

The Role of Hemoglobin Heme Loss in Heinz Body Formation: Studies with a Partially Heme-Deficient Hemoglobin and with Genetically Unstable Hemoglobins

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ABSTRACT A number of mutant hemoglobins are inordinately unstable, denaturing in circulating red cells into Heinz bodies, resulting in congenital Heinz body hemolytic anemia (CHBHA). We have emphasized that most such hemoglobins involve amino acid substitutions at sites neighboring the heme group of the β -polypeptide chain, and have shown that heme binding to globin is diminished thereby. Thus, hemes were progressively lost from four unstable hemoglobins (Köln, Hammersmith, San Francisco, and Zürich) as they precipitated into Heinz bodies at 50°C.

The role of heme loss, especially from beta chains, in Heinz body formation was supported by studies with a hemoglobin synthesized to contain hemes only on its alpha chains ($\alpha_2^{\text{heme}}\beta_2^0$). The behavior of this compound, postulated to be an intermediary in the formation of Heinz bodies, mimicked that of the genetically unstable hemoglobins in several ways: (a) it precipitated at 50°C into typical coccoid Heinz bodies; (b) as also observed with CHBHA hemoglobins this denaturation was virtually prevented by the heme ligands, cyanide or carbon monoxide, which inhibit further heme loss; it was potentiated by oxidation of hemes to the ferri-state, which accentuates heme loss; (c) the thiol groups of $\alpha_2^{\text{heme}}\beta_2^0$ were hyperreactive, forming mixed disulfides with glutathione and membrane sulfhydryls at rates similar to those of CHBHA hemoglobins and 10 or more times that of normal hemoglobin A; (d) heme repletion of the protein molecules by the addition of crystalline hemin to either $\alpha_2^{\text{heme}}\beta_2^0$ or to the genetically unstable hemoglobins, prevented their precipitation into Heinz bodies

and normalized their aberrant electrophoretic behaviors; and (e) during Heinz body formation at 50°C both $\alpha_2^{\text{heme}}\beta_2^0$ and the genetically unstable hemoglobins released free α^{heme} -chains into solution, suggesting that the bulk of the whitish, Heinz body precipitate is naked β^0 -chains.

We conclude that heme loss from mutant beta chains is an early step in Heinz body formation in several of the unstable hemoglobinopathies. The resulting heme-depleted compounds, of which synthetic $\alpha_2^{\text{heme}}\beta_2^0$ is a prototype, are unstable, cleaving into β^0 -chain precipitates (the bulk of the Heinz body material) and soluble, free α^{heme} -chains (demonstrated previously in hemolysates from many patients with CHBHA).

INTRODUCTION

A number of mutant hemoglobins are inordinately unstable, denaturing in circulating red cells into Heinz bodies and precipitating in vitro when heated to 50°C (1). Patients harboring these hemoglobins suffer from the syndrome of congenital Heinz body hemolytic anemia (CHBHA) which consists of: (a) chronic hemolytic anemia; (b) the presence of circulating red cells with inclusion (Heinz) bodies, more obvious after splenectomy; and (c) frequently the excretion of urine darkened by the presence of large quantities of pyrrolic pigments loosely termed "dipyrroles" (2-4). When familial the disease is transmitted from one parent, as when hemoglobin Köln ($\beta 98$ valine \rightarrow methionine) is found in the affected individuals (5). Sporadic cases involving hemoglobins such as Hammersmith ($\beta 42$ phenylalanine \rightarrow serine) have also been reported and produce equally severe disease as in the familial cases (6). The unstable hemoglobins are all precipitated by heating at

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50°C for brief periods, the basis of a simple screening test for CHBHA (1).

From evidence that hemoglobin Köln binds its heme groups less avidly than hemoglobin A, one of us has suggested that heme groups might inordinately be lost from mutant globin polypeptide chains of CHBHA hemoglobins, predisposing them to denaturation into Heinz bodies (8, 9). It was emphasized that the amino acid alteration in Köln, as well as three other unstable hemoglobins under study at that time, Hammersmith (6), Zürich (7), and Gun Hill (10), were all in the vicinity of the heme group of the beta chain of hemoglobin. Since then several more unstable hemoglobins associated with CHBHA have been isolated and their mutations elucidated. As we have emphasized previously (31), when examined using the Perutz model (11) of beta chain structure, nearly all the unstable hemoglobins reported to date appear to have mutations in the vicinity of the beta chain heme group.¹ This consistency supports the view (8, 9, 16) that heme loss from beta chains might frequently underlie the denaturation of CHBHA hemoglobins into Heinz bodies. The present studies: (a) document inordinate heme loss from several genetically unstable hemoglobins during their precipitation into Heinz bodies, and (b) demonstrate that a synthetic hemoglobin containing hemes only on its alpha chains ($\alpha_2^{\text{heme}}\beta_2^{\circ}$) behaves like CHBHA hemoglobins in numerous ways. Since this synthetic compound would be the postulated intermediary compound if CHBHA hemoglobins lose their beta chain hemes before precipitation, its mimicry of the latter's properties supports the role of heme loss from beta chains in the pathogenesis of Heinz body formation in CHBHA. These studies have been reported in preliminary form elsewhere (17).

