The Structure of a Hemoglobin Carrying Only Two Hemes*

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ABSTRACT: Sequential addition of hemin to globin gives rise to three distinct intermediates in the formation of reconstituted hemoglobin which are postulated to have the prosthetic group:polypeptide chain ratios 1:4, 2:4, and 3:4, respectively. The quantitatively favored intermediate postulated to contain two hemes per four polypeptide chains was isolable as a single peak on CM Sephadex, and the properties of this intermediate have been studied in the present work. The absorption spectra of the cyan-met and carbon monoxy derivatives closely resemble those of the corresponding hemoglobins in the heme absorption region, and quantitation of the spectral intensities revealed a heme:polypeptide chain ratio of 1:2, or 1 mole of heme/mole of globin. The sedimentation coefficients for the cyan-met derivative are comparable with those reported for hemoglobin, with a similar concentration dependence indicating partial dissociation at low concentrations. Molecular weight estimates from equilibrium sedimentation and from sedimentation diffusion (assuming the diffusion coefficient to be identical with that of hemoglobin) yielded values of 58,000, in agreement with previous results by gel filtration, indicating that the molecule is a dimer consisting of two globin subunits. The location of the two associated heme molecules was established by converting the intermediary compound to hemoglobin by the addition of labeled hemin (or by the addition of nonlabeled heme to labeled intermediate), followed by separation of the polypeptide chains. Specific binding sites for hemin located on α chains were occupied, whereas the corresponding sites located on β chains were all free. The structure of the intermediate can therefore be written as $\alpha^h\beta\alpha^h\beta$, with the possibility that the molecule exists in solution as a partly dissociated species.

Storage or acid pH did not lead to any appreciable heme transfer from one type of polypeptide chain to another in the presence of cyanide, but rapid exchange of heme from the β chains of ferrihemoglobin onto the α chains of globin took place when cyanide was absent.

The reaction of native globin having the formula $\alpha\beta$ (Winterhalter and Huehns, 1964) with the cyanide derivative of hemin gives cyanmethemoglobin with the formula $\alpha^h\alpha^h\beta^h\beta^h$ (where h denotes a molecule of hemin). This reaction involves two processes: the dimerization of $\alpha\beta$ subunits and the insertion of four heme molecules into their respective crevices on the polypeptide chain. Recently, three intermediary compounds resulting from the stepwise combination of globin with hemin have been separated by starch gel electrophoresis (Winterhalter, 1966). These compounds were observed to migrate on starch gel approximately one-fourth, one-half, and three-fourths of the distance between globin and hemoglobin, and were postulated to have the prosthetic group:polypeptide chain ratio of 1:4, 2:4, and 3:4, respectively. All of these intermediary compounds could be converted into hemoglobin by the addition of excess hemin. The compound having an anodic mobility halfway between globin and hemoglobin was quantitatively favored, and was isolated by column chromatography. Its molecular weight on gel filtration was similar to that of natural hemoglobin. The present paper deals with the structure and physical properties of the latter compound, which we have designated as intermediary compound II.

Materials and Methods

All chemicals used were of the highest purity available. Determination of pH values was carried out at 20°. Phosphate buffers for the preparation of cyanmethemoproteins were 0.01 M in phosphate and contained 100 mg of KCN/l. For the carbonmonoxyhemoglobin preparation the buffers were 0.01 M phosphate saturated with carbon monoxide. The pH 4.7 buffer was 0.1 M sodium acetate containing 100 mg of KCN/l. Starch gel electrophoresis was carried out at 4° according to Poulik (1957). Protein concentrations for globin were calculated on the basis of an extinction coefficient $E^{1\%}_{1\,cm}$ 8.5 at 280 mμ (Gibson and Antonini, 1963). ICII[1] was prepared as previously described (Winterhalter, 1966). Hemin was reduced to heme in dilute solution by addition of 1% by volume of a freshly prepared 1% sodium dithionite solution (Gibson and Antonini, 1963), and converted into carbonmonoxyheme by bubbling carbon monoxide through

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[1] Intermediary compound II.

the solution. Cyan-methemoproteins were reduced in the same manner and converted into carbonmonoxyhemoproteins by storage in a CO atmosphere with gentle shaking.

Ultraviolet and visible spectra were measured on a Beckman DK 2A spectrophotometer fitted with a temperature-regulated cell holder. All measurements were made at 4° in pH 7.2 phosphate buffer containing 0.1 M NaCl in addition to KCN or CO, with dry nitrogen purging of the cell compartment to prevent condensation on the cell faces. The heme content was estimated spectroscopically by standard methods for the determination of the concentration of one absorbing species in the presence of a second (see, for example, Jaffé and Orchin (1962)). In place of the usual single wavelengths, the integrated areas under the heme and hemoprotein absorption bands were measured and used for the calculation in order to minimize the effects of spectral changes occurring when hemin is bound to globin. The limits of integration were arbitrarily chosen for the heme and hemoprotein bands; 350–650 and 260–295 mμ, respectively.

