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Modification of the Heme·Heme Oxygenase System with Iron and Cobalt Tetrasulfonated Phthalocyanines

ŁUCJA OSTROPOLSKA, HELENA PRZYWARSKA-BONIECKA and HANNA SWIRSKA
Institute of Chemistry, University of Wrocław, Poland
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Abstract

Modification of heme·heme oxygenase by iron(III) and cobalt(II) tetrasulfonated phthalocyanines has been performed. New compounds have been isolated and their properties have been investigated by difference spectroscopy, electrophoresis, molecular weight estimation, electron paramagnetic resonance (EPR) and carboxymethylation at histidyl groups. Spectrophotometric titration data indicate the ratio of the reagents in this process to be 1:1. The visible absorption spectra show the main peak at 650 nm for the iron compound and 682 nm for the cobalt one. Electrophoresis and molecular weight estimation show both complexes to be monomers. Cobalt(II) tetrasulfonated phthalocyanine, under aerobic conditions with heme oxygenase protein, undergoes autooxidation to the cobalt(III) complex, as has been proved by EPR and spectroscopic data. Iron and cobalt phthalocyanine modified heme·heme oxygenase with excess dithionite is reduced at the phthalocyanine ligand. In the presence of oxygen, the reduction product transforms into oxygenated Fe(III)L–heme oxygenase or Co(III)–heme oxygenase, respectively. Reduction of the iron(III) model complex with ascorbic acid under anaerobic conditions leads to degradation of the phthalocyanine moiety, while Co(III)–heme oxygenase with ascorbic acid is reduced to Co(II)L–heme oxygenase. As has been shown by carboxymethylation of the heme oxygenase protein at the histidine residues, the predominant binding site of both phthalocyanine complexes is the heme-binding histidyl residue. There is evidence that there is a second binding site with lower affinity towards Co(II)L on the heme oxygenase protein. Iron and cobalt tetrasulfonated phthalocyanines are not able to displace heme from the heme·heme oxygenase complex. In this reaction the iron complex undergoes degradation and the cobalt one gives a hybrid compound with heme·heme oxygenase.

Heme oxygenase protein complexes with iron and cobalt tetrasulfonated phthalocyanines do not exhibit activity in their oxidative degradation.

Introduction*

Heme oxygenase is a microsomal enzyme protein which plays an essential role in physiological heme metabolism [1]. It is not a hemo-protein by nature, but it easily binds with heme to form a heme·heme oxygenase complex. The absorption spectrum of this complex in its ferric form resembles those of methemoglobin and methmyoglobin [2, 3]. The heme bound to heme oxygenase undergoes oxidative conversion to biliverdin when the complex is incubated with NADPH–cytochrome *c* reductase in the presence of oxygen [4]. According to the hypothesis of Yoshida *et al.* [5], the ferric heme bound to heme oxygenase is first reduced to the ferrous state by NADPH–cytochrome *c* reductase, followed by oxygenation with molecular oxygen to form the oxygenated form of the complex. Then a second electron may be transferred to this complex yielding a heme-bound activated oxygen species which indicates heme degradation by hydroxylation of a methene bridge carbon atom to form hydroxyheme. Incorporation of the two further atoms of oxygen from molecular oxygen results in ring opening of the hydroxyheme to yield biliverdin and iron with the methene carbon atom being liberated as carbon monoxide [6].

Heme oxygenase was found to oxidize not only the natural hemin IX, but also a number of its synthetic analogs [7]. However, the enzymatic oxidation of heme is stereo-selective because of an α-bridge and this is independent of the flanking substituents and in every case only α-biliverdin isomers were obtained.

Recently, Yoshida and Kikuchi have studied the reaction of a purified heme oxygenase with cobaltic protoporphyrin IX, in the presence of NADPH–

*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; heme·heme oxygenase = complex of heme with heme oxygenase protein; CM-oxygenase = carboxymethylated heme oxygenase protein.

