The incorporation of CN–hemin into three human adult apohemoglobin species (apohemoglobin, α-apohe- 
moglobin, and apohemoglobin modified at its β93 sulf-
hydryl with p-hydroxymercuribenzoate) has been mon-
tored at micromolar concentrations in 0.05 M potassium 
phosphate buffer, pH 7.0, at 10 °C. In all cases, Soret 
spectral blue shifts accompanied CN–protohemin 
formation but not CN–deuterohemoglobin formation. 
This finding in conjunction with isofocusing studies provided 
evidence of a CN–protopseudo-α-hemoglobin intermediate, 
the formation of which appeared to be a direct conse-
quence of CN–protoprotein-α heme pocket interactions. 
The kinetics of full reconstitution of CN–protohemin 
and CN–deuterohemoglobin revealed four distinct phases 
that apparently correlated with heme insertion (Phase I), 
local structural rearrangement (Phase II), global confor-
mational response (Phase III), and irreversible histidine 
iron bond formation (Phase IV). These phases exhibited 
rates of 7.8–22 × 10^3 M^−1 s^−1, 0.19–0.23 s^−1, 0.085–0.12 s^−1, 
and 0.008–0.012 s^−1, respectively. Partial (50%) reconsti-
tution with CN–protoprotein, in contrast, revealed only 
three kinetic phases (with Phase III missing) of heme 
incorporation into native and p-hydroxymercuribenzo-
ate-modified apohemoglobin. Furthermore, the absence 
of Phase III slowed the rate of proximal bond formation. 
These findings support the premise that irreversible as-
sembly of CN–protopseudo-α-hemoglobin is deterred by the 
presence of a heme-free β partner, the consequence of 
which may be that intermolecular heme transfer is en-
couraged under conditions of heme deficiency in vivo.

The structural, functional, and subunit assembly properties 
of human hemoglobin have been intensely investigated (1–5). 
Yet the precise nature and sequence of events that occur during 
hemoglobin formation are still unknown. Not only is the mode(s) of combination of mitochondrial Fe-protoporphyrin IX 
(heme) with cytoplasmic nascent α and β polypeptide chains 
unknown, but the actual dimer precursor (or precursors) re-
main undefined. Three distinct pathways of hemoglobin tet-
ramer assembly may be proposed, that of assembly through a 
-1 hemec-containing heterodimer (αββ), a heme-globin pair (semi-
hemoglobin; αββ or αββ), or a heme-free α dimer (apohemo-
globin; αββ). The detection of α hemoglobin (αββ) and α apohem-
globin (αββ) chains as well as semi-α-hemoglobin (αββ) and 
apohemoglobin in vivo has served to strengthen the plausibility 
of these three assembly mechanisms (6, 7).

Gibson and Antonini (8, 9) carried out pioneering studies that 
involved the binding of a monomeric heme moiety to an 
apohemoglobin species isolated from normal adult hemolysate 
(Hb A). Their rapid kinetic investigations resulted in the de-
velopment of a model which proposed that hemoglobin forma-
tion occurred via a reversible intermediate complex. In-
dependent kinetic studies (10–12) have supported this classical model, 
which postulated a two-step kinetic mechanism involving a 
rapid second order heme insertion event followed by a slower 
first order process attributed to structural rearrangement and 
the irreversible formation of a histidine-iron bond. Experiment-
inal variables that altered the heme insertion process (meso-
and deuter-derivatives of CN– or CO–heme) as well as those 
amised at modulating apohemoglobin structural response (pH 
changes; introduction of polyanions) were explored, and no 
evidence of an ordered sequence (either α or β subunit) of heme 
binding was found.

