Expression, purification, and characterization of human hemoglobins Gower-1 ($\zeta_2\epsilon_2$), Gower-2 ($\alpha_2\epsilon_2$), and Portland-2 ($\zeta_2\beta_2$) assembled in complex transgenic–knockout mice

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Embryonic ζ- and ϵ-globin subunits assemble with each other and with adult α- and β-globin subunits into hemoglobin heterotetramers in both primitive and definitive erythrocytes. The properties of these hemoglobins—Hbs Gower-1 ($\zeta_2\epsilon_2$), Gower-2 ($\alpha_2\epsilon_2$), and Portland-2 ($\zeta_2\beta_2$)—have been incompletely described as they are difficult to obtain in quantity from either primary human tissue or conventional expression systems. The generation of complex transgenic–knockout mice that express these hemoglobins at levels between 24% and 70% is described, as are efficient methods for their purification from mouse hemolysates. Key physiological characteristics—including P_{50}, Hill coefficient, Bohr effect, and affinity for 2,3-BPG—were established for each of the 3 human hemoglobins. The stability of each hemoglobin in the face of mechanical, thermal, and chemical stresses was also determined. Analyses indicate that the ζ-for-α exchange distinguishing Hb Portland-2 and Hb A alters hemoglobin O_2-transport capacity by increasing its P_{50} and decreasing its Bohr effect. By comparison, the ϵ-for-β exchange distinguishing Hb Gower-2 and Hb A has little impact on these same functional parameters. Hb Gower-1, assembled entirely from embryonic subunits, displays an elevated P_{50} level, a reduced Bohr effect, and increased 2,3-BPG binding compared to Hb A. The data support the hypothesis that Hb Gower-2, assembled from reactivated ϵ globin in individuals with defined hemoglobinopathies and thalassemias, would serve as a physiologically acceptable substitute for deficient or dysfunctional Hb A. In addition, the unexpected properties of Hb Gower-1 call into question a common hypothesis for its primary role in embryonic development. (Blood. 2001;97: 1099-1105)

Introduction

Physiologically meaningful human hemoglobins assemble from 2 α-like and 2 β-like globin subunits. The 3 genes that encode α-like globins (5′-ζ-α2-α1-3′) and the 5 genes that encode β-like globins (5′-ϵ-^{G}γ-^{A}γ-δ-β-3′) are expressed in a developmental sequence that parallels their structural arrangement.[1,2] Coordinated switching of the α-like and β-like genes results in the high-level expression of Hb Gower-1 ($\zeta_2\epsilon_2$), Hb F ($\alpha_2\gamma_2$), and Hb A ($\alpha_2\beta_2$) during the embryonic, fetal, and adult developmental stages, respectively. Other structurally defined hemoglobins—Gower-2 ($\alpha_2\epsilon_2$), Portland-1 ($\zeta_2\gamma_2$), and Portland-2 ($\zeta_2\beta_2$)—are expressed at relatively low levels in primitive and definitive erythroid cells, primarily during embryonic and early fetal development.[1-8] Their heterotetrameric structures predict that each of these semi-embryonic hemoglobins will display properties compatible with human physiology.

Unlike Hb A ($\alpha_2\beta_2$) and Hb F ($\alpha_2\gamma_2$), the properties of semi-embryonic Hbs Gower-2 ($\alpha_2\epsilon_2$) and Portland-2 ($\zeta_2\beta_2$), as well as fully embryonic Hb Gower-1 ($\zeta_2\epsilon_2$), remain largely undefined.[3-8] Intact erythrocytes from 35-mm crown–rump embryos (approximately 7-week gestation), containing a complex mixture of embryonic, semi-embryonic, and fetal hemoglobins, have been shown to bind O_2 strongly.[9] However, it is difficult to purify individual hemoglobins from primitive erythroid cells because they are produced in low numbers during an early and relatively brief developmental window[4,5] and because they contain a highly heterogeneous population of hemoglobin heterotetramers.[4,5,9] Recently, a yeast expression system was developed to generate Gower hemoglobins for in vitro analysis.[10] As with bacteria, yeast culture systems express relatively low levels of functional hemoglobin, which must be rigorously purified from incompletely processed globins and from inaccurately assembled heterotetramers.[11-13] Nevertheless, yeast-expressed Gower hemoglobins appear to exhibit high O_2 affinities when studied under defined conditions.[10,14] A more comprehensive functional and structural characterization of Gower and other low-abundance hemoglobins would be facilitated by the generation of a system that expresses high levels of fully functional heterotetramers.