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cytochrome *c* reductase [8]. They have found that Co–heme which was bound to the heme oxygenase protein could not be reduced with the NADPH–cytochrome *c* reductase system with oxidative cleavage to give biliverdin. On the contrary, the Fe–heme bound to heme oxygenase was readily reducible under the same conditions. According to the authors' suggestion, the iron is essential for the oxidative cleavage of metalloporphyrin by the heme oxygenase system. The Co–heme oxygenase complex closely resembles those of cobalt hemoglobin and cobalt myoglobin, which supports an idea of the authors that heme oxygenase may have a heme-binding crevice similar to those of myoglobin and hemoglobin [3, 4].

Earlier we have shown that iron and cobalt tetrasulfonated phthalocyanine, being metalloporphyrin analogs, may be incorporated into the heme crevice of apohemoglobin and apomyoglobin to give complexes whose properties resemble those of the appropriate native hemoproteins [9–11]. In their reduced form these complexes are able to combine reversibly with molecular oxygen. This modification results in a marked increase in the helix content of these proteins, which points to the stabilization of their secondary structure by formation of metal phthalocyanine complexes. On the basis of the results presented above we have undertaken studies of the interaction of iron and cobalt tetrasulfonated phthalocyanines with heme oxygenase in the presence of some reducing agents in the NADPH–cytochrome *c* reductase system.

Experimental

Preparation of microsomes from pig liver and purification of heme oxygenase was performed by the procedure described by Yoshida and Kikuchi [3]. Purified heme oxygenase was identified by electrophoresis on sodium dodecyl sulfate–polyacrylamide gel (10%, 0.6 × 10 cm), as a single protein band. The molecular weight of this protein was estimated according to the method of Wever and Osborn [12]. It was found to be about 32 000. Partial purification of NADPH–cytochrome *c* reductase was performed by the method of Yoshida and Kikuchi [3]. The activity of NADPH–cytochrome *c* reductase was assayed according to the method of Omura and Takesue [13]. The preparation of the carboxymethylhistidine derivative of heme oxygenase was performed by iodacetylation of the enzyme at pH 5.5, as described previously by Crestfield *et al.* [14]. The preparation and purification of iron and cobalt tetrasulfonated phthalocyanines have been described earlier [15]. Stock solutions were obtained by weighing appropriate amounts of the solid and dissolving the latter in 100 ml of a suitable buffer.

Synthesis of the Complexes of the Metal Tetrasulfonated Phthalocyanines with Heme Oxygenase

The heme oxygenase dissolved in 0.01 M phosphate buffer, pH 7.4, containing solution A (the mixture of 1 mM EDTA, 0.1% Triton X-100 and 0.1% sodium cholate in 1 dm^3 of 0.01 M potassium phosphate buffer, pH 7.4) was treated with a twofold excess of iron or cobalt tetrasulfonated phthalocyanine (Fe(III)L or Co(II)L). The reaction mixture was allowed to stand for three days at 4 °C and then was separated by gel filtration on Sephadex G-50. The protein fractions were identified spectrophotometrically at 280 nm; the iron and cobalt phthalocyanine complexes gave bands at 650 or 682 nm, respectively. The blue–green protein fractions were dialyzed to water and lyophilized. The reduced forms of the complexes were prepared by addition of a few milligrams of sodium dithionite to their buffered solutions and removing the excess of the reductant on a Sephadex G-25 column.

The concentrations of the heme·heme oxygenase and its phthalocyanine derivatives were determined from their molar absorption coefficients in the phosphate buffer (pH 7.4) containing solution A. Molar absorption coefficients for heme·heme oxygenase, Fe(III)L–heme oxygenase and Co(II)L–heme oxygenase were found to be: $\epsilon_{405} = 14.0 \times 10^4$ M^{-1} cm^{-1}, $\epsilon_{650} = 5.1 \times 10^4$ M^{-1} cm^{-1} and $\epsilon_{682} = 2.84 \times 10^4$ M^{-1} cm^{-1}, respectively. The molar absorption coefficient of the heme oxygenase at 280 nm in phosphate buffer, pH 7.4, was 3.0×10^4 M^{-1} cm^{-1}.

The activity of heme oxygenase and its phthalocyanine complexes in the heme degradation process was determined by measuring the bilirubin formation on the basis of the increase in absorbance at 468 nm, closely according to the method described earlier by Yoshida and Kikuchi [3].