Sedimentation coefficients were measured in a Beckman–Spinco Model E Ultracentrifuge at 10° in 0.01 M phosphate buffer containing 0.1 M NaCl in addition to 100 mg/l. of KCN. The Wratten 77A filter was replaced with two Jena Glaswerk filters (OG 590/3 and OG 550/3) to avoid absorbance due to heme. The filter combination had zero transmittance below 580 mμ and about 80% transmittance from 620 to above 1000 mμ, with a sharp cut (37% T) at 592 mμ. Photographs were recorded on Kodak RS Pan film. All sedimentation coefficients were corrected to water at 20°.

Radioactive hemin was prepared by injection of 50 μc of $^{59}FeCl_3$ subcutaneously into a rabbit that previously had been made anemic by phlebotomy. The rabbit was sacrificed 10 days after injection of the isotope, and hemin was crystallized from the whole blood (Labbe and Nishida, 1957).

The polypeptide chains of hemoglobin were separated by incubating 1 μmole of carbonmonoxyhemoglobin with 8 μmoles of *p*-mercuribenzoate for 16 hr. The chains were isolated by chromatography on CM-cellulose (Bucci and Fronticelli, 1965). Since this resulted in complete decomposition of hemoglobin into α and β chains as evidenced by starch gel electrophoresis, a stepwise elution procedure of the CM-cellulose column was used. Up to 100 mg of protein was extensively dialyzed against a pH 6.42 phosphate buffer and then applied to a 18 × 100 mm column which had been previously equilibrated with pH 6.0 phosphate buffer. The first elution step was carried out with pH 6.7 phosphate buffer. After 90 ml of effluent had been collected, the second step was initiated with 0.02 M Na_2HPO_4. Analysis of the eluates by starch gel electrophoresis showed clear separations of α and β chains. For the separated chains protein concentration was calculated on the basis of an extinction coefficient $E^{1\%}_{1\,cm}$ 8.4 at 540 mμ for the carbonmonoxy derivatives (Bucci and Fronticelli, 1965). Radioactivity was counted on a well-type scintillation detector equipped with a 2-in. NaI (Tl) crystal connected to a two-channel Tri-Carb liquid scintillation spectrometer Model 314 E.

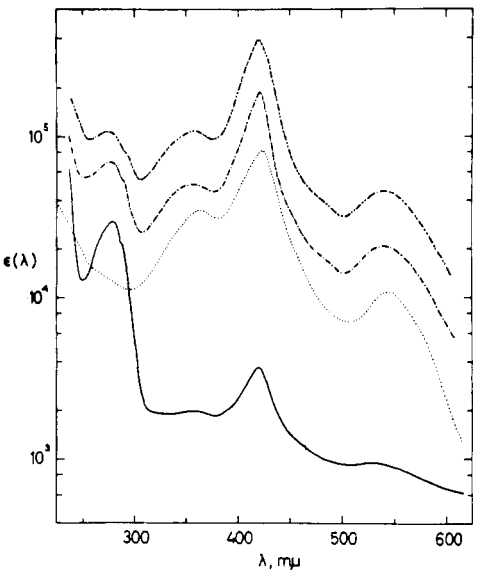

FIGURE 1: Extinction coefficient (liters per mole centimeter) *vs.* wavelength (millimicrons) for globin (———) and the cyan-met forms of hemin (· · · ·), ICII (– · – ·), and natural hemoglobin (– · · –) in 0.01 M sodium phosphate buffer containing 100 mg/l. of KCN and 0.1 M NaCl (pH 7.2).

Results

Spectral Properties. In the region 300–600 mμ, the spectrum of cyan-met ICII shows a close qualitative correspondence with the spectrum of natural cyanmethemoglobin (Figure 1). A similar relationship is observed in the case of the corresponding carbonmonoxy proteins (Figure 2). For globin, the extinction coefficient at 280 mμ, determined after correcting for the calculated heme content, was $E^{1\%}_{1\,cm}$ 8.6, in good agreement with the literature (Gibson and Antonini, 1963) value of 8.5. The extinction coefficient of cyan-met ICII was estimated at 280 mμ from the absorbance of a solution of known concentration (determined by quantitative amino acid analysis for lysine and histidine), giving $E^{1\%}_{1\,cm}$ 11.1. The spectral similarity between ICII and natural hemoglobin can be seen more clearly in the difference spectra shown in Figure 3. The sample and reference solutions were normalized by dilution of one or the other solution until equal absorbance was observed at 540 mμ, and the difference spectrum was recorded. The absorbance differences outside the region of protein absorption are small, amounting to about 5% of the total absorbance, and are confined mainly to the Soret band at 420 mμ. Similar small differences are also observed between natural and reconstituted hemoglobin, as shown. Presuming that