METHODS

Preparation of unstable hemoglobins. Hemoglobins Köln, Hammersmith, Zürich, and San Francisco were purified from hemolysates of heterozygous donors within 72 hr of phlebotomy.² The mutant hemoglobins were separated from hemoglobin A by column chromatography on CM—Sephadex-50 at 4°C, Köln at pH 6.0 with a linear gradient of NaCl from 0 to 0.4 mole/liter, the others with

a nonlinear gradient as previously described (30). The purified proteins were concentrated by vacuum dialysis to approximately 5 mg/ml before use.

The heme-deficient hemoglobin $\alpha_2^{\text{heme}}\beta_2^{\circ}$, suspected of being an intermediary when CHBHA hemoglobins precipitate into Heinz bodies, was prepared as previously described (18). Briefly, globin was purified from fresh hemoglobin A by a modification of the acid-acetone method of Rossi Fanelli, Antonini, and Caputo (19), as described elsewhere (20). Hemin was prepared according to the method of Labbe and Nishida (21), and was dissolved in 0.1 N NaOH to a concentration of 10 mg/ml; subsequent dilution to the desired concentration was made in 0.01 M Na_2HPO_4 containing 10 g KCN/liter, after the pH of this buffer had been adjusted to 7.5 with 0.01 M NaH_2PO_4 . To prepare $\alpha_2^{\text{heme}}\beta_2^{\circ}$ a half-stoichiometric amount of hemin was added to globin; that is, 2.0 μ moles of hemin were added per 64 mg of globin. The synthetic compound was isolated on a 50×0.9 cm CM—Sephadex 50 column equilibrated with 0.01 M Na_2HPO_4 containing 100 mg KCN/liter and adjusted to pH 6.8 with concentrated H_3PO_4 . The protein, previously dialyzed against the same buffer, was added at a concentration of 20 mg/ml and eluted by a nonlinear gradient obtained by addition of 0.02 M Na_2HPO_4 containing 100 mg KCN/liter to a constant volume mixing chamber containing the original pH 6.8 buffer. Reflecting the fact that α -chains of globin have an eightfold greater affinity for hemes than do β -chains (22), the addition of half-stoichiometric amounts of heme to globin produces predominantly $\alpha_2^{\text{heme}}\beta_2^{\circ}$, as documented in our previous studies (23, 24). Previous evidence has also been presented that synthetic $\alpha_2^{\text{heme}}\beta_2^{\circ}$ exists as a tetramer of α - and β -chains (23) and when reacted with excess hemin becomes physiologically identical with normal hemoglobin A (25). Carbonmonoxy- and oxyderivatives of $\alpha_2^{\text{heme}}\beta_2^{\circ}$ were obtained from the cyanmet- compound by addition of small amounts of dithionite and subsequent dialysis in buffer saturated with O_2 or CO (23). Heme-deficient CHBHA hemoglobins were converted into fully heme-saturated compounds by addition of excess hemin solution (see above). Hemes in excess of the stoichiometric amount of four per hemoglobin tetramer were removed by chromatography on DEAE columns with 0.1 M phosphate buffer (pH 7.0) as previously described (25).

Incubation procedures. Isolated CHBHA hemoglobins and $\alpha_2^{\text{heme}}\beta_2^{\circ}$ were incubated as 0.01 M phosphate-buffered (pH 7.4) solutions either at 37°C or 50°C. The latter temperature leads to precipitation of the mutant unstable hemoglobins into typical coccoid Heinz bodies (9), which underlies its use in the convenient screening procedure for CHBHA (1). Following centrifugation at 25,000 *g*, heme losses from the supernatant solutions of incubated hemo-

¹ Exceptions include hemoglobins Torino α^{43} phenylalanine \rightarrow valine in which a mutation occurs in close apposition to the α -chain heme group at the homologous site as that found in the β -chain of hemoglobin Hammersmith (12), and hemoglobins Riverdale-Bronx β^{24} glycine \rightarrow arginine (13), Philly β^{35} tyrosine \rightarrow phenylalanine (14), and Bibba α^{136} leucine \rightarrow proline (15). The latter mutations may be in areas involved in α - and β -chain contact, thereby affecting the tetramerization of these chains into normal hemoglobin, $\alpha_2^{\text{heme}}\beta_2^{\text{heme}}$ (see discussion).

