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Complexes of Iron Tetrasulfonated Phthalocyanine with Separated Human α and β-Globins*

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The modification of separated α and β-chains of hemoglobin has been performed by iron tetrasulfonated phthalocyanine incorporation in place of heme. New model compounds have been isolated and their structure and properties have been investigated by difference spectroscopy, molecular weight estimation and circular dichroism measurements.

Visible absorption spectra of iron(III) model complexes show the main absorption band at 650 nm. Incorporation of Fe(III)L into α and β-globins results in a change in their quarternary structure shown by molecular weight estimation. α-globin is transformed from dimer to monomer and β-globin from dimer to tetramer. Interaction of Fe(III)Lαglobin with Fe(III)Lβglobin leads to the formation of tetrameric phthalocyanine hemoglobin [Fe(III)Lαglobin–Fe(III)Lβglobin]$_2$. Combination of α and β-globins with iron phthalocyanine derivative increases helicity in both proteins. Reconstituted artificial hemoglobin shows a CD spectrum quite different from the sum of the individual spectra of its subunits which points to its more ordered or differently ordered structure. This fact suggests chain-chain stabilizing interactions between subunits within the tetramer. Like the native α and β chains their phthalocyanine analogues are nonequivalent in their properties. The rate of reduction by ascorbic acid and oxygenation are markedly higher in the case of phthalocyanine β-chain. Reconstituted phthalocyanine hemoglobin shows a higher stability than its subunits. Hydrid phthalocyanine-heme hemoglobins have been prepared from modified α and β chains and native α and β subunits. Investigations of these compounds are in progress.

Introduction

The preparation and study of the properties of hemoglobins containing prosthetic groups other than protoheme are of considerable interest in helping to define the structure/function relation in these compounds. Many investigations have been carried out in this field [1–5]. Earlier we showed that substitution of heme in hemoglobin by metal tetrasulfonated phthalocyanines gives complexes whose properties resemble those of the native species [6–8].

Advances in understanding of the physiological action of hemoglobin have arisen from studies of isolated hemoglobin and its subunits. Results have conclusively demonstrated that α and β chains are structurally non-equivalent and this is the cause of their functional differences. Significant differences observed in the ligand binding properties of α and β chains results from non-equivalence in the heme environment [9–12]. It has been shown that heme-free α and β globins differ in molecular weight, secondary structure and sulfhydryl reactivity from the heme-containing separated sub-units they derive from [13].

In this paper we report the results concerning the preparation, structure and properties of the metal tetrasulfonated phthalocyanine substituted α and β chains of hemoglobin as well as their interactions with heme-containing subunits.

Experimental

Materials

Human hemoglobin was prepared by the Rossi-Fanelli and Antonini method [14]. Globin was obtained using the method of Rossi-Fanelli *et al.* [15]. The preparations of α and β subunits as well as α and β globins were carried out as described previously [16, 17]. The preparation and purification of iron tetrasulfonated phthalocyanine were described earlier [18]. The stock solutions were

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

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obtained by weighing appropriate amounts of solid and dissolving the latter in 100 ml of water.

The protein concentrations were determined spectrophotometrically on a Specord spectrophotometer. The molar absorptivities for α and β globins determined by dry weight at 280 nm were 10×10^3 and 15×10^3, respectively.

The synthesis of the iron phthalocyanine substituted α and β chains involved the incubation of the appropriate globin with double excess of the iron tetrasulfonated phthalocyanine in 0.1 *M* phosphate buffer pH 7.5 for three days at 4 °C. The reaction mixture was separated by gel filtration on a Sephadex G50. The fractions were identified by absorption spectroscopy at 280 and 650 nm. The reduced form of the complexes was prepared by addition of a few milligrams of sodium dithionite to the solution and removal of the excess of the reductant on a Sephadex G50 column, in argon atmosphere. The concentrations of the complexes were determined from the molar absorption coefficients calculated for one monomeric subunit ($\epsilon_{650} = 3.7 \times 10^4\ M^{-1}\ cm^{-1}$, $\epsilon_{678} = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$, respectively, for the oxidized and reduced forms). The reduction of the modified chains to the ferrous form for the examination of the oxygenation process was carried out in a syringe with a minimal amount of dithionite under spectroscopic control. The cyanmet form of the artificial α and β chains was prepared by addition of 1.5 molar excess of KCN and potassium ferricyanide to their buffered solutions and separation of the cyanide complex on a Sephadex G25 column equilibrated with 0.1 *M* phosphate buffer pH 7.5.