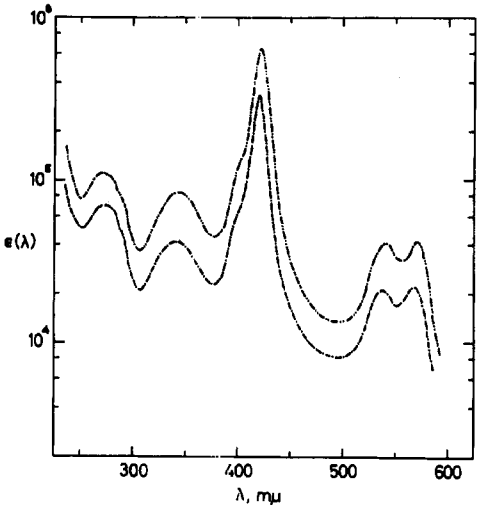

FIGURE 2: Extinction coefficient (liters per mole centimeter) *vs.* wavelength (millimicrons) for the carbonmonoxy forms of ICII (– · – · –) and natural hemoglobin (– · · · –) in 0.01 M sodium phosphate buffer containing 0.1 M NaCl and saturated with CO (pH 7.2).

the heme environment is similar in ICII and hemoglobin, at least as reflected in the 540-mμ absorption band, a value for the extinction coefficient of ICII can be computed from the excess protein absorbance due to ICII in the difference spectrum. This value was $E^{1\%}_{1\,cm}$ 11.0 at 280 mμ, in good agreement with the value determined from quantitation by amino acid analysis.

The heme content of both cyan-met ICII and cyanmethemoglobin was calculated from the integrated areas under the relevant absorption bands (see Materials and Methods), obtaining the values 2.0 and 4.0 moles of heme/mol wt 62,000, respectively, using hemoglobin as a defined standard (observed values: 2.20 and 4.34). From the ratio of the single-wavelength absorbancies at 275 and 542 mμ, the corresponding observed values were 2.1 and 4.0 moles of heme/mol wt 62,000. The values of the peak ratios in the cyan-met form for ten measurements of six different preparations of ICII are shown in Table I, together with the values observed by us for natural and reconstituted hemoglobin.

Electrophoretic Behavior. The starch gel electrophoresis of the carbonmonoxy derivatives showing the formation of intermediate compounds by stepwise addition of carbonmonoxyheme to globin are illustrated in Figure 4. They are qualitatively similar to those obtained for the cyan-met proteins by stepwise addition of cyanhemin to globin (Winterhalter, 1966). Carbonmonoxy ICII could also be obtained by reduction of cyan-met ICII followed by saturation with CO, and was fully convertible into carbonmonoxyhemoglobin by addition of carbonmonoxyheme (Figure 5). The anodic mobility of the carbonmonoxy proteins was slightly smaller than the one observed for the cyan-met proteins.

Sedimentation Behavior of ICII. ICII sediments as a single, symmetrical peak in the velocity ultracentrifuge (Figure 6), and a 1:1 mixture of ICII and hemoglobin also sediments as single peak (Figure 7). In the latter case, the boundary is not symmetrical, and broadening of the leading edge can be observed in the photograph.