The following reagents were purchased from Serva Feinbiochemia, Heidelberg, F.R.G.: hemin, bilirubin, iodacetic acid, chymotripsinogen, carboxypeptidase A, ovalbumin and bovine serum albumin. DEAE-cellulose (DE 23 and DE 32) were products of Whatman Biochemicals, Maidstone, U.K.. Sephadex G-25, G-50 and G-100 were purchased from Pharmacia Fine Chemicals, Upsala.

Absorption and difference spectra were performed on a Cary recording spectrometer with a cell compartment thermostatically controlled at 10 °C or on a Specord recording spectrophotometer. The measurements were made under equilibrium conditions.

Molecular weight estimation was performed by dodecyl sulfate polyacrylamide gel electrophoresis, in 10% gel, as according to Weber and Osborn [12]. The following proteins were used as reference substances: cytochrome *c* (12 400), myoglobin (17 800), chymotrypsinogen (25 000), carboxypeptidase A (34 000) and serum albumin (67 000).

Electron paramagnetic resonance (EPR) X-band spectra of the complex of cobalt tetrasulfonated phthalocyanine with heme oxygenase protein in 0.01 M phosphate buffer (pH 7.4) containing solution A, in its oxygenated and reduced forms, were performed at liquid nitrogen temperature on a JEOL JES-ME-3X spectrometer at 9.12 GHz.

Results

Interaction of Iron and Cobalt Tetrasulfonated Phthalocyanines with Heme Oxygenase Protein

Purified heme oxygenase reacts with the iron and cobalt tetrasulfonated phthalocyanines to give blue complexes. The reaction was followed by the difference spectroscopy method. New phthalocyanine compounds in 0.01 M phosphate buffer containing solution A exhibit characteristic absorptivities at 650 nm and 682 nm, respectively (Fig. 1). At the same time, a lowering of absorptivity at 635 nm and 632 nm is observed in the difference spectra of the mixtures of protein with Fe(III)L and Co(II)L, respectively. Additionally, in the spectrum of the cobalt complex, a slight increase in absorptivity is seen at 410 nm.

Free Fe(III)L exhibits in water solutions an absorption spectrum with a characteristic intense band at 635 nm ascribed to the dimer, which is the dominating form under these conditions. The visible spectrum of Co(II)L in water solutions is formed by superposition of at least two bands of comparable intensity at 625 nm and 670 nm. Veprek-Siska *et al.* [16] suggested that the band at 625 nm belongs to the Co(II)L monomer and the other one to its oxygenated form. Bernauer and Fallab [17] assigned them to the dimer and the monomer in equilibrium. Probably the positions of the dimer and the unoxygenated monomer are close together. In phosphate buffer containing solution A the band characteristic of the dimer (or unoxygenated monomer) is observed

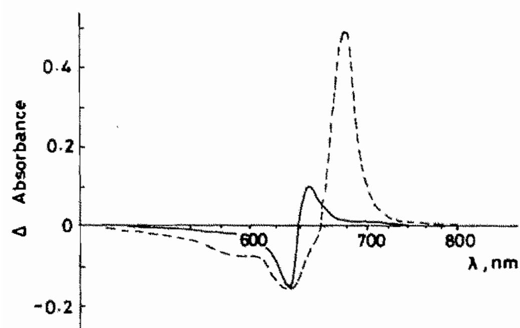

Fig. 1. Difference spectra of Fe(III)L plus heme oxygenase (——) and Co(II)L plus heme oxygenase (– – –) mixtures against the same solutions unmixed. Concentrations: Fe(III)L = Co(II)L = 1×10^{-5} M, heme oxygenase = 3.5×10^{-6} M.