The hybrid heme–phthalocyanine hemoglobins were obtained by incubation of mixtures of the heme-containing α and β chain with its phthalocyanine analogue, in equal proportions at 4 °C for three days. The hybrid complex was separated from the reaction mixture chromatographically and identified by a spectroscopic method.

Methods

Absorption Spectroscopy

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

Molecular Weight Estimations

The approximate molecular weights of the model complexes were determined by gel filtration on a Sephadex G75 column according to the method of Andrews [19]. 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).

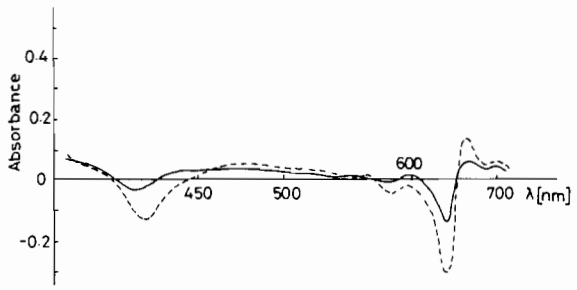

Fig. 1. Difference spectra of the mixtures of Fe(III)L + αHb (——) and Fe(III)L + βHb (- - - -), against the same solutions unmixed, in phosphate buffer pH 7.5.

Circular Dichroism Measurements (CD)

The circular dichroism spectra were recorded using a model ORD/UV-5 Japan Spectropolarimeter with CD attachment. The solutions were prepared by dissolving the appropriate amounts of lyophilized preparation in Tris-HCl buffer pH 7 or 0.02 *M* phosphate buffer.

Results

Interaction of Iron Tetrasulfonated Phthalocyanine [Fe(III)L] with Separated α and β Chains and their Apoproteins

The interaction of Fe(III)L with heme-free and heme-containing α and β subunits of human hemoglobin was followed using the difference spectroscopy method. The difference spectra of the Fe(III)L mixtures with heme-containing α and β chains are presented in Fig. 1. The intensity decrease of the Soret band suggests that heme is removed from the protein crevice. In the visible region both spectra exhibit a negative peak at 635 nm and a positive one at 650 nm. The position of the negative peak corresponds to the main absorption band of Fe(III)L. The appearance of the positive band is indicative of the reaction between Fe(III)L and the protein. The same changes are observed in the visible part of the difference spectra of Fe(III)L mixtures with α and β globins. These results point to the formation of the complex of Fe(III)L with α and β globins with a phthalocyanine derivative in place of heme. Stoichiometric titration of the α chain with Fe(III)L indicates the formation of this complex at a molar ratio of the reactants of 1:1.

As is the case for the reconstituted α and β subunits from heme and the appropriate globins, the iron phthalocyanine modified α chain is less stable than the modified β chain.

Both artificial α and β chains are reduced with dithionite and ascorbic acid as demonstrated by characteristic changes in their absorption spectra which

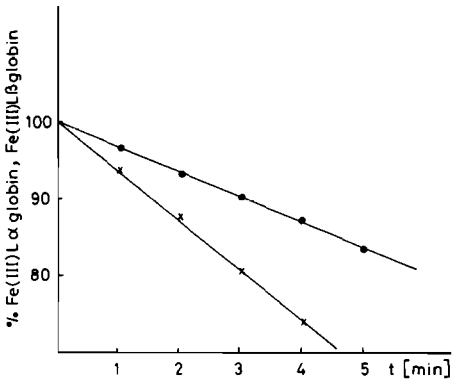

Fig. 2. Reduction of Fe(III)Lαglobin (•) and Fe(III)Lβglobin (×) by ascorbic acid, in 0.1 *M* phosphate buffer, pH 7.5. Concentrations of both complexes and ascorbic acid = 3×10^{-6} *M*. Temp. = 15 °C.