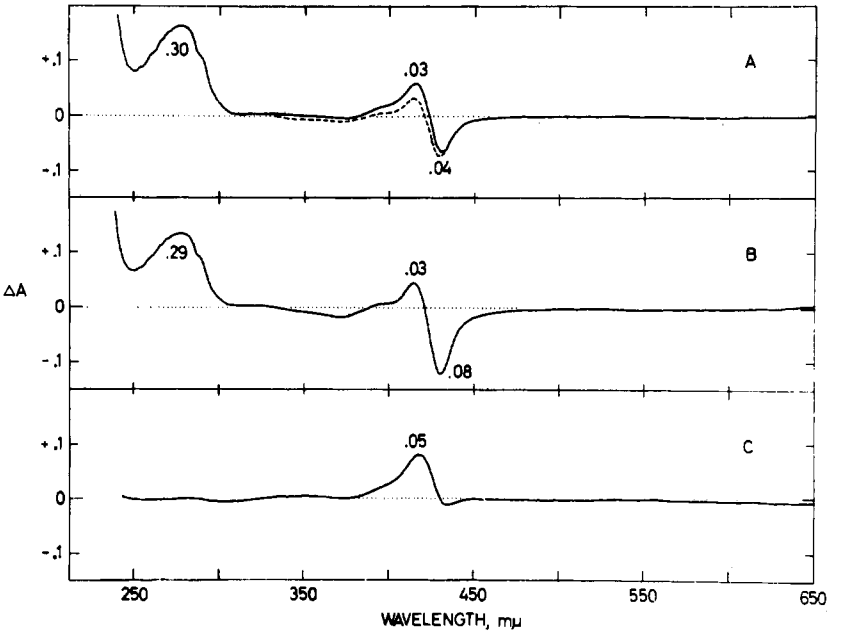

FIGURE 3: Difference spectra normalized at 540 mμ. (A) ICII (two samples) *vs.* reconstituted hemoglobin. (B) ICII *vs.* natural hemoglobin. (C) Reconstituted *vs.* natural hemoglobin. The numbers on the curves indicate the relative excess absorbance $\Delta A/A_{ICII}$ at 280 mμ (left) and referred to 421 mμ (center).

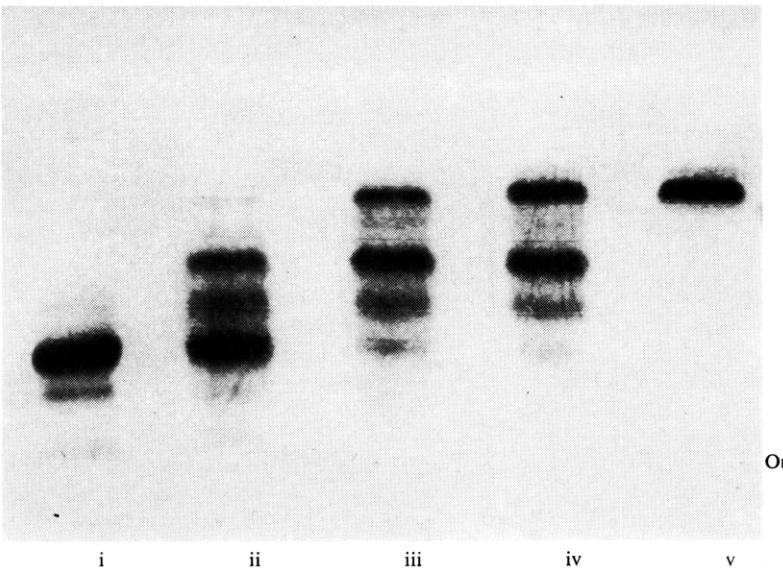

FIGURE 4: Starch gel electrophoresis. (i) Globin A; (ii) globin A plus 1 mole of carbonmonoxy heme/2 moles of globin; (iii) as ii with 2 moles of carbonmonoxy heme; (iv) as ii with 3 moles of carbonmonoxy heme; (v) as ii with 4 moles of carbonmonoxy heme.

TABLE I: Spectral Peak Ratios and Root Mean Square Errors for ICII and Natural and Reconstituted Hemoglobin in the Cyan-met Form.

	275:540	420:540	420:275	360:275
ICII (10)	3.6 ± 0.2	10.8 ± 0.7	3.0 ± 0.1	0.74 ± 0.01
Reconstituted Hb (4)	2.4 ± 0.2	9.7 ± 1.5	3.9 ± 0.4	1.04 ± 0.02
Hb (3)	2.6 ± 0.1	10.4 ± 0.3	4.1 ± 0.3	1.05 ± 0.02

The mixture had an $s_{20,w}$ of 4.20 S at a total concentration of 2.1% protein, well within the range of values reported for hemoglobin (Rossi-Fanelli *et al.*, 1964). In Figure 8, the concentration dependence of the sedimentation velocity of ICII is shown together with that of globin. The sedimentation coefficients for ICII are approximately 10% lower than those reported for hemoglobin under similar conditions (Rossi-Fanelli *et al.*, 1964), and the concentration dependence above 1% protein concentration is approximately the same. Below 1% protein concentration, the results suggest partial dissociation of the molecule into smaller subunits, as has been reported by a variety of authors for hemoglobin (Rossi-Fanelli *et al.*, 1964). However, molecular weight determinations of ICII at three concentrations (0.2, 0.3, and 0.4% protein)[2] in the equilibrium ultracentrifuge with interference optics yielded an average molecular weight of 58,000, with no apparent concentration dependence in 0.01 M phosphate buffer containing 100 mg of KCN/l.

[2] Through the courtesy of Dr. P. Moser.

