Assembly of Human Hemoglobin (Hb) β- and γ-Globin Chains Expressed in a Cell-free System with α-Globin Chains to Form Hb A and Hb F*

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Rates of in vitro synthesis of radiolabeled γ and β chains made in a cell-free transcription/translation system were similar, but expressed globin chains were unstable. The addition of unlabeled β or γ chains at the start of chain synthesis generated radiolabeled β2 or γ2 and γ2 chains, respectively. If unlabeled α-globin chains were added at the start of chain synthesis, then approximately equal amounts of radiolabeled αβ or αγ bands were generated. If unlabeled Hb A or Hb F was added to reactions containing radiolabeled αβ or αγ prior to electrophoresis, then radiolabeled Hb A or Hb F tetramers, respectively, were generated. If α chains were added after synthesis of radiolabeled γ chains made in the presence of unlabeled γ chains, then little radiolabeled αγ formed. In contrast, if α chains were added after synthesis of radiolabeled β chains made in the presence of unlabeled β chains, then radiolabeled αβγ formed. These findings suggest that β and γ chains associate with α chains during or soon after translation. This would prevent the formation of unstable monomers as well as stable γ2 dimers and suggests that α chains may bind to nascent non-α chains, acting as folding catalysts to promote functional tetrameric hemoglobin formation in vivo.

Assembly in vitro of human Hb1 subunits (α and non-α chains) into stable Hb heterotetramers (e.g. αβγ2 or αγ2γ) using purified globin chains has been explored and a 3-step mechanism proposed (1–8). The α chains are in monomer/dimer equilibrium-favoring monomers, whereas non-α chains are in monomer/tetramer equilibrium-favoring tetramers (9, 10). It is generally assumed that dissociation of these oligomeric subunits into monomers must occur before these two different chains can combine to form αβ or αγ dimers, which then associate to form tetrameric Hb (αββ2 or αγγ2) (11, 12). In addition, the assembly of αβ or αγ dimer was postulated to be the rate-limiting step for assembly in vivo and has been theorized to be governed by electrostatic attractions between α- and non-α partner subunits (8, 12). Furthermore, from in vitro studies it is known that Hb F formation using purified γ- and α-globin chains is very slow compared with Hb A using purified β- and α-globin chains (11). In fact, our previous studies showed approximately a 103-fold slower rate of assembly in vitro for Hb F compared with Hb A (11). The slow rate of Hb F formation in vitro is caused by stable γ2 dimer formation and is unlikely to occur in erythroid precursors (11). We also found that even at low concentrations (<5 μM), γ chain dimers do not dissociate readily into monomers, resulting in decreased assembly with α chains. Furthermore, our results showed that the assembly of [Ile116→His]γ chains with α chains was similar to that of β chains, whereas the assembly of [Thr112→Cys]γ with α chains was similar to wild type γ chains (11). These findings indicate that amino acid differences between [Ile116→His]γ and [His116→β] at αγ, γ2, and αβγ interaction sites, respectively, are responsible for the different assembly rates in vitro between Hb F and Hb A. These results also indicate that dissociation of γ2 dimers to monomers limits the formation in vitro of Hb F and suggest that γ chains assemble in vivo with α chains prior to forming stable γ2 dimers, possibly binding to α chains as partially folded nascent γ chains prior to or soon after release from polypeptides. To evaluate the ability of β- and γ-globin with α-globin chains in vivo, we expressed β and γ chains in a wheat germ-coupled cell-free transcription/translation system using cDNA expression vectors. In addition, we added α and γ or β chains as well as Hb F or Hb A to reactions at different times and assessed the formation of radiolabeled assembled homo-/heterodimers and tetrameric globins.

EXPERIMENTAL PROCEDURES

Non-α-Globin cDNA Expression Vectors—The plasmids pcDNA β and pcDNA γ contain the SP6 promoter and cDNAs coding for the human β- or γ-globin chains, respectively. They were constructed from pcDNA3 and pHe2 by subcloning each cDNA (11) into the HindIII/XbaI sites of pcDNA3. Transcription in vitro by SP6 RNA polymerase generates β- or γ-globin mRNA, which is translated in a commercially available wheat germ cell-free transcription/translation system. The sequence and insertion site of the β or γ chain cDNAs in the expression vectors were confirmed by automated DNA sequence analysis using dye-tagged terminators.