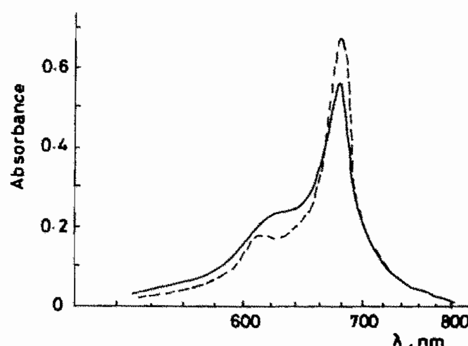

Fig. 2. Absorption spectra of the complex between heme oxygenase protein and Co(II)L: under aerobic conditions (——); oxidized with potassium ferricyanide and dialyzed (– – –).

at 632 nm. The oxygenated form of the complex is found to absorb at 675 nm.

The shape of the difference spectrum of the Fe(III)L–heme oxygenase system points to the shift of the dimer–monomer equilibrium state towards Fe(III)L monomer formation, due to monomer incorporation into the protein crevice. In the case of cobalt tetrasulfonated phthalocyanine, three complexes are in equilibrium: dimer, monomer and oxygenated monomer. The position of the band characteristic of its complex with heme oxygenase protein corresponds to that of the oxygenated monomer. However, in contrast to free O_2–Co(II)L, oxygen cannot be removed from its complex with protein by bubbling argon through the solution. As is shown by the absorption spectra presented in Fig. 2, oxidation of the cobalt phthalocyanine complex with heme oxygenase by potassium ferricyanide does not change the position of its characteristic band at 682 nm, which suggests the oxidation state three for the cobalt ion in this compound. This suggestion was confirmed by the EPR method. No EPR spectrum was observed either in the polycrystalline state or in buffered solution.

Spectrophotometric titration data indicate the formation of the metal phthalocyanine–heme oxygenase complexes at a molar ratio of the reactants of approximately 1:1, calculated for the monomer of the metal tetrasulfonated phthalocyanine (Fig. 3).

The results of the molecular weight estimation by dodecyl sulfate polyacrylamide electrophoresis are shown in Fig. 4. The plot of the mobility against molecular weight indicates that molecular weights of Fe(III)L–heme oxygenase and Co(III)L–heme oxygenase are about 31 000. This value is comparable to that of the heme·heme oxygenase complex (32 000).

Properties of Heme Oxygenase Protein Complexes with Fe(III)L and Co(II)L

The iron and cobalt phthalocyanine complexes of heme oxygenase protein reduced with excess

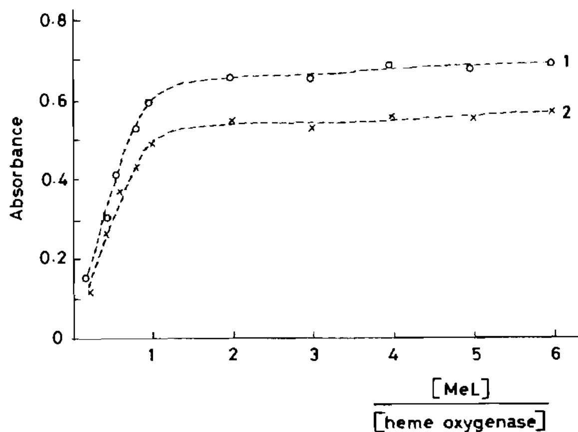

Fig. 3. Stoichiometric titration of heme oxygenase with Fe(III)L (1) and Co(II)L (2). The changes in absorption were measured at 650 and 682 nm, respectively, as a function of the molar ratio of Fe(III)L/protein and Co(II)L/protein. Concentrations: protein = 1×10^{-5} M, Fe(III)L = Co(II)L = 2×10^{-6}–6×10^{-5} M.

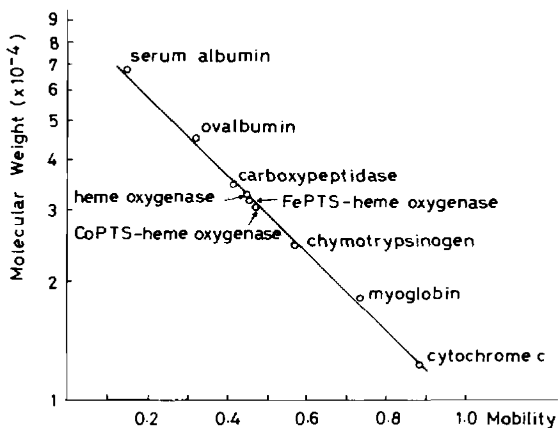

Fig. 4. Polyacrylamide gel electrophoresis of the Fe(III)L–heme oxygenase, Co(III)L–heme oxygenase, heme oxygenase protein, serum albumin, ovalbumin, carboxypeptidase, chymotrypsinogen, myoglobin and cytochrome *c*. Plot of molecular weights against the mobilities.