Location of the Heme Groups in ICII. When ICII was converted into hemoglobin by the addition of [^{59}Fe]hemin and the chains were subsequently separated, virtually all the radioactivity was found on the β chains and no radioactivity was incorporated into the α chains, thus indicating that all specific binding sites for hemin located on α chains were occupied (Figure 9). If (inversely) 2 μmoles of radioactively labeled hemin were mixed with 2 μmoles of globin and ICII isolated from this mixture was subsequently converted into hemoglobin by addition of nonlabeled hemin, virtually all radioactivity was found on the α chains and none on the β chains (Figure 9) indicating that all binding sites on β chains were free. In both instances the specific activity of the labeled chains was at least two orders of magnitude higher than in the other type of chain. In addition, no dilution of the radioactive label was observed on incorporation, implying that all α (or β) chains were labeled. If hemoglobin carrying the label only on one type of polypeptide chain was dialyzed against pH 4.7 buffer containing cyanide (conditions under which a considerable number of polypeptide chains appear to exist in monomeric

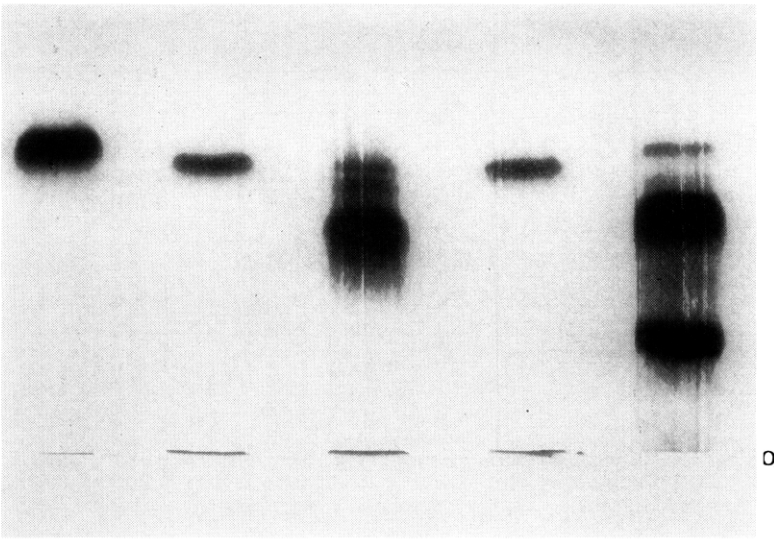

FIGURE 5: Starch gel electrophoresis. (i) Cyan-methemoglobin marker. (ii) Carbonmonoxy hemoglobin prepared from oxyhemoglobin. (iii) Mixture of ICII and hemoglobin after conversion into the carbonmonoxy form. The band between hemoglobin and ICII is the compound postulated to have three prosthetic groups per four polypeptide chains. (iv) Same sample as in iii but after addition of excess carbonmonoxy heme. (v) Mixture of globin, ICII, and hemoglobin, the latter two in the cyan-met form.

form, Guidotti *et al.*, 1963) for 24 or 48 hr, respectively, no appreciable transfer of labeled hemin from one type of polypeptide chain to the other could be observed.

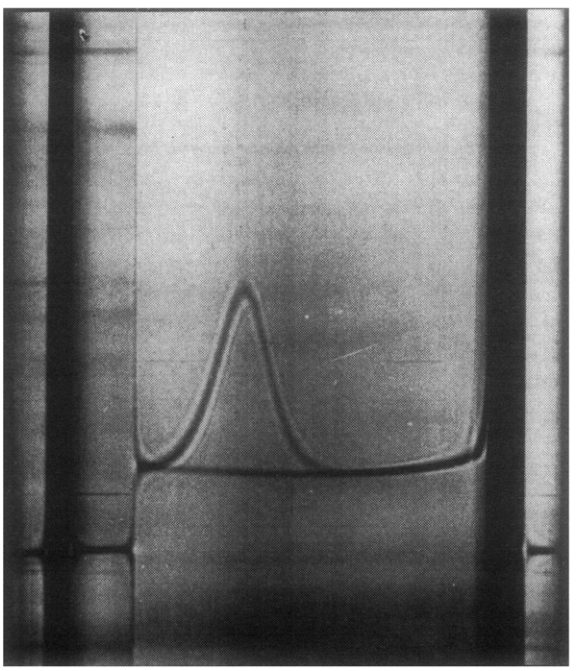

FIGURE 6: Sedimentation velocity pattern of ICII after 90 min at 59,780 rpm in 0.01 M phosphate plus 0.1 M NaCl plus KCN (100 mg/l.). Protein concentration 1.6%, bar angle 65°.

Similarly no transfer of label from one type of polypeptide chain to the other took place upon storage of asymmetrically labeled hemoglobin for 3 weeks in pH 7.2 phosphate, at 4° in the presence of CN. Incubation of ferrihemoglobin with globin at 4° in the absence of CN, however, gave rise to the progressive appearance of a protein band having the characteristics of ICII (Figure 10).

Discussion

The observations of Gibson and Antonini (1963) postulating the existence of an intermediate compound in the reaction of heme with globin have been recently substantiated by the separation of several intermediates on starch gel (Winterhalter, 1966). The present results and those of Winterhalter (1966) show that one of these intermediate compounds (ICII) has two hemes per four polypeptide chains, a molecular weight nearly identical with that of hemoglobin, and an absorption spectrum differing only minimally from that of hemoglobin in the region of pure heme absorption.

