Asymmetrical Hemoglobin Hybrids. An Approach to the Study of Subunit Interactions†

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ABSTRACT: The properties of asymmetrical hemoglobin hybrids of the type $\alpha_2\beta^A\beta^S$ have been studied. Mixtures of two hemoglobins of unlike charge were analyzed by isoelectric focusing on polyacrylamide gel. When liganded hemoglobins were studied, a prominent middle band appeared initially but disappeared after about 40 min. In contrast, when comparable mixtures were deoxygenated and then analyzed anaerobically, a stable middle band was clearly demonstrated. With the use of [^{3}H]hemoglobins S and A, the middle band was identified as the hybrid $\alpha_2\beta^A\beta^S$. In like manner hybrids were demonstrated in various mixtures of A, S, C, E, F, and canine hemoglobins. In a mixture containing equal amounts of two parent hemoglobins, the hybrid approached half the total indicating that the stability of the hybrid is equivalent to that of the parent hemoglobins. Measuring the formation of hybrid at intervals following admixing of two deoxyhemoglobins permitted an estimation of a first-order rate constant for the dissociation of deoxyhemoglobin tetramer into dimers. This rate was markedly reduced by the addition of 2,3-diphosphoglycerate to the mixture and was enhanced at high pH. These observations are consistent with the structural–functional formulation recently presented by Perutz. Mixtures of cyanmethemoglobin and oxyhemoglobin when deoxygenated failed to form a stable hybrid. Likewise no intermediate band was found in solutions of deoxyhemoglobin partially saturated with carbon monoxide or nitric oxide.

Among the many physical–chemical properties which distinguish deoxyhemoglobin from liganded forms of hemoglobin is the extent to which the tetramer dissociates into dimers, according to the following reaction

$$\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta$$

The dissociation equilibrium constant for oxyhemoglobin is about 2×10^{-6} M (heme) (Guidotti, 1967a; Briehl and Hobbs, 1970; Edelstein *et al.*, 1970; Kellett and Schachman, 1971). The dissociation of deoxyhemoglobin into dimers is less by at least several orders of magnitude (Benesch *et al.*, 1962, 1964; Kellett and Schachman, 1971; Thomas and Edelstein, 1972). The conformational isomerization involved in the removal of ligand from hemoglobin is associated with a marked increase in binding energy between $\alpha\beta$ dimers. This is probably due in large part to intersubunit salt bonds which stabilize the deoxy tetramer (Perutz, 1970). In view of these considerations, it seems reasonable to suppose that when two structurally different liganded hemoglobins are mixed, unlike dimers can reassociate to form asymmetrical hybrid molecules. Thus, if equal amounts of A and S oxyhemoglobins are mixed and if the hybrid tetramer were equally as stable as the two parent tetramers, the distribution of the hemoglobins at equilibrium would be: $\alpha_2\beta_2^A$, 2 $\alpha_2\beta^A\beta^S$, $\alpha_2\beta_2^S$. As Guidotti *et al.* (1963) pointed out, failure to demonstrate such a hybrid by methods dependent on separation by charge does not argue against its existence. During the separation procedure, the liganded hybrid would dissociate and the resultant dimers would then sort with like dimers. Subsequently, Guidotti (1967b) reported osmotic pressure measurements indicating that such hybrids do indeed exist. Macleod and Hill (1973) have demonstrated electrophoretically and chromatographically the hybrid hemoglobin $\alpha_2\beta^A\beta^S$ by the use of the cross-linking reagent *p,p'*-difluoro-*m,m'*-dinitrodiphenyl sulfone. Recently Bunn (1972) and Park (1973) have used gel electrofocusing to demonstrate a variety of asymmetrical hemoglobin hybrids. This report presents further data on the properties of these hybrids. We have examined the effect of heme ligands, pH, and organic phosphates on the stability of the hybrid. These results permit an estimation of the rate of dissociation of the deoxyhemoglobin tetramer into dimers.

