# Modification of the Human Serum Albumin–Heme System with Metal Tetrasulfonated Phthalocyanines

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## Abstract

Modification of human serum albumin by cobalt and iron tetrasulfonated phthalocyanines has been performed. New compounds have been isolated and their structure and properties have been investigated by difference spectroscopy, molecular weight estimation and circular dichroism measurements.

Spectroscopic studies of the interaction of phthalocyanine derivatives with albumin and its M and C fragments have shown that there are two high affinity sites towards phthalocyanine on albumin. The strongest one is located in fragment M and that of lower affinity in fragment C. They react with phthalocyanine at the molar ratio of phthalocyanine to protein 1:1 and 2:1, respectively. The reaction of cobalt phthalocyanine with the heme complexes of M and C fragments leads to displacement of heme from the heme–M fragment complex, which suggests the same binding site for heme and phthalocyanine. Slight displacement of heme from the heme–C fragment complex occurs in spite of the phthalocyanine–C fragment complex formation. Evidence is presented that the predominant binding site of phthalocyanine is close to that of aspirin, *i.e.*, to lysine 199, and that both sites strongly interact such that modification of one prohibits reaction at the other. Spectroscopic investigation of the iron and cobalt phthalocyanine modified albumin together with previous structural studies of the phthalocyanine modified hemoproteins suggests that the iron or cobalt are involved in the bond to the protein.

## Introduction

The heme-albumin complex appears as an intermediate in the plasma heme degradation process. In the reaction of this complex with hemopexin, heme is delivered and transferred to the degradation site [1, 2]. Hemopexin exhibits a stronger binding of heme as compared to albumin. The mechanism of the heme transport in serum is not yet known in detail. Approaches to the clarification of the structural bases for the biological function of albumin in this process by various methods have generated a considerable literature. One of the main problems is the identification of the heme binding centers. Rosenfeld and Surgenor [3] suggested two high-affinity centers for heme binding in the albumin molecule. Beaven *et al.* [4] demonstrated that the albumin molecule had only one strong binding site, as well as several weaker ones. The strong binding center differs from those of bilirubin and fatty acid. Many attempts have been made to split albumin into its various fragments and to study their affinity for ligands characteristic for albumin [5–15]. The structural and functional parameters of albumin have been reviewed by Peters [16]. Meloun *et al.* [17] described the preparation of three CNBr fragments, M, N and C, of the albumin molecule with preserved disulfide bonds. Hrkal *et al.* [18] studied the interaction of heme with albumin and CNBr fragments by difference spectra, circular dichroism and stopped-flow measurements; they concluded that the main heme binding site of the albumin molecule is not contained completely in any of the fragments and that it might be formed by cooperation of the M and C fragments. The comparable reactivity of the binding sites of the M fragment and albumin supports the idea about the role of the middle part of albumin molecule in heme binding.

Gerig *et al.* [19, 20] have shown that two lysine residues on albumin are highly reactive towards such amino group reagents as tribenzenosulfonato and its derivatives. There are also a number of weaker binding sites between lysines and the examined sulfonates. The lysine residues are also very reactive towards aspirin. Under conditions of low aspirin concentration, a single lysine residue of human albumin is selectively acetylated by aspirin [21]. Walker has identified this predominant site of acetylation as lysine 199 [22]. Competition experiments have indicated that selectively acetylated albumin exhibits only one highly reactive site for sulfonate binding. Another one is blocked by aspirin lysine 199. On the other hand, arylation of albumin with the examined sulfonate blocks the primary site for

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acetylation by aspirin. These results suggest mutual interaction of both highly reactive binding sites on albumin.

The present results deal with the interaction of albumin and its CNBr fragments with Co(II)L* and Fe(III)L* as well as aspirin competition in phthalocyanine binding process.

## Experimental

*Materials*

The human serum albumin used in this study was obtained from Wytwórnia Surowic i Szczepionek, Warszawa, Poland. Samples were purified by starch gel electrophoresis. Protein concentrations were measured by spectrophotometry, taking the absorbance of a 1 mg/ml solution at 280 nm as a 0.55 [4].