The close correspondance of the spectra of hemoglobin and ICII in the region of pure heme absorption is reflected both in the cyan-met and in the carbonmonoxy forms, suggesting that the major contributions to the spectral changes occurring on addition of heme are due to heme–protein interactions of the same nature as those in hemoglobin. The heme interaction environment appears to be closely similar in both α and β chains, for if this were not the case larger changes in the difference spectrum would be expected between ICII and hemoglobin. For ICII *vs.* hemin normalized

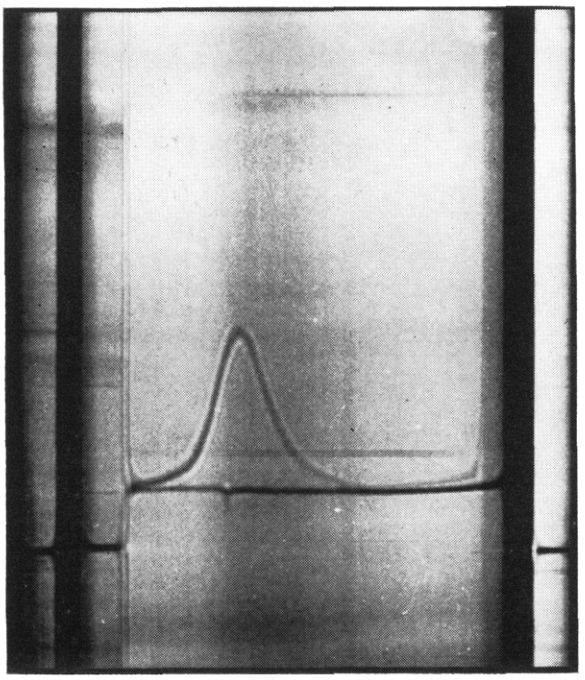

FIGURE 7: Sedimentation velocity pattern of a 1:1 mixture of ICII and hemoglobin after 80 min at 59,780 rpm. Total protein concentration 2.1% in 0.01 M phosphate plus 0.1 M NaCl plus KCN (100 mg/l.) (pH 7.4), bar angle 70°.

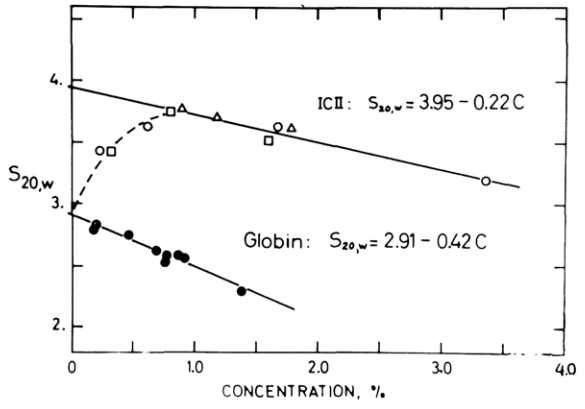

FIGURE 8: Concentration dependence of the sedimentation coefficients of ICII (present work) and globin (plotted from the data given by Winterhalter and Huehns, 1964).

at 540 mμ, for example, the differences amount to about 20% of the total absorbance near 370 mμ and 30% near 420 mμ, as contrasted with differences of the order of 2 and 5% observed between ICII and hemoglobin at the same wavelengths. The fact that ICII, in which no β-chain-bound heme exists, has a minimal difference spectrum with hemoglobin in the heme absorption region suggests that completion of the mole-