Materials and Methods

Blood specimens were obtained from individuals with the following hemoglobin phenotypes: AA, SS, CC, and AE. Hemolysates were prepared by the method of Drabkin (1946). Organic phosphates were removed as described by Benesch *et al.* (1969). Hemoglobins A, C, E, and S were purified by chromatography on DEAE-cellulose (Huisman and Dozy, 1965). Visible absorbance spectra were measured with a Perkin-Elmer 350 recording spectrophotometer. For one experiment cyanmethemoglobin A was prepared by the addition of a 1.5-equiv excess of $K_3Fe(CN)_6$ to oxyhemoglobin in 0.1 M potassium phosphate buffer (pH 6.6) followed a 1.5-equiv excess of KCN. These reactants were separated from the cyanmethemoglobin by passing the mixture through a long column containing G-25 Sephadex in 0.1 M NaCl–0.05 M Tris (pH 7.5). For another experiment, radioactive A and S hemoglobins were prepared by Dr. Gabriel Cividalli as follows. Reticulocytes from one patient with microangiopathic hemolytic anemia (hemoglobin A) and from another with sickle cell anemia were incubated with tritium-labeled leucine at 37° for 1 hr. The [^{3}H]hemoglobins were each purified by DEAE-cellulose chromatography. Solutions of 2,3-diphosphoglycerate were prepared and analyzed as described previously (Bunn *et al.*, 1971).

Deoxyhemoglobin was prepared by passing a stream of 99.997% pure nitrogen through a hydrator and then across solutions of oxyhemoglobin (10 mg/ml) during agitation by a rapid wrist action shaker. In most experiments, a 0.2-equiv amount of sodium dithionite was then added anaerobically to

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the hemoglobin in order to ensure complete deoxygenation. Solutions of deoxyhemoglobin were mixed anaerobically by transfer from one flask to another under positive nitrogen pressure as described by Benesch *et al.* (1962). Generally, Tris, bistris (bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane) (General Biochemicals, Inc.), or potassium phosphate buffers were used for these incubation studies.

Hemoglobin solutions were analyzed by isoelectric focusing on polyacrylamide gel (Drysdale *et al.*, 1971). Samples (0.020 ml) containing 0.20 mg of hemoglobin were applied to gels in glass cylinders (3.5 i.d. × 100 mm). The rate of separation of hemoglobins was enhanced by the use of a pulse power supply. A maximum of 0.20 W was applied to each gel. Generally, separations were complete within 90 min. Solutions of deoxyhemoglobin were analyzed in a controlled atmosphere glove box (Labconco, Inc.) containing 100% nitrogen or argon. Reagents and apparatus were passed in and out of the box *via* a vacuum chamber. Dissolved oxygen was removed from the anolyte and catholyte by thoroughly equilibrating the solutions with nitrogen. Prior to application of samples, traces of oxygen were removed from the gels by the addition of 10 ml of 0.1% sodium dithionite to the catholyte. Upon completion of each run, unstained gels were photographed with a MP3 Polaroid camera. Hemoglobin bands were quantitated by scanning at selected wavelengths with a Gilford recording spectrophotometer. When carboxy- or oxyhemoglobins were analyzed, gels were scanned at 540 nm, whereas gels containing deoxyhemoglobin were scanned at 555 nm. In an experiment in which mixtures of carboxy- and deoxyhemoglobins were analyzed, the patterns were quantitated as explained in legend of Figure 9. As shown in Figure 1, there was excellent agreement between the relative amounts of two hemoglobins applied to the gels and that quantitated by spectrophotometric scanning.

Hemoglobin was eluted from gels as follows. Sliced segments of gels containing hemoglobin bands were minced and then incubated in 1.0 ml of Drabkin's solution–water (1:10). After 2 hr the gels had completely decolorized. The solution was centrifuged and the supernatant was analyzed from 600 to 400 nm with a Perkin-Elmer 350 recording spectrophotometer.

Solutions of buffer saturated at room temperature with carbon monoxide and nitric oxide were prepared as described by Antonini and Brunori (1971), giving concentrations of approximately 1.0 and 2.0 mM, respectively. Care was taken to exclude NO from any contact with oxygen.

Results

When a mixture containing equal amounts of carboxyhemoglobins A and S in 0.05 M bistris buffer (pH 7.2) were analyzed by gel electrofocusing, three prominent bands were initially visualized prior to the focusing of the bands at their isoelectric points. As the hemoglobins descended further into the gel, the middle band became progressively less prominent. As shown in Figure 2, 40 min after application of the hemoglobin mixture to the gel, the middle band was faint and, by 60 min, no longer visible. Identical results were obtained with solutions of oxyhemoglobins. This transient demonstration of a middle band in mixtures of liganded hemoglobin confirms results of Park (1973) and represents presumptive evidence that hemoglobin hybrids of the type $\alpha_2\beta^A\beta^S$ exist in solution in equilibrium with the two parent hemoglobins. However, the marked lability of the middle band makes it very difficult to identify or to study its properties.

