Modification of Isolated α and β Chains of Human Hemoglobin by the Metal Tetrasulfonated Phthalocyanines. Studies on Hybrid Phthalocyanine–Heme Intermediates*

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Abstract

α and β chains of hemoglobin have been modified with cobalt(II) tetrasulfonated phthalocyanine in place of heme. They display properties very similar to those of iron(II) phthalocyanine modified α and β chains. Mixed together they form tetrameric cobalt-(II) phthalocyanine hemoglobin.

Incorporation of Co(II)L into α and β globins results in stabilization of the protein structure, which is shown by a marked increase in its helicity content. Cobalt phthalocyanine substituted α and β chains are able to combine reversibly with oxygen giving more stable oxygenated species than their native analogues. The rate of both processes is lower in the case of the modified α chain. Recombination of the phthalocyanine α and β chains with the alternate heme containing chains gives tetrameric hybrid hemoglobins. These comprise two phthalocyanine modified subunits and two heme containing subunits. The helicity content of the tetrameric hybrid hemoglobin calculated for one subunit is lower than the arithmetic mean of helicities for its isolated subunits. This suggests a destabilizing chain–chain interaction within the tetramer. Unlike in the separated subunits, oxygen binding by hybrid hemoglobins is irreversible. Deoxygenation by argon bubbling leads to the formation of inactive species which in oxygen atmosphere undergo irreversible oxidation with destruction of the complex.

Introduction

Recently, the modification of separated α and β chains of hemoglobin has been performed by iron tetrasulfonated phthalocyanine incorporation in place of heme [1]. We have shown that such modification results in a change in quaternary structure of the protein transforming α globin from dimer to monomer and β globin from dimer to tetramer. Combination of α and β globins with iron phthalocyanine derivative increases helicity in both proteins. Interaction of Fe(III)L α globin with Fe(III)L β globin leads to a tetrameric phthalocyanine hemoglobin [Fe(III)L α globin–Fe(III)L β globin]$_2$, with a stability higher than that of its isolated artificial α and β subunits. It was also found that native α and β subunits give with their phthalocyanine modified analogues hybrid phthalocyanine–heme hemoglobins.

In continuation of these studies we now report the results of modification of α and β chains by cobalt(II) tetrasulfonated phthalocyanine, as well as results of studies of the structure and properties of the artificial phthalocyanine–heme intermediates.

Experimental

Materials

α and β subunits, as well as α and β globins were prepared according to the method of Geraci *et al.* [2]. Iron and cobalt tetrasulfonated phthalocyanines were prepared and purified as described previously [3]. Stock solutions were obtained by dissolving appropriate amounts of solid in 100 ml of water.

Protein concentrations were determined spectrophotometrically on a Specord spectrophotometer. Molar absorptivities for α and β globins determined by dry weight at 280 nm were 10^4 and 1.5×10^4 M^{-1} cm^{-1}, respectively. The average molar absorptivity for heme containing α and β chains at 578 nm was 1.53×10^4 M^{-1} cm^{-1}.

The synthesis of metal substituted α and β chains has already been described [1]. The reduced form of the complexes was prepared in argon atmosphere, adding a few milligrams of sodium dithionite to the solution and removing the excess reductant on a

*Abbreviations: L = tetrasulfonated phthalocyanine ligand ($C_{32}H_{12}N_8(SO_3Na)_4$) Co(II)L, Fe(III)L = cobalt(II) and iron(III) tetrasulfonated phthalocyanines. Co(II)Lαglobin, Co(II)Lβglobin, Fe(III)Lαglobin, Fe(III)Lβglobin: complexes of cobalt(II) and iron(III) tetrasulfonated phthalocyanines with α and β globins. αHb and βHb = α and β chains of hemoglobin.

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TABLE I. Circular Dichroism Parameters of α and β Chains and Their Phthalocyanine Derivatives at 222 and 208 nm.