The successful high-level expression of fully processed and fully assembled human Hbs A, F, S, and C in complex transgenic–knockout mice[15-17] suggested that a similar strategy might be used to generate large quantities of less common human hemoglobins for in vitro analysis. A key step in this process was the generation of adult mice expressing high levels of human (h) embryonic ζ- and ϵ-globins in their definitive erythrocytes.[18] These mice were subsequently used to generate lines expressing hybrid mouse–human semi-embryonic hemoglobins at 100% levels.[19] Comprehensive in vitro and in vivo evaluation demonstrated that hemoglobins

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assembled from mα- and hε-globin subunits ($m\alpha_2h\epsilon_2$) displayed O_2-binding properties similar to those of control Hb $m\alpha_2h\beta_2$. This result strengthened the hypothesis that physiologically important characteristics of fully human Hb Gower-2 ($\alpha_2\epsilon_2$) might be similar to those of Hb A ($\alpha_2\beta_2$).[19] In contrast, substantial differences in the properties of Hbs $h\zeta_2m\beta_2$ and $h\alpha_2m\beta_2$ suggested that the physiological characteristics of fully human Hb Portland-2 ($\zeta_2\beta_2$) and Hb A might differ in several important respects.[19] In addition to providing an estimate of the biochemical and physiological properties of the semi-embryonic hemoglobins, these studies also indicated the potential value of human embryonic globin subunits as substitutes for adult globin subunits in individuals with defined thalassemias or hemoglobinopathies.

The current study extends our previous work by assessing key biochemical and physiological properties of *fully human* semi-embryonic and embryonic hemoglobins purified from complex transgenic–knockout mice. We describe a strategy for generating mice expressing high levels of human Hbs Gower-1 ($\zeta_2\epsilon_2$), Gower-2 ($\alpha_2\epsilon_2$), and Portland-2 ($\zeta_2\beta_2$), as well as specific methods for their rapid and efficient purification. The key biochemical characteristics of each hemoglobin are subsequently determined, including their O_2 affinities, subunit cooperativities, and changes in O_2 affinity in response to allosteric modifiers and variations in ambient pH. We also assess the stability of each of these hemoglobins in response to defined mechanical, chemical, and thermal stresses. Based on the data, we speculate on the evolutionary basis for hemoglobin switching and the potential value of these poorly understood hemoglobins to patients with congenital α- and β-globin chain defects.

Materials and methods

Transgenic and knockout mice

The generation and characterization of transgenic mice expressing high levels of human α, β, ζ, and ε globins have previously been described.[18-21] Mice with heterozygous knockout of their endogenous α-globin genes (genotype $m\alpha^{+/-}$) or β-globin genes (genotype $m\beta^{+/-}$) were generously provided by Y. W. Kan and Judy Chang (University of California, San Francisco)[22] and O. Smithies (University of North Carolina, Chapel Hill),[23] respectively. All mouse husbandry and experimentation was performed using protocols approved by the IACUC of the University of Pennsylvania.

Hemoglobin purification

Whole blood was collected from decapitated mice in 200 μL phosphate-buffered saline (PBS)–heparin (20 U/mL) or PBS–EDTA (27 mM), and the hemoglobins were promptly converted to the carbonmonoxy form by bubbling the sample with CO. Erythrocytes were subsequently washed twice with excess PBS–EDTA (2.7 mM), and the cell pellets were stored in aliquots at −80°C. Lysate was prepared in approximately 3-fold excess buffer A (see below) and clarified by ultracentrifugation at 20°C in a TLA-100 rotor at 40 000 rpm for 20 minutes (Beckman, Fullerton, CA). Hemolysates were fractionated over an SP/H 4.5 × 100 Poros column (PerSeptive Biosystems, Foster City, CA) using a BioCAD Sprint perfusion chromatography system (Framingham, MA). Hb Gower-1 was purified using buffer A (40 mM Bis-Tris, 5 mM EDTA, pH 6.5) and buffer B (buffer A + 200 mM NaCl) at 2 mL/min using a linear 10% to 60% buffer B gradient. Hb Portland-2 was similarly purified using a 10% to 40% buffer B gradient. To purify Hb Gower-2, buffers were adjusted to pH 6.8, and a nonlinear 30% to 50% buffer B gradient was used. Fractions collected in 96-well microtiter plates were analyzed at A_{540} on a SpectraMAX plate reader (Molecular Devices, Sunnyvale, CA), pooled, and concentrated at 4°C over a Centricon YM-10 filter (Millipore, Bedford, MA). Hb A, prepared from human hemolysate, was used as a control in all experiments.

Electrophoretic analysis

The identity and purity of each hemoglobin preparation was verified by denaturing Triton–acid–urea[24,25] and nondenaturing cellulose acetate electrophoresis[19] using methods recommended by the manufacturer (Helena Laboratories, Beaumont, TX).

Oxygen equilibrium curves

Purified CO-hemoglobins were resuspended to a final concentration of approximately 7.5 μM in P_{50} buffer (50 mM Bis-Tris, pH 7.4, 100 mM NaCl, 5 mM EDTA) and converted to the oxy form by photolysis under 100% O_2 using an ice water–cooled rotary condenser.[26] Conversion to the oxyhemoglobin form was judged complete by an A_{540}:A_{576} ratio of less than 0.95. Oxygen equilibrium curves (OECs) were subsequently determined on a HEMOX analyzer (TCS, Southampton, PA) at 20°C. Studies of 2,3-bisphosphoglycerate binding (2,3-BPG; Sigma, St Louis, MO) were carried out in P_{50} buffer (pH 7.4), whereas Bohr effect studies were carried out in P_{50} buffer adjusted to defined pH values.