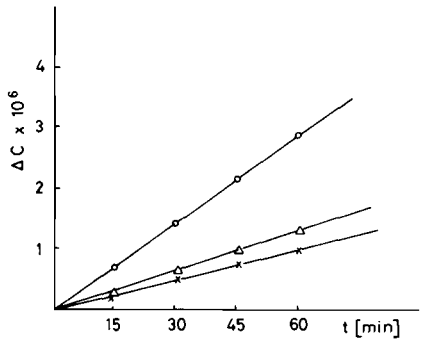

Fig. 3. Oxygenation of Fe(II)Lαglobin (×), Fe(II)Lβglobin (○) and [Fe(II)Lαglobin–Fe(II)Lβglobin]$_2$ (△), in air atmosphere. Concentrations of all complexes = 8×10^{-6} *M*.

are identical with those of the iron phthalocyanine modified hemoglobins [6]. In Fig. 2 the results of the reduction of the iron phthalocyanine substituted α and β chains are presented. In order to prevent the oxidation process the complexes examined were reduced in their much more stable cyanomet form. The percent reduction is calculated from the expression: $(\Delta E \times 100)/(E_o - E_r)$ at 650 nm, where E_o is the absorbance of a completely oxidized complex and E_r is the absorbance of a completely reduced form. It is shown that the rate of the reduction is markedly greater in the artificial β chain.

Like the native α and β chains their phthalocyanine analogs, when reduced, are capable of reversible combination with molecular oxygen. This is shown by a marked increase in their main absorption bands. In this case, however, the oxygenation process is much slower than for the native α and β chains. Likewise, the removal of oxygen from the oxygenated species is more difficult. In Fig. 3 are shown the results of the oxygenation process of

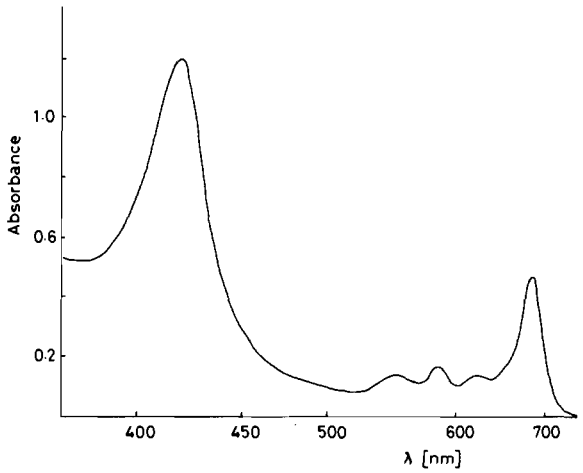

Fig. 4. Absorption spectrum of oxygenated hybrid hemoglobin [Fe(II)L(O_2)αglobin–(O_2)βHb]$_2$ in 0.1 *M* phosphate buffer, pH 7.5.

Fe(II)Lαglobin and Fe(II)Lβglobin in air atmosphere at 15 °C. The results indicate that the rate of oxygenation is higher for the modified β chain.

When the iron phthalocyanine derivative is added to the equimolar mixture of α and β globins at 4 °C, the tetrameric species [Fe(III)Lαglobin–Fe(III)Lβ-globin]$_2$ is formed. Dimeric α and β globins alone do not combine in these conditions [13]. An identical species is formed in an equimolar mixture of Fe(III)Lαglobin and Fe(III)Lβglobin. The rate of oxygen binding by a mixed complex is up to 50% of the rate for the Fe(III)Lβglobin.