	$[\theta]_{222} \times 10^{-6}$ deg cm^2 dmol^{-1}	$[\theta]_{208} \times 10^{-6}$ deg cm^2 dmol^{-1}	$[\theta_m]_{222}$[a] $\times 10^{-6}$ deg cm^2 dmol^{-1}
α globin	−1.4	−1.6	
β globin	−2.0	−1.8	
α hemoglobin	−3.2	−3.0	
β hemoglobin	−3.9	−3.2	
Co(II)Lαglobin	−2.5	−2.4	
Co(II)Lβ globin	−3.0	−2.8	
Fe(III)Lαglobin	−2.26	−2.4	
[Co(II)Lβglobin−αHb]$_2$	−2.6	−2.1	−3.1
[Fe(III)Lαglobin−βHb]$_2$	−2.7	−2.5	−3.08

[a] $[\theta_m]$ = mean ellipticity values of the hybrid complexes calculated from the ellipticities of the isolated subunits at 222 nm.

Sephadex G50 column. For the examination of the oxygenation process, the modified chains were reduced in a syringe using a minimal amount of dithionite under spectroscopic control. Concentrations of the cobalt phthalocyanine modified α and β chains were determined from the molar absorption coefficient for one monomeric subunit ($\epsilon_{695} = 2.2 \times 10^4$ M^{-1} cm^{-1}).

Hybrid heme–phthalocyanine hemoglobins were obtained by incubation of mixtures of heme containing α or β chains with its phthalocyanine analogue at a molar ratio of 1:1, at 4 °C for three days. The hybrid complexes were separated from reaction mixtures chromatographically on a Sephadex G75 column and identified by a spectroscopic method. The molar absorption coefficients of the cobalt phthalocyanine–heme hemoglobins at 695 and 418 nm are 2.2×10^4 and 12.8×10^4, respectively.

Absorption Spectroscopy

Absorption and difference spectra were recorded on a Cary 15 recording spectrometer with a cell compartment thermostatically controlled at 15 °C or on a Specord recording spectrophotometer.

Molecular Weight Estimation

Approximate molecular weights of the model complexes were determined by gel filtration on a Sephadex G75 column according to the method of Andrews [4]. The results are shown by plot of the ratio V_e/V_o against molecular weights of known proteins and model phthalocyanine complexes (V_e = protein elution volume, V_o = column void volume). The following proteins were used as reference substances: cytochrome c (MW 12400), myoglobin (MW 17800), chymotrypsin (MW 25000), ovalbumin (MW 45000), and serum albumin (MW 67000). Column size (2 × 50 cm) was equilibrated with 0.02 M potassium phosphate buffer, pH 7.

Oxygen Dissociation Experiments

Removal of oxygen from the oxygenated α and β chains, their cobalt phthalocyanine analogues and cobalt phthalocyanine–heme hemoglobins, in oxygen dissociation experiments, was performed by argon bubbling through the solutions, at 4 °C in 0.1 M phosphate buffer, pH 7. The rate of argon flow was approximately constant. Complex concentration in all solutions was 13.5×10^{-6} M. Reactions were followed at 695 and 418 nm. No denaturation was observed under experimental conditions.

Circular Dichroism Measurements

Circular dichroism spectra were recorded using a model ORD/UV-5 Japan Spectropolarimeter with CD attachment. Solutions were prepared by dissolving appropriate amounts of lyophilized preparation in Tris-HCl buffer pH 7 or 0.02 M phosphate buffer. Ellipticities of the model complexes are given per mol of bound metal tetrasulfonated phthalocyanine (or heme). All ellipticity values are collected in Table I.

Results

Interaction of Cobalt Tetrasulfonated Phthalocyanine (Co(II)L) with Separated α and β Chains and Their Apoproteins

The interaction of Co(II)L with heme-free and heme-containing α and β subunits of human hemoglobin was followed using the difference spectroscopy method. Difference spectra of the Co(II)L mixtures with the β chain and its apoprotein are presented in Fig. 1. Identical difference spectra were obtained for the system with the α chain and its apoprotein. The results indicate the formation of a complex of Co(II)L with α and β globins containing a phthalocyanine derivative in place of heme. The characteristic absorption band of these complexes appears at 695 nm.