² The donors of hemoglobins Köln and Hammersmith have been previously described (1, 6); samples of their blood were generously provided by Doctors J. V. Dacie and M. C. Brain. Blood containing hemoglobin Zürich was provided by Dr. P. G. Frick from a previously described patient (7). Blood containing hemoglobin San Francisco was supplied by Dr. J. D. Heywood from a patient with moderately severe CHBHA, whose child is similarly affected. The mutation in this unstable hemoglobin has not yet been fully elucidated; it involves a beta chain alteration, but is electrophoretically distinguishable from the other unstable hemoglobins used in this study. All bloods were drawn aseptically into NIH formula-A acid-citrate-dextrose (ACD) and kept at 4°C until used.

globins was assessed using a recording spectrophotometer.³ The ratio of optical density at 540 m μ (mainly protein-bound heme) to that at 280 m μ (mainly protein) was used as a measure of heme:globin ratio. This "540/280 ratio" is 0.44 \pm 0.02 (SD) for hemoglobin A and drops to 0.29 \pm 0.02 when 50% of hemes are lost as in the synthetic compound $\alpha_2^{\text{heme}}\beta_2^{\circ}$ (23). Decreasing values of the 540/280 ratio probably mainly reflect heme loss from globin, since heme not bound to globin has a fivefold diminished extinction coefficient at 540 m μ (23). Diminution of the 540/280 m μ ratio would also occur to the extent that methemoglobin and hemichrome derivatives of subunit α - or β -chains (29) are formed during the short periods of incubation utilized. This effect was minimized by addition of one drop of neutralized KCN to the supernatant solutions before spectrophotometry.

Precipitated hemoglobin was quantitated by assaying the difference in 280 m μ absorbency before and after incubation; supernatant solutions from centrifuged (25,000 g) samples were utilized. Alternatively gravimetric analysis was utilized as previously described (9). For the latter the precipitate from a known quantity of hemoglobin was collected by centrifugation at 25,000 g, washed twice with distilled water, and dried to constant weight *in vacuo* over P₂O₅; the method was similar to that used by Jandl, Engle, and Allen (26). The presence of free subunit α - or β -chains in incubated hemolysates was detected by pH 8.6 starch-gel electrophoresis at 4°C as previously described (23).

Studies of sulfhydryl reactivity of unstable hemoglobins. Free sulfhydryl groups of the CHBHA hemoglobins and of $\alpha_2^{\text{heme}}\beta_2^{\circ}$ were titrated by the method of Boyer (27), utilizing paramercuribenzoate (PBM)⁴ as titrant. The relative degrees of binding of glutathione to hemoglobin A and the unstable hemoglobins were measured as previously described (9). Briefly, 8 \times 10⁻⁸ moles of chromatographically purified heme protein were reacted with 7 \times 10⁻⁷ moles of G³⁵S^H (about 4 μ Ci)⁵ in a final volume of 1.1 ml of 0.1% EDTA, buffered to pH 7.4 with phosphate buffer. Following incubation at 37°C for various periods, the heme protein was precipitated with 5 ml of ice-cold 10% trichloroacetic acid (TCA), and the precipitate washed sequentially with 3 ml of 5% TCA, 0.4 ml of absolute ethanol, and 0.5 ml of ether. Weights of the dried precipitates indicated nearly complete (>90%) recoveries. The precipitates were dissolved in 1 ml of 0.1 N NaOH, and aliquots were pipetted in triplicate into aluminum planchets, dried, and counted with 20-25% efficiency in an automated gas-flow counter. Triplicate determinations agreed to within \pm 3%. Ancillary studies previously reported from this laboratory (9) have shown that virtually all bound radioactivity in these incubations reflects mixed-disulfide formation of GSH with thiols of the beta chain of hemoglobin, most probably the freely titratable one at the 93rd position.

Methods for assessing the binding of $\alpha_2^{\text{heme}}\beta_2^{\circ}$ to red cell ghosts were similar to those utilized and reported in past studies with hemoglobin Köln. Briefly, normal red cell ghosts were prepared by osmotic lysis by slight modification of the method described by Dodge, Mitchell, and Hanahan (28). Ghosts with their sulfhydryl groups blockaded were prepared by adding equal volumes of 4 mM PMB⁴ in isotonic saline to a suspension of 4 \times 10⁸ ghosts/mm³. After incubation for 1 hr at 37°C the ghosts were washed twice

in the same 30 mOsmol media used in the preparation of the ghosts. Another aliquot of ghosts was treated identically except isotonic saline replaced PMB. To assess binding of hemoglobin A and $\alpha_2^{\text{heme}}\beta_2^{\circ}$ to red cell membranes, these proteins were labeled with ⁵⁶Fe as follows. Stoichiometric and half-stoichiometric amounts, respectively, of hemin-⁵⁶Fe were mixed with purified globin, and the labeled heme-proteins were then purified by column chromatography as described above for the preparation of unlabeled $\alpha_2^{\text{heme}}\beta_2^{\circ}$. The labeled proteins were incubated for 2 hr with either control or PMB-treated ghosts at 37°C. The final suspension contained 14 mg of labeled heme-protein and 8 \times 10⁹ ghosts. After incubation the centrifuged (25,000 g) ghost pellets were washed three times in 30 mOsmol ghost buffer and then counted in an automated, well type gamma spectrometer.