Mixing of the phthalocyanine modified α and β subunits with the alternate heme-containing subunits leads to the formation of hybrid hemoglobins. The hybrid complexes were reduced with dithionite and isolated from the reaction mixtures by chromatography on a Sephadex G75 column. The absorption spectra of both hybrid compounds in the oxygenated form are presented in Fig. 4. In the Soret region they exhibit the band which is characteristic for heme-containing subunits. In the visible portion of the spectrum one can identify the band belonging to their phthalocyanine containing analogues. The approximate molar ratio for these two kinds of subunits, calculated from the molar absorption coefficients of their main bands ($\epsilon_{418} = 12.8 \times 10^4$, $\epsilon_{678} = 6.2 \times 10^4$) is about 1:1.

Molecular Weight Estimation

In Fig. 5 are presented the results of the gel filtration molecular weight determination of the iron phthalocyanine substituted α and β chains, the reconstituted phthalocyanine hemoglobins as well as hybrid heme–phthalocyanine hemoglobins

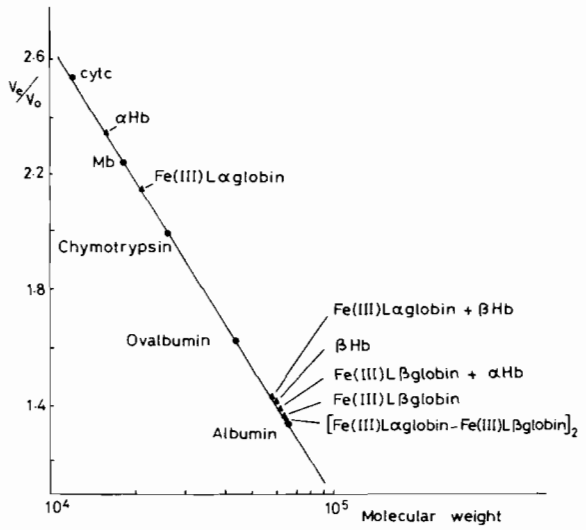

Fig. 5. Gel filtration molecular weight estimations of iron phthalocyanine substituted α and β chains, reconstituted phthalocyanine hemoglobin and hybrid hemoglobins, compared with native α and β chains. V_e/V_o represents the ratio of protein elution volume (V_e) to column void volume (V_o). Column size = 2 × 50 cm equilibrated with 0.02 *M* potassium phosphate, pH 7.6.

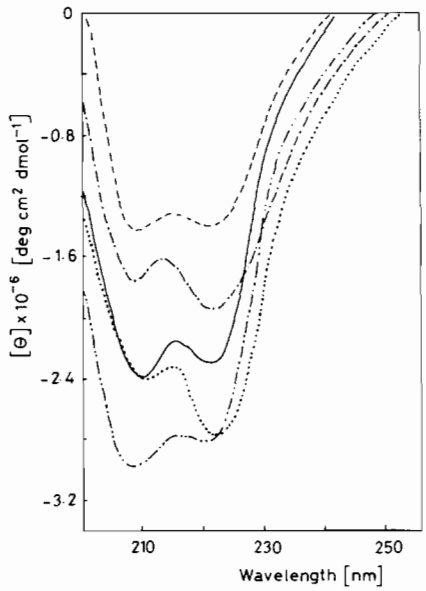

Fig. 6. CD spectra of Fe(III)Lαglobin (———), Fe(III)Lβglobin (–·–·–·–) and [Fe(III)Lαglobin–Fe(III)Lβglobin]$_2$ (·····) compared with α globin (------) and β globin (–·–·–).

in comparison with the native α and β chains. The column was calibrated with cytochrome c, myoglobin, chymotrypsin ovalbumin and serum albumin, as molecular weight standards. The plots of the ratio of elution volume to exclusion volume V_e/V_o, *versus* molecular weight shows that the molecular weight of Fe(III)Lαglobin is 21500, of Fe(III)Lβglobin 64500 and that of [Fe(III)Lαglobin–Fe(III)Lβglobin]$_2$ 63500. These results suggest that the artificial α chain is a monomer and the two other complexes are tetramers, like their native, heme-containing analogues. The molecular weight of the hybrid complexes is about 61000 which points to their tetrameric form.

