Vol. 104, No. 2, 1982 **BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS**
January 29, 1982 Pages 793-798

ENZYMATIC REDUCTION OF SYNTHETIC HEMIN TO HEME AND ITS APPLICATION TO STUDY ON OXYGENATION IN AQUEOUS MEDIUM

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Received December 3, 1981

The enzyme system consisting of glucose-6-phosphate, glucose-6-phosphate dehydrogenase, ferredoxin, ferredoxin-NADP-reductase, and NADP was used to reduce various synthetic iron(III) porphyrins to iron(II) in aqueous buffer (pH7.0) at 25°C. The oxygenation reactions of the thus prepared iron(II) porphyrin complexes were examined and it was found that only the iron(II) picket fence porphyrin-mono(1-lauryl-2-methylimidazole) complex incorporated in liposomes of phosphatidylcholine can form a stable oxygen adduct at 25°C in neutral aqueous medium.

Recently many synthetic hemoglobin models are prepared (1-4) and we have found that the meso-tetra(α,α,α,α-(o-pivalamidophenyl))porphinato iron(II) (picket fence porphyrin iron(II))-mono(1-lauryl-2-methylimidazole) complex incorporated in liposomes can bind oxygen reversibly under semiphysiological conditions (5). Vasilenko et al. reported that the protoheme mono(L-leucyl-L-histidine methyl ester)amide could bind oxygen in aqueous medium having liposomes, but they did measurement without removing excess reductant ($Na_2S_2O_4$) (6). Various organic and inorganic reductants are used to reduce iron(III) porphyrins to iron(II) (7-10). But many troubles were found to remove excess reductant and/or reacted reductant residues (11,12). Here we wish to report the application of the enzyme system, which has been developed by Hayashi et al. (13) to reduce met myoglobin to myoglobin <u>in vitro</u> and found not to interfer the measurement of oxygenation, to the reduction of water soluble and insoluble synthetic hemins to hemes in aqueous medium at 25°C. And thus prepared iron(II) porphyrin complexes were studied in the reaction with oxygen at 25°C.

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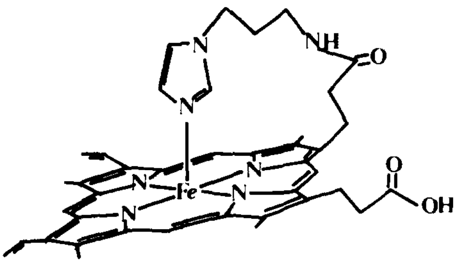
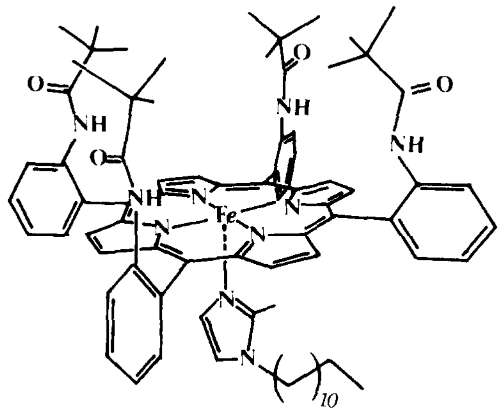


(I)

iron protoporphyrin IX mono(1-imidazolylpropyl)amide



(III)

iron picket fence porphyrin-mono-(1-lauryl-2-methylimidazole) complex

MATERIALS AND METHODS

NADP(sodium salt), glucose-6-phosphate, glucose-6-phosphate-dehydrogenase (typeXV), ferredoxin (typeIII), ferredoxin-NADP-reductase, and catalase (thymol free) were purchased from Sigma. 0.05M Phosphate buffer (pH7.0) was used as solvent for reduction. Iron(III) protoporphyrin mono(1-imidazolylpropyl)amide chloride and iron(III) protoporphyrin mono(L-leucyl-L-histidine methyl ester)amide were prepared according to the literature (14). Iron(III) picket fence porphyrin bromide was synthesized through the literature method (15). Egg yolk phosphatidylcholine was purified (5,16). Poly(1-vinyl-2-methylimidazole) ($\overline{Mn}$=15000) was synthesized by polymerizing the corresponding monomer.

For the reduction, the following mixture was normally used : iron(III) porphyrin=1.8x10^{-7}mole, NADP=0.20 mg, glucose-6-phosphate=1.1 mg, ferredoxin=0.012 mg, ferredoxin-NADP-reductase=0.017 mg, glucose-6-phosphate dehydrogenase=0.0020 mg, catalase=0.01 mg. The total volume of the solution was 3.00 ml. In this mixture, the initial molar ratio of glucose-6-phosphate to iron(III) porphyrin was 2.3. The solutions of the each component were prepared immediately before use and added to the hemin solution. Finally the glucose-6-phosphate dehydrogenase solution was added to start the reaction. The reduction was carried out under N_2 at 25°C. The reactions were followed by measuring visible absorption spectra of an iron porphyrin.