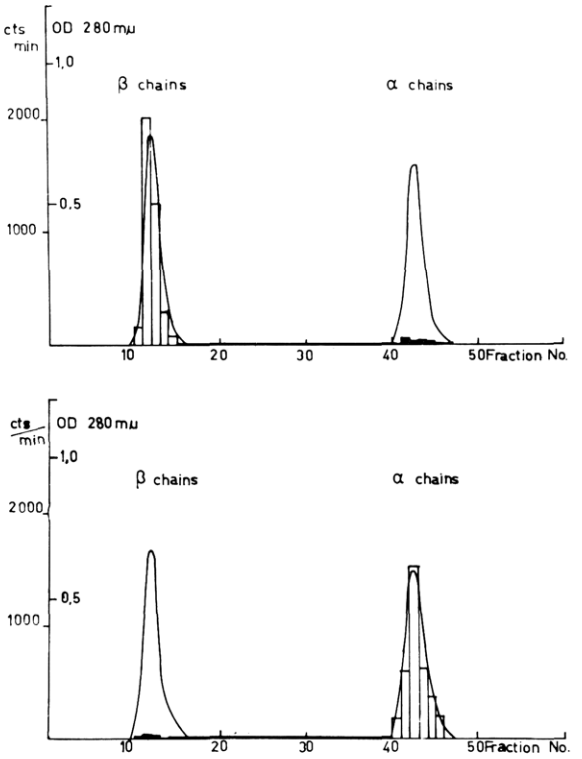

FIGURE 9: Separation of polypeptide chains by chromatography on a CM-cellulose column. The rectangular blocks represent counts per minute. Top part: Polypeptide chains from hemoglobin obtained through conversion of ICII into hemoglobin by addition of excess [^{59}Fe]hemin. Bottom part: Polypeptide chains from hemoglobin obtained through conversion of [^{59}Fe]-hemin-labeled ICII into hemoglobin by addition of excess nonlabeled hemin.

cule to hemoglobin by the addition of two more hemes to the β chains results in small (if any) further spectral changes. Since similar spectral anomalies occur in the difference spectrum between natural and reconstituted hemoglobin, it is possible that small amounts of denatured material could contribute to the difference spectrum observed between ICII and natural or reconstituted hemoglobin, and between natural and reconstituted hemoglobin. However, some differences between the two types of chains must exist in the heme-interaction environment in view of their different affinities for heme, although it is not necessary that these differences be reflected in the absorption spectra within the limitations of the present measurements. The observation that the change in anodic mobility between the carbonmonoxy and cyan-met forms of ICII appears to be proportional to the changes observed in the corresponding hemoglobin derivatives implies that the charge distribution is similar in the two forms.

The molecular weight of ICII as determined by gel filtration has been shown to be indistinguishable from

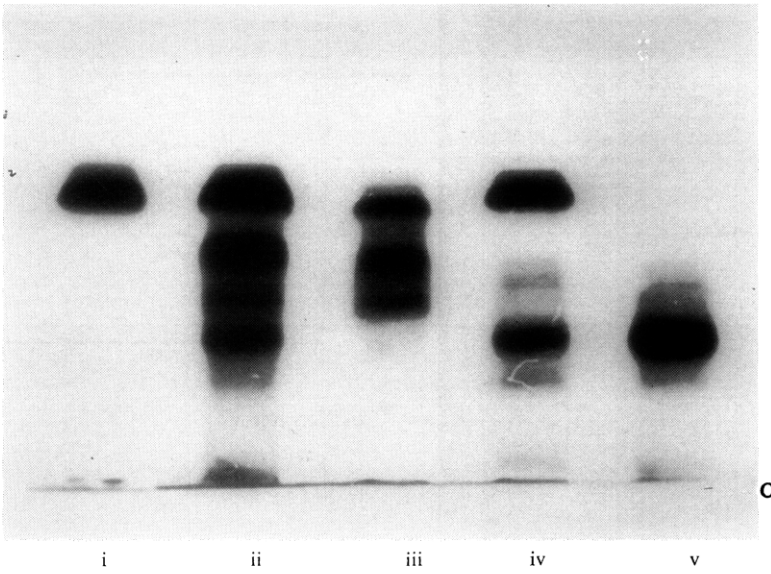

FIGURE 10: Starch gel electrophoresis. (i) Cyan-methemoglobin. (ii) Methemoglobin–globin mixture (1:1) incubated in 0.01 M PO_4 (pH 7.4) for 48 hr at 4°, no cyanide. (iii) Marker containing cyan-methemoglobin ICII and ICI. (iv) Methemoglobin–globin mixture (1:1) incubated in 0.01 M PO_4 (pH 7.4) for 48 hr at 4° (100 mg of KCN/l.). (v) Globin marker containing small amounts of heme-carrying impurities (also visible in iv).

that of natural hemoglobin (Winterhalter, 1966). In support of these measurements, we find sedimentation coefficients and molecular weights as determined in the ultracentrifuge to be in essential agreement with the idea that ICII is composed of four polypeptide chains. From the values $D_{20} = 4.31$ (average of three values on human hemoglobin given in Rossi-Fanelli *et al.* (1964)) and $s^0_{20,w} = 3.95$ S, a molecular weight of 58,300 can be computed from the Svedberg relationship. This calculation assumes, of course, that the diffusion coefficients of ICII and natural hemoglobin are the same, but the results are in excellent agreement with the molecular weight of 58,000 obtained by equilibrium sedimentation. The actual molecular weight of ICII may be closer to that of hemoglobin than indicated by the determined molecular weight, as indicated by the gel filtration studies (Winterhalter, 1966). We note that ultracentrifuge molecular weights ranging between 59,400 and 66,800 have been reported for human hemoglobin (s/D), and corresponding sedimentation coefficients ranging between 4.1 and 4.6 (extrapolated to zero protein concentration (Rossi-Fanelli *et al.*, 1964)). The observation that the 1:1 mixture of ICII and hemoglobin sedimented with an asymmetric boundary shows a molecular weight difference, yet this difference may be small in view of the sedimentation coefficient of the mixture, 4.2 at 2% protein concentration.