The preparation and purification of metal tetrasulfonated phthalocyanines were described earlier [23]. Stock solutions were obtained by weighing appropriate amounts of solid and dissolving the latter in 100 ml of appropriate buffer (0.1 M phosphate buffer, pH 7.6 or 0.1 M carbonate buffer, pH 9.3).

Hemine chloride was obtained from Biomed, Kraków and used without further purification. The appropriate amount of the preparation was dissolved in 0.01 N alkali, rapidly diluted ten times, and used in this form within about 1 h. The concentration was determined spectrophotometrically in 0.01 N NaOH using a milimolar absorption coefficient of 58.4 $mM^{-1}\,cm^{-1}$ at 385 nm [4].

The fragments of the human plasma albumin were isolated from the cyanogen bromide hydrolysate of the carboxamidomethylated albumin as previously described [17].

Acetylated human albumin was prepared according to the procedure of Pinckard *et al.* [21].

The phthalocyanine complexes of albumin and its fragments were prepared by incubation of the appropriate protein with metal tetrasulfonated phthalocyanine at a molar ratio of protein to phthalocyanine of 1:1 or 1:2, in buffered solution at pH 7.6 for three days at 4 °C. The reaction mixture was separated by gel filtration on a Sephadex G-100 column. The fractions were identified by absorption spectroscopy at 280 nm and at a wavelength characteristic for a given phthalocyanine complex (685 nm and 650 nm for the cobalt and iron complexes, respectively). The blue protein fractions were lyophilized to crystalline state in the case of the albumin complexes or to the more concentrated solutions in the case of the phthalocyanine complexes of the fragments. The concentrations of the albumin complexes were determined from the molar absorption coefficients (CoL–HSA $\epsilon_{685} = 4.0 \times 10^4\ M^{-1}\,cm^{-1}$, FeL–HSA $\epsilon_{650} = 3.5 \times 10^4\ M^{-1}\,cm^{-1}$).

*Methods*

*Absorption spectroscopy*

Absorption and difference spectra were obtained with a Specord recording spectrophotometer. The measurements were made under equilibrium conditions.

*Circular dichroism measurements*

Circular dichroism (CD) spectra were recorded using a model ORD/UV-5 Japan Spectropolarimeter with CD attachment. The solutions were prepared by dissolving an appropriate amount of a lyophilized preparation in phosphate buffer, pH 7.6.

*Molecular weight estimation*

Molecular weights of the model complexes were determined by gel filtration on Sephadex G-75 column according to the method of Andrews (see Stellwagen *et al.*) [24]. The following proteins were used as reference substances: cytochrome c ($M_r$ 12400), myoglobin ($M_r$ 17600), ovalbumin ($M_r$ 45000), albumin ($M_r$ 69000) and transferrin ($M_r$ 80000).

## Results

*Interaction of Iron and Cobalt Tetrasulfonated Phthalocyanines (Fe(III)L and Co(II)L) with Human Serum Albumin (HSA)*

Human serum albumin undergoes a pH-dependent conformation change in neutral or slightly alkaline solution [25, 26]. This transition has been designated as the neutral-to-base or N–B transition. The N form occurs mainly at about pH 7 and changes into the basic B form with an increase to pH 9.

The interactions between metal phthalocyanine complexes and albumin were followed by difference spectroscopy experiments at 0.1 M phosphate buffer (pH 7.0 or 7.6) and 0.1 M carbonate buffer (pH 9.2), corresponding to existence of the N and B forms of the protein, respectively. In both conditions, difference spectra of identical shape were obtained for the same phthalocyanine complex.

Visible difference spectra of Fe(III)L–HSA and Co(II)L–HSA systems are presented in Fig. 1. They show positive bands at 650 nm and 685 nm for iron and cobalt phthalocyanine–albumin complexes, respectively, as well as negative bands at wavelengths characteristic for unbonded Fe(III)L and Co(II)L (635 nm and 625 nm, respectively).