The liposomes having iron porphyrins were prepared by modifying the normal method for preparing normal liposomes (5,17). The solutions having multilayer liposomes were sonicated for 30-60 min at 5°C under N_2.

For studying the reactions of thus prepared iron(II) porphyrin complex solutions with oxygen, we bubbled oxygen for 10-15 sec through solution and then measured visible absorption spectra.

RESULTS AND DISCUSSION

Reduction of Synthetic Iron(III) Porphyrins to Iron(II)

Firstly, we tried to reduce two water soluble iron(III) porphyrin complexes, that is, protohemin mono(1-imidazolylpropyl)amide (I) and protohemin-poly(1-vinyl-2-methylimidazole) complex (II) (18), to iron(II).

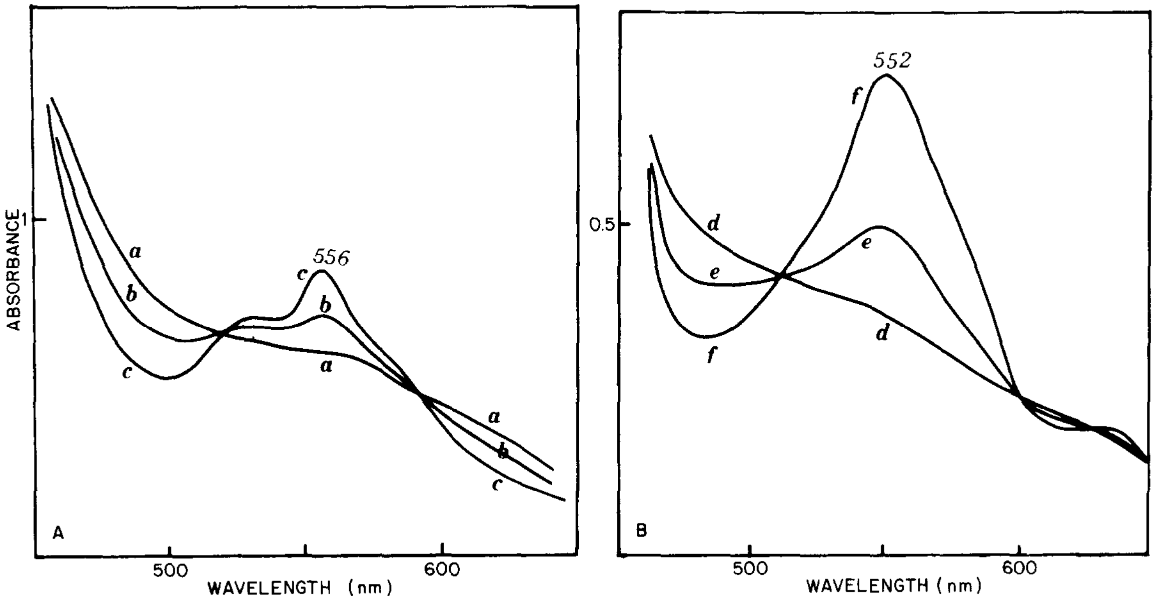


Figure 1. Spectral changes during the enzymatic reduction of protohemin-mono(1-imidazolylpropyl)amide (A) and of the protohemin-poly(1-vinyl-2-methylimidazole) complex (B) in 0.05M phosphate buffer (pH7.0) at 25°C. (A): a (time=0 min), b (15 min), c(80 min); (B): d (0 min), e (2.5 hr), f (7 hr).

The changes of the absorption spectra in the visible region are shown in Figure 1(A) and 1(B), respectively. The absorption bands due to iron(III) (a and d) decreased with increasing new absorption bands (c and f), which could be assigned to the corresponding iron(II) complexes (4,14,18). The time for reducing 95% of iron(III) to iron(II) was 80 min and 400 min for I and II, respectively, in comparison to 80 min for met myoglobin and 200 min for met hemoglobin under the same conditions. When the mixture of an organic solvent such as ethylene glycole or N,N-dimethylformamide and phosphate buffer was used as solvent, the reaction was retarded with increasing the organic solvent content. At lower temperature (-20°C) no reduction was observed.