Stability determinations

Mechanical. Using a modified version of a previously described method,[27] Hbs were diluted to approximately 13 μM with 10 mM potassium phosphate buffer (pH 8.0) and converted to the oxyhemoglobin form (see above). Aliquots (2 mL) were shaken for defined intervals at a setting of 2000 on a Maxi-Mix III type 65800 shaker (Thermolyne, Dubuque, IA), and denatured hemoglobins were precipitated by a 5-minute desktop spin. The soluble hemoglobin was determined by A_{542} spectrophotometry of the supernatant.

Chemical. Purified hemoglobins were diluted to approximately 0.1 mM in buffer (0.1 mM Tris, pH 7.4) and converted to the oxyhemoglobin form as described above. Aliquots diluted 10-fold in prewarmed Tris buffer containing 17% (vol/vol) isopropanol were incubated at 37°C for 5 minutes.[28] Precipitated hemoglobins were clarified by desktop centrifugation, and the A_{542} of the supernatant was determined.

Thermal. Purified hemoglobins were diluted to approximately 50 μM in buffer (0.1 mM Tris, pH 7.4) and converted to the oxyhemoglobin form as described above. Test and control aliquots were incubated for 2 hours at 50°C and 4°C, respectively, and spun for 10 minutes on a desktop centrifuge.[29] The supernatant was diluted 10-fold with developer solution [11.9 mM $NaHCO_3$, 0.77 mM KCN, 0.61 mM $K_3Fe(CN)_6$], insoluble hemoglobins were precipitated by desktop centrifugation, and the A_{540} of the supernatant was determined.

Results

Generation of adult mice expressing high levels of human embryonic and semi-embryonic hemoglobins

The construction of transgenes and the generation of mice expressing hζ, hα, hε, and hβ globins in definitive erythrocytes has previously been described.[18-20] The high-level, developmental-stage inappropriate expression of transgenic hζ and hε globins was achieved by linking their encoding genes to transcriptional control elements from the hα and hβ globin genes, respectively (Figure 1A).[18] Full-length genes encoding hα and hβ globins, containing their native transcriptional control elements, were anticipated to be expressed at high levels in adult erythrocytes and consequently were not modified.[19,20] Each transgene was linked to a micro β-locus control region to insure its high-level, integration position-independent expression.[18-20,30] Single lines expressing high levels of each transgenic globin were identified by phenotypical screening of hemolysates using denaturing globin electrophoresis.[24,25] These lines were used in the experiments described in the current work.

A breeding strategy was designed to generate mice expressing high levels of human embryonic or semi-embryonic hemoglobins with minimal mouse globin background (Figure 1B). We had

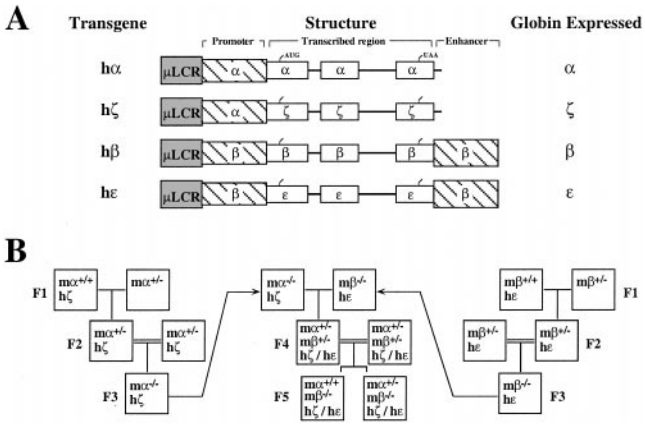

Figure 1. Generation of transgenic mice expressing high levels of human embryonic and semi-embryonic hemoglobins. (A) Structures of human transgenes. Exons are depicted as open boxes, with the positions of the translational initiation and termination codons indicated by tick marks. Globin-gene origins of promoter and enhancer elements (cross-hatched) are also indicated. All transgenes were linked to an identical micro β-LCR cassette (shaded).[30] The common name for each transgene and the human globin it expresses are indicated to the left and right of the diagram, respectively. (B) Mating strategy for generating mice expressing human Hb Gower-1 ($\zeta_2\epsilon_2$). Partial globin genotypes of selected mice from 5 generations (F1-F5) are depicted. The strategy facilitates the generation of mice expressing high levels of human Hb $\zeta_2\epsilon_2$ from progenitors expressing hζ or hε globin or containing heterozygous deletion of their endogenous mα or mβ globin genes. A similar strategy was used to generate mice expressing high levels of human Hbs Gower-2 ($\alpha_2\epsilon_2$) and Portland-2 ($\zeta_2\beta_2$). m, mouse; h, human; +/+, homozygous; +/−, heterozygous; −/−, nullizygous.

previously noted that the expression of each of the 4 human globin transgenes increased substantially in mice carrying one or more knockout mutations of the related endogenous adult globin gene homologue.[18,19] The level of hζ and hα induction was sufficient to rescue the viability of mice with homozygous-lethal deletions of their endogenous mα-globin genes,[18,31,32] whereas the viability of mice with homozygous-lethal deletions of the mβ-globin genes could be rescued by the expression of either transgenic hε or hβ globins.[18,31,32] We reasoned that the assembly of hemoglobin heterotetramers from transgenic human α-like and β-like globins would be similarly enhanced in mice carrying both mα- and mβ-globin knockout alleles.