*Abbreviations: L = tetrasulfonated phthalocyanine ligand [$C_{32}H_{12}N_8(SO_3Na)_4$]; Fe(III)L = iron(III) tetrasulfonated phthalocyanine; Co(II)L = cobalt(II) tetrasulfonated phthalocyanine; HSA = human serum albumin. Fe(III)L–HSA and Co(II)L–HSA, complexes of iron and cobalt tetrasulfonated phthalocyanines with human serum albumin.

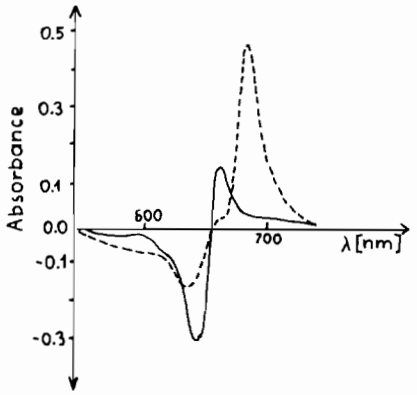


Fig. 1. Difference spectra of Fe(III)L + albumin (——) and Co(II)L + albumin (- - - -) mixtures against the same solutions unmixed. Concentrations: Fe(III)L = Co(II)L = albumin = $4 \times 10^{-5}$ M.

Both phthalocyanine derivatives used in the modification of albumin exist in water solutions in two forms, with the monomeric and dimeric being in equilibrium. However, iron tetrasulfonated phthalocyanine in experimental conditions appears mainly in the more stable dimeric form. The position of the negative band in the difference spectra of both reaction mixtures corresponds to the dimeric form of the appropriate phthalocyanine. On the other hand, characteristic peaks of Fe(III)L–HSA and Co(II)L–HSA complexes are close to those of Fe(III)L and Co(II)L monomers at 650 nm and 675 nm, respectively [27].

Spectrophotometric titration of albumin solution with cobalt tetrasulfonated phthalocyanine is presented in Fig. 2. There is no sharply defined endpoint, although at a Co(II)L:HSA ratio close to unity the slope decreases, but absorptivity continues to rise at higher molar ratios. This suggests further binding of phthalocyanine molecules with albumin. The shape

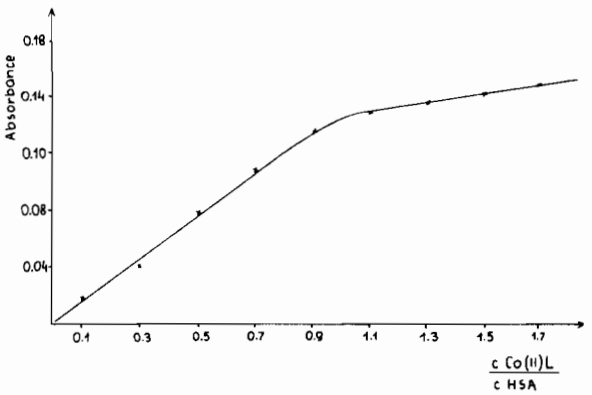


Fig. 2. Stoichiometric titration of albumin with Co(II)L. The changes in absorption were measured at 685 nm as a function of the molar ratio of Co(II)L/HSA. Concentration: HSA = $8 \times 10^{-5}$ M, Co(II)L = $8 \times 10^{-6}$ M – $1.6 \times 10^{-5}$ M.

of the titration curve suggests the existence of additional binding sites on the protein.

The complexes of albumin with metal tetrasulfonated phthalocyanines undergo reduction on addition of dithionite as is shown by characteristic changes in their absorption spectra. The main absorption peak is shifted to longer wavelengths, *i.e.*, to 690 nm and 720 nm for the iron and cobalt complexes, respectively.