In contrast, if a mixture of oxyhemoglobins A and S were deoxygenated and then analyzed anaerobically, a prominent

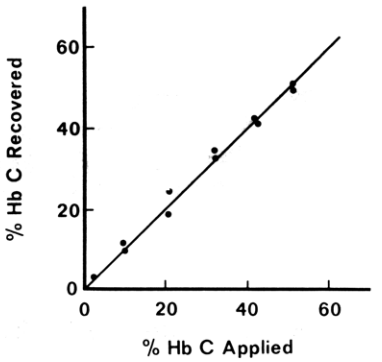


FIGURE 1: Quantitation of mixtures of A and C hemoglobins following gel electrofocusing. Mixtures containing known amounts of the two oxyhemoglobins were applied to gels. The percentage of hemoglobin C measured by scanning the gels at 540 nm is plotted against the percentage of hemoglobin C in the mixtures applied to the gels. The straight line has a slope of 1.0.

middle band was observed as shown in Figure 3. Since deoxyhemoglobin does not dissociate readily into dimers, the hybrid is fixed as a stable tetramer. The identity of this middle band as the hybrid $\alpha_2\beta^A\beta^S$ was established with the use of radioactive hemoglobins. After mixture of unlabeled hemoglobin A and [^{3}H]hemoglobin S was deoxygenated and then separated on gels, the three bands were eluted, analyzed spectrophotometrically (as the cyanmethemoglobin derivative) and counted. As shown in Table I, the specific activity of the middle band was approximately half of the radioactive parent hemoglobin. Similar results were obtained in the converse experiment where mixtures of unlabeled hemoglobin S and [^{3}H]hemoglobin A were analyzed (Table I). Hybrid hemoglobin was also demonstrated in mixtures of A and C, S and C, A and E as well as A and F_{II}, S and F_I, S and F_{II}, S and canine (Bunn, 1972). As shown in Figure 4, the deoxygenated hybrid is stable over several hours. In a mixture containing equal amounts of two parent hemoglobins, the hybrid approached half of the total. This is expected if the hybrid tetramer were equally as stable as the parent hemoglobins. The distribution of the three components at equilibrium should follow the binomial expansion $a^2 + 2ab + b^2 = 1$, where a and b are the initial fractions of parent hemoglobins. As Figure 3 shows, this distribution was also found in mixtures containing unequal amounts of the two parent hemoglobins. As the deoxygenated hemoglobins remain on the gels for several hours, the

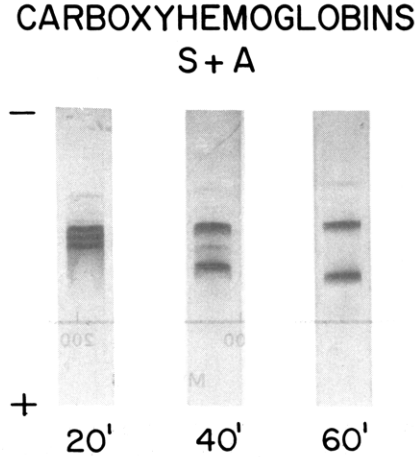
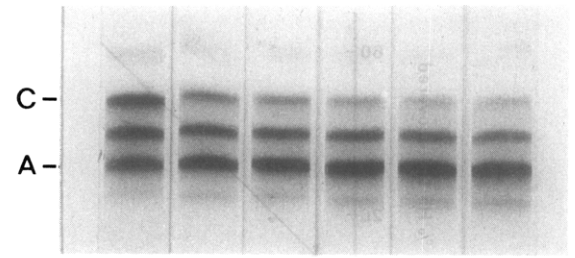


FIGURE 2: Separation of a mixture containing equal amounts of carboxyhemoglobins A and S. The gels were removed and photographed at 20, 40, and 60 min after application of the samples.



