper heme incorporation during the assembly of the αβ tetramer. One of the chief advantages of our protocol is that we can express soluble rHb without co-expressing molecular chaperones like AHSP, and without the need for additional reconstitution or heme-incorporation steps. Moreover, our plasmid construct contains a combination of four unique restriction enzyme cut-sites flanking the α- and β-globin genes, which facilitates the swapping of alternative α- and β-globin variants to produce different tetrameric combinations.

**Supporting Information**

**Figure S1 Optimization of HH rHb expression in E. coli.** (a) 16% SDS-PAGE image showing the expression profile of HH rHbs in different E. coli strains. Lane M, BioRad size standards; Lanes SF and ISF correspond to the soluble and insoluble fraction of rHbs under each of five different E. coli strains: (A) JM109 (DE3), (B) BL21 (DE3) pLysS, (C) BL21StarTM (DE3), (D) BLR (DE3), (E) OrigamiTM (DE3). Lane BHb, Bovine Hb standard; (b) Densitometric analysis of rHbs at different expression conditions. Graph shows the soluble versus insoluble fraction ratios on the y axis, different expression conditions on the x axis.

**References**

1. Dickerson RE, Gei I (1983) Hemoglobin. California: Benjamin/Cummings.
2. Ho C (1992) Proton nuclear magnetic resonance studies on hemoglobin: cooperative interactions and partially ligated intermediates. Adv Protein Chem 43: 153–312.
3. Ho C, Lakin JA (2000) In Encyclopedia of Life Sciences. London: Nature Publishing Group.
4. Weber RE, Fago A (2004) Functional adaptation and its molecular basis in vertebrate hemoglobins, neuroglobins and cytoglobins. Respir Physiol Neurobiol 144: 141–159.
5. Kim HW, Shen TQ, Sun DP, Ho NT, Madrid M, et al. (1994) Restoring allosterism with compensatory mutations in hemoglobin. Proc Natl Acad Sci USA 91: 11547–11551.
6. Kim HW, Shen TQ, Sun DP, Ho NT, Madrid M, et al. (1995) A novel low oxygen affinity recombinant hemoglobin (96Val→Asp) switching quaternary structure without changing the ligation state. J Mol Biol 248: 867–882.
7. Tsai CH, Shen TQ, Ho NT, Ho C (1999) Effects of substitutions of lysine and aspartic acid for asparagine at 108 and of tryptophan for valine at 96 on the structural and functional properties of human normal adult hemoglobin: roles of αβ1 and αβ2 subunit interfaces in the cooperative oxygenation process. Biochemistry 38: 9751–9761.
8. Cheng Y, Shen TQ, Simplaceanu V, Ho C (2002) Ligand binding properties and structural studies of recombinant and chemically modified hemoglobins altered at β93 cysteine. Biochemistry 41: 11901–11913.
9. Chang CK, Simplaceanu V, Ho C (2002) Effects of amino acid substitutions at β131 on the structure and properties of hemoglobin: evidence for communication between αβ1 and αβ2 subunit interfaces. Biochemistry 41: 5644–5655.
10. Brittain T (2002) Molecular aspects of embryonic hemoglobin function. Mol Aspects Med 23: 293–342.
11. Jesen TH, Komiyama NH, Tame J, Pagnier J, Shi A, et al. (1994) Production of human hemoglobin in Escherichia coli using cleavable fusion protein expression vector. Methods Enzymol 231: 347–364.
12. Weber RE, Jesen TH, Malhe H, Tame J (1993) Mutant hemoglobins (α19-Ala and β55-Ser): functions related to high-altitude respiration in geese. J Appl Physiol 75: 2666–2655.
13. Komiyama NH, Miyazaki G, Tame J, Nagai K (1995) Transplanting a unique allosteric effect from crocodile into human hemoglobin. Nature 373: 244–246.
14. Campbell KL, Roberts JE, Watson LN, Stetfeld J, Sloan AM, et al. (2010) Substitutions in woolly mammoth hemoglobin confer biochemical properties adaptive for cold tolerance. Nat Genet 42: 536–540.
15. Olson JS, Eich RF, Smith LP, Warren JJ, Knowles BC (1997) Protein engineering strategies for designing more stable hemoglobin-based blood substitutes. Artif Cells Blood Substit Immobil Biotechnol 25: 227–241.
16. Nagai K, Thogersen HC (1984) Generation of θ-hemoglobin by sequence-specific protolysis of a hybrid protein produced in Escherichia coli. Nature 309: 810–812.