The concentration dependence of the sedimentation coefficient of ICII suggests a partial dissociation of the molecule below protein concentrations of 1%, similar to the behavior of hemoglobin under comparable conditions (Rossi-Fanelli *et al.*, 1964). In analogy with the case of hemoglobin, this dissociation might be represented as $2\alpha^h\beta \rightleftharpoons \alpha^h\beta\alpha^h\beta$, with the equilibrium largely in favor of the dimer. From the sedimentation coefficient concentration dependence, an approximate dissociation constant of the order of 10^{-5} M can be computed by assuming the above model. Further studies will be necessary, however, to clarify the exact nature of the dissociation, which is not clearly understood even in the case of hemoglobin (Rossi-Fanelli *et al.*, 1964).

The radioactive-labeling experiments and molecular weight estimates provide a clear demonstration that the molecule contains two hemes per four polypeptide chains. Further, these experiments demonstrate that the two hemes in ICII are bound only to α chains, since no label could be observed in the β chains when radioactively labeled ICII was converted to hemoglobin by addition of nonlabeled heme, and no label could be observed in α chains when nonlabeled ICII was converted into hemoglobin by the addition of labeled heme. Hence the correct formula for ICII is $\alpha^h\beta\alpha^h\beta$.

Under the present experimental conditions leading to the formation of cyan-methemeproteins, α chains have a higher affinity for hemin than β chains. Similar conclusions can be drawn for methemoglobin from the data recently reported by Bunn and Jandl (1966). These authors have been able to demonstrate that exchange of heme groups between human methemoglobins A and F readily takes place, whereas little or no transfer of radioactivity took place between the two hemoglobins in their oxy-, deoxy-, carbonmonoxy- and cyan-met forms. However, the exchange between the α chains of the two methemoglobins was about four times slower than between the non-α chains.

The fact that incubation of ferrihemoglobin with

globin in the absence of ligand led to a rapid transfer of heme groups from ferrihemoglobin to globin, further supports the concept of the differential affinity for heme between the two types of chains. For each two molecules of heme released from ferrihemoglobin two molecules of ICII were formed, one from β-chain heme-depleted ferrihemoglobin and one from two globin molecules each carrying a single heme on the α chain.

Upon storage of asymmetrically labeled cyan-methemoglobin for 3 weeks at 4°, we detected no transfer of label from one type of polypeptide chain to the other. This is in keeping with the idea that only very little dissociation of cyan-metheme from its crevice on the polypeptide chain into solution is taking place. This behavior could not be influenced by incubating asymmetrically labeled cyan-methemoglobin for 24 and 48 hr in pH 4.7 buffer, although these conditions favor the dissociation of hemoglobin into single-chain subunits. Recently it has been reported by Banerjee and Cassoly (1966) that incubation of human methemoglobin with horse apomyoglobin for 1 week at pH 7.0 in 0.1 M phosphate buffer leads to the formation of a hemoglobin carrying only two hemes per four polypeptide chains. Assuming that this compound has a composition similar to the ICII described in the present work, it would appear that transfer of heme from the β chains to the apomyoglobin has taken place.

From the formula of ICII it can be deduced that the formula for the postulated intermediate having three prosthetic groups per four polypeptide chains must be $\alpha^h\alpha^h\beta^h\beta$, and the one for the postulated compound containing only one heme group per four polypeptide chains can be either $\alpha^h\beta(+\alpha\beta)$ or $\alpha^h\alpha\beta\beta$.

However, if a compound of the composition $\alpha^h\beta$ existed, it would be one-half ICII, and would presumably associate with another molecule of the same composition to give rise to ICII, and its separation as a clear band on starch gel would therefore not be probable.

A further implication of the higher affinity of α chains for heme is given by the following considerations. The stimulating effect of heme on the biosynthesis of the protein moiety of hemoglobin has been demonstrated by several authors in a variety of systems (Hammel and Bessman, 1965; Grayzel *et al.*, 1966). In view of the difference in affinity for heme between the α and β chains, it is conceivable that the synthetic rate of one type of chain is affected more than the rate of the other in the presence of heme. Accepting the general idea (Huehns and Shooter, 1962; Winterhalter, 1964; Colombo and Baglioni, 1966) that the synthetic rate of one chain type is controlled by the availability of the other (which would have to be in short supply in order to be effective as a control mechanism), heme could have a regulatory effect on the synthetic rate of one polypeptide chain. Therefore heme would influence the synthetic rate of one type of chain directly and of the others indirectly, thereby assuring (at the polysomal level) a synchronous production of both.

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