% C in mixture	48	37	34	29	25	22
% C exp.	24	14	11	8	6	5
% C rec.	27	18	15	10	8	7
% hyb. exp.	50	46	45	41	37	34
% hyb. rec.	43	38	37	38	33	29

FIGURE 3: Demonstration of the hybrid hemoglobin $\alpha_2\beta^A\beta^C$. Mixtures containing varying amounts of oxyhemoglobin A and oxyhemoglobin C were deoxygenated and then applied to the gels anaerobically. Sixty minutes later they were photographed and scanned at 555 nm. The amount of C and hybrid hemoglobins recovered (determined from the scanning data) is compared with that expected from the binomial distribution $a^2 + 2ab + b^2 = 1$.

middle band decreased slowly (Figure 4). This is a reflection of the slow rate of dissociation of the deoxygenated hybrid into dimers of unlike charge. Because of reassociation of dimers, this kind of experiment does not permit any calculation of the velocity of subunit dissociation of deoxyhemoglobin.

The rate of dissociation of deoxyhemoglobin into dimers could be estimated by measuring the appearance of hybrid following the mixture of deoxygenated hemoglobins of unlike

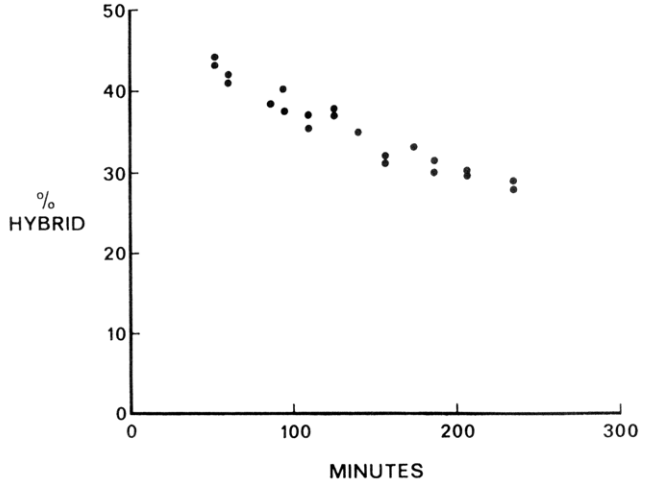


FIGURE 4: The decay in the deoxygenated hybrid hemoglobin during prolonged gel electrofocusing. Mixtures containing equal amounts of A and C oxyhemoglobins were deoxygenated and then applied to gels anaerobically. The gels were removed at intervals thereafter and quantitated by scanning at 555 nm.

TABLE I: Identification of the Middle Band as $\alpha_2\beta^A\beta^S$ by Use of Tritium-Labeled Hemoglobins.[a]

	Sp Act.[b] (cpm/mg)			Sp Act of Hybrid to [^{3}H]Hb
	Hb A	"Hybrid"	Hb S	
[^{3}H]Hb A + unlabeled Hb S	16,100	9800	760	0.60
	14,500	8000	610	0.55
	14,700	6150	700	0.42
Unlabeled Hb A + [^{3}H]Hb S	380	8900	16,700	0.53
	380	7600	14,400	0.52

[a] A mixture containing equal amounts of labeled oxyhemoglobin A and unlabeled oxyhemoglobin S (and *vice versa*) in 0.1 M bistris buffer (pH 7.2) was deoxygenated and then analyzed anaerobically. The measurement of radioactivity and hemoglobin content of the bands is described in the text. [b] Specific activity is defined here in the conventional way: cpm per mg of total hemoglobin. Therefore these values are proportional to cpm per mol of hemoglobin tetramer.

charge. In the following experiments, hemoglobins A and C were used. Because of the wide difference in their isoelectric points, these hemoglobins could be rapidly separated. The assumption that these two hemoglobins dissociate equally into dimers seems reasonable since they differ by a single residue on the surface of the molecule ($\beta^{6Glu\rightarrow Lys}$), one which is not at an interface between subunits. Furthermore, the two hemoglobins have identical oxygen binding curves and reactivity to 2,3-diphosphoglycerate and CO_2 (Bunn, 1972). In the experiment shown in Figure 5, mixtures of phosphate-free deoxyhemoglobins A and C were incubated at room temperature, pH 7.2, for varying intervals before anaerobic application to gels. A progressive increase in the hybrid was observed over a 4-hr period. If the rate at which deoxyhemoglobins A, C and the hybrid $\alpha_2\beta^A\beta^C$ dissociate into dimers is indeed identical

$$\alpha_2\beta_2 \xrightarrow{k} 2\ \alpha\beta$$

then the rate of appearance of hybrid following the mixture of equal amounts of A and C deoxyhemoglobins is: $dY_H/dt = k(^1/_2 - Y_H)$, where Y_H is the fraction of total hemoglobin as hybrid. Therefore $\ln(1 - 2Y_H) = -kt$. As shown in Figure 6, the rate at which deoxyhemoglobin dissociates into dimers was