### *Molecular Weight Estimation*

The results of the molecular weight estimation of Fe(III)L–HSA and Co(II)L–HSA show that the molecular weight of the iron complex is approximately 70 000 and that of the cobalt complex is 71 600. These values are comparable to that of human serum albumin, which demonstrates that upon binding of Co(II)L or Fe(III)L to HSA the protein remains monomeric.

### *Circular Dichroism Spectra (CD)*

The conformational properties of the model complexes were examined by CD technique in phosphate buffer at pH 7.6. For comparative purposes the CD spectrum of albumin was recorded at the same conditions (Fig. 3). Both phthalocyanine complexes exhibit negative UV Cotton effects at 207 nm and 220 nm. The ellipticity values expressed in deg cm$^2$ dmol$^{-1}$ are: $\theta_{207} = -3.5 \times 10^5$ for both complexes and albumin and $\theta_{220} = -3.4 \times 10^5$, $-3.43 \times 10^5$ and $-3.5 \times 10^5$ for Fe(III)L–HSA, Co(II)L–HSA and albumin, respectively. Visible CD spectra of the phthalocyanine complexes are characterized by a main negative band for each complex which appears

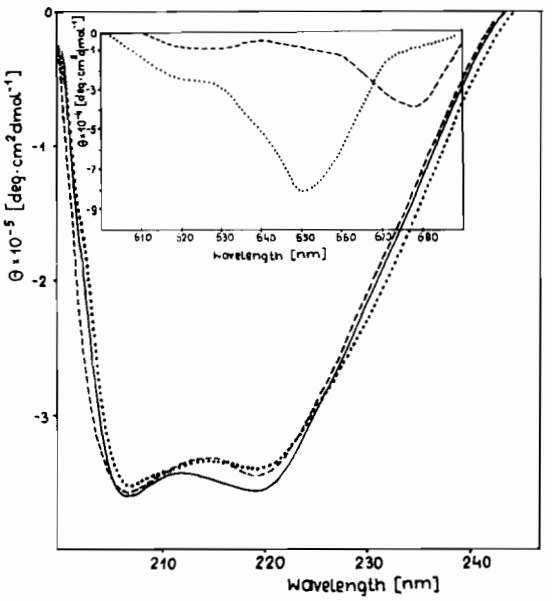


Fig. 3. CD spectra of albumin (——), Co(II)L–HSA (- - - -), and Fe(III)L–HSA (·····), in ultraviolet and visible regions.

at 650 nm $\theta = 8.0 \times 10^4$ and 680 nm $\theta = 4 \times 10^4$ for Fe(III)L–HSA and Co(II)L–HSA, respectively. Visible CD spectra are associated with the absorption bands of the examined complexes observed at 650 nm and 685 nm, respectively.

*Interaction of the Cobalt Tetrasulfonated Phthalocyanine with CNBr Albumin Cleavage Fragments*

In order to identify metal phthalocyanine binding centers in albumin complexes, specific cleavage of albumin was performed. After previous blockade of the cysteine residues, the specific cleavage of albumin with cyanogen bromide divides each molecule into three fragments: N (1–123), M (124–298) and C (299–585). Spectroscopic studies of the mixtures of the cobalt phthalocyanine derivative with albumin fragments suggest complex formation between Co(II)L and the M and C peptide fragments. Visible difference spectra show one positive peak at 685 nm and 680 nm for the M and C fragments, respectively (Fig. 4). Complex formation with the N fragment was not observed under these experimental conditions. Spectrophotometric titration data presented in Fig. 5 indicate the formation of albumin fragment complexes with the cobalt phthalocyanine derivative at the peptide; the Co(II)L molar ratios were 1:1 and 1:2 for the M and C fragments, respectively. While the dependence of the difference absorption at 685 nm of Co(II)L–M complex on Co(II)L/peptide M exhibits sharp break at the molar ratio value 1, similar dependence at 680 nm of the C fragment complex shows a smoother character which suggests its lower stability.