Design of methods to purify human hemoglobins from transgenic hemolysates

A combination of genetic and biochemical strategies was used to facilitate the preparation of human Hbs from transgenic mice. We screened more than 125, 335, and 89 candidate pups expressing Hbs Gower-1, Gower-2, and Portland-2, respectively, without identifying any $m\alpha^{-/-}/m\beta^{-/-}$ mice expressing 100% of the desired human hemoglobins (data not shown). On the other hand, a substantial proportion of these pups displayed either $m\alpha^{+/-}/m\beta^{-/-}$ or $m\alpha^{+/+}/m\beta^{-/-}$ genotypes (more than 25 pups expressing each hemoglobin; data not shown). Hbs Gower-1 ($\zeta_2\epsilon_2$), Gower-2 ($\alpha_2\epsilon_2$), and Portland-2 ($\zeta_2\beta_2$) were expressed in the these complex transgenic–knockout mice as 37%, 24%, and approximately 70% of total hemoglobin, respectively, corresponding to the expression of human hemoglobin in the range of approximately 20 to 80 mg/mouse (data not shown). These high levels of expression facilitated the task of hemoglobin purification, as did the fact that $m\alpha^{+/-}/m\beta^{-/-}$ or $m\alpha^{+/+}/m\beta^{-/-}$ mice each assembled only a single contaminant hemoglobin species ($m\alpha_2h\epsilon_2$ or $m\alpha_2h\beta_2$).

A method was subsequently established for isolating each of the desired human hemoglobin heterotetramers using cation-exchange chromatography. Human Hbs Gower-1 ($\zeta_2\epsilon_2$), Gower-2 ($\alpha_2\epsilon_2$), and Portland-2 ($\zeta_2\beta_2$) were purified from contaminant hybrid Hbs $m\alpha_2h\epsilon_2$, $m\alpha_2h\epsilon_2$, and $m\alpha_2h\beta_2$, respectively, in single-step processes using NaCl gradients at defined pH levels (Figure 2, and data not shown). The identities and purities of the eluted human hemoglobins were subsequently verified by nondenaturing[19] and denaturing[24,25] electrophoretic methods (Figure 2 and data not shown). The large quantities of high-purity human embryonic and semi-embryonic hemoglobins efficiently prepared by this method were sufficient to permit their detailed physiological and biochemical evaluation and to provide substantial banked product for future functional and structural studies.

Embryonic and semi-embryonic hemoglobins exhibit elevated O_2 affinities

The O_2-binding affinities of human Hbs $\zeta_2\epsilon_2$, $\alpha_2\epsilon_2$, and $\zeta_2\beta_2$ were determined on 3 or more occasions under standard conditions by