Expression of β- or γ-Globin Chains in a Wheat Germ Cell-free Transcription/Translation System—Expression of β- or γ-globin chains in a cell-free coupled transcription/translation system was performed using a TNT™ SP6-coupled wheat germ extract system kit (Promega, Madison, WI) containing [35S]methionine (Amersham Biosciences). A typical 50-μl reaction contained a 2-μg DNA template and was incubated for 30–60 min at 30 °C. For tetramer assembly studies, 6 ng of human α-globin chain, human Hb (Hb A or Hb F), human β chains, or recombinant γ chains were added to the reaction either at zero time or 30 min after radiolabeled chain synthesis. Synthesized β- or γ-globin chains, as well as those assembled with α chains to form radiolabeled αβ or αγ...
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RESULTS

Synthesis of Radiolabeled \( \gamma \) or \( \beta \)-Globin Chains and Assembly of Homodimers/Tetramers in a Cell-Free System—Results from in vitro transcription/translation using \( \gamma \) or \( \beta \)-globin chain expression vectors showed a single radiolabeled \(-16\text{-kDa}\) band after SDS-PAGE (Fig. 1, lane 2 in panels A and B) comigrating with purified \( \gamma \) or \( \beta \)-globin chains, respectively, whereas plasmids lacking cDNAs showed no band (Fig. 1, lane 1 in panels A and B). However, after cellulose acetate electrophoresis, radioactivity corresponding to newly synthesized \( \gamma \) or \( \beta \)-chains was only seen at the origin (Fig. 1, lane 2 in panels C and D).

When unlabeled purified \( \gamma \) chains were added at zero time to reactions containing \( \gamma \) cDNA, two radiolabeled bands were observed after cellulose acetate electrophoresis following a 30-min incubation with \[^{35}\text{S}\]-labeled methionine (Fig. 1, panel C, lane 3). The major band corresponds to \( \gamma_4 \) homodimers, whereas traces of a second band comigrating with recombinant \( \gamma_4 \) chains was also detected. In contrast, when unlabeled \( \beta \) chains were added at zero time to the \( \beta \) cDNA reaction, a single radiolabeled band was observed after cellulose acetate electrophoresis following a 60-min incubation with \[^{35}\text{S}\]-labeled methionine, which corresponds to \( \beta_4 \) tetramers (Fig. 1, panel D, lane 3). These results indicate that monomeric forms of newly synthesized \( \beta \) and \( \gamma \)-globin chains are unstable, precipitate, and remain at the origin during electrophoresis at room temperature; however, the addition of excess unlabeled non-\( \alpha \) chains facilitates formation of homodimers and/or tetramers and prevents precipitation of newly synthesized globin chains during electrophoresis.

Assembly of Radiolabeled \( \gamma \) or \( \beta \)-Globin Chains in a Cell-Free System with Unlabeled \( \alpha \)-Chains—When unlabeled human \( \alpha \)-globin chains were added at zero time to the \( \beta \) cDNA reaction, a single radiolabeled band was seen after cellulose acetate electrophoresis that migrated close to the \( \alpha_4 \) marker (Fig. 2, panel B, lane 1). When unlabeled human \( \alpha \)-globin chains were added at zero time to the \( \gamma \) cDNA reaction, a single radiolabeled band was observed after cellulose acetate electrophoresis that migrated close to the \( \alpha_2 \beta_2 \) marker (Fig. 2, panel B, lane 3). The single radiolabeled bands generated in these reactions with \( \alpha \)-globin chains added at zero time that migrate less than \( \alpha_2 \beta_2 \) and \( \gamma_4 \) heterodimers, respectively.