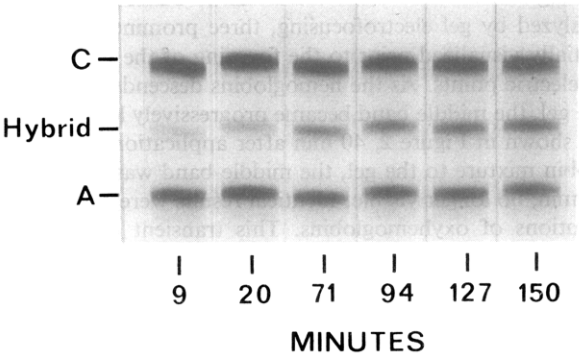


FIGURE 5: The rate of formation of deoxy hybrid. Equal amounts of deoxyhemoglobin A and C in 0.1 M bistris buffer (pH 7.2) were admixed and incubated at 24° for varying intervals prior to application to gels. Gels were scanned at 555 nm.

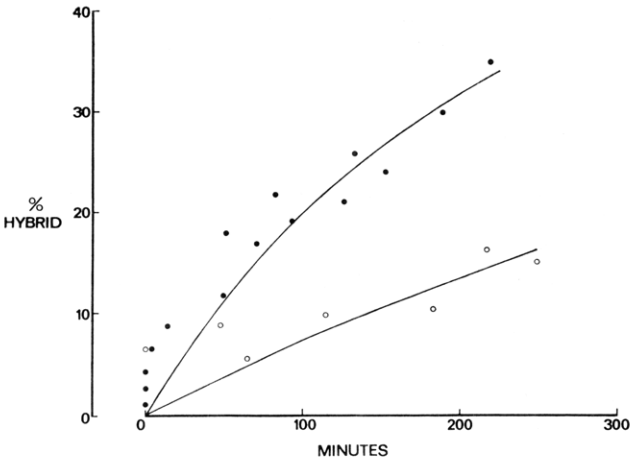


FIGURE 6: The rate of formation of deoxy hybrid in the absence (●) and presence (○) of 1 mM 2,3-diphosphoglycerate. Otherwise, experimental conditions are as stated in Figure 5. The unbroken lines correspond to first-order rate constants of 0.30 hr^{-1} (no 2,3-diphosphoglycerate) and 0.10 hr^{-1} (1 mM 2,3-diphosphoglycerate) for the dissociation of deoxyhemoglobin into dimers.

found to be strongly influenced by the presence of 2,3-diphosphoglycerate. In its absence, $k = 0.3$ hr^{-1} at pH 7.2, 24°. In the presence of 1 mM 2,3-diphosphoglycerate the rate was reduced about threefold. As shown in Figure 6, an appreciable amount of hybrid (2–7%) was observed in specimens which were applied to gels immediately after mixing ("zero time"). This may have been due in part to further incubation on the gels (approximately 20 min) prior to separation. Furthermore, the mixture was exposed to a somewhat elevated pH (~8.0) prior to separation. As shown below, subunit dissociation is enhanced at higher pH.

The effect of pH on the rate of hybrid formation from phosphate-free hemoglobins is shown in Figure 7. At pH 6.0 the rate is relatively slow, comparable to that of hemoglobin in the presence of 2,3-diphosphoglycerate (pH 7.2) (Figure 6). In contrast, hybrid formed relatively rapidly at pH 8.0.

We looked for the presence of hybrid in mixtures of hemoglobins which were partially bound by heme ligands. A mixture containing equal amounts of oxyhemoglobin S and CN methemoglobin A (0.1 M bistris, pH 7.2) was first deoxygenated and then analyzed anaerobically. As Figure 8 shows,