This was unexpected because detailed protein chemical studies 
(13–15), which involved half-equivalency titration of apo-
hemoglobin with heme, had led to the conclusion that α chains 
have a greater preference than their β chain counterparts for heme. 
Furthermore, Soret spectral kinetic studies focused on 
CO–heme (16) and CN–hemin (17) binding to preassembled 
semi-hemoglobins (semi-hemoglobins prepared in vitro by 
heme-chain transfer; Ref. 18) have further documented differ-
ences in the heme affinity of α and β chains. In this report, the 
incorporation of CN–protoprotein and CN–deuterohem into 
human apohemoglobins has been monitored, and results indi-
cate that a significant difference in binding of α and β subunits 
of apohemoglobin does exist, a finding consistent with human 
hemoglobin assembly through a semi-α-hemoglobin interme-
diate in vitro and most probably in vivo.

EXPERIMENTAL PROCEDURES

Preparation and Characterization of Apohemoglobin Protein Spe-
"cies—Human adult hemoglobin and its isolated α heme subunit were 
prepared (19) and characterized as previously reported (20, 21). Re-
moval of heme was accomplished by treatment with acid acetone as 
described (22) with modifications (23). Final solutions of the hemo-
globins were suspended in 0.05 M potassium phosphate buffer, pH 7.0, 
and concentrations were determined (ε_{280nm} = 12.7 mm^−1 cm^−1, on a sub-
unit basis) in a Cary 2200 spectrophotometer (Varian Instruments).

Nativeness of these species was confirmed by carrying out a heme 
titration at a single wavelength. In addition, reconstituted CN– 
protoprotein and CN–deuterohemoglobin were chromatographed 
on Biogel P-6 (Bio-Rad) and subjected to spectral measurements which 
confirmed maxima of 420 and 409 nm, respectively.

Static Measurements of CN–Hemin Incorporation—Heme titrations 
over a Soret spectral region of 400 to 450 nm were performed in the 
following manner. Protoprotein and deuterohem (Porphyrin Products 
Inc.) were dissolved in a minimal amount of 0.1 N NaOH and distilled 
water, and their concentrations were determined (ε_{280nm} = 50 mm^−1 
cm^−1 and ε_{382nm} = 57 mm^−1 cm^−1 for protoprotein and deuterohem, 
respectively). These solutions were converted to the cyanide derivative
by adding excess KCN. Increments of the stock CN–hemin solutions were added to both sample and reference cells, and data acquisition was carried out by Lab Calc Software (Galactic). Confirmation of apohemoglobin nativeness was obtained from isoelectric focusing studies (Omega Horizontal Electrophoretic System; Isolab Inc.) on an agarose gel at pH levels 6 and 8, with the gel surface maintained at 10°C. The samples were focused for 1 h at 1100 V at the end of which the gel was fixed in a 10% trichloroacetic acid solution followed by staining with a heme specific stain, o-dianisidine. Titration of apohemoglobin with p-hydroxymercuibenzoate (PMB; Sigma) was performed according to the method of Boyer (24) and revealed that an end point was achieved when half-equivalent amount of PMB was bound to the apohemoglobin dimer. The concentration of PMB was determined \( f_{p_M} = 16.9 \text{ mM}^{-1} \text{ cm}^{-1} \), and the titration was monitored at 255 nm. Subsequent preparations of PMB-apohemoglobin were made by adding half-equivalent amounts of a concentrated PMB solution to the apohemoglobin sample. The integrity and stability of PMB-apohemoglobin were evaluated in the UV region (see “Results and Discussion”).