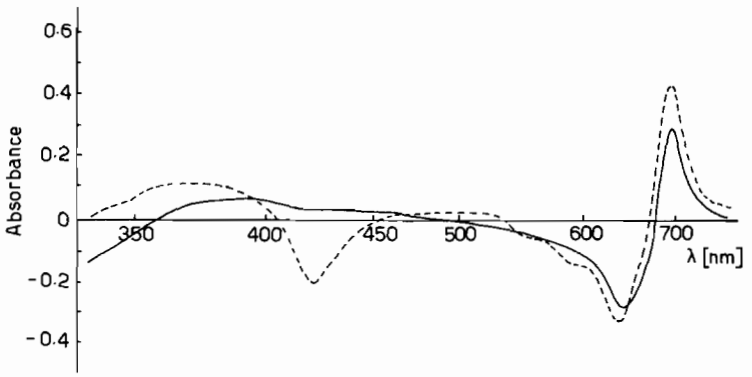

Fig. 1. Difference spectra of mixtures of Co(II)L + βHb and Co(II)L + βglobin against the same solutions unmixed, in phosphate buffer pH 7.5.

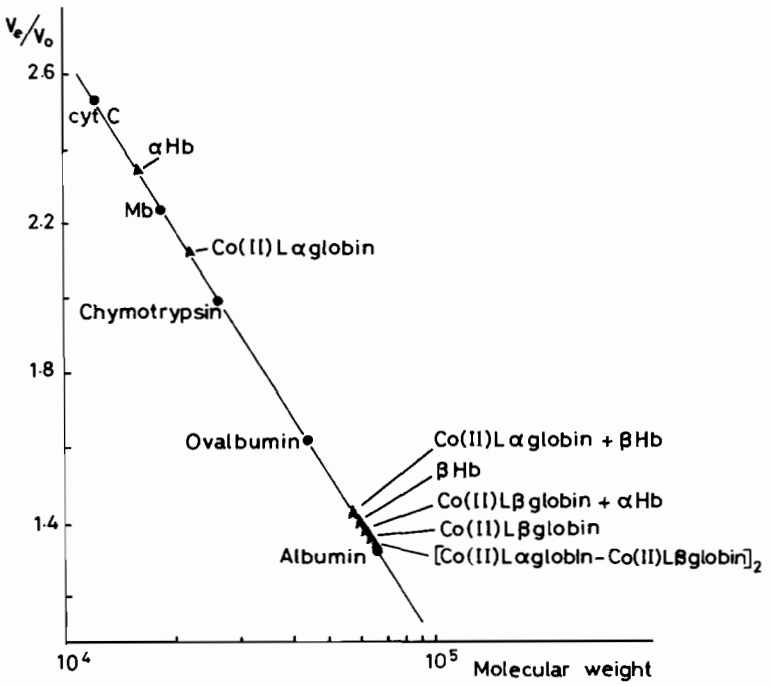

Fig. 2. Gel filtration molecular weight estimations of cobalt phthalocyanine substituted α and β chains, reconstituted phthalocyanine hemoglobin and hybrid hemoglobins, compared with native α and β chains. V_e/V_o represents the ratio of protein elution volume to column void volume. Column size = 2 × 50 cm equilibrated with 0.02 M potassium phosphate, pH 7.6.

Properties of cobalt phthalocyanine modified α and β chains are very similar to those of iron(II) phthalocyanine substituted α and β subunits. They are capable of combining reversibly with molecular oxygen as indicated by a marked increase in their main absorption band. The rate of oxygen binding by both model complexes is much lower than that for the native α and β chains. Iron phthalocyanine substituted α and β subunits behave similarly [1]. Likewise, dissociation of oxygen is more difficult in the case of the model complexes (Fig. 6 A,B).

Mixing equimolar solutions of Co(II)Lαglobin and Co(II)Lβglobin leads to the formation of a tetrameric artificial hemoglobin [Co(II)Lαglobin–Co(II)-Lβglobin]$_2$. The rate of oxygen binding by this compound is the average of that of the modified α and β chains.

Results of molecular weight determination of the cobalt phthalocyanine substituted α and β chains by gel filtration suggest that the modified α chain is a monomer (MW 24000) and that the modified β chain is a tetramer (MW 66000), like their phthalocyanine containing analogues (Fig. 2).