Assembly of Radiolabeled \( \gamma \) or \( \beta \)-Globin Chains with Unlabeled \( \alpha \)-Chains to Form Heterotetramers—Our contention that the single radiolabeled bands in Fig. 2 (panel B, lanes 1 and 3)
are radiolabeled αβ and αγ heterodimers, respectively, is supported by studies in which unlabeled Hb A or Hb F is added after the 30 min chain synthesis just prior to electrophoresis. Both radiolabeled bands comigrated with the corresponding heterotetramers after the addition of unlabeled Hb A or Hb F just prior to electrophoresis, respectively, (Fig. 2, panel B, lanes 2 and 4), indicating that the addition of Hb A and Hb F shifted the equilibrium from newly synthesized radiolabeled heterotetramers to heterotetramers. Mobility of the radiolabeled αβ and αγ bands on cellulose acetate electrophoresis depended on amounts of unlabeled Hb A and Hb F added; the higher the amount, the closer the mobility to the corresponding tetrameric hemoglobins A and F (Fig. 3). The concentration of unlabeled hemoglobins A and F needed to generate band mobility midway of unlabeled Hb A and Hb F were then added, and reactions containing α chains, reactions containing αγ dimers (panel B) were also similar to rates of globin chain synthesis (panel A).

Effects of Hp binding on electrophoretic mobility of radiolabeled heterotetramers and heterotetramers. Panel A, unlabeled Hp was added after the 30 min incubation period to transcription/translation reactions containing β-globin cDNA in the presence of unlabeled α-globin chains, in the absence (lane 2) or presence of Hb A (lane 4). Reactions were then subjected to cellulose acetate electrophoresis followed by autoradiography. Radiolabeled αβ dimers formed in the presence of unlabeled α chains (lane 1) or radiolabeled Hb A heterotetramers formed in the presence of unlabeled α chain plus Hb A (lane 3) are shown without Hp addition. Panel B, same as panel A except γ cDNA vector was used and unlabeled Hb F instead of Hb A added to form radiolabeled Hb F heterotetramers. Radiolabeled αγ dimers (lane 1) and radiolabeled Hb F heterotetramers (lane 3) are also shown without Hp addition.

**Time Course for Synthesis and Assembly of Radiolabeled Globin with Unlabeled α-Chains**—Time courses for incorporation of 35S-labeled methionine into radiolabeled γ and β chains were the same in the presence or absence of unlabeled α chain addition at zero time (data not shown) (as assessed by relative band intensity after SDS-PAGE) and plateaued after about 40 min (Fig. 5, panel A). In addition, rates of radiolabeled αγ or αβ dimer formation were similar in reactions containing unlabeled α chains measured by relative intensity after cellulose acetate electrophoresis (Fig. 5, panel B). Rates of formation of αβ and αγ dimers (panel B) were also similar to rates of globin chain synthesis (panel A).

Effects of Unlabeled α-Globin Chain Addition on Radiolabeled Heterodimer Formation after a 30-min Synthesis Period in the Presence of Unlabeled Non-α-Globin Chains—To test the effects of newly synthesized and assembled γ chain monomers and dimers on assembly with α chains, reactions containing unlabeled γ-globin chains added at zero time were stopped after a 30 min γ-globin chain synthesis period by the addition of puromycin. Unlabeled α chains or Hb F were then added, and samples were electrophoresed on SDS-PAGE or cellulose acetate membranes followed by autoradiography. Results from cellulose acetate electrophoresis of γ cDNA reactions containing unlabeled γ chains at zero time followed by the addition of unlabeled α chains just prior to electrophoresis showed a single radiolabeled band comigrating with γ dimers with no evidence for formation of radiolabeled αγ dimers (Fig. 6, panel A, lane 2).
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Fig. 5. Time course for chain synthesis and heterodimer formation. Time course is shown for radiolabeled chain synthesis assessed by SDS-PAGE (panel A) and for radiolabeled heterodimer formation assessed by cellulose acetate electrophoresis (panel B). Unlabeled α-globin chains were added at zero time only in experiments in panel B. Results are expressed as relative formation (%) where 100% represents maximum values at the plateau of each reaction.

Fig. 6. Effects of unlabeled α-globin chain addition on radiolabeled heterodimer formation after a 30-min synthesis period in the presence of unlabeled non-α chains. In panel A, reactions containing γ-globin cDNA vector were incubated with 35S-labeled methionine for 30 min in the presence of unlabeled γ-globin chains added at zero time (lanes 1 and 2). Reactions were stopped by the addition of puromycin, and unlabeled α-globin chains (lane 2) were added just prior to electrophoresis on cellulose acetate membranes and then subjected to autoradiography. In panel B, β-globin cDNA and unlabeled β chains were used. Reactions were stopped by the addition of puromycin, and α-globin was added (lane 2) just prior to cellulose acetate electrophoresis. In panel C, reactions contained γ cDNA vector and unlabeled γ globin at zero time. Reactions were stopped after 30 min by the addition of puromycin, and unlabeled α-globin chains and Hb F were added just prior to electrophoresis (lane 1) or incubated for an additional 30 min prior to electrophoresis (lane 2).