Kinetic Measurements of CN–Hemin Incorporation—All kinetic measurements were carried out in a Kinetic Instruments stopped flow device online to OLIS 3820 data acquisition software. The pathlength of the reaction cell was 20 mm, and the instrument had a dead time of 2 ms. All measurements were performed in 0.05 M potassium phosphate buffer, pH 7.0 at 10°C, by mixing equal volumes of the respective CN–hemin and apohemoglobin in a 1:1 or 1:2 ratio. The reaction of a apohemoglobin was carried out only under equimolar conditions. The time courses were monitored, at their respective absorption maxima over a variety of time frames (0.02–300 s) to permit the collection of a sufficient number of data points. Each time course consisted of an average of three runs, and a minimum of three independent trials was performed allowing the determination of standard deviations. Standard fitting routines allowed the isolation of multiple phases for each time course. Apparent first (and second order) rate constants were derived from standard plots of log absorbance and \( 1/(\text{CN–hemin}) \) versus time, respectively. A slow reaction (inaccessible by rapid kinetic technique) was observed when CN–protohemin and apohemoglobin were in a 1:2 ratio and its baseline was obtained in a Cary 2200 spectrophotometer after manual mixing of the two reactant solutions.

RESULTS AND DISCUSSION

Soret absorption spectra of hemoglobins, which are sensitive not only to the type and state of ligand on the central iron of heme but also the heme environment of the protein itself, have been used to study heme binding kinetics (8–12, 16, 17). In addition, an increased preference of the \( \alpha \) over the \( \beta \) subunit for heme is postulated to be the basis for the occurrence of semi-\( \alpha \)-hemoglobins. The present study represents a novel method of monitoring for the existence of this kinetic intermediate during reconstitution of hemoglobin. Static and kinetic Soret spectral changes of apohemoglobin model systems that occur upon incorporation of two distinct heme moieties, the native CN–protohemin and its less hydrophobic derivative, CN–deuterohemin, have been monitored.

Static Spectral and Isofocusing Studies of Apohemoglobin—The change in absorption spectra of apohemoglobin upon binding of CN–protohemin (Fig. 1, top panel) and CN–deuterohemin (Fig. 1, bottom panel) was followed in the Soret region between 400 and 450 nm. Titration curves (Fig. 1, insets) of absorbance changes at 420 and 409 nm for CN–protohemin and CN–deuterohemin, respectively, clearly indicate an end point corresponding to one heme bound per monomer subunit. Under the standard experimental conditions of 0.05 M potassium phosphate buffer, pH 7, at 10°C incremental addition of CN–protohemin to apohemoglobin (5 \( \mu \text{M} \)) resulted in a significant blue spectral shift (5 ± 0.5 nm) until half-saturation (one heme/apohemoglobin dimer) was reached (Fig. 1, top panel), and then no further spectral shift was observed with additional CN–protohemin.

Wavelength dependence has been observed by Kawamura-Konishi and Suzuki (25) upon addition of a caffeine adduct of hemin to apohemoglobin and was reported to be a consequence of caffeine–heme binding to the \( \alpha \) subunit of apohemoglobin. In addition, the blue shift seen here is consistent with CN–protosemi-\( \alpha \)-hemoglobin formation, because the Soret spectra of this semihemoglobin is more blue-shifted than that of its semi-\( \beta \)-hemoglobin counterpart in both CO–protoheme (16, 18) and more importantly the CN–protohemin form (17).

CN–deuterohemin incorporation into apohemoglobin, on the other hand, revealed a titration whose spectra were wavelength-independent over the region of study from 400 to 450 nm. The CN–deuterohemin lacks the vinyl groups in positions 2 and 4 of protohemin, and this would be expected to alter

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1 The abbreviation used is: PMB, p-hydroxymercuibenzoate.
heme-protein contacts and consequently spectral properties (see below). Furthermore, electrophoretic studies from the laboratories of Winterhalter et al. (13) and Cassoly and Banerjee (18) indicate that CN–deuterohemoglobin reconstitution does not exhibit αβ chain differences. Random heme binding could obscure any spectral shifts and would also preclude preferential formation of a CN–deuterosemi-α-hemoglobin.

Isofocusing studies (Fig. 2) reveal that a semihemoglobin intermediate is present only during CN–protohemin incorporation into apohemoglobin. These results are in general agreement with earlier zonal electrophoresis studies (13). Furthermore, the cathodic heme-containing component observed here has been previously identified as semihemoglobin (16, 18). CN–protohemin incorporation into apohemoglobin reveals no semihemoglobin formation; a fact that corresponded well with the lack of detectable wavelength dependence (Fig. 1, bottom panel) during titration.