Circular Dichroism Spectra (CD)

The far ultraviolet CD spectra of Fe(III)Lαglobin, Fe(III)Lβglobin and [Fe(III)Lαglobin–Fe(III)Lβglobin]$_2$ in comparison with α and β globins are shown in Fig. 6. The ellipticity values in all CD experiments are expressed in degrees cm^2 per dmole. The ellipticity value for Fe(III)Lαglobin at 222 nm is -2.3×10^6, whereas that of α globin is -1.3×10^6. This demonstrates an increase in helix content of about 76% upon combination of α globin with Fe(III)L. The ellipticity for Fe(III)Lβglobin at 222 nm is -2.8×10^6. It can be seen that this is about 47% more negative than that of β globin (-1.9×10^6). The ellipticity values at 208 nm for Fe(III)Lαglobin and Fe(III)Lβglobin are -2.4×10^6 and -2.95×10^6, respectively, while those for α and β globins are -1.4×10^6 and -1.75×10^6, respectively. The reconstituted phthalocyanine hemoglobin–[Fe(III)Lαglobin–Fe(III)Lβglobin]$_2$ shows a CD spectrum with ellipticity -2.75×10^6 at 222 nm and -2.4×10^6 at 208 nm. A higher content of the β-structure compared with the α-structure in the model α and β chains points to the marked conformational changes of the proteins due to the substitution of the heme moiety by the phthalocyanine derivative.

Conclusions

In previous papers [6, 7, 20] we have shown that it is possible to obtain artificial hemoglobins with metal tetrasulfonated phthalocyanines in place of heme.

The results just presented demonstrate that the combination of apoproteins of isolated α and β subunits with iron tetrasulfonated phthalocyanine leads to the formation of close analogues of the native α and β chains, Fe(III)Lαglobin and Fe(III)-Lβglobin. Both ferric complexes exhibit a characteristic absorption band at 650 nm and ferrous ones at 678 nm.

Incorporation of the iron tetrasulfonated phthalocyanine into α globin results in a decrease in its molecular weight to the value corresponding to that of the monomer (about 21500). A free α globin in the same conditions occurs as a dimer. Combination of the iron tetrasulfonated phthalocyanine with β

globin brings about an increase in molecular size from dimeric to tetrameric (64500). The same is observed in the reaction of heme with α and β globins.

Interaction of Fe(III)Lαglobin with Fe(III)Lβ-globin leads to the formation of the tetrameric compound [Fe(III)Lαglobin–Fe(III)Lβglobin]$_2$.

Combination of α and β globins with Fe(III)L causes a significant increase in helicity in both proteins, as shown by UV CD spectra. In contrast to heme-containing α and β chains their phthalocyanine analogues differ in helicity content, that of Fe(III)L-βglobin being higher. Reconstituted artificial hemoglobin [Fe(III)Lαglobin–Fe(III)Lβglobin]$_2$ shows a far UV CD spectrum different from the sum of the individual CD spectra for its subunits. This fact suggests that the combination of the modified α and β chains results in a change in their conformations due to chain-chain stabilizing interaction to produce a more ordered or differently ordered structure of the subunits within the tetramer.

Like the native α and β chains, their phthalocyanine analogues are non-equivalent in their properties. It is shown that reduction of the iron phthalocyanine modified β chain by ascorbic acid occurs at a markedly greater rate than that of the α chain. Likewise, the rate of oxygen binding is higher in the case of the β chain. Reconstituted phthalocyanine hemoglobin displays higher stability than its subunits. It is more resistant to autoxidation as well as to irreversible denaturation. The rate of oxygenation in this case is a close arithmetic mean of the values determined for separated Fe(III)Lαglobin and Fe-(III)Lβ globin.

Recombination of the phthalocyanine modified α and β subunits with the alternate heme-containing subunits gives hybrid hemoglobins [Fe(III)Lα-globin–βHb]$_2$ and [Fe(III)Lβglobin–αHb]$_2$. These compounds are stable in their ferric forms. When reduced with dithionite they combine with molecular oxygen. Deoxygenation by argon bubbling through the solution leads to the formation of inactive species which finally undergo irreversible denaturation. Further investigations of the hybrid phthalocyanine-heme hemoglobins are in progress.

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