If reactions were incubated in the presence of α chains and Hb F for an additional 30 min prior to cellulose acetate electrophoresis, then only trace amounts of radiolabeled Hb F were seen (Fig. 6, panel C, lane 2). These results suggest that the formation of αγ or αγ2 requires the addition of α-globin chains at zero time prior to initiation of the transcription/translation reaction. In contrast, radiolabeled βγ heterotetramers formed in β cDNA reactions containing unlabeled β chains at zero time (Fig. 6, panel B, lane 1). The addition of unlabeled α chains just prior to electrophoresis resulted in the formation of radiolabeled αβγ heterotetramers and no βγ bands (Fig. 6, panel B, lane 2). These results indicate that during synthesis of radiolabeled γ-globin chains the unlabeled γ-globin chains form unstable monomers and/or stable γ2 dimers that do not dissociate readily to monomers and, therefore, do not form αγ heterodimers following the addition of unlabeled α chains after chain synthesis. In contrast, stable βγ tetramers formed during chain synthesis dissociate readily into monomers and assemble with α chains, resulting in the formation of Hb A as shown in in vitro studies (6, 8, 11). These findings are consistent with our previous results of in vitro assembly showing only trace amounts of Hb F formation after an additional 30-min incubation using purified γ and α chains. In contrast, we showed that β chains associate readily with α chains to form functional Hb A (11).

Effects of Unlabeled α-Globin Chains and/or Hb F Addition at Zero Time on Radiolabeled αγ Heterodimer, γ2 Homodimer, and Hb F Formation—Because γ chains form stable γ2 homodimers in vitro, which stabilize newly synthesized γ-globin chains in the cell-free system, it was important to know how unlabeled γ and α chains compete for assembly with newly synthesized and/or nascent γ chains to form γ2 homodimers and/or αγ heterodimers. Therefore, we studied the effects of unlabeled Hb F added at zero time to γ cDNA reactions on the formation of radiolabeled Hb F. It is known that αγ dimers are in equilibrium with α2γ tetramers (Hb F) that can dissociate into α and γ monomers (15). Dissociated monomers should be able to associate with newly synthesized radiolabeled γ-globin chains in this cell-free system to form radiolabeled αγ or radiolabeled γ2 dimers. As shown earlier (Fig. 2, lane 2), the addition of unlabeled α-globin chains at zero time resulted in newly synthesized γ-globin chains forming radiolabeled αγ dimers (Fig. 7, panel A, lane 1). When unlabeled human Hb F was added at zero time in the absence of added α chains, a single radiolabeled band was detected that comigrated with Hb F after cellulose acetate electrophoresis (Fig. 7, panel A, lane 3). No radiolabeled γ2 band was generated, and the intensity of the Hb F band was less than that of the αγ heterodimer band generated by the addition of α chains at zero time (Fig. 7, panel A, compare band intensity in lane 3 versus lane 1). In addition, after adding Hb F at zero time in the absence of α chain addition (lane 3) radiolabeled material was present at the origin, indicating that some of the radiolabeled unassociated γ chain monomers precipitated. This may be caused by the limited amounts of α- and γ-globin chains generated from dissociation of Hb F even though both chains can stabilize newly synthesized γ-globin chains. Increasing the concentration of Hb F 10-fold had no effect on increasing the formation of radiolabeled γ2 homodimer bands (data not shown). In contrast, when unlabeled α-globin chains plus Hb F were added at zero time to reactions containing γ chain expression vector, a major radio-
labeled Hb F band appeared with no γ2 dimer band or material at the origin (Fig. 7, panel A, lane 2). Amounts of γ chain synthesized in reactions containing α chains with Hb F or just Hb F alone were the same as assessed by SDS-PAGE (Fig. 7, panel B, compare intensity in lanes 2 and 1). These results indicate that if α and γ chains are present as a result of Hb F dissociation only αγ dimers form. These findings suggest that unlabeled γ chains have a lower affinity than α chains for newly synthesized γ-globin chains or that the slow dissociation of unlabeled Hb F to α and γ monomers was rate-limiting, therefore generating less radiolabeled Hb F than that observed in the α plus Hb F reaction. We believe that in the absence of α chains, the newly synthesized γ chains (after translation) are unstable and readily precipitate during electrophoresis because of their low concentration. However, when unlabeled α and/or γ chains are added at zero time, they assemble with newly synthesized radiolabeled γ chains to form αγ heterodimers or γ2 homodimers, respectively. These more stable forms prevent precipitation during electrophoresis.

