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ASSIGNMENT OF THE QUATERNARY STRUCTURE OF Fe/Zn HYBRID HEMOGLOBINS: IMPLICATIONS FOR ALLOSTERIC THEORIES

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SUMMARY

The quaternary structures of $\alpha_2Zn\beta_2Fe(III)$, $\alpha_2Zn\beta_2Fe(III)N_3^-$, $\alpha_2Zn\beta Fe(II)CO$, $\alpha_2Fe(III)\beta_2Zn$, $\alpha_2Fe(III)N_3^-\beta_2Zn$ and $\alpha_2Fe(II)CO\beta_2Zn$ have been assigned using U.V. Circular dichroism, subunit dissociation measurements, and ligand binding rates. A "T" structure is obtained for all these Zn/Fe hybrids. These results suggest that a complete R → T quaternary structure change does not occur until the third ligand binds. These observations, combined with previous studies of mixed valence Hb hybrids, place severe constraints on two state models of hemoglobin allostery.

INTRODUCTION

Metal substitution has provided a valuable tool for the investigation of interactions between the porphyrin, metal, ligand, and polypeptide in hemoglobin (1). The preparation of mixed-metal hybrid hemoglobins, wherein the iron atoms of only one pair of subunits are replaced by another metal, holds special promise as it permits the examination of intermediate ligation states which previously have been accessible only via the mixed-valency hybrids (2-5). Of particular interest to us, has been the effect of changes at the metal center on the quaternary or allosteric structure of the hemoglobin tetramer. By selection of the appropriate metals and ligands, one may envision the construction of hemoglobins containing complementary subunits of unlike

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Abbreviations used: Hb, hemoglobin; DMF, N, N-dimethylformamide; IHP, inositol hexaphosphate; PIPES, piperazine-N, N^1-bis[2-ethane sulfonic acid].

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tertiary structure. The characterization of the quaternary structure of such molecules is of obvious interest.

In the present communication, we report the investigation of the quaternary conformational state of the complementary hybrids: $\alpha_2Zn\beta_2Fe$ and $\alpha_2Fe\beta_2Zn$. Although these species have been previously prepared, no studies of their solution (quaternary) structure have appeared. Since previous studies have shown that the five coordinate Zn-porphyrin displays a geometry very similar to that of deoxy-Fe(II)porphyrin (6), and that totally Zn substituted hemoglobin exists in the T state (7,8), these hybrids should be very appropriate models for the diliganded hemoglobin intermediates.

MATERIALS AND METHODS

Human hemoglobin was purchased from Sigma. Isolated subunits were obtained by ion-exchange chromatography (9) following reaction of CO-hemoglobin with p-mercuribenzoate (10). Heme-free subunits were prepared by acid/butanone extraction (11) and combined with the complementary CO-subunit to yield the half-filled intermediates (12). Fe/Zn hybrid hemoglobins were obtained by combining the half-filled intermediates with Zn(II)protoporphyrin IX (prepared by reaction of $ZnCl_2$ with protoporphyrin IX (Sigma) in refluxing DMF (13) and purified by chromatography on a column of Polyamid CC 6.6 (Brinkman Instruments, Inc.) eluted with pyridine). The hybrid hemoglobins were purified by ion-exchange and gel permeation chromatography, and finally by isoelectric focusing in a granulated polyacrylamide bed in the pH range 6-8. Methemoglobin was obtained by ferricyanide oxidation. ZnHb was prepared as previously described (14,15).

Analytical gel filtration experiments were conducted on a 2.5 x 85 cm column of Bio-Gel P-100, 100-200 mesh, eluted with 50mM Tris, 100mM KCl buffer, pH 7.5. Ferritin (Sigma, Type I) and $K_3Fe(CN)_6$ or N-phenylglycine were used as markers. Elution was monitored at 280 nm.

Circular dichroism (CD) spectra were recorded and plotted as previously described (8). Kinetic measurements were made with a Dionex (Durrum) stopped flow system. UV-visible spectra were recorded on a Cary-118 spectrophotometer.