RESULTS

Heme loss from CHBHA hemoglobins during Heinz body formation. During heat-induced (50°C) formation of Heinz bodies, the unstable hemoglobins, Zürich, Hammersmith, and Köln, lose hemes. In Fig. 1 this loss is depicted by the progressive diminution in their ratio of heme to globin, measured at 540 and 280 m μ , respectively.⁶ Hemoglobin A (solid circles) resists denaturation and concomitantly maintains a stable heme-to-globin ratio. The tendency of CHBHA hemoglobins to lose hemes correlates well with the severity of the clinical syndromes associated with them. Thus, hemoglobin Zürich loses hemes only after prolonged heating (open circles, Fig. 1), and *in vivo* causes little hemolysis and no anemia (30) unless patients are stressed with certain oxidant drugs, such as the sulfonamides (7). Conversely, hemoglobins Hammersmith and Köln produce a chronic, moderately debilitating Heinz body hemolytic anemia (1, 6), and *in vitro* immediately lose hemes. Indeed, both are partially heme deficient before incubation, simply following chromatographic isolation.⁷ A third hemoglobin, San Francisco (not shown), behaves similarly, causing moderate hemolysis in the patient; it also appears heme

³ Oxidation of the unstable hemoglobins to their methemoglobins or subunit hemichrome states (29) would also diminish the 540/280 m μ ratio. The extent of this potential artefact was judged minimal since addition of buffered KCN, which converts the oxidized pigments into cyanmethemoglobins, altered the ratio by no more than 5%. This is consistent with the observation that methemoglobin levels never exceeded 20% in the incubated solutions, and was less than 10% with all hemoglobins other than Hammersmith; such a level would depress the 540/280 m μ ratio at the most by roughly 6% (or 0.03 in Fig. 1). In addition the formation of hemichromes must have been minimal, if occurring at all, since no evidence of their characteristic 530 m μ peak and 565 m μ shoulder were perceptible in spectra from incubated solutions. Nevertheless, it is acknowledged that decreasing 540/280 m μ ratios can only semi-quantitatively reflect actual heme loss from the unstable hemoglobins.

⁷ Another unstable hemoglobin, Gun Hill, also produces a moderately severe Heinz body hemolytic anemia and has been reported to be heme deficient after isolation (10).

³ Cary model 15.

⁴ Sigma Chemical Co., St. Louis, Mo.

⁵ Schwarz Bioreserch, Mount Vernon, N. Y. Available as 90% radiochemically pure G³⁵S^H μ Ci/mg.

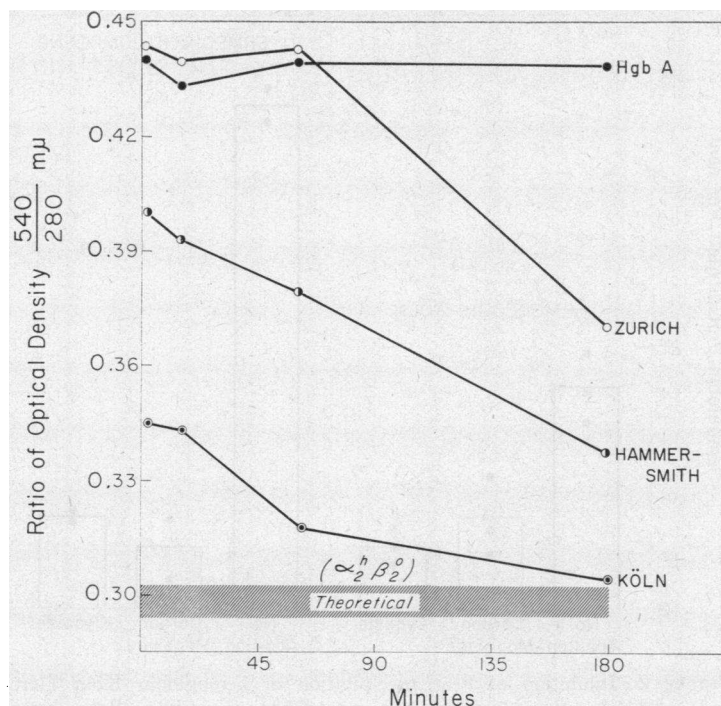


FIGURE 1 Heme loss from various oxyhemoglobins incubated at 50°C. Unlike hemoglobin A (solid circles), Heinz body-forming hemoglobins progressively lose hemes as measured by decreasing 540/280 $m\mu$ ratios approaching that of a heme protein completely devoid of beta chain hemes, $\alpha_2^h\beta_2^o$ (hatched bar). The bar depicts the approximate value of a 50:50 mixture of $\alpha_2^{hemo}\beta_2^o$ in the cyanmet- and oxy- forms.

deficient when isolated, e.g., 540/280 $m\mu$ ratio = 0.32 (31). With incubation all three progressively diminish in 540/280 $m\mu$ ratio which ultimately approaches the value observed for a synthetic hemoglobin containing no hemes at all on its beta chains, $\alpha_2^{hemo}\beta_2^o$ (hatched bar, Fig. 1).