**DISCUSSION**

Studies on the in vitro assembly of Hb A show that the αβ2 tetramer assemblies via a stable αβ dimer intermediate as follows in Equation 1.

\[
2\alpha + 2\beta \rightarrow 2\alpha\beta = \alpha_2\beta_2 \quad \text{(Eq. 1)}
\]

This pathway reflects the relative stability of the αβ2 tetramer and equilibration with αβ dimer (2αβ = α2β2 or 2αγ = α2γ2) and involves stable protein-protein interactions of α and β chains at α1β1 interfaces as well as α1γ1 interfaces of Hb F (1). The α1β1 or α1γ1 interface forms first at low subunit concentrations in vitro, implying that this interface is energetically more stable than all the other subunit interfaces combined. This equilibrium exists under physiological conditions, is an important determinant of hemoglobin function, and shifts depending on various conditions such as pH, ionic strength, and Hb concentration. Subunit dissociation to dimers upon dilution has been studied by a variety of methods, all of which involve measurement of the average molecular weight of Hb dimer and tetramer (16). Our present results show that in the presence of excess α chains, heterodimers rather than heterotetramers form because of the low concentration of newly synthesized globin chains generated in this cell-free system (10^-6 M) compared with the dissociation constant \( K_d \) of tetrameric Hb (10^-6 M) (16–18).

The biosynthesis of α- and non-α-globin polypeptide chains to form heterodimers and tetramers in vivo is normally balanced. How the linear amino acid sequence in polypeptides promotes protein folding leading to formation in vivo of functional tetramers after transcription/translation is not completely understood. We found differences in the assembly of purified γ and α chains to form Hb F and of β and α chains to form Hb A in vitro compared with expression of both tetramers in bacteria and yeast (11). The difference between in vitro and in vivo assembly can be explained by our present experimental results suggesting that nascent γ-globin chains assemble with free α chains to form αγ heterotetramers during translation, or soon after, but prior to formation of stable γ2 homodimers (11). Furthermore, our previous studies in vitro showed a 10^4-fold slower rate of stable γ2 dimer assembly with α chains to form Hb F compared with Hb A formation by α- and β-globin chains. This large rate difference can be explained by the fact that γ2 homodimers do not dissociate readily to monomers like β2 or β4 chains (11).

However, if γ chains are in the monomeric state, they can associate with α chains as β chains (11). Our present results using this in vitro cell-free system show that in the presence of added unlabeled α chains prior to translation, newly synthesized γ-globin chains do not form unstable γ chain monomers or stable γ2 homodimers. The chains assemble with α chains and form radiolabeled αγ heterodimers that can then form Hb F tetramers. Our present results also show that γ or β chains can be stabilized by the formation of homodimers or homotetramers in the absence of α chains. In addition, it was expected that in the presence of unlabeled α- and γ-globin chains that newly synthesized γ chains would form not only αγ heterodimers but also γ2 homodimers. However, our results show that the addition of unlabeled α-globin chains or α-globin chains and Hb F at zero time produced only radiolabeled αγ or Hb F, respectively, and no γ2 or γ4. These results suggest that α chains associate with newly synthesized γ-globin chains faster than γ-globin chains, even though γ-globin chains can form stable homodimers.