RESULTS AND DISCUSSION

Recent papers (8, 16-18) have established the utility of circular dichroism as a qualitative indicator of hemoglobin quaternary states. T state derivatives typically display mod-

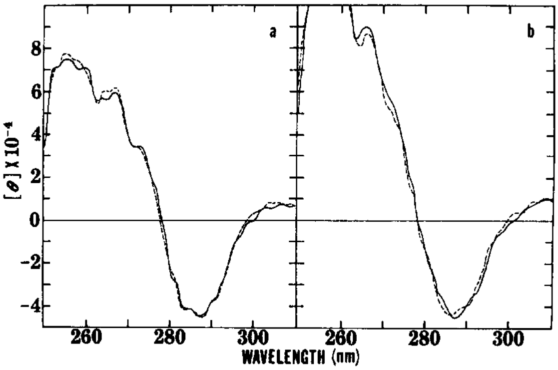

FIGURE 1: UV region CD spectra of ∿ 25 μM (tetramer) solutions of aquomet hybrids in the presence (– – –) and absence (———) of IHP. a,α_2Znβ_2Fe(III)H_2O. b,α_2Fe(III)$H_2O\beta_2$Zn. 50 mM PIPES, 180 mM NaCl, pH 6.5 Molar ellipticities (deg cm^2/decimole) are in terms of tetramers.

erately negative ellipticities in the diagnostic region near 287 nm, while R state hemoglobins show weak, positive ellipticities (17,18). The CD spectra in the ultraviolet region of the FeIII/Zn(II) hybrids are shown in Figs. 1 and 2. The negative ellipticity at 287 nm which appears unaffected by the substitution of N_3^- for water as the sixth ligand in the ferric subunits, is similar to that observed for T state hemoglobins. The addition of a 4-fold molar excess of IHP, known to switch metHb from R to T (17), causes no significant spectral changes.

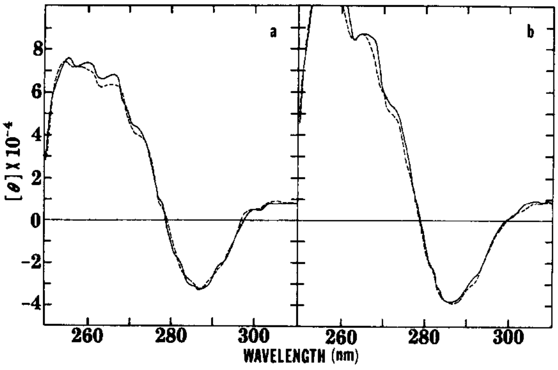

FIGURE 2: UV region CD spectra of ∿ 25 μM (tetramer) solutions of azidomet hybrids in the presence (– – –) and absence (———) of IHP. a,α_2Znβ_2Fe(III)N_3^-. b,α_2Fe(III)$N_3^-\beta_2$Zn. Conditions as in Fig. 1.

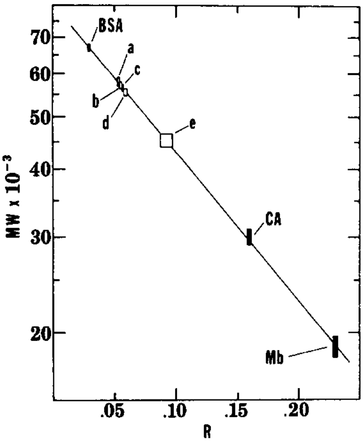

FIGURE 3: Gel filtration plot of hemoglobin derivatives. Solid error bars: standards, BSA (bovine serum zlbumin), CA (carbonic anhydrase), Mb (myoglobin). Outlined error bars: a,$\alpha_2Zn\beta_2FeCO$; b,ZnHb; c, $\alpha_2Zn\beta_2Fe(III)$; d, $\alpha_2Fe(III)\beta_2Zn$; e, met Hb. Conditions as in methods.

Liganded R state hemoglobins dissociate into dimers at moderate salt concentrations and neutral pH. Measured values for $K_{4,2}$, the tetramer-dimer dissociation constant, are on the order of 2 to 10μM (19-22). In contrast, $K_{4,2}$ for T state derivatives such as deoxyHb or IHP-bound metHb ranges from 10^{-9} to 10^{-12} M (20). Thus, at concentrations in the micromolar range, R state hemoglobins should be largely dissociated, while T state hemoglobins should remain essentially tetrameric. These observations suggest that allosteric states may be indirectly, but unambiguously determined by measuring the degree of dissociation. Gel permeation chromatography is well suited for this task, as the apparent molecular weights of hemoglobin derivatives determined by this method directly reflect the tetramer-dimer equilibrium. Fig. 3 presents the data for a series of such experiments. The $\log_{10}$ M.W. of the three standard proteins, bovine serum albumin, carbonic anhydrase, and equine myoglobin, is linearly related to the partition coefficient, R , as expected.