Circular Dichroism Spectra

Far ultraviolet CD spectra of the cobalt phthalocyanine substituted α and β chains in comparison with α and β globins are shown in Fig. 3. The results

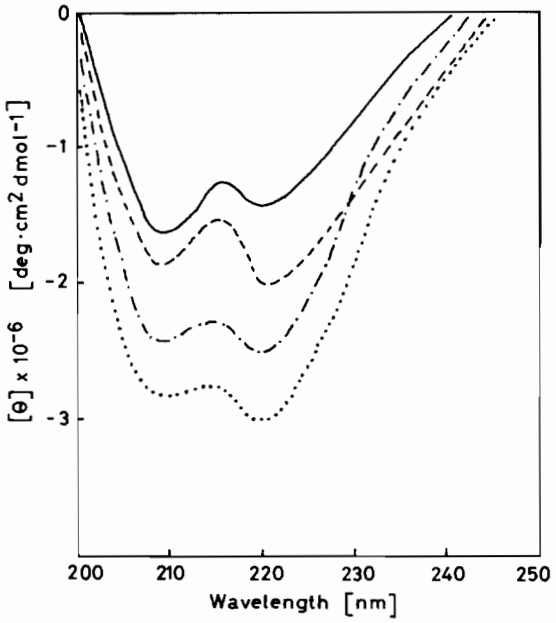

Fig. 3. CD spectra of Co(II)Lαglobin (———) and Co(II)Lβ-globin (·····) compared with αglobin (-------) and βglobin (–·–·–).

demonstrate an increase in helix content of the protein due to Co(II)L incorporation. Ellipticity values are close to those of the iron phthalocyanine substituted α and β subunits, respectively (Table I) [1].

Interaction of the Cobalt and Iron Phthalocyanine Modified α and β Subunits with Alternate Heme-containing Subunits

Cobalt phthalocyanine modified α and β subunits, like their iron analogues, when mixed with alternate heme-containing subunits give hybrid phthalocyanine–heme hemoglobins. The absorption spectrum of such hybrid hemoglobin isolated from the reaction mixture is presented in Fig. 4. This spectrum exhibits the bands characteristic for heme-containing subunits at 418, 540 and 578 nm, as well as the main absorption band of its cobalt phthalocyanine containing analogues at 695 nm. The approximate molar ratio for these two kinds of subunits, calculated from the molar absorption coefficients of their main absorption bands, is about 1:1.

Results of molecular weight determinations show that the molecular weights of both hybrid complexes are about 62000, indicating a tetrameric form. Data from spectrophotometric investigations and molecular weight estimations suggest that the hybrid complexes involve two metal phthalocyanine modified subunits and two alternate heme containing ones.

Reconstitution of the artificial phthalocyanine–heme hemoglobins leads to conformational changes of the proteins, as observed in their CD spectra.

The far ultraviolet CD spectra of $[Fe(III)L\alpha\text{-globin}-\beta Hb]_2$ in comparison with Fe(III)αglobin and βhemoglobin, are shown in Fig. 5A. The mean ellipticity value (θ_m) of the hybrid complex, calculated from the ellipticities of the isolated subunits at 222 nm, differs from the experimental one by about 10% (Table I).

In Fig. 5B the far ultraviolet CD spectra of $[Co(II)L\beta\text{globin}-\alpha Hb]_2$ are shown in comparison with Co(II)Lβglobin and the native α subunit. The mean ellipticity value of the hybrid complex at 222 nm is higher than that found experimentally by about 14%.

The formation of the phthalocyanine–heme hemoglobins also causes changes in the functional properties of the isolated α and β subunits, as well as in their phthalocyanine analogues. Oxygen binding by the reduced form of the iron phthalocyanine–heme hemoglobin or cobalt(II) phthalocyanine–heme hemoglobin, is irreversible. Deoxygenation by argon bubbling through the solution leads to the formation of inactive species which in oxygen atmosphere undergo irreversible oxidation with destruction of the prosthetic group and denaturation of the protein. In Fig. 6A are shown the results of the oxygen dissociation process of the oxygenated α subunit, Co(II)Lβglobin, and the hybrid hemoglobin

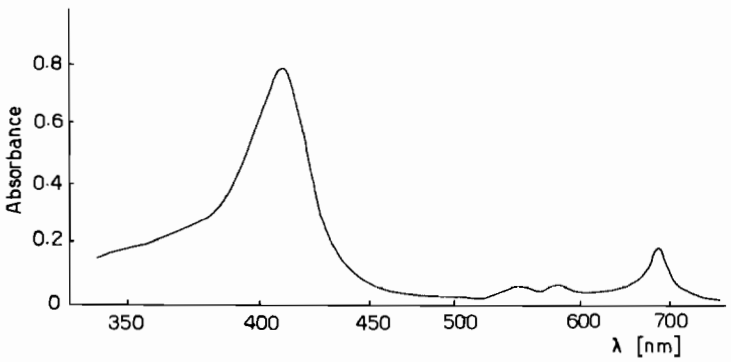

Fig. 4. Absorption spectrum of oxygenated hybrid hemoglobin $[Co(II)L(O_2)\alpha\text{globin}-(O_2)\beta Hb]_2$ in 0.1 M phosphate buffer pH 7.5.