This intermediary compound, which is a prototype of the moieties which would form if hemes are lost preferentially from mutant β -chains, was synthesized and found to behave very similarly to the genetically unstable hemoglobins in several ways. It copiously precipitates into Heinz bodies at 50°C (left bar, right portion of Fig. 2). As with hemoglobin Köln (left, Fig. 2), this precipitation is inhibited by the heme ligands, cyanide or carbon monoxide, which suppress further heme loss (22). The genetically unstable hemoglobins release free α^{hemo} -chains into the supernatant solution while forming Heinz bodies (17); $\alpha_2^{hemo}\beta_2^o$ reacts similarly (Fig. 3). After 90 min at 50°C (middle channel, Fig. 3), when approximately 25% of $\alpha_2^{hemo}\beta_2^o$ has precipitated, much of the remaining soluble heme protein migrates more slowly, electrophoretically like marker-free α^{hemo} -chains (right channel,

Fig. 3). No free soluble β^o -chains could be detected in these experiments.⁸

The importance of heme loss in the denaturation of CHBHA hemoglobins into Heinz bodies was further substantiated by utilizing the observations of Bunn and Jandl (22) that heme loss from globin polypeptide chains is markedly accentuated when hemes are in the ferri-state. When $\alpha_2^{hemo}\beta_2^o$ is heated at 50°C for 45 min, Heinz body generation approximately doubles if hemes are in the higher oxidation state (e.g. $\alpha_2^{ferrihemo}\beta_2^o = 23\%$ precipitated; $\alpha_2^{ferrihemo}\beta_2^o = 48\%$ precipitated). That heme loss from alpha chains occurs in this latter situation is documented by the progressive accumulation of $\alpha_2^{hemo}\beta_2^{hemo}$ (i.e. hemoglobin A) in the soluble fraction; that is, ferrihemes released from alpha chains reattach to the

⁸ Using previously described techniques (18) of gel filtration through Sephadex G-100 columns, protein with an apparent molecular weight consistent with α -chain monomers was observed in the supernatant solutions of $\alpha_2^{hemo}\beta_2^o$ heated for 90 min at 50°C. Normal hemoglobin ($\alpha_2^{hemo}\beta_2^{hemo}$) was generated by adding free β^{hemo} -chains to these supernatants as also described in our recent studies with Hemoglobin Zürich (30).

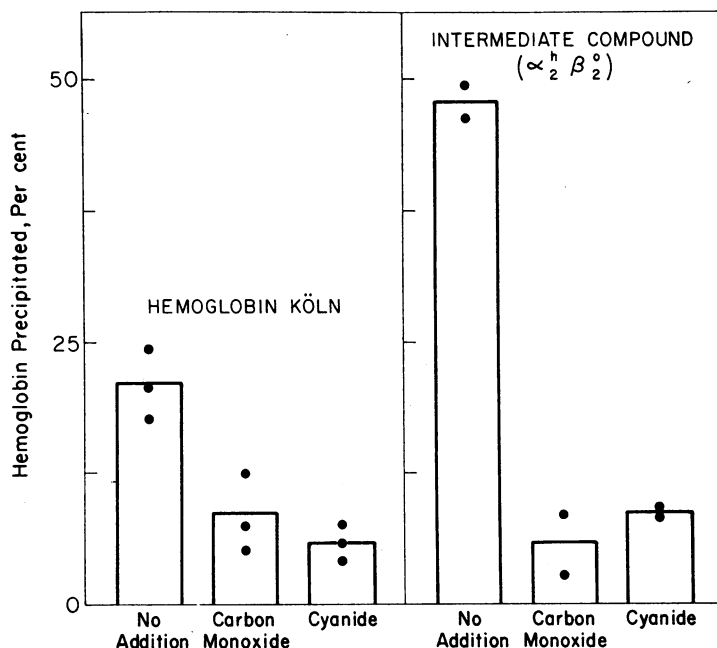


FIGURE 2 Inhibition of heat precipitation of hemoglobin Köln (left) and $\alpha_2^{\text{heme}}\beta_2^{\text{o}}$ (right) by cyanide or carbon monoxide. Both heme-proteins copiously precipitate when heated at 50°C for 2 hr unless the heme ligands, cyanide or carbon monoxide, are present.

naked beta chains of other $\alpha_3^{\text{heme}}\beta_2^{\text{o}}$ molecules, thereby forming hemoglobin A.