Interaction of heme–M and heme–C fragment complexes with Co(II)L leads to substitution of heme in the heme–M fragment by Co(II)L (Fig. 6). Difference spectra of the appropriate mixtures show positive bands characteristic for Co(II)L–M and Co(II)L–C complexes at 685 nm and 680 nm, respectively, which points to the formation of the

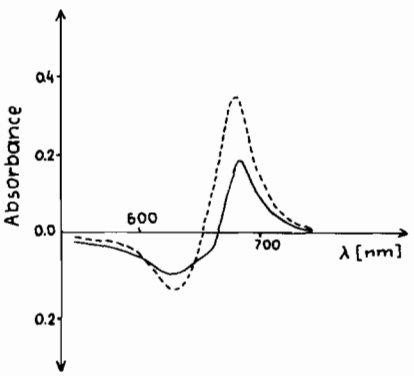


Fig. 4. Difference spectra of Co(II)L + M fragment (——) and Co(II)L + C fragment (- - - -) mixtures against the same solutions unmixed. Concentrations: M fragment = C fragment = Co(II)L = $2.5 \times 10^{-5}$ M.

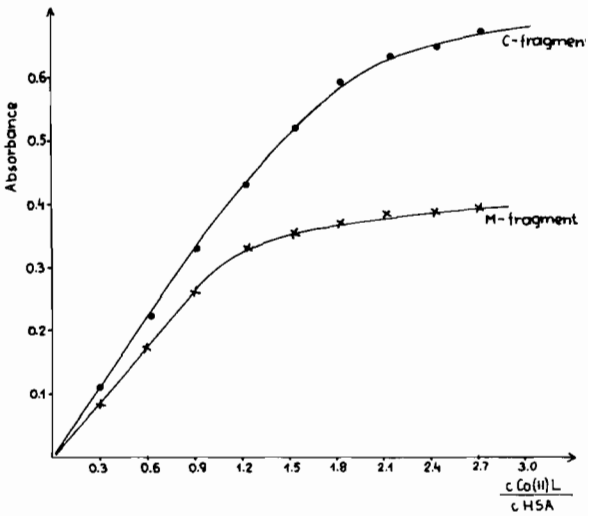


Fig. 5. Stoichiometric titration of M fragment with Co(II)L (–X–) and C fragment with Co(II)L (–•–). The changes in absorption were measured at 685 nm (M fragment) and 680 nm (C fragment), as a function of the molar ratio of Co(II)L/M fragment and Co(II)L/C fragment, respectively. Concentrations: C fragment = N fragment = $2.5 \times 10^{-5}$ M, Co(II)L = $8 \times 10^{-6}$ M–$4.8 \times 10^{-5}$ M.

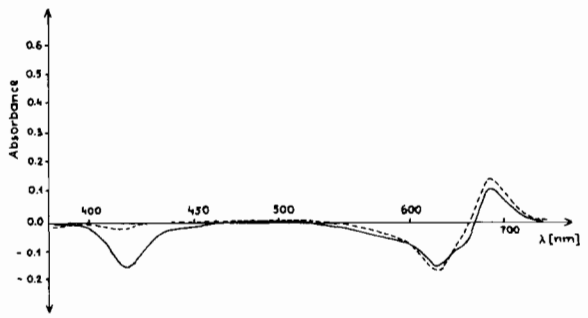


Fig. 6. Difference spectra of Co(II)L + heme–M fragment complex (——) and Co(II)L + heme–C fragment complex (- - - -) mixtures against the same solutions unmixed. Concentrations: heme–M fragment = heme–C fragment = Co(II)L = $1.44 \times 10^{-5}$ M.

complexes of Co(II)L with the M and C fragments. At the same time two negative bands for each complex are observed. One at 625 nm, characteristic for the dimeric form of cobalt tetrasulfonated phthalocyanine and the second characteristic for heme complexes of the M and C fragments at 416 and 410 nm, respectively. The intensities of the bands in the region characteristic for the phthalocyanine absorption, in equilibrium state, are almost the same. However, in the Soret region, the negative band at 410 nm is minimal compared to that at 416 nm, which suggests only insignificant displacement of heme from the heme–C fragment complex by Co(II)L, in spite of the complex formation between the phthalocyanine derivative and the C fragment in an extent

comparable with that of Co(II)L–M complex formation.