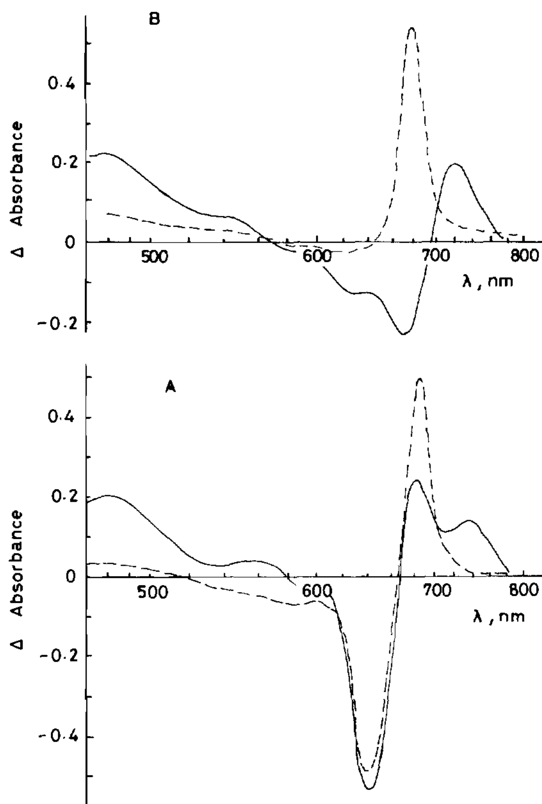

Fig. 5. Difference spectra of (A) Fe(III)L–heme oxygenase plus $Na_2S_2O_4$ and (B) Co(III)L–heme oxygenase plus $Na_2S_2O_4$: in argon (——); and under aerobic conditions (– – –). Concentrations: Fe(III)L–heme oxygenase = Co(III)L–heme oxygenase = 8×10^{-6} M.

dithionite under anaerobic conditions exhibit the characteristic difference spectra presented in Fig. 5A, B. The difference spectrum of the iron phthalocyanine derivative shows a negative band at 650 nm and three positive bands at 480, 685 and 740 nm. A decrease in the absorptivity at 650 nm and the appearance of a band at 685 nm points to the reduction of the iron ion in Fe(III)L–heme oxygenase to the oxidation state two. The band at 685 nm represents the absorption region of the iron(II) tetrasulfonated phthalocyanine derivatives. Two other positive bands are characteristic of many systems containing metal tetrasulfonated phthalocyanine and excess dithionite. They were suggested to present an intermediate reduction product involving a phthalocyanine ligand–dithionite complex [9, 15]. These bands disappear in the presence of oxygen because of reoxidation of the phthalocyanine ring. Simultaneously, the band at 685 nm increases as a result of oxygen binding by Fe(II)L–heme oxygenase. The difference spectrum of the cobalt phthalocyanine complex with excess dithionite under anaerobic conditions does not exhibit this band, indicating reduction of the cobalt ion in Co(III)L–heme oxygenase. The appearance of the bands of an intermediate at 457 and 720 nm, with simultaneous decrease in absorptivity at 630 and 682 nm, points to the reduction of the phthalocyanine ligand in the cobalt phthalocyanine complexes. Under aerobic conditions, reoxidation of the phthalocyanine ring takes place. The bands of the intermediate disappear and the positive band of Co(III)L–heme oxygenase at 682 nm is reconstituted. At 630 nm a negative band slowly disappears. It probably belongs to Co(II)L non-specifically bonded to the protein, which undergoes decomposition upon reduction and reoxidation of its phthalocyanine ligand.