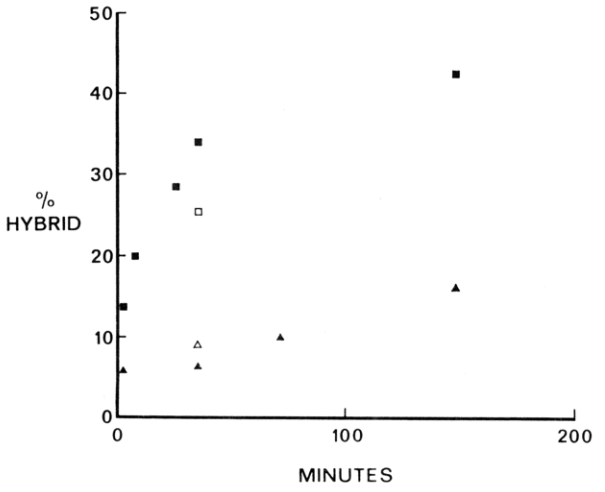
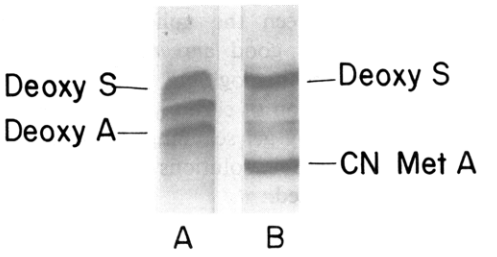


FIGURE 7: The effect of pH on the rate of formation of deoxy hybrid: (■) 0.1 M Tris, pH 8.0; (□) 0.1 M phosphate, pH 8.0; (▲) 0.1 M bistris, pH 6.0; (Δ) 0.1 M phosphate, pH 6.0. Deoxyhemoglobins A and C were admixed and incubated at 24° for varying intervals prior to application to gels. Gels were scanned at 555 nm.



A. Oxy A + Oxy S ⟶ Deoxygenated

B. CN Met A + Oxy S ⟶ Deoxygenated

FIGURE 8: Failure of cyanmethemoglobin to form stable hybrid with deoxyhemoglobin. Equal amounts of hemoglobins were deoxygenated and analyzed anaerobically: (A) oxyhemoglobin A and oxyhemoglobin S; note prominent hybrid; (B) cyanmethemoglobin A and oxyhemoglobin S; no hybrid band was observed. The faint band at the position of deoxyhemoglobin A probably represents the small amount of cyanmethemoglobin A which had been reduced by the dithionite used to purge the gels of oxygen (see Materials and Methods).

almost no hybrid could be demonstrated. Since oxyhemoglobin and cyanomethemoglobins probably have identical tertiary and quaternary structures, in the mixture of the liganded hemoglobins, the hybrid $(\alpha\beta)^{oxy}(\alpha\beta)^{CN\,met}$ should coexist with the two parent hemoglobins. However, upon deoxygenation it is likely that the relatively unstable hybrid dissociated, permitting reassociation of like dimers. As Park (1970) has shown, deoxyhemoglobin can be readily separated from fully liganded hemoglobin by isoelectric focusing. This represents a graphic demonstration of the alkaline Bohr effect (Park, 1970). Solutions of deoxyhemoglobin (0.1 M bistris, pH 7.2) were treated with subsaturating amounts of carbon monoxide prior to anaerobic analysis. A purple band representing deoxyhemoglobin was readily separated from the red carboxyhemoglobin band. The isoelectric points of the two forms differed by about 0.1 pH unit. As shown in Figure 9, at approximately 25, 50, and 75% saturation, no intermediate

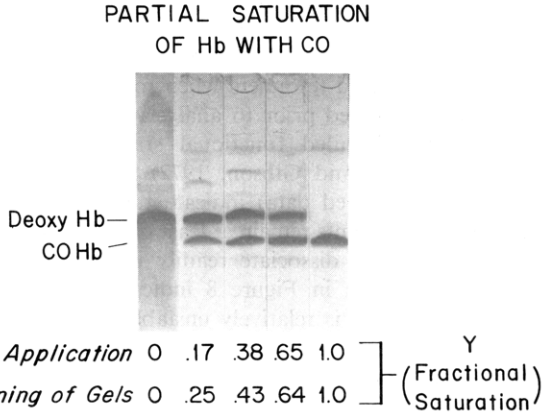


FIGURE 9: Analysis of solutions of hemoglobin A partially saturated with carbon monoxide. Measured amounts of water saturated with carbon monoxide (~1 mM) were added to solutions of deoxyhemoglobin in a tonometer attached to a 2-mm cuvet. The fractional saturation was determined spectrophotometrically. After the hemoglobin solutions were separated by gel electrofocusing, they were scanned at 540 and 555 nm. The distribution of carboxyhemoglobin and deoxyhemoglobin was calculated with the use of the following extinction coefficients (mM, heme). Deoxyhemoglobin: 555 nm, 13.04; 540 nm, 10.28. Carboxyhemoglobin: 555 nm, 11.33; 540 nm, 14.27 (van Assendelft, 1970).

bands were observed between the unliganded and fully liganded forms. There was good agreement between the fractional saturation of the hemoglobin solutions prior to separation and the distribution of carboxy- and deoxyhemoglobin bands as measured by gel scanning. Identical results were obtained when hemoglobin solutions partially saturated with nitric oxide were analyzed.