In addition, Komar et al. (19) demonstrated that incomplete human α-globin molecules of 140, 100, and 86 amino acid residues are capable of co-translational heme binding with an approximately equal efficiency, whereas polypeptide chains of 75, 65, and 34 amino acid residues display a significantly weaker, or just nonspecific, affinity for heme. This indicates that nascent α chains having 86 amino acids possess a structure that allows interaction with heme or that heme binding to nascent globin chains promotes formation of the proper tertiary structure of the growing polypeptide chain on polyribosomes (19, 20). It is not clear how α- and non-α-globin genes are transcribed at nearly equal rates; however, α-globin mRNA, whether by virtue of a higher rate of transcription or greater stability, accumulates in slight excess compared with β-globin mRNA during normal adult erythropoiesis. In fact, α-globin mRNA tends to be 25–50% more abundant than β-globin mRNA in normal erythrocytes (21).

Furthermore, in red cells the concentration of hemoglobin is very high (5 mM) even though free globin chain concentrations are not known. From these results, we propose that nascent non-α chains undergo folding and in the presence of a chain monomers assemble with already folded α-globin chains thereby preventing the formation of γ2 or apo β2 homodimers. This would result in the formation of stable α-non-α globin heterodimers, which then leads to the formation of functional hemoglobins in vivo. Current attempts are focused on demonstrating α chain interaction with nascent γ chains during translation to form functional Hb F using cell-free systems.

**REFERENCES**

1. Bunn, H. F., and Forget, B. G. (1986) Hemoglobin: Molecular, Genetic and Clinical Aspects, pp. 13–140, Saunders, Philadelphia
2. McGovern, P., Reisberg, P., and Olson. (1978) J. Biol. Chem. 251, 7871–7879
3. Friedman, F., and Beyshok, S. (1979) *Ann. Rev. Biochem.* **48**, 217–250
4. McDonald, M. J. (1981) *J. Biol. Chem.* **256**, 6487–6490
5. Bunn, H. F., and McDonald, M. J. (1983) *Nature* **306**, 498–500
6. Kawahara, Y., and Nakamura, S. (1983) *J. Biochem.* **94**, 1851–1856
7. Mrabet, N. T., McDonald, M. J., Turci, S., Sarkar, R., Szabo, A., and Bunn, H. F. (1986) *J. Biol. Chem.* **261**, 5222–5228
8. McDonald, M. J., Turci, S. M., Mrabet, N. T., Himmelstein, B. P., and Bunn, H. F. (1987) *J. Biol. Chem.* **262**, 5951–5956
9. Bucci, E., Fronticelli, C., Chiancone, E., Wyman, J., Antonini, E., and Rossi-Fanelli, A. (1965) *J. Mol. Biol.* **17**, 29–46
10. Antonini, E., Bucci, E., Fronticelli, C., Chiancone, E., Wyman, J., and Rossi-Fanelli, A. (1966) *J. Mol. Biol.* **12**, 183–192
11. Adachi, K., Zhao, Y., Yamaguchi, T., and Surrey S. (2000) *J. Biol. Chem.* **275**, 12424–12429
12. Bunn, H. F. (1987) *Blood* **69**, 1–6
13. Ascoli, F., Rossi-Fanelli, M. R., and Antonini, E. (1981) *Methods Enzymol.* **232**, 292–321
14. Taspis, A., Thellet J., and Rosa, J. (1978) *Biochem. Biophys. Res. Commun.* **85**, 5211–5516
15. Mrabet, N., Shaeffer, J. R., McDonald, M. J., and Bunn, H. F. (1986) *J. Biol. Chem.* **261**, 1111–1115
16. Turner, B. W., Pettigrew, D. W., and Ackers G. K. (1981) *Methods Enzymol.* **76**, 596–628
17. Williams, R. C., and Kim, H. (1976) *Biochemistry* **15**, 2207–2211
18. Bunn, H. F. (1969) *J. Clin. Invest.* **48**, 126–138
19. Komar, A. A., Kommer, A., Krasheninikov, I. A., and Spirin, A. S. (1997) *J. Biol. Chem.* **272**, 10646–10651
20. Komar, A. A., Kommer, A., Krasheninikov, I. A., and Spirin, A. S. (1993) *FEBS Lett.* **326**, 261–263
21. Benz, E. J., Jr., and Forget, B. G. (1975) *Prog. Hematol.* **9**, 107–155