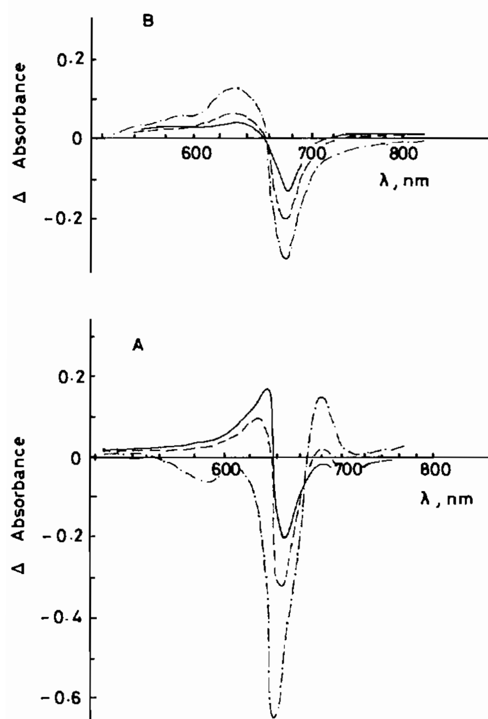

Fig. 6. Difference spectra of (A) Fe(III)L–heme oxygenase plus ascorbic acid and (B) Co(II)L–heme oxygenase plus ascorbic acid in argon: immediately after mixing the reagents (——); after the reaction had run for 2 h (– – –); and after 24 h under aerobic conditions (—·—). Concentrations: Fe(III)L–heme oxygenase = Co(III)L–heme oxygenase = 2×10^{-8} M.

Visible difference spectra of the mixtures of Fe(III)L–heme oxygenase and Co(III)L–heme oxygenase with ascorbic acid are presented in Fig. 6A. In an argon atmosphere, the difference spectrum of the iron complex at the start of the reaction exhibits a positive band at 625 nm which disappears slowly as the reaction continues. At the same time, the absorptivity at 650 nm significantly decreases, which suggests partial degradation of the phthalocyanine ligand. In the presence of oxygen, the intermediate transforms slowly into Fe(II)L–heme oxygenase and, further, to its oxygenated form. That is observed by the appearance of the band at 685 nm. The band at 625 nm probably belongs to an intermediate formed between Fe(III)L–heme oxygenase and ascorbic acid.

The difference spectrum of the Co(III)L–heme oxygenase–ascorbic acid system under anaerobic conditions exhibits an intense negative band at 682 nm and a broad positive one at 632 nm. The shape of this spectrum suggests reduction of the cobalt ion in Co(III)L–heme oxygenase to the oxidation state two. This spectrum does not change in the presence of oxygen (Fig. 6B).

Iron and cobalt tetrasulfonated phthalocyanines as their complexes with heme oxygenase protein virtually do not undergo oxidative degradation when they are incubated with the NADPH–cytochrome *c* reductase system in air.

Reaction with Additional Ligands

Examination of the difference spectra shows that, contrary to free Fe(III)L, its complex with heme oxygenase protein does not coordinate detectably with cyanide and histidine. On the other hand, the complex of Fe(III)L with histidine also does not react with heme oxygenase protein. These results suggest that Fe(III)L is coordinated axially with the histidyl residue of the oxygenase protein.

Interaction between the Co(II)L–histidine complex and heme oxygenase protein gives slow decomposition of the phthalocyanine moiety, which is shown in the difference spectrum by the disappearance of its main absorption band (Fig. 7). Instead, the reaction of Co(III)L–heme oxygenase with histidine leads to complex formation which is observed in its difference spectrum as an increase in absorptivity at 682 nm.