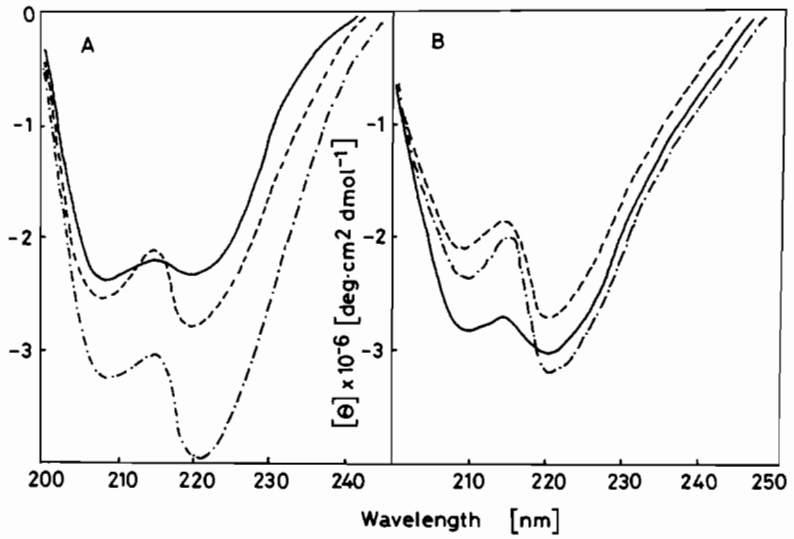

Fig. 5. CD spectra of iron and cobalt phthalocyanine–heme hemoglobin compared with native α and β subunits and their iron phthalocyanine modified analogues. A: [Fe(III)Lαglobin–βHb]$_2$ (-------), Fe(III)αglobin (———) and βhemoglobin (–·–·–); B: [Co(II)Lβglobin–αHb]$_2$ (-------), Co(II)Lβglobin (———), αhemoglobin (–·–·–).

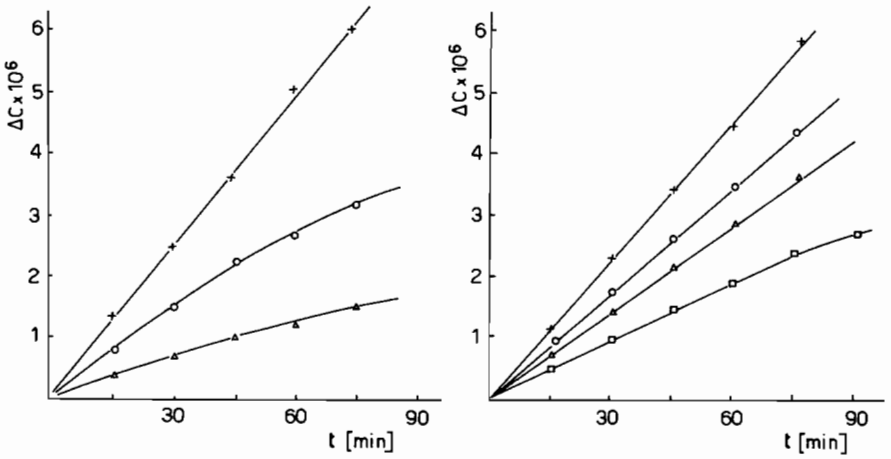

Fig. 6. Oxygen dissociation process of the oxygenated native α and β chains, their cobalt phthalocyanine analogues and cobalt(II)phthalocyanine–heme hemoglobins. A: The reaction run in buffered solutions of αhemoglobin (×), Co(II)Lβglobin (○), and [Co(II)Lβglobin–αHb]$_2$(△), observed at 695 nm. B: The reaction run in buffered solutions of β hemoglobin (×), Co(II)Lαglobin (○), and [Co(II)Lαglobin–βHb]$_2$, observed at 695 nm (△) and 418 nm (□). Temp. 4 °C, pH 7.0, concentrations of all complexes = 13.5 × 10^{-6} M.