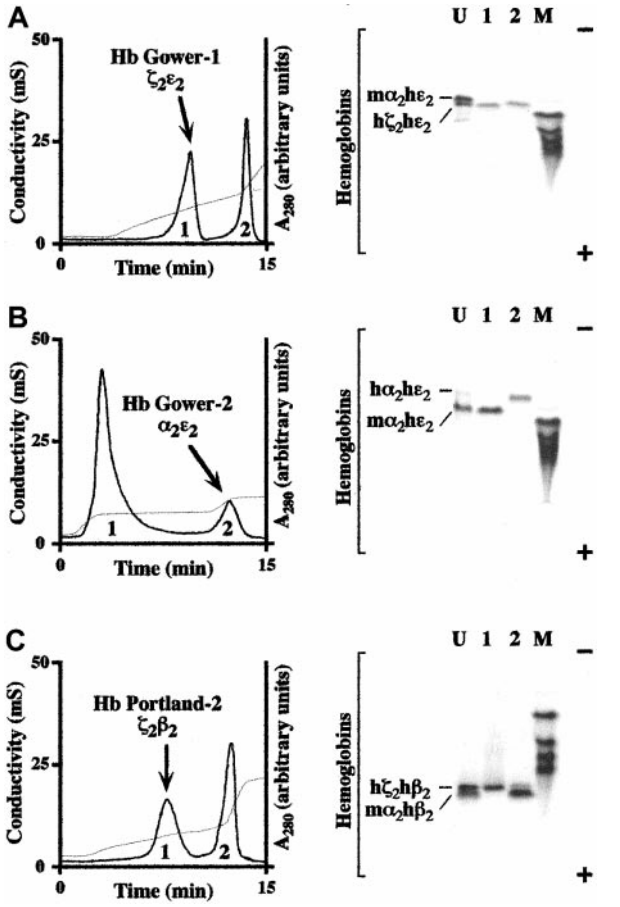

Figure 2. Purification of human embryonic and semi-embryonic hemoglobins. (A) Human Hb Gower-1 ($\zeta_2\epsilon_2$). (Left) Hemolysate prepared from adult $m\alpha^{+/-}/m\beta^{-/-}$/hζ/hε complex transgenic–knockout mice was resolved over a Poros SP/H column using a linear 20 to 120 mM NaCl gradient (pH 6.5). The positions of human Hb $\zeta_2\epsilon_2$ (peak 1) and contaminant hybrid mouse–human Hb $m\alpha_2h\epsilon_2$ (peak 2) are indicated (arbitrary A_{280} units). Eluate conductivity (in mS) is depicted by a gray line. (Right) Aliquots of unfractionated (U) lysate and eluate corresponding to peaks 1 and 2 were resolved by nondenaturing cellulose acetate electrophoresis. A control lane contains a mixture of human Hbs A, F, S, and C. The migration of constituent hemoglobins is indicated to the left, and gel polarity to the right. (B) Human Hb Gower-2 ($\alpha_2\epsilon_2$). Resolution of hemolysate from an adult $m\alpha^{+/-}/m\beta^{-/-}$/hα/hε complex transgenic–knockout mouse into human Hb $\alpha_2\epsilon_2$ (peak 2) and contaminant hybrid mouse/human Hb $m\alpha_2h\epsilon_2$ (peak 1) using a nonlinear 60- to 100-mM NaCl gradient (pH 6.8). (C) Human Hb Portland-2 ($\zeta_2\beta_2$). Resolution of hemolysate from an adult $m\alpha^{+/-}/m\beta^{-/-}$/hζ/hβ complex transgenic–knockout mouse into human Hb $\zeta_2\beta_2$ (peak 1) and contaminant hybrid mouse/human Hb $m\alpha_2h\beta_2$ (peak 2) using a linear 20- to 80-mM NaCl gradient (pH 6.5).

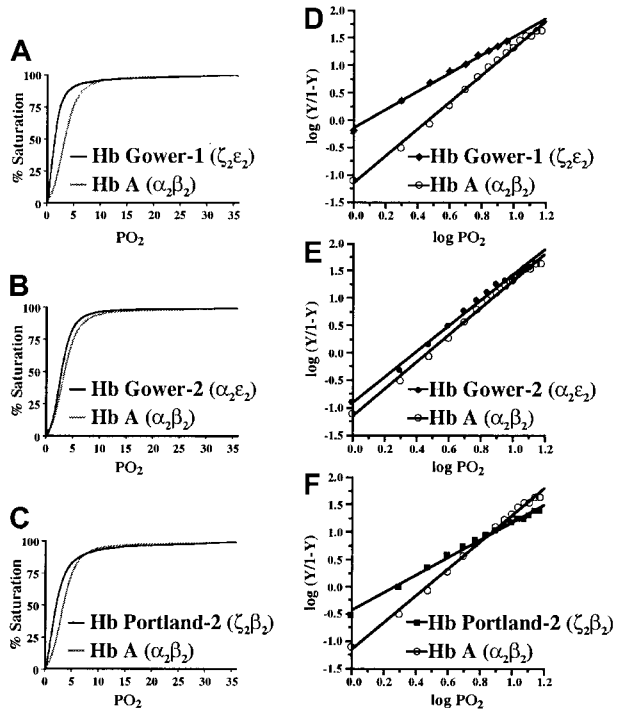

Figure 3. Oxygen equilibrium curves and Hill coefficients for human embryonic and semi-embryonic hemoglobins. Oxygen equilibrium curves are displayed in panels A-C. (A) Human Hb Gower-1 ($\zeta_2\epsilon_2$). (B) Human Hb Gower-2 ($\alpha_2\epsilon_2$). (C) Human Hb Portland-2 ($\zeta_2\beta_2$). OECs were established for affinity-purified human hemoglobins under standard assay conditions ("Materials and methods"). Representative curves (black) are displayed with an OEC from control human Hb A for reference (gray). P_{50} values derived from analyses of these curves are included in Table 1. Hill coefficients are displayed in panels D-F. (D) Human Hb Gower-1 ($\zeta_2\epsilon_2$, ◆). (E) Human Hb Gower-2 ($\alpha_2\epsilon_2$, ●). (F) Human Hb Portland-2 ($\zeta_2\beta_2$, ■). Hill plots constructed from OECs of human hemoglobins in panels A to C are illustrated along with control human Hb A (O). Hill coefficients derived from these curves are included in Table 1.

HEMOX analysis (Figure 3A-C, Table 1).[19] Each of the hemoglobins displayed a higher O_2 affinity than control Hb A, whose P_{50} of 3.2 torr was highly reproducible. The P_{50} values of Hbs Gower-1 ($\zeta_2\epsilon_2$) and Portland-2 ($\zeta_2\beta_2$) (1.4 and 1.9 torr, respectively) were approximately one-half the P_{50} for control Hb A. In contrast, Hb Gower-2 ($\alpha_2\epsilon_2$) exhibited a P_{50} of 2.7 torr, only marginally different from that of Hb A. These results were substantiated in independent experiments, using different temperature and buffer conditions, in which the relative P_{50} values of the 4 human hemoglobins were preserved (data not shown).[14] Hill coefficients derived from OEC analyses indicated substantially reduced subunit cooperativity for Hbs Gower-1 ($\zeta_2\epsilon_2$) and Portland-2 ($\zeta_2\beta_2$) (Hill n = 1.7 and 1.6, respectively) relative to control Hb A (Figure 3D-F; Table 1). In contrast, Hb Gower-2 ($\alpha_2\epsilon_2$) (n = 2.3) displayed subunit cooperativity much closer to our observed measure for Hb A (n = 2.9), which reproduced its accepted value (n = 2.8-3.0).[1] These results indicate that the O_2-binding properties of Hb $\alpha_2\beta_2$ heterotetramers are not materially affected by a β-to-ϵ exchange (converting Hb A to Hb Gower-2), whereas an α-to-ζ exchange (converting Hb A to Hb Portland-2) has a more substantial impact on both O_2 affinity and subunit cooperativity.