Interpretation of Soret spectral shifts for β-apohemoglobin is precluded by the fact that this species exists as a dimer (29–32). An alternate approach would be to modify the β subunit of apohemoglobin, a challenging endeavor because the heme-free protein is rather unsuitable for extensive protein chemical manipulation. Nonetheless, modification of apohemoglobin has been reported, and fortuitously, the most successful was that of the site-specific modification of the reactive β93 (F9) cysteine residue (15, 27). Furthermore, a reagent attached to this β93 (due to the residue being adjacent to the proximal histidine β92) would be expected to be a “reporter group” of the β chain heme insertion event.

Static Titrations of PMB-Apohemoglobin—The sulfhydryl reagent, PMB, has been shown to bind rapidly and specifically to...
the β93 (F9) cysteine residue of apohemoglobin at one PMB bound per apohemoglobin dimer. Investigation of the UV spectral region (240–290 nm) upon addition of PMB to apohemoglobin (Fig. 4, left panel) revealed two significant spectral changes. One corresponds to the formation of a mercaptide bond in the 250–260 nm region (24), and the other (in the 280 nm region) may correlate with changes in the β chain heme environment (see kinetic studies below). Under conditions of 0.05 M potassium phosphate buffer, pH 7.0, with increments of stock PMB solution (1500 μM) in the UV region between 240 and 300 nm resulted in an end point of 0.5 equivalents of PMB per apohemoglobin dimer as previously reported (27). Center panel, UV spectra of apohemoglobin before and after the addition of 0.5 equivalents of PMB. The upper spectra consists of a series of nine consecutive scans taken at intervals of 3 min each. The consecutive scans revealed the stability of the bound reagent and that of the now modified apohemoglobin. Right panel, CN–protohemin titration of PMB-apohemoglobin (5 μM) confirmed the heme binding capacity of this modified apohemoglobin. Arrows specify a spectral shift identical to that seen for native unmodified apohemoglobin (see Fig. 1), and this titration demonstrated one equivalent heme bound per apohemoglobin monomer. Experimental conditions were identical to those for Fig. 1.

Kinetics of CN–Hemin Binding to Apohemoglobin—This present kinetic investigation was aimed at evaluating the heme incorporation process in vitro and attempting to extrapolate these findings to the in vivo event. Our current studies of static titrations have demonstrated that the CN–protohemin–protein binding involves a spectrally definable intermediate (presumably semi-o-hemoglobin) that is not seen in CN–deuterohemin–protein association and that this intermediate is most discernible up to half-saturation (one heme per apohemoglobin dimer). Taking this into account CN–protohemin and CN–deuteroheomin were mixed in a 1:1 and 1:2 ratio with apohemoglobin in 0.05 M potassium phosphate buffer, pH 7.0, at 10 °C, and the change in Soret absorbance (at 420 and 409 nm, respectively) was followed in a stopped flow device. All four reactions were multiphasic, and the resultant rate plots are presented (Fig. 5). The kinetics of full reconstitution of CN–protohemin and CN–deuteroheomin are displayed in rows 1 and 3, respectively, whereas those that promote partial (50%) reconstitution are in rows 2 and 4, respectively.