17. Nagai K, Thogersen HC (1987) Synthesis and sequence-specific protolysis of hybrid proteins produced in Escherichia coli. Methods Enzymol 153: 461–481.
18. Ollis PO, Lee SC (1993) Recent advances in heterologous gene expression in Escherichia coli. Curr Opin Biotechnol 4: 320–325.
19. Hoffman SJ, Loecker DL, Rochjem HC, Cozart PE, Durfee SL, et al. (1990) Expression of fully functional tetrameric human hemoglobin in Escherichia coli. Proc Natl Acad Sci USA 87: 8492–8496.
20. Chang SY, McGary EC, Chang S (1989) Methionine aminopeptidase gene of Escherichia coli is essential for cell growth. J Bacteriol 171: 4047–4072.
21. Shen TJ, Ho NT, Simplaceanu V, Zou M, Green BN, et al. (1993) Production of unmodified human adult hemoglobin in Escherichia coli. Proc Natl Acad Sci USA 90: 4108–4112.
22. Shen TJ, Ho NT, Zou M, Sun DP, Costam PF, et al. (1997) Production of human normal adult and fetal hemoglobins in Escherichia coli. Protein Eng 10: 1085–1097.
23. Hernan RA, Hui HL, Andracki ML, Noble RW, Sligar SG, et al. (1992) Human hemoglobin expression in E. coli: importance of optimal codon usage. Biochemistry 31: 8619–8626.
24. Vasour-Godblon C, Hamdan D, Marder MC, Baudin-Creuzot V (2006) High-yield expression of soluble human θ-hemoglobin complexed with its molecular chaperone. Protein Eng Des Sel 19: 91–97.
25. Kihm AJ, Kong Y, Hong W, Russell JE, Rouza S, et al. (2002) An abundant erythrocyte protein that stabilizes free θ-hemoglobin. Nature 417: 758–763.
26. Del D, Kong Y, Eaton SA, Weiss MJ, Mackay JP (2002) Biophysical characterization of the θ-hemoglobin binding protein θ-hemoglobin stabilizing protein. J Biol Chem 277: 40602–40609.
27. Storz JF (2007) Hemoglobin function and physiological adaptation to hypoxia in high-altitude mammals. J Mamal 88: 24–31.
28. Storz JF, Kelly JR (2000) Effects of spatially varying selection on nucleotide diversity and linkage disequilibrium: insights from deer mouse globin genes. Genetics 158: 367–379.
29. Storz JF, Sabatino SJ, Hoffmann FG, Gering EJ, Moriyama H, et al. (2007) The molecular basis of high-altitude adaptation in deer mice. PLoS Genet 3: e45.
30. Storz JF, Runck AM, Sabatino SJ, Kelly JR, Ferrand N, et al. (2009) Evolutionary and functional insights into the mechanism underlying high-
altitude adaptation of deer mouse hemoglobin. Proc Natl Acad Sci USA 106: 14450–14455.
31. Storz JF, Runck AM, Moriyama H, Weber RE, Fago A (2010) Genetic differences in hemoglobin function between highland and lowland deer mice. J Exp Biol 213: 2563–2574.
32. Sharp PM, Li WH (1987) The codon adaptation index—a measure of directional synonymous codon usage bias, and its potential applications. Nucleic Acids Res 15: 1281–1295.
33. Pieragostini E, Bullo R, Scaloni A, Bramante G, Di Luccia A (2005) The α chains of goat hemoglobins: old and new variants in native Apulian breeds. Comp Biochem Physiol B Biochem Mol Biol 142: 18–27.
34. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.
35. Weber RE (1981) Cationic control of O₂ affinity in lugworm erythrocruorin. Nature 292: 386–387.
36. Weber RE (1992) Use of ionic and zwitterionic (Tris/BisTris and HEPES) buffers in studies on hemoglobin function. J Appl Physiol 72: 1611–1615.
37. Wilkinson DL, Harrison RG (1991) Predicting the solubility of recombinant proteins in Escherichia coli. Biotechnology (N Y) 9: 443–448.
38. Jones PG, VanBogelen RA, Neidhardt FC (1987) Induction of proteins in response to low temperature in Escherichia coli. J Bacteriol 169: 2082–2095.
39. Jones PG, Mitta M, Kim Y, Jiang W, Inouye M (1996) Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in Escherichia coli. Proc Natl Acad Sci USA 93: 76–80.
40. Jones PG, Inouye M (1994) The cold-shock response - a hot topic. Mol Microbiol 11: 811–818.
41. Lee SJ, Xie A, Jiang W, Etchegaray JP, Jones PG, et al. (1994) Family of the major cold-shock protein, CspA (CS7.4), of Escherichia coli, whose members show a high sequence similarity with the eukaryotic Y-box binding proteins. Mol Microbiol 11: 833–839.