Table 1. Properties of human hemoglobins

Hb	Structure	P_{50}*	Hill†	Bohr‡	2,3-BPG§
Gower-1	$\zeta_2\epsilon_2$	1.4 ± 0.06	1.7 ± 0.24	−0.25	0.09
Gower-2	$\alpha_2\epsilon_2$	2.7 ± 0.10	2.3 ± 0.02	−0.51	0.17
Portland-2	$\zeta_2\beta_2$	1.9 ± 0.17	1.6 ± 0.06	−0.10	0.30
A	$\alpha_2\beta_2$	3.2 ± 0.14	2.9 ± 0.36	−0.54	0.29

*torr; †log(Y/1 − Y)/log PO_2; ‡Δlog P_{50}/ΔpH in the alkaline region; §apparent binding constant (mM) calculated from half-saturation point.

The P_{50} values of embryonic and semi-embryonic hemoglobins display disparate responses to changes in pH and [2,3-BPG]

The effect of pH on the O_2-binding affinities of the 3 human embryonic and semi-embryonic hemoglobins was determined by measuring the P_{50} values for each in a series of buffers with defined pH levels ranging from 6.0 to 8.2 (Figure 4A; Table 1). The 2 human hemoglobins containing ζ-globin subunits displayed attenuated Bohr effects [Hbs $\zeta_2\epsilon_2$ (−0.10 $\Delta\log P_{50}/\Delta PO_2$) and $\zeta_2\beta_2$ (−0.25 $\Delta\log P_{50}/\Delta PO_2$)], whereas the Bohr effect of Hb Gower-1 ($\alpha_2\epsilon_2$) and control Hb A ($\alpha_2\beta_2$) were nearly identical (−0.51 vs −0.54 $\Delta\log P_{50}/\Delta PO_2$, respectively). The apparent binding constant of 2,3-BPG for each hemoglobin was estimated by establishing the half-saturation point using buffers with defined concentrations of the allosteric modifier (Figure 4B; Table 1). 2,3-BPG appears to bind to Hb Portland-2 ($\zeta_2\beta_2$) and control Hb A with equal affinity (0.30 mM and 0.29 mM, respectively) while binding to Hbs Gower-1 ($\zeta_2\epsilon_2$) and Gower-2 ($\alpha_2\epsilon_2$) with substantially higher avidity (0.09 mM and 0.17 mM, respectively). These results indicate that the inclusion of embryonic globin subunits may have an impact on the biochemical function of intact heterotetramers, permitting speculation on the evolutionary pressures favoring the conservation of globin gene switching.

Human embryonic and semi-embryonic hemoglobins display different physical stabilities

Hbs Gower-1, Gower-2, and Portland-2 were assessed for their stability in the setting of defined mechanical,[27] chemical,[28] and thermal stresses.[29] Hbs A and S were evaluated in parallel as stable and unstable hemoglobin controls, respectively (Figure 5). The stabilities of the various hemoglobins by each of the 3 methods were in general agreement: Hbs Gower-1 ($\zeta_2\epsilon_2$) and Portland-2 ($\zeta_2\beta_2$) were equally or less stable than Hb S, whereas the stability of Hb Gower-2 ($\alpha_2\epsilon_2$) was generally intermediate between the 2 control hemoglobins. Hence, the low stability of Hb Gower-1 appears to be well adapted to the short survival of primitive erythrocytes, whereas the higher stability of Hb Gower-2 may facilitate its expression in long-lived definitive erythrocytes.