The initial part of all four time courses is dominated by a second order process that is designated Phase I. This is the heme insertion event, and studies with an array of monomeric heme derivatives (8–12, 16, 17, 25) have yielded rates in the order of 10^7 M^-1 s^-1. As expected the rates of CN–protohemin insertion (Table I) for both full and half-saturation (10 and 14 × 10^-3 M^-1 s^-1, respectively) were 1.3–2-fold faster than the rate of entry of the less hydrophobic CN–deuteroheomin derivative (full and half-saturation yields rates of 7.8 and 7.1 × 10^7 M^-1 s^-1, respectively). Interestingly enough, the formation of CN–protosemihemoglobin was 1.4 times more rapid than that of the CN–protohemoglobin. This faster rate could result from an increased accessibility of the α chain for heme and is consistent with the finding that the β subunit structure is more rigid (less accommodating; Ref. 29) possibly due to the presence of its D-helix (33). All subsequent phases (Phases II–IV) were found to be first order in nature and almost certainly attributed to structural changes in the apohemoglobin molecule. Phase II exhibited a rate of 0.21 s^-1, which was invariant with the type of CN–hemin derivative or the degree of reconstitution achieved. Phase III (0.085 s^-1) was approximately 2.5-fold slower than Phase II for all reactions except that it was apparently missing in the formation of CN–protosemi-o-hemoglobin (Fig. 5, row 2, III). This suggests that the absence of this phase is related to lack of CN–hemin insertion into the β chain partner. The final phase of the reaction (Phase IV) displayed a rate of 0.013 s^-1 (6.5-fold slower than Phase III) except in the case of CN–protosemi-o-hemoglobin (Fig. 5, row 2, IV) where the rate obtained was 0.008 s^-1. This slower rate for Phase IV is of interest because recent studies have assigned this rate of reaction to the formation of the bond between the central iron of heme and the proximal histidine (F-8) in myoglobin (34, 35).

Thus, it appears that binding half-saturating amounts of CN–protohemin to apohemoglobin results in a process that allows faster heme insertion, that lacks one of two discernible first order structure rearrangement components, and that pos-
sibly results in a 1.5-fold decrease in the rate of iron-histidine bond formation. This bond formation ensures irreversible heme incorporation and prevents the possibility of heme exchange between the subunits of apohemoglobin (36–38). The 1.5-fold decrease in this rate of bond formation for CN–proto semi-hemoglobin would therefore allow more time for such a heme transfer (from α to β) to occur.

Kinetics of CN–Hemin Binding to α-Aphohemoglobin—CN–protohemin (Fig. 6, row 1) and CN–deuterohemin (Fig. 6, row 2) were mixed in a 1:1 ratio with α-aphophemoglobin in 0.05 M potassium phosphate buffer, pH 7.0, at 10 °C, and both reactions yielded four independent kinetic phases (Table I). The rate of CN–protohemin insertion (Phase I) into this monomeric apohemoglobin was 1.4-fold faster than that for CN–deuterohemin entry yielding values of 12 and 8.4 × 10^{-3} M^{-1} s^{-1}, respectively. Phase II exhibited a rate (0.20 s^{-1}) similar to that seen for apohemoglobin in the case of both CN–hemin, presumably indicative of a similar event in the presence and the absence of a partner chain. The additional structural event (designated as Phase III) was present for the incorporation of both CN–protohemin and CN–deuterohemin into α-aphohemoglobin yielding values of 0.087 and 0.099 s^{-1}, respectively. These rates are comparable with those seen for full incorporation of CN–hemin into apohemoglobin (Fig. 5, rows 1 and 3, III). The final Phase IV exhibited rates (0.012 s^{-1}) comparable with those of the fully reconstituted parent hemoglobins.

This current investigation is of considerable interest because it allows comparison with the earlier study of Leutzinger and Beychok (11) in which these workers demonstrated that the kinetics of CN–protohemin incorporation into α-aphohemoglobin is multiphasic. Their three mixed phases can be readily correlated with the four phases seen here for CN–protohemin and CN–deuterohemin incorporation. Their Soret spectral, fluorescence quenching, and far UV circular dichroism studies revealed a process in which heme entry (Phase I) was followed by structural rearrangements local (Phase II) and global (Phase III). Although these workers postulated that the His (F8)-iron bond formation preceded these structural changes, recent evidence (34, 35) suggests that this step (Phase IV) occurs later in the overall heme incorporation process. Taken together these investigations suggest that the heme pocket of α-aphohemoglobin is quite accessible and can readily accommodate both CN–
protohemin and CN–deuterohemin, that this α-apohemoglobin monomer is capable of undergoing structural rearrangements comparable with those observed during full reconstitution of its apohemoglobin parent, and that if these structural adjustments are permitted allow a stable linkage between its proximal histidine and the heme iron to be formed at a normal rate.