Supplementation of $\alpha_3^{\text{heme}}\beta_2^{\text{o}}$ with enough crystalline hemin to provide the normal stoichiometry of hemoglobin (four hemes per $\alpha_2\beta_2$ tetramer) produces physiologically normal hemoglobin A (25). This completed material no longer precipitates at 50°C. Likewise, if hemoglobins Köln and San Francisco are treated with excess crystal-

line hemin before incubation at 50°C, their precipitation into Heinz bodies is virtually prevented (Table I). We have presented evidence elsewhere that such treatment also normalizes their aberrant electrophoretic behavior (31).

Altered thiol reactivity of heme-depleted hemoglobins. Previously we showed that two unstable hemoglobins, Köln and Hammersmith, excessively form mixed disulfides between their beta chain thiols and glutathione and also with red cell membrane thiols; $\alpha_3^{\text{heme}}\beta_2^{\text{o}}$ is similarly

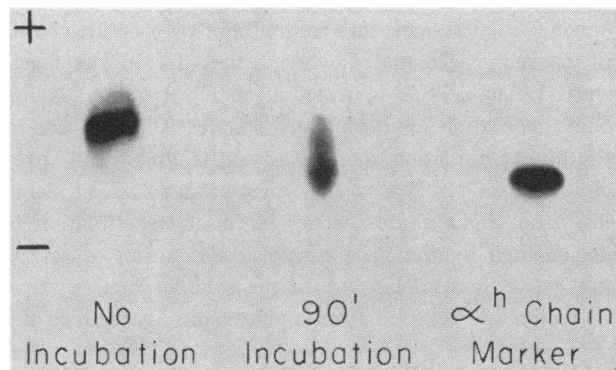


FIGURE 3 Formation of free α^{heme} -chains from $\alpha_2^{\text{heme}}\beta_2^{\text{o}}$ at 50°C. Starch-gel electrophoresis at pH 8.6; amido black stain. Incubation for 90 min (middle channel) produces a heterogeneous mixture with components exhibiting slower anodal migration than unincubated $\alpha_2^{\text{heme}}\beta_2^{\text{o}}$ (left channel), one identical with marker free α^{heme} -chains used as a marker (right channel).

TABLE I
Effect of Heme Repletion on Precipitation of CHBHA Hemoglobins*

Hemoglobin	Experiment	Hemoglobin precipitated		Heme repletion effect: inhibition of precipitation
		Untreated	Heme repleted	
		%		%
Köln	1	15	2	87
	2	12	1	92
San Francisco	1	19	2	89
	2	15	3	80

* Two aliquots of unstable oxyhemoglobins were incubated at 50°C for 30 min. To one aliquot enough crystalline hemin was added before heating to produce a normal stoichiometric heme: globin ratio (four hemes per $\alpha_2\beta_2$ tetramer). For details see text.

hyperreactive (Fig. 4). Hemoglobins Köln and Zürich bind 3–5 times more G³⁵S³⁵SH in mixed disulfide linkage than does hemoglobin A; $\alpha_2^{\text{heme}}\beta_2^0$ (closed circles, Fig. 4) is even more active in this regard. As in our previous studies (9), protein-bound label was found solely on beta chains and could be specifically released by mixed disulfide-splitting reagents.

As with CHBHA hemoglobins (9), $\alpha_2^{\text{heme}}\beta_2^0$ also excessively attaches to red cell membrane thiols in mixed disulfide linkage (Table II). Significantly more ⁵⁵Fe-labeled $\alpha_2^{\text{heme}}\beta_2^0$ binds to normal red cell ghosts than does hemoglobin A-⁵⁵Fe. This enhanced binding is inhibited if thiols of the red cell membranes are blocked by paramercuribenzoate (PMB) before incubation with labeled $\alpha_2^{\text{heme}}\beta_2^0$. Conversely, the slight attachment of hemoglobin A to red cell ghosts is probably not by disulfide bonding since preblockade of the membrane thiols with PMB is noninhibitory in this case.

DISCUSSION

The present studies document that the predilection of several CHBHA hemoglobins to precipitate into Heinz bodies reflects a diminished affinity of their mutant beta chains for heme groups. Three separate lines of evidence provide this documentation.

(a) All CHBHA hemoglobins studied lose hemes concomitantly with their precipitation into Heinz bodies at 50°C, and ultimately this loss approaches 50% of the available hemes (Fig. 1). It is unclear whether these hemoglobins are heme deficient in vivo; however, our findings (30), as well as those of others (32), that free α^{heme} -chains exist in fresh hemolysates from such patients suggests that heme loss, followed by cleavage of α - and β -chains into monomers, does occur in vivo.