[Co(II)Lβglobin–αHb]$_2$, observed at 695 nm. In Fig. 6B are shown the results of the same process for the oxygenated β subunit, Co(II)Lαglobin, and hybrid hemoglobin [Co(II)Lαglobin–βHb]$_2$, observed at 418 and 695 nm. It is evident from the experimental data that the rate of oxygen dissociation is lower for the hybrid than for separated α and β chains or their phthalocyanine models. On the other hand, it is higher for hybrids with a β chain than with a α one. Following the reaction course at the main absorptivities for heme and phthalocyanine containing subunits in the hybrid hemoglobin indicates a higher oxygen dissociation rate in the case of the phthalocyanine containing chains. The reverse picture is observed for the separated subunits of the same hybrid.

Conclusions

Continuing our studies on the modification of separated α and β subunits of hemoglobin by heme substitution with metal phthalocyanine derivatives, we have obtained artificial α and β chains with cobalt tetrasulfonated phthalocyanine in place of heme. They have been identified as Co(II)Lαglobin and [Co(II)Lβglobin]$_4$. Both complexes exhibit char-

acteristic absorptivity at 695 nm. They display properties very similar to those of iron(II) tetrasulfonated phthalocyanine modified α and β chains. Mixed together they form a tetrameric cobalt phthalocyanine hemoglobin, [Co(II)Lαglobin–Co(II)Lβglobin]$_2$.

Incorporation of Co(II)L into α and β globins results in stabilization of their structure as shown by a significant increase in helicity in both proteins. Reconstituted cobalt(II) phthalocyanine hemoglobin shows higher stability than its separated subunits.

Cobalt(II) phthalocyanine modified α and β chains are able to combine reversibly with molecular oxygen giving oxygenated intermediates. The rate of oxygen binding as well as oxygen dissociation is much lower for the model compounds than for the native α and β chains. Moreover, the rate of both processes is lower for the modified β chain.

Mixing iron or cobalt phthalocyanine modified α and β subunits with alternate native heme chains leads to the formation of hybrid phthalocyanine–heme hemoglobins. Absorption spectra of these complexes exhibit absorption bands characteristic for both kinds of subunits, *i.e.* heme and phthalocyanine containing ones. Spectroscopic data as well as results of molecular weight determination suggest that the mixed complexes are tetramers and comprise two metal phthalocyanine modified subunits and two alternate heme containing subunits.

Recombination of the phthalocyanine–heme hemoglobins leads to conformational changes of the protein as shown by the CD spectra. The experimental average ellipticity value of the hybrids at 222 nm calculated for one subunit is lower by about 12% than the arithmetic mean of the values determined for separated heme and phthalocyanine containing subunits of these hybrids. This fact suggests some destabilization of the helical structure due to the chain–chain interaction.

Phthalocyanine–heme hemoglobins are unstable compared to their unmixed analogues. When reduced with dithionite, they combine with molecular oxygen to give more stable oxygenated species. However, contrary to the case in their separated subunits, oxygen binding by hybrid hemoglobins is irreversible. Deoxygenation by argon bubbling leads to the formation of inactive species which in an oxygen atmosphere undergo irreversible oxidation and finally denaturation. According to theory, the formation of the stable oxygen adduct by a reduced iron porphyrin is possible only when the oxygen molecule lies with its axis parallel to the porphyrin plane, but it transfers an electron to an oxygen molecule when its axis is perpendicular to the porphyrin plane [5]. Probably, in the case of phthalocyanine–heme hemoglobins the oxygen dissociation causes a change of the protein crevice shape in such a way as to prevent the oxygen molecule from being parallel to the phthalocyanine ring.

The observation of the oxygen dissociation reaction for the phthalocyanine and heme containing subunits within tetrameric hybrid hemoglobin indicates a higher rate of this process for the phthalocyanine modified chain than for the native heme containing ones. The rate of the same process for the isolated subunits of this hybrid is higher for the heme containing chains. This fact confirms a chain–chain interaction in hybrid hemoglobins.

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