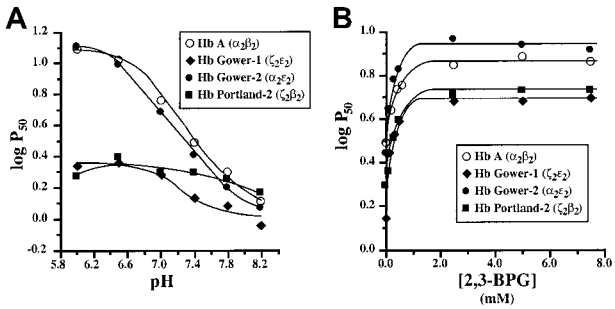

Figure 4. Biochemical properties of human embryonic and semi-embryonic hemoglobins. (A) Effect of pH on O_2 binding (Bohr effect). The P_{50} of each hemoglobin was determined in standard buffers adjusted to defined pH levels. Bohr effect values (included in Table 1) were calculated from best-fit curves of values from the alkaline range. $\zeta_2\epsilon_2$ (◆), $\alpha_2\epsilon_2$ (●), $\zeta_2\beta_2$ (■), $\alpha_2\beta_2$ (○). (B) Effect of allosteric modifiers on O_2 binding. The P_{50} values of human hemoglobins were determined in standard buffers containing defined concentrations of 2,3-BPG. The affinity of each Hb for 2,3-BPG (indicated in Table 1) was derived from the half-saturation point of each curve. $\zeta_2\epsilon_2$ (◆), $\alpha_2\epsilon_2$ (●), $\zeta_2\beta_2$ (■), $\alpha_2\beta_2$ (○).

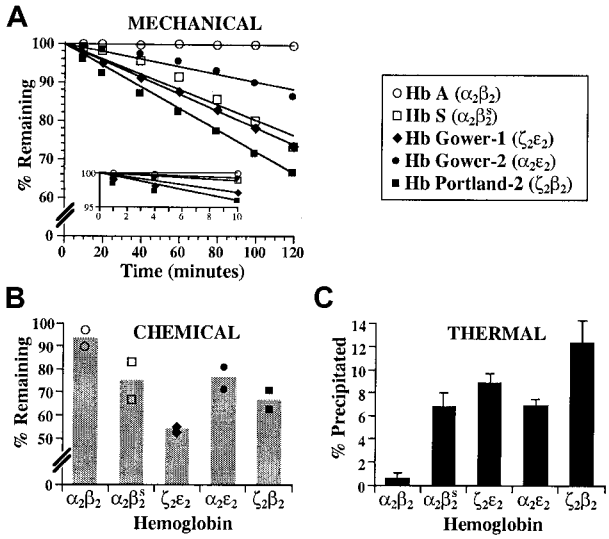

Figure 5. Stabilities of human hemoglobins to defined perturbations. (A) Mechanical stability. The stabilities of the human hemoglobins exposed to mechanical stress were determined as described in "Materials and methods." The percentage soluble hemoglobin is plotted as a function of time. Early time points are displayed on an expanded scale (inset). (B) Chemical stability. The stabilities of the human hemoglobins exposed to 17% isopropanol are illustrated. Bars represent the average of duplicate determinations using independently prepared isopropanol solutions. Symbols are the same as in panel A. (C) Thermal stability. The relative stabilities of the human hemoglobins incubated at 50°C were determined and plotted as a function of precipitated hemoglobin. Symbols are the same as in panel A.

Discussion

In spite of their developmental sobriquets, there is considerable overlap in the temporal expression of embryonic ζ and ϵ globins and adult α and β globins, respectively. Human α and β globins are expressed at moderate levels in developing embryos and fetuses,[33-36] whereas varying amounts of embryonic ζ and ϵ globins and their encoding mRNAs can be detected in fetal erythrocytes and in adult-stage reticulocytes, respectively.[37,38] The developmental integrity of embryonic globin gene expression is further compromised in patients with certain congenital genetic disorders. Low-level ζ-globin expression persists in adults heterozygous for the α-thalassemia $-^{SEA}$ deletion,[39] whereas fetuses and infants with defined trisomies may express easily detectable levels of ϵ globin.[40-43] The temporal overlap of embryonic and adult globin expression, particularly during intrauterine development, favors the assembly of Hbs Gower-2 and Portland-2. Remarkably few studies have directly addressed the biochemical and physiological properties of either of these hemoglobins or of the embryonic hemoglobin Gower-1, despite their assembly in normal primitive and definitive erythrocytes.

Although it is uncertain whether Hbs Gower-2 and Portland-2 play a defined role in human development or are simply incidental to developmental overlapping of globin gene expression, a full understanding of their properties, along with the properties of Hb Gower-1, would be valuable for several purposes. First, structure–function analyses of all 3 hemoglobins are likely to provide additional insight into the biochemistry of abundant, thoroughly studied hemoglobins such as Hbs A and F. Second, knowledge of its properties would provide a context for assessing the role of Hb Gower-1 in normal development and would help focus speculation on the evolutionary pressures favoring absolute phylogenic conservation of developmental globin gene switching. Third, a direct application of the analyses of Hbs Gower-2 and Portland-2 stems from the possibility that reactivation of the ζ and ϵ genes would produce globins that could substitute for deficient or abnormal α- or β-globin chains, respectively, in individuals with defined thalassemias and hemoglobinopathies.[18,19] The implications of the current work on each of these 3 issues are considered below.