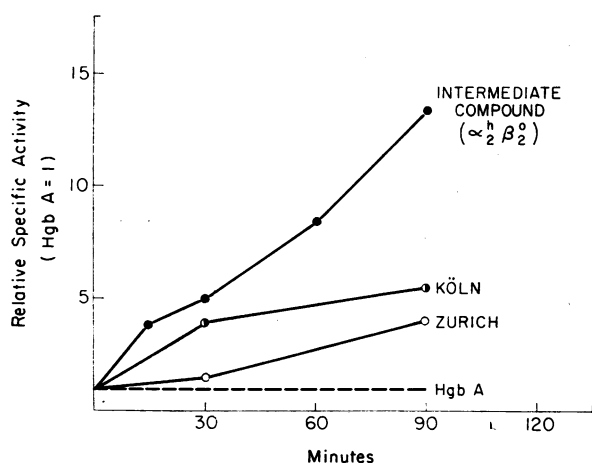


FIGURE 4 Binding of G³⁵S³⁵SH to hemoglobins. The unstable hemoglobins, Köln, Zürich, and $\alpha_2^{\text{heme}}\beta_2^0$ bind G³⁵S³⁵SH more readily than does hemoglobin A (dotted line).

TABLE II
Diminished Binding of $\alpha_2^{\text{heme}}\beta_2^0$ -Fe⁵⁵ to SH-Depleted (PMB-Treated) Red Cell Membranes*

Heme-protein	Experiment	Bound protein- ⁵⁵ Fe		
		Untreated ghosts	PMB-treated ghosts	PMB effect: % inhibition
$\alpha_2^{\text{heme}}\beta_2^0$	1	70	22	69
	2	64	19	70
Hemoglobin A	1	8	7	12
	2	7	7	0

* Two aliquots of normal red cell ghosts, one of which had sulfhydryl groups blocked by prior incubation with PMB, were incubated for 2 hr at 37°C in identical amounts with either ⁵⁵Fe-labeled hemoglobin A or $\alpha_2^{\text{heme}}\beta_2^0$ of known specific activity. After removal of unreacted heme protein by washing, ghost radioactivity was assessed and converted to micrograms of heme-protein.

(b) Perhaps the strongest evidence that heme depletion underlies the instability of some CHBHA hemoglobins is the observation that simple addition of excess hemin to these hemoglobins markedly retards their denaturation into Heinz bodies (Table I). In addition, a previously perplexing observation that hemoglobin Köln migrates in multiple bands and electrophoretically more slowly than hemoglobin A, despite a mutation which should lead to no net charge alteration, becomes explicable on the basis of heme deficiency. Synthetic, heme-deficient hemoglobins with variously deficient ratios of heme:globin similarly migrate as separate bands, all more slowly than hemoglobin A (18). Addition of hemin to them as well as to hemoglobins Köln and San Francisco corrects their aberrant electrophoretic behavior, all then migrating as solitary bands identically with hemoglobin A (31). The multiplicity of electrophoretic bands of the unstable hemoglobins suggests that they exist as moieties with several heme:globin ratios, e.g., $\alpha_2^{\text{heme}}\beta_2^0$ and $\alpha_2^{\text{heme}}\beta^{\text{heme}}\beta^0$. Schneider et al. have suggested that another CHBHA hemoglobin, Sabine, may be heme deficient and have presented pH 8.6 electrophoretograms which show a heterogeneous, slowly migrating heme protein (33). The effect of heme repletion on the electrophoretic behavior of hemoglobin Sabine would therefore also be interesting.

(c) Finally, a hemoglobin synthesized to contain heme groups only on its alpha chains, $\alpha_2^{\text{heme}}\beta_2^0$, behaves identically with the available genetically unstable CHBHA hemoglobins in several respects. This compound is the homologue of the postulated intermediary compound in the denaturation of CHBHA hemoglobins if, indeed, beta chain hemes are released before precipitation. Thus, $\alpha_2^{\text{heme}}\beta_2^0$ and the CHBHA hemoglobins (a) both precipitate into typical coccoid Heinz bodies at 50°C; (b) if both are treated with excess hemin

prior to incubation at 50°C, precipitation is virtually eliminated; (c) both progressively release free α^{heme} -chains into supernatant solutions as Heinz bodies are generated, and Heinz body precipitates derived from both are whitish suggesting heme-deficient β^0 -chains make up the bulk of the precipitates; (d) in both, weakening heme binding by oxidizing hemes to the ferriform (22), potentiates Heinz body formation, and conversely the ligands cyanide and carbon monoxide, by strengthening heme attachment (22), inhibit Heinz body generation; and (e) finally, both $\alpha_2^{\text{heme}}\beta_2^0$ and the available CHBHA hemoglobins manifest hyperreactivity of their beta chain thiol groups, by excessively forming mixed disulfides with glutathione, and by attaching inordinately to red cell membranes through mixed disulfide bonds between their thiol groups and those of the membrane.