### *Interaction of Acetylated Human Serum Albumin and its M Fragment with Cobalt and Iron Tetrasulfonated Phthalocyanines*

As is shown by the difference spectra presented in Fig. 7, selective acetylation of albumin under defined conditions [21] inhibits Co(II)L and Fe(III)L binding by protein. These results suggest that the group on the albumin that is acetylated by aspirin selectively is one of those which react with phthalocyanine derivatives. According to the Walter's results, the predominant site of acetylation is lysine 199. However, the same experiment performed with the acetylated M peptide fragment of albumin gives different results. The difference spectrum exhibits an intense positive peak at 685 nm characteristic for the Co(II)L–M fragment complex, which points to phthalocyanine binding by the M peptide in spite of modification of the protein by aspirin (Fig. 8). This

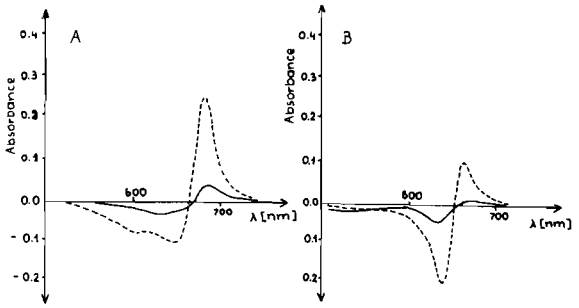


Fig. 7. Difference spectra of the mixtures of Co(II)L (a) and Fe(III)L (b) with aspirin acetylated albumin (——) and unacetylated albumin (- - - -). Concentrations: acetylated albumin = unacetylated albumin = Co(II)L = Fe(III)L = $1.7 \times 10^{-5}$ M.

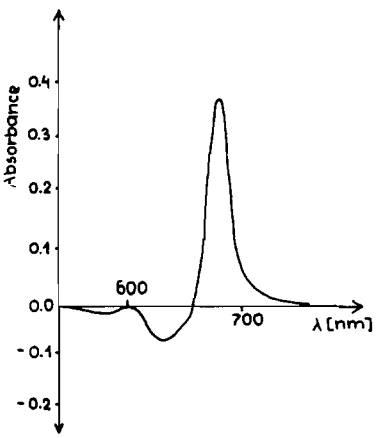


Fig. 8. Difference spectrum of Co(II)L with aspirin acetylated M fragment mixture against the same solutions unmixed. Concentrations: acetylated M fragment = Co(II)L = $1.6 \times 10^{-5}$ M.

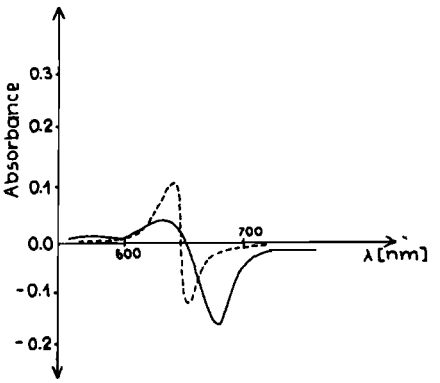


Fig. 9. Difference spectra of the mixtures of (a) Co(II)L–albumin (——) and (b) Fe(III)L–albumin (- - - -) complexes with aspirin against the same solutions unmixed. Concentrations: Co(II)L–albumin = aspirin = $3 \times 10^{-5}$ M, Fe(III)L–albumin = aspirin = $6 \times 10^{-5}$ M.

fact suggests that the high affinity sites of both compounds are different, but they are close together in the native albumin.