Discussion

These experiments demonstrate naturally occurring hemoglobin hybrids such as $\alpha_2\beta^A\beta^S$. These asymmetrical molecules are tetramers composed of unlike dimers. This type of hemoglobin hybrid must be distinguished from two other kinds. Valency hybrids are molecules in which the hemes of the α and β chains have different oxidation states such as $\alpha_2^{Fe^{3+}}\beta_2^{Fe^{2+}}$. These chemically modified hemoglobins have been carefully studied as possible analogs of partially liganded native hemoglobins. Secondly, the term "hybrid" is often used in designating new hemoglobins formed from the reassocation of subunits in mixtures of hemoglobins exposed to extremes of pH (Vinograd and Hutchinson, 1960) or in certain salt solutions (Tomita *et al.*, 1973). Under such conditions a mixture of human (A) and canine hemoglobins can form the hybrids $\alpha_2^A\beta_2^{Can}$ and $\alpha_2^{Can}\beta_2^A$. In contrast to the asymmetrical hybrids studied in this report, the other two types of molecules are symmetrical tetramers readily demonstrated by conventional electrophoretic or chromatographic methods.

The demonstration of naturally occurring hybrids in mixtures of unlike hemoglobins is in agreement with recent studies of Macleod and Hill (1973) and Park (1973). In experiments on functionally normal hemoglobins such as human A, S, C, E, and F and canine, the amount of hybrid formed from a mixture of two hemoglobins was predictable from the binomial distribution $(a_2 + 2ab + b^2)$ (Figures 3 and 4). This strongly implies that the stability of the mixed-hybrid hemoglobin was equivalent to that of the parent hemoglobins. The experimental results of Macleod and Hill (1973) are in accord with this conclusion and do not indicate (as they have suggested) that the stability of the hybrid hemoglobin exceeds that of the symmetrical tetramers. In contrast, we have found that certain variants having high oxygen affinity and lacking subunit cooperativity (hemoglobins Bethesda ($\alpha_2\beta_2^{145His}$), Kempsey ($\alpha_2\beta_2^{99Asn}$), and Malmö ($\alpha_2\beta_2^{97Gln}$) do not form stable mixed-hybrid tetramers when mixed with hemoglobin A and deoxygenated prior to analysis (H. F. Bunn, unpublished data). Detailed functional studies of hemoglobins Bethesda (Olson and Gibson, 1972) and Kempsey (Q. H. Gibson, unpublished data) indicate that when fully deoxygenated these variants remain at least partially in the oxy or R conformation and dissociate readily into dimers. Likewise, the results shown in Figure 8 indicate that the tetramer $(\alpha\beta)^{deoxy}(\alpha\beta)^{CNmet}$ is relatively unstable. This finding may be relevant to recent studies of Bookchin and Nagel (1971) on the participation of various cyanmethemoglobins in gelation with deoxyhemoglobin S.

Experiments in which deoxyhemoglobins A and C (or A and E) were mixed and then analyzed after incubation provide a measure of the rate of dissociation of deoxyhemoglobin into dimers. Two factors limit the precision of these data. First, a small amount of hybrid (2–7%) was always found in solutions which were immediately applied to the gels after mixing. If the separating system were ideal, no detectable hybrid should have been found. It is unlikely that incomplete deoxygenation was responsible for the appearance of zero-time hybrid. Varying the amount of dithionite over a tenfold range had no effect on the results. Furthermore, the amount of hybrid formed during incubations at 0° was considerably less than incubations carried out at 24°. Since the affinity of hemoglobin for oxygen is greatly increased at low temperature, an artifact due to the presence of small amounts of oxygen would have been amplified. A second source of error in the estimation of rate constants for subunit dissociation is due to the fact that the hybrid dissipates slowly during the process of electrofocusing (Figure 4). In order to minimize this error, the gels were removed as soon as the separations were adequate for scanning. These two systematic errors go in opposite directions—the first tends to overestimate hybrid, while the second leads to falsely low values. Neither of these is apt to be of sufficient magnitude to give grossly anomalous results.