ZnHb, a known T state derivative (7,8) exhibits a hydrodynamic molecular weight of 57,100 ± 700. The R state derivative, metHb, by comparison shows an apparent molecular weight of 45,200 ± 1500, indicative of considerable dissociation. The greater uncertainty in the metHb measurement reflects how slight variations in concentration on the micromolar scale can shift the tetramer-dimer equilibrium of R state derivatives, and result in different average molecular weights. The $\alpha_2Zn\beta_2Fe$ and $\alpha_2Fe\beta_2Zn$ aquomet hybrids display molecular weights of 56,800 and 55,700, respectively, while the $\alpha_2Zn\beta_2FeCO$ hybrid shows a molecular weight of 57,500. The molecular weights of neither the hybrids nor of ZnHb showed any significant variation over a two-fold change in concentration. These results suggest that very small amounts of (dissociable) R state hemoglobin are in equilibrium with the non-dissociating T state Zn hybrids. A minimum value of L = 5 is suggested by the results.

Both the CD and the gel permeation chromatography are structural probes of the allosteric state. As a functional probe, we have examined the kinetics of cyanide binding to the ferric subunits. The results are shown in Table I. Phosphate-stripped

TABLE I

Pseudo First Order Rate Constants for the Reaction of Hemoglobin Derivative with Cyanide

Derivative	Rate Constant (sec^{-1})	
	Phosphate-Stripped	≥ 4 fold excess IHP
met Hb	2.9	1.8
$\alpha_2Fe(III)\beta_2Zn$	1.6	1.7
$\alpha_2Zn\beta_2Fe(III)$	1.3	-

Reaction in 50mM PIPES, 0.14M NaCl, pH 6.6. Hemoglobin concentration 1μM (tetramer). Cyanide concentration 200μM (as CN^-). 20° C.

metHb exhibits a 38% decrease in the CN^- binding rate, upon the addition of IHP. The aquomet Fe/Zn hybrids display CN^- binding rates similar to that of IHP-bound metHb. Also, the $\alpha_2^{Fe}\beta_2^{Zn}$ hybrid shows no decrease in the binding rate upon addition of IHP. At the hemoglobin concentrations used in these experiments (~ 1μM), metHb would be expected to be only about 11% tetrameric. The observed IHP effect is thus primarily due to the assembly of dimers into T state tetramers.

The results of the kinetic experiments are in good agreement with the CD and gel permeation based assignment of a T state structure to the Fe/Zn hybrids. Using the Zn hybrids as a model for diliganded hemoglobins, the present results strongly support the suggestion of Hoffman and Gibson (23), that hemoglobin remains in a "T" state when two ligands are added. The switch to R would occur after the binding of the third ligand. Such a switch is indeed predicted by the MWC model (24,25), for $L_0 = T/R = 10^5$ and $c = {}^{K}T/K_R = 0.01$. In this case, $L_2 = L_0c^2 = 10$, while $L_3 = 0.1$. A more surprising result of the present study is that while $Zn_2Fe^+_2$ is assigned a T structure, the highly analagous $Fe(II)_2Fe(III)_2$ mixed valency hybrid previously was assigned an R structure, based on both functional and NMR criteria (26,27). Moreover, Zn(II) and high-spin Fe(II) porphyrins show virtually identical stereochemistry and coordination chemistry (6,28). This apparent contradiction requires either that the structurally homologous Zn(II) and Fe(II) porphyrins dictate different quaternary structures via an unknown mechanism, or that the previous assignment of an R state to the mixed valency hybrids is misleading. Preliminary studies (29) of the mixed valency hybrids, by CD, gel permeation, and NMR, indeed show some T state characteristics. This discrepancy in structure assignment by different techniques cannot be reconciled with a pure two state model. Thus, it appears that structural studies of mixed metal hybrids may offer a powerful tool for testing current theories of hemoglobin action. Such studies are continuing in our laboratory (29), and elsewhere (23).

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