Many well-established studies have identified functionally crucial amino acid residues in the α- and β-globin chains. Our data generally confirm the role of these residues, but they also emphasize the importance of distant residues on specific hemoglobin functions through their likely influence on the high-order structure of the globin molecule. In general, Hb Gower-2 and Hb A function similarly, suggesting that many of the 36 amino acid differences between the β- and ϵ-globin chains (of 146 total residues) cluster in functionally silent domains (Table 1). The 2 hemoglobins exhibit similar O_2-binding characteristics and display Hill coefficients consistent with normal hemoglobin function (Figures 3B, E), reflecting the conservation of 13 of 17 and 15 of 16 residues comprising the α1β1 and α1β2 interfaces, respectively.[44,45] Similarly, the robust Bohr effect common to Hb Gower-2 and Hb A (Figure 4A) might be anticipated from the conservation of 82Lys, 143His, and 146His in both the ϵ- and β-globin chains.[46-48] Unexpectedly, Hb Gower-2 and Hb A displayed markedly different affinities for 2,3-BPG (Figure 4B) despite their identity at all 4 residues believed to mediate this property (1Val, 2His, 82Lys, and 143His).[46,49,50] Although the half-saturation method provides only an estimate of the strength of this interaction, it is likely that one or more differences in amino acid content elsewhere in the ϵ- and β-globin chains alter the spatial arrangement, and hence the function, of the 4 conserved residues. A less likely explanation is that a fifth, unrecognized residue, differing between the β- and ϵ-globin chains, directly participates in 2,3-BPG binding. Crystallographic evaluation of Hb Gower-2, available in quantity from the transgenic–knockout mice, would be useful in evaluating these 2 possibilities.

In comparison to the β-like globin chains, the 57 (of 141) amino acid differences between the ζ- and α-globin chains appear to occupy functionally sensitive regions (Table 1). Hb Portland-2 and Hb A, differing only in the identity of their α-like chains, display substantially different P_{50} values and Hill coefficients (Figure 3C, F) despite sharing 14 of 16 and 14 of 15 residues at the α1β1 and α1β2 interfaces, respectively. Remarkably, 2 of the 3 interface substitutions are conservative, emphasizing the unpredictable functional effects of changes in high-order globin subunit structure resulting from spatially distant amino acid substitutions. Although both globins share a common 122His, the α1Val-ζ1Ser exchange would be expected to substantially blunt the Bohr effect exhibited by Hb Portland-2,[46,51] a prediction that is experimentally observed (Figure 4A). A direct comparison of Hb Portland-2 function with its high-resolution structure, when available, may yield important new information relative to the function of human heterotetramers.

The observation that the properties of Hb Gower-1, comprising developmental stage-concordant ζ- and ϵ-globin subunits, are strikingly different from those of Hb A suggests a strong evolutionary basis for Hb switching (Figures 3A, 4A-B; Table 1). A widely held, yet largely unsubstantiated, explanation for this process suggests that embryonic and fetal hemoglobins have been evolutionarily selected to facilitate the *trans*-placental delivery of O_2 during intrauterine development. This hypothesis has been challenged by studies that do not detect the predicted elevated rates of fetal wastage in pregnant women with high-affinity hemoglobins.[52,53] By demonstrating that the O_2-binding properties of Hb Gower-1

differ from those of Hb F, our data support the latter dissenting opinion. In contrast to Hb F, which exhibits a normal O_2 affinity (in the absence of 2,3-BPG), a relatively high Bohr effect, and poor 2,3-BPG binding, we have found that Hb Gower-1 displays a high O_2 affinity, a reduced Bohr effect, and relatively tight 2,3-BPG binding. Were their developmental roles strictly limited to O_2-transport, the O_2-binding properties of Hbs Gower-1 and F might be anticipated to be more similar. An alternative explanation for this difference is that the evolutionary basis for developmental hemoglobin switching reflects properties of Hb Gower-1 that are independent of its gaseous-exchange function. This possibility is underscored by recent reports indicating that in addition to its O_2-transporting function, Hb A may serve an important role in the regulation of vascular tone and blood flow.[54] It is clear that the functional properties of Hb Gower-1, reflecting poorly understood evolutionary demands and manifesting as phylogenic conservation of hemoglobin switching, merit additional study.

We have previously proposed that Hbs Portland-2 ($\zeta_2\beta_2$) and Gower-2 ($\alpha_2\epsilon_2$) might be suitable substitutes for Hb A in individuals with defined hemoglobinopathies and thalassemias.[18,19] These 2 hemoglobins would assemble from existing adult globin chains in adults in whom the embryonic globin genes were reactivated. The functional identity between Hb Gower-2 ($\alpha_2\epsilon_2$) and Hb A ($\alpha_2\beta_2$) indicates that the former hemoglobin would act in a physiologically valuable manner in definitive erythrocytes. Evidence that the ϵ- and γ-globin genes are independently regulated[55-58] raises the possibility that different classes of agents, with different toxicities, might be used to separately reactivate expression from the 2 genes. This consideration is particularly important in patients with severe β-thalassemia determinants in whom γ-globin reactivation by current agents is toxicity-limited.[59,60] The observation that Hb Gower-2 will serve a physiologically important role in individuals with any of several hemoglobinopathies and thalassemias should serve as an impetus to identify agents that might be useful in this regard.

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Expression, purification, and characterization of human hemoglobins Gower-1 ($\zeta_2\varepsilon_2$), Gower-2 ($\alpha_2\varepsilon_2$), and Portland-2 ($\zeta_2\beta_2$) assembled in complex transgenic–knockout mice

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