Because of the high degree of stability of deoxyhemoglobin under physiological conditions, no one has succeeded in measuring experimentally the equilibrium between tetramer and dimers. Using an indirect approach, Thomas and Edelstein (1972) have estimated that in 0.1 M phosphate (pH 7.0), the dissociation equilibrium constant is 3×10^{-12} M (heme). The value for liganded hemoglobin is about 7×10^5 greater under similar conditions. From measurements of combinations of CO hemoglobin with haptoglobin, Nagel and Gibson (1971) have estimated that the velocity for the dissociation of liganded tetramer into dimers is approximately 2 sec^{-1}. If liganded and unliganded dimers combine at a comparable rate, then the estimated rate of dissociation of deoxyhemoglobin would be 2 $sec^{-1}/7 \times 10^5$ or 0.01 hr^{-1}. This value is within an order of magnitude of the rate constant which we have obtained experimentally.

Recently, Park (1973) has measured the rate of dissociation of deoxyhemoglobin into dimers using methodology very similar to ours. The rate constants which she obtained are in reasonable agreement with those reported here, allowing for differences in experimental conditions.

Our experiments indicate that the rate at which deoxyhemoglobin dissociates into dimers is decreased by 2,3-diphosphoglycerate. Furthermore, over a pH range of 6–8, the rate is proportional to pH. These results are entirely compatible with the recent structural studies of Perutz (1970). From high-resolution X-ray data, he has concluded that deoxyhemoglobin is stabilized by a series of inter- and intrasubunit salt bonds including the binding of 2,3-diphosphoglycerate at a specific site and the binding of protons to residues involved in the alkaline Bohr effect. Therefore, the removal of protons or 2,3-diphosphoglycerate from hemoglobin would tend to destabilize the deoxy conformation and enhance dissociation of the tetramer into $\alpha\beta$ dimers.

The dissociation of hemoglobin tetramer into dimers is a prerequisite for its binding to haptoglobin (Nagel and Gibson, 1971). Recently Nagel and Gibson (1972) found that phosphate-free deoxyhemoglobin is capable of slow binding to haptoglobin. Binding was almost completely abolished in the presence of inorganic phosphate. These observations fit well with our measurements of the effect of 2,3-diphosphoglycerate on the dissociation of deoxyhemoglobin into dimers.

Acknowledgments

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Involvement of a Tryptophan Residue in the Binding Site of *Escherichia coli* Galactose-Binding Protein†

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ABSTRACT: The galactose-binding protein, a component of the β-methyl galactoside transport system of *Escherichia coli*, undergoes a conformational change upon the binding of substrate. A variety of spectrophotometric techniques were employed in order to probe the nature of this change. All known substrates of the β-methyl galactoside transport system cause an alteration in the ultraviolet absorbance of the galactose-binding protein. The ultraviolet difference spectra produced by these substrates are similar but not identical and resemble solvent perturbation difference spectra of *N*-acetyltryptophan ethyl ester. In addition, solvent perturbation difference spectroscopy reveals that the exposure of external chromophores is not affected by binding of substrate. These results indicate that the substrate interacts directly with a tryptophan residue present in the binding site of the protein, and further, that the active site tryptophan is not accessible to the bulk solvent either in the presence or absence of substrate. However, substrate protects the protein from fluorescence quenching by KI. It is concluded that the alteration in surface charge of the protein which has been shown to accompany the binding of substrate causes this differential quenching of an external tryptophan. A mutant galactose-binding protein which does not exhibit an increase in electrical charge upon substrate binding is not protected from the quenching effects of KI by substrate, even though interaction of substrate with the active-site tryptophan still occurs. Thus, the substrate-dependent conformational change of the galactose-binding protein does not result in extensive refolding of the polypeptide chain, but rather is restricted to small alterations in the active site and an increase in the surface charge of the protein.

The galactose-binding protein from *Escherichia coli* is a periplasmic protein (Anraku, 1968a–c) necessary for the proper functioning of the β-methyl galactoside transport system (Boos, 1969, 1972; Boos and Sarvas, 1970; Lengeler *et al.*, 1971). Although the role of this protein in the transport mechanism is not understood, a conformational change has

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