Acetylation of albumin that has been pretreated with Co(II)L or Fe(III)L under conditions which favour modification of only the most reactive sites leads to displacement of the phthalocyanine derivative from its albumin complex; this is demonstrated by the difference spectra of the appropriate mixtures (Fig. 9). The positive peak observed in the region of dimeric phthalocyanine absorption points to the dimerization of the monomeric phthalocyanine molecules liberated from the phthalocyanine–albumin complex.

## Conclusions

Spectroscopic investigations of the systems involving human serum albumin and iron or cobalt tetrasulfonated phthalocyanines, under conditions of low phthalocyanine concentration, show complex formation between the protein and the phthalocyanine derivative at the molar ratio 1:1. Molecular weights of both phthalocyanine–albumin complexes correspond to that of the native albumin monomer and its heme derivative. The spectrophotometric titration curve suggests the presence of some weaker phthalocyanine binding sites on albumin. Spectroscopic studies of the interaction between cobalt tetrasulfonated phthalocyanine and cyanogen bromide albumin fragments demonstrate that phthalocyanine binds specifically to the isolated M and C fragments at phthalocyanine to peptide molar ratios of 1:1 and 1:2, respectively. Both binding centers are the high affinity ones, but the smoother character of the spectrophotometric titration curve in the case of fragment C points to the weaker phthalocyanine binding on this fragment.

High affinity sites of albumin and its fragments bind with the monomeric form of phthalocyanines; this is demonstrated by the position of the main absorption band of the complexes formed in this process.

The combination of albumin with metal tetrasulfonated phthalocyanines virtually does not change the shape of the protein CD spectra in the ultraviolet, but a small decrease of the α-helical structure is observed. That points to some destabilization of the secondary structure of the protein by the phthalocyanine binding. In the visible region, circular dichroism bands are associated with the absorption bands of the metal phthalocyanines used. The unbound cobalt and iron tetrasulfonated phthalocyanines do not exhibit the CD spectra.

The reaction of cobalt tetrasulfonated phthalocyanine with the complex of heme with the M fragment leads to the displacement of heme by Co(II)L. This fact implies that the heme and phthalocyanine binding sites on albumin M fragment are the same. A different result is obtained in the reaction of Co(II)L with the heme complex of the C fragment. Displacement of heme occurs in to minimal extent, in spite of Co(II)L–C fragment complex formation to a degree comparable with that of the M fragment. This fact suggests that the phthalocyanine binding site on the C fragment is not the same as the heme binding site, but it is probably very close to or overlaps it. In such a situation, phthalocyanine binding can produce conformational perturbation of the heme binding center and the relase of some heme molecules.

Absorption spectra of the examined complexes are nearly identical with those of some apohemoprotein complexes with Co(II)L and Fe(III)L, which suggests that phthalocyanine binding is similar in both types of compounds [27–29]. Structural studies of the latter complexes have shown that the phthalocyanine metal ion is involved in the bond to the protein. It can be assumed then that, in the case of the examined phthalocyanine–albumin complexes, cobalt and iron ions are implicated in the binding to albumin.

To identify some of the groups of albumin modified by cobalt and iron tetrasulfonated phthalocyanines, competition experiments were performed. Under conditions of low aspirin concentration, acetylation of albumin appears to be specific for lysine-199 [21, 22]. Spectroscopic studies show that acetylated albumin is only slightly reactive towards phthalocyanine complexes, but its M fragment modified by aspirin exhibits Co(II)L binding ability to an extent comparable to that of the unmodified protein. These results indicate that the predominant binding sites of aspirin and Co(II)L on albumin are not the same, but both sites are merely close to each other and strongly interacting, such that modification of one prevents reaction at the other. Structural perturbations caused by CNBr cleavage of albumin followed by conformational changes in the protein make it possible for the fragment M to bind aspirin and phthalocyanine at the same time. Aspirin is able to displace Co(II)L and Fe(III)L from their complexes with albumin. Presumably there is competition between the strong sites for aspirin and phthalocyanine. The mode of interaction between these two sites could involve steric exclusion or conformational changes in the protein.

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