If, however, as may be the case during half-saturation of apohemoglobin with CN–protohemin (see above), conformational restraints (presumably due to $\alpha\beta$ coupling) are present, then this rate of bond formation is diminished.

**Kinetics of CN–Hemin Binding to PMB-Apohemoglobin—** The static Soret absorption changes accompanying titration with CN–protohemin were identical for apohemoglobin (Fig. 1, top panel) and PMB-apohemoglobin (Fig. 4, right panel), and the overall kinetic profile of CN–hemin binding to PMB-apohemoglobin (Fig. 7) was comparable with that of apohemoglobin (Fig. 5) but not the rates. All four time courses (full reconstitution, Fig. 7, rows 1 and 3; partial (50%) reconstitution, Fig. 7, rows 2 and 4 for CN–protohemin and CN–deuterohemin, respectively) reveal heme insertion rates (Phase I) 1.7-fold more rapid for CN–protohemin and 1.5-fold more rapid for CN–deuterohemin than seen for unmodified apohemoglobin. Furthermore, the difference between the heme insertion rate of CN–protohemin and CN–deuterohemin increased to 1.8-fold, whereas the rate of formation of CN–protohemin-semi-α-hemoglobin (Fig. 7, row 2) actually decreased when compared with that of the fully reconstituted species (Fig. 7, row 1); a finding heretofore only seen with CN–deuterohemin insertion. Phase II exhibited a rate of 0.23 s$^{-1}$ for all four time courses and is remarkably similar to that seen for both apohemoglobin and α-apohemoglobin. Phase III (0.12 s$^{-1}$) was 1.9-fold slower than Phase II for reactions involving full reconstitution and either missing or much slower (2.5-fold) for half-reconstitution with CN–protohemin and CN–deuterohemin, respectively. Phase IV displayed rates of 0.008 (15-fold slower than Phase III) and 0.011 s$^{-1}$ (10-fold slower than Phase III) for CN–protohemin and CN–deuterohemin binding irrespective of the degree of reconstitution. In fact, full reconstitution of PMB-apohemoglobin displayed a Phase IV rate 1.4-fold slower than that for reconstitution of unmodified apohemoglobin. Thus, even the presence of Phase III could not restore proximal bond formation to its original rate when PMB is bound.

These present studies of CN–hemin incorporation into PMB-apohemoglobin showed that β93 (F9) sulfhydryl modification not only accelerated but also accentuated the difference in the rate of heme insertion (Phase I) of CN–protohemin and CN–
deuterohemin. It appears that even though subunit accessibility has been enhanced, the vinyl groups continue to play a key role in the kinetics of CN–hemin binding. Phase II consistently reflected local protein-heme interactions, the majority of which, interestingly enough, are on the proximal (F8) side of the α and β chains (26). Although the rate of Phase III of PMB-apohemoglobin increased during complete reconstitution, it continued to be absent during partial reconstitution of this modified protein. This demonstrated that PMB alone is able to alter αβ coupling (PMB has been reported to affect dimer coupling in hemoglobin; Ref. 39) but not enough to allow the α subunit to respond as it would if decoupled (Fig. 6) from its β partner. This absence of Phase III inevitably resulted in a slowed rate of proximal bond formation for the α subunit. This finding is not a direct consequence of the presence of PMB because the residue adjacent to the proximal (F8) histidine, is a sulfhydryl, only in the case of the β subunit.