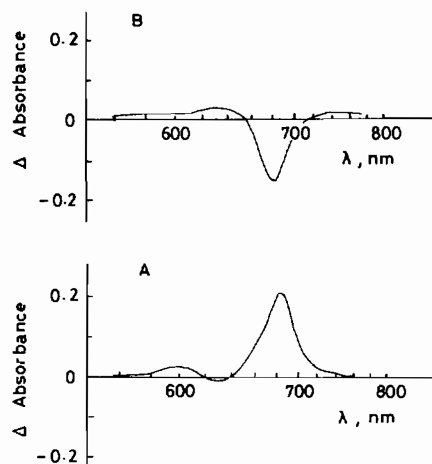

Fig. 7. Difference spectrum of (A) Co(III)L–heme oxygenase plus histidine, (B) Co(II)L–histidine plus heme oxygenase protein. Concentrations: (A) complex = 3.1×10^{-5} M, histidine = 13 mM; (B) complex = 1.8×10^{-5} M, heme oxygenase protein = 3.1×10^{-5} M.

Effects of Carboxymethylation of Heme Oxygenase Histidyl Residues on Iron and Cobalt Tetrasulfonated Phthalocyanine Binding

In order to identify the iron and cobalt tetrasulfonated phthalocyanides binding sites on heme oxygenase protein, selective carboxymethylation of its histidyl residues was performed. As is shown by the difference spectrum presented in Fig. 8, carboxymethylated heme oxygenase reacts with cobalt tetrasulfonated phthalocyanine to give a result similar to that with the unmodified protein. Appearance of the

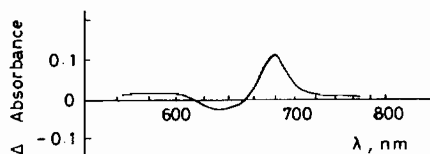

Fig. 8. Difference spectrum of Co(II)L plus heme oxygenase protein carboxymethylated at the histidine residues. Concentrations: CM-oxygenase = Co(II)L = 3.25×10^{-6} M.

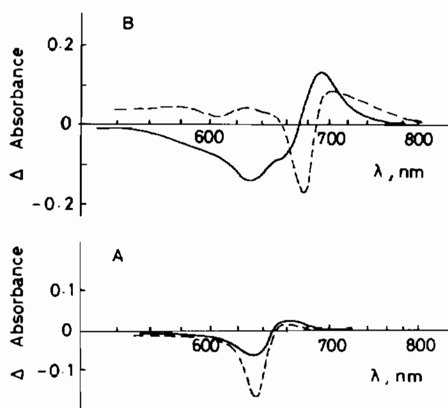

Fig. 9. Difference spectra of (A) Fe(III)L plus heme·heme oxygenase and (B) Co(II)L plus heme·heme oxygenase: immediately after mixing the reagents (——); and after 2h (– – –). Concentrations: Fe(III)L = 7.3×10^{-6} M, Co(II)L = 1.2×10^{-5} M, heme·heme oxygenase = 1.4×10^{-5} M.

band at 680 nm points to Co(II)L binding by the modified protein. However, this reaction is twice as slow and occurs with low efficiency, compared to that with the unmodified protein. These results are indicative of two different Co(II)L binding sites on the protein. One of them, the more active, is the heme-binding histidyl group; the weaker one (which is probably close to the first) has not been identified at present. Identification of this site will be possible after more detailed structural studies, which are in progress. Moreover, no reaction is observed with Fe(III)L, which confirms the suggestion that only the histidyl residue of the protein is a Fe(III)L binding site.

Interaction of Fe(III)L and Co(II)L with the Heme·Heme Oxygenase Complex

Interaction between Fe(III)L and the heme·heme oxygenase complex leads to slow degradation of the phthalocyanine compound, as is demonstrated by the difference spectrum of the appropriate mixture, where a strong negative band of Fe(III)L appears at 635 nm (Fig. 9A). No positive band and no change in the heme absorption region are observed. This result may indicate that formation of the complex between Fe(III)L and heme oxygenase protein by heme displacement from the heme·heme oxygenase complex, does not occur in this system.

The reaction between Co(II)L and heme·heme oxygenase is more complicated. The difference spectrum of the appropriate reaction mixture is shown in Fig. 9B. As in the case of the iron complex, no change is observed in the Soret part of this spectrum, which suggests that heme is not substituted by Co(II)L. Instead, in the region characteristic of the cobalt phthalocyanine complexes, a new positive band arises at 690 nm. At the same time, the intensities of the bands characteristic of free cobalt phthalocyanine complexes at 632 and 675 nm decrease. This points to hybrid complex formation between Co(II)L and the protein. The spectrum changes during the course of the reaction.