The primary site of binding of heme to the β -chains of hemoglobin is to a histidine present as the 92nd amino acid of the beta chain. Previously one of us demonstrated that the neighboring cysteine at $\beta 93$ is blocked in hemoglobin Köln by combination with glutathione in mixed disulfide linkage (8, 9). It was suggested that the alteration in conformation of the beta chain which would result by such combination might underlie the demonstrated diminished avidity of hemoglobin Köln for its heme groups (9). The further findings that heme binding by hemoglobin A could be weakened by artificially blockading its $\beta 93$ thiols (34) and that Heinz body formation at 50°C was potentiated thereby (9) supported this view. However, the present studies support another view; that heme loss from mutant beta chains in CHBHA hemoglobins is primary, and that excessive reactivity of $\beta 93$ thiols results therefrom. That is, synthetic $\alpha_2^{\text{heme}}\beta_2^0$ forms mixed disulfides with glutathione and with membrane thiols at rates 10–35 times that of heme-replete hemoglobin A.*

The blockade of membrane thiols resulting from their binding to soluble $\alpha_2^{\text{heme}}\beta_2^0$ (Table II) might represent a previously neglected mechanism of red cell destruction in CHBHA. Previous evidence has indicated that membrane thiols are crucial to red cell viability *in vitro* (35) and *in vivo* (36). If blockaded with sulfhydryl inhibitors such as PMB or *N*-ethylmaleimide, red cells become leaky to cations, osmotically fragile, and are rapidly removed from the circulation by reticuloendothelial tissues, especially the spleen. When hemoglobin precipitates into Heinz bodies in circulating red cells the cells become stuck in narrow areas of the microvasculature, tethered by their nondeformable inclusion bodies. This

* Since the $\beta 93$ sulfhydryl group of hemoglobin is normally reactive whereas the other beta chain thiol is buried (11), it is assumed that the binding of G^{SH} and membrane thiols to unstable hemoglobins involves the $\beta 93$ position.

process has been visualized especially in the spleen (37). The present studies indicate that denaturing hemoglobin even while still soluble, (e.g. $\alpha_2^{\text{heme}}\beta_2^0$) may also affect the stability of red cell membranes by attaching to their thiol groups; this presumably could diminish *in vivo* survival even before inclusion bodies are formed.

We conclude that heme is crucial to globin stability, and that its loss from mutant beta chains is an important mechanism of hemoglobin denaturation in the congenital Heinz body hemolytic anemias. The mechanism by which heme loss predisposes to globin instability is obscure, although others have demonstrated that the conformation of globin is altered when hemes are removed; that is, the helical content of the globin decreases (38). The heme-depleted polypeptide chains in CHBHA hemoglobins are therefore partially unfolded, and may further "unwind" during their precipitation into Heinz bodies.

It seems likely that precipitation of the mutant CHBHA hemoglobins into Heinz bodies involves two steps. The first involves the tendency for mutant polypeptide chains (usually beta chains) to lose their heme groups, which in turn reflects an alteration in conformation of the heme pocket due to neighboring amino acid substitutions. It has been emphasized that mutations in this same area need not necessarily lead to heme loss and hemoglobin instability, but may cause methemoglobinemia instead (16). If heme loss does occur, however, the resulting excessively freed hemes most likely show up as the dark urinary dipyrrolic pigment characteristic of many cases of CHBHA (1–4). The second step reflects the fact that the resulting intermediary compound, $\alpha_2^{\text{heme}}\beta_2^0$, is unstable, cleaving into soluble heme-containing α -chains, and into insoluble heme-deficient β -chains, which make up the bulk of the Heinz body material. Both soluble intermediary compound and precipitated Heinz bodies attach to red cell membrane thiols in mixed disulfide linkage, reflecting the hyperreactivity of the thiols of beta chains when devoid of their hemes. The resulting blockade of membrane thiols predisposes the red cell to entrapment and destruction in reticuloendothelial organs, especially the spleen.

Although the above sequence of denaturation is thought to underlie most CHBHA hemoglobinopathies, new observations (14) on another, recently described unstable hemoglobin, Philly (β^{P} tyrosine \rightarrow phenylalanine) suggest another mechanism as well. Mutation in this hemoglobin, and perhaps in hemoglobin Riverdale-Bronx (13) as well, is not near the heme pocket, but is in a region where alpha and beta chains make contact. The linkage between these subunits evidently diminishes thereby, as free alpha and beta chain monomers (with hemes attached) have been demonstrated during precipitation of this CHBHA hemoglobin (14). Unlike

the unstable hemoglobins examined in the present studies, hemoglobin Philly and Bronx-Riverdale¹⁰ Heinz bodies are reddish (heme replete) rather than white (heme deficient) and affected patients do not excrete dark "dipyrrolic" urine. These observations suggest that excessive monomer formation, even in the absence of heme loss, may be another, albeit less common, cause of unstable hemoglobin denaturation into Heinz bodies.

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