In conclusion—CN–hemin incorporation, although not seen under normal physiological conditions, may nonetheless allow insight into the probable sequence of events leading up to hemoglobin tetramer formation in vivo. CN–deuterohemin appears to randomly bind to the α and β chains of apohemoglobin and as such does not promote either Soret spectral shifts or anomalous kinetic behavior when incorporated into the apohemoglobin models employed here. Furthermore, absence of the vinyl groups may impair the heme insertion (Phase I) process (possibly due to heme orientation stereospecificity factors; Ref. 40) but does not impede structural rearrangements (Phases II and III) nor timely proximal histidine-iron bond formation (Phase IV).

The presence of the porphyrin 2,4 vinyl groups, on the other hand, has interesting consequences. CN–protoporphin promoted Soret spectral shifts upon binding in all apohemoglobin models. The invariant G5 phenylalanine of the α subunit appears to be a likely candidate for involvement in this spectral shift, especially because the G5 residue of its β chain partner is not reported to interact with the vinyl groups (4, 26). Furthermore, the magnitude of Soret spectral shift, the presence of Phase III, and the rate of Phase IV were all governed by whether the α apohemoglobin was free as a monomer or sequestered in an apohemoglobin dimer, implying that αβ interplay is primarily responsible. The G-helical segments are reported to be essential for αβ coupling of apohemoglobin, and thus it would appear that amino acid residues in this region account for static enhancements and kinetic restraints imposed on the preassembled α subunit. Although the B-helical region may be important (26, 41, 42), focusing on the FG and G regions, where the majority of interface contacts in both apohemoglobin and hemoglobin reside, may be informative. In this region, four α chain residues (FG5, G4, G5 and G8) account for 11 out of 13 vinyl contacts, whereas three β chain residues (FG5, G4, and G8) are responsible for 5 out of 14 vinyl contacts. Furthermore, these same residues not only interact with the heme moiety but also contribute one αGβ1 and six αGβ2 interface contacts in hemoglobin.

A possible scenario, consistent with the kinetics of PMB-apohemoglobin, would be that the FG5 residue is involved in Phase III (the bulky PMB could enhance this residue’s overall interaction; Table I). Movement of this FG5 valyl residue could reorient the G-helix, strengthen the αGβ1 contact, and prime the αGβ2 region for tetramer assembly (a process encouraged by heme binding to the β subunit). Histidine bond formation would be inevitable. At half-saturating amounts during semi-apohemoglobin formation, however, the momentum of these structural movements is lost, and the rate of irreversible proximal bond formation is impaired.

In vivo heme and globin production are delicately balanced processes so that ample quantities of both are available for hemoglobin formation in the typical precursor red blood cell (3); yet the exact manner in which four nascent globin chains and four Fe-protoporphyrin-IX groups combine to form the heme-containing tetramer is still a mystery. As with all complex biochemical phenomena, isolation of given reactions have aided in understanding this process. Previous studies have attempted to fine tune aspects of assembly through kinetic investigations of association of heme-containing α and β subunits (20, 43, 44); however, another plausible pathway of assembly may be that of combination of heme-containing and heme-free partner chains. If this is indeed the case then the heme-containing partner must be the α subunit. Evidence for this is overwhelming and consistent with findings that heme is readily inserted (possibly cotranslationally; Ref. 45) into α1 (1, 11), that both α1 and α3 are found in vivo (6, 7), and that β chains are present only in the case of severe α-thalassemia (HbH disease; Ref. 3). This stable viable α3 species may then combine with its heme-free ribosomal bound β partner to form a stable semi-α-hemoglobin. The results presented here suggest that its preassembled counterpart may also be converted into hemoglobin by either acceptance of a heme moiety from another semihemoglobin precursor or by binding heme directly.

The existence of the former pathway is intriguing, not only because both semi-α-hemoglobin and apohemoglobin have been found in vivo but also because it implies that the α chain plays a key role in heme currency exchange.

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