Conclusions

The interaction between heme oxygenase protein and iron(III) and cobalt(II) tetrasulfonated phthalocyanines leads to formation of the blue complexes Fe(III)L–heme oxygenase and Co(III)L–heme oxygenase at a molar ratio of 1:1. The new compounds exhibit characteristic absorptions at 650 and 682 nm, respectively. Electrophoresis and molecular weight estimation show both complexes to be monomers.

Free Co(II)L under aerobic conditions binds molecular oxygen to give the oxygen adduct; this reaction is reversible. After its combination with heme oxygenase protein, autoxidation of the cobalt ion takes place. The oxidation state three of the cobalt ion in this complex was confirmed spectroscopically after its oxidation with potassium ferricyanide, as well as by examination of the X-band EPR spectrum.

Iron and cobalt phthalocyanine complexes with heme oxygenase protein are reduced by excess dithionite. In both cases the reduction process involves an intermediate stage where dithionite attacks the complexes at the phthalocyanine ligand edge. At this stage a brown intermediate is formed, which was suggested to be a metal chelate with a reduced phthalocyanine ligand [15]. It is stable under anaerobic conditions. In the presence of oxygen, reoxidation of the phthalocyanine ring occurs. Under these conditions the iron intermediate changes slowly into the ferrous phthalocyanine complex, with a characteristic absorption band at 685 nm, and the cobalt one into the original Co(II)L–heme oxygenase. In the case of the iron complex, dithionite attacks both the phthalocyanine ring and the iron ion.

When ascorbic acid is used as a reducing agent, the reduction process is different in the case of the iron and cobalt complexes. The iron complex, at the first stage of the reduction process, forms an unstable intermediate, which under anaerobic conditions undergoes slow degradation with destruction of the

phthalocyanine ring. In the presence of oxygen, oxygenated Fe(II)L–heme oxygenase is formed. Co(III)L–heme oxygenase under the same conditions is reduced to Co(II)L–heme oxygenase with a characteristic absorption band at 632 nm. It does not change in the presence of oxygen, which may be due to blockade of the sixth coordination place of the cobalt ion resulting from conformational change of the protein or from complex formation with the reducing agent.

Fe(III)L–heme oxygenase does not coordinate detectably with additional ligands such as cyanide and histidine. Likewise, the complex of Fe(III)L with histidine does not react with heme oxygenase protein. This suggests that Fe(III)L is coordinated axially with a histidyl residue of the heme oxygenase protein. This assumption was confirmed by the fact that Fe(III)L does not react with heme oxygenase protein upon its carboxymethylation at the histidine residues. However, Co(III)L–heme oxygenase reacts with histidine to give a complex. Interaction between heme oxygenase protein and the complex of Co(II)L with histidine leads to slow decomposition of the phthalocyanine moiety. In contrast to Fe(III)L, cobalt tetrasulfonated phthalocyanine reacts with carboxymethylated heme oxygenase protein to give a complex, but the efficiency of this reaction is low compared with the unmodified protein. These results are indicative of the existence of two Co(II)L binding sites on the protein: one with higher reactivity involving a heme-binding histidyl group, and a weaker site which is probably close to the first one. Identification of this site will be possible after selective modification of the other amino acid residues.

Iron and cobalt phthalocyanine derivatives are not able to displace heme from the heme·heme oxygenase complex. Interaction of Fe(III)L with heme·heme oxygenase leads to slow degradation of the phthalocyanine complex. Cobalt tetrasulfonated phthalocyanine coordinates with heme oxygenase protein, in spite of its combination with the heme moiety, to give a hybrid complex. This fact once again suggests the existence of the second binding site for Co(II)L.

Interactions between cobalt tetrasulfonated phthalocyanine and heme oxygenase protein are complicated and their full explanation requires further detailed studies, which are in progress.

Heme oxygenase protein complexed with iron and cobalt tetrasulfonated phthalocyanines does not exhibit enzyme activity in oxidative degradation in the presence of the NADPH–cytochrome *c* reductase system in air.

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