For the reduction of the water insoluble iron(III) porphyrin complexes, that is, iron(III) picket fence porphyrin-mono(1-lauryl-2-methylimidazole) (III) and protohemin mono(L-leucyl-L-histidine methyl ester)amide (IV), the complexes were solubilized in water by incorporating them in liposomes of phosphatidylcholine. Liposomes were selected because other surfactants

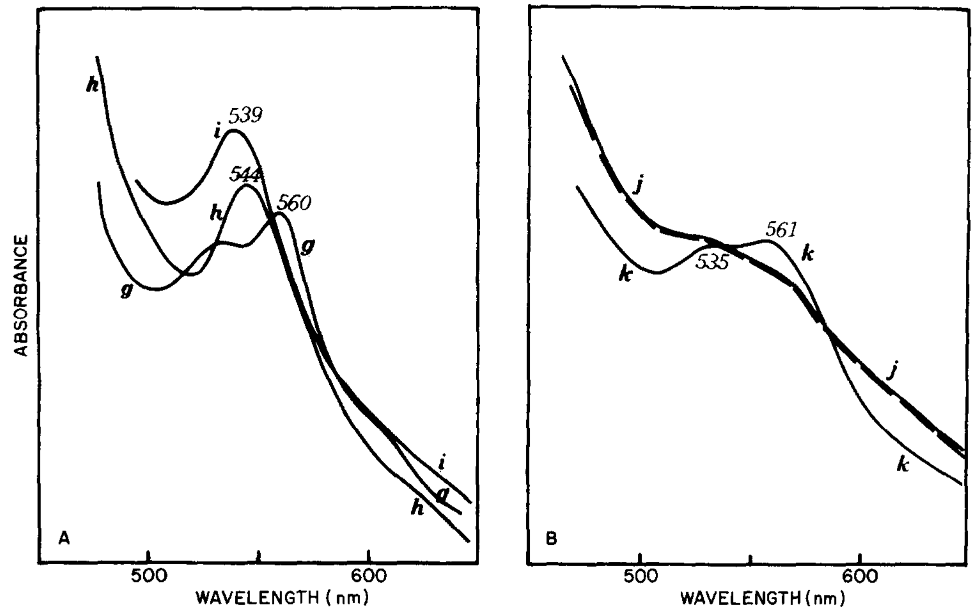


Figure 2. Visible absorption spectra of iron picket fence porphyrin-mono(1-lauryl-2-methylimidazole) complex (A) and iron protoporphyrin-mono(L-leucyl-L-histidine methyl ester)amide (B) in 0.05M phosphate buffer (pH7.0) having 1.3 wt/vol% liposomes of phosphatidylcholine at 25°C. The corresponding iron(II) complexes were prepared enzymatically (see text). (A): g (iron(II)), h (oxygen adduct), i (after bubbling CO through h), (B): j (iron(III)), k (iron(II)), ----- (after bubbling O_2 through k).

such as Triton X-100 and cetyltrimethyl ammonium bromide retarded the redox reaction. In the case of the liposome-iron(III) porphyrin and the enzyme components, the enzyme may be incorporated into liposomes (19). The changes of the visible absorption spectra during the reduction are shown in Figure 2(A) and 2(B) for III and IV, respectively. The rate of reduction in the normal mixture was so slow, so that we added the amount of the reducing system as ten times as much as in the normal mixture except iron(III) porphyrin. The time for reducing 95% of hemin to heme was 150 min for III or IV at 25°C. The absorption maxima for each iron(II) porphyrin complexes are in agreement with those found in the literatures (6,14,20).

Thus, it was found that the enzyme system was useful not only for the reduction of met myoglobin but also for synthetic hemins to hemes in aqueous medium at 25°C.

Reaction of Iron(II) Porphyrin Complexes with Oxygen

When the solutions of the iron(II) porphyrin complexes of I, II or IV were exposed to oxygen at 25°C in 0.05M phosphate buffer (pH7.0), a rapid oxidation was observed without oxygen adduct formation. The protoheme mono(L-leucyl-L-histidine methyl ester) amide (IV) incorporated in liposomes prepared by the enzymatic reduction could not form an oxygen adduct at 25°C, in spite of the report by Vasilenko et al. (6). This may be due to the difference in the reduction method used, because the latters did not remove excess reductant ($Na_2S_2O_4$) before measuring the reaction with oxygen.

In accordance with the report (18), it was confirmed that the protoheme-poly(1-vinyl-2-methylimidazole) complex (II) prepared by the enzymatic reduction could reversibly bind oxygen at -20°C in the phosphate buffer (pH7.0)-ethylene glycole (1/1(vol/vol)) mixture. The absorption maximum at 556 nm was changed reversibly to those of the oxygen adduct at 545 and 577 nm by bubbling oxygen or nitrogen gas.

In the case of the iron(II) picket fence porphyrin-mono(1-lauryl-2-methylimidazole) complex (III) incorporated in liposomes, the formation of the stable oxygen adduct was found at 25°C. As shown in Figure 2(A), the deoxy iron(II) (g̲ , absorption maxima: 560, 533 nm) could be oxygenated (h̲, 544 nm). The oxygenated spectrum h̲ could be reversibly changed by bubbling N_2 for 5-7 min through the solution to the spectrum g̲ . And by bubbling CO through the solution h̲ the spectrum of the iron(II) CO complex (i̲, 539 nm) was observed, indicating no irreversible oxidation. The absorption bands of these complexes are in agreement with those reported in toluene or benzene (20). This result agrees with our previous report (5). The half life time of the oxygen adduct at 25°C in 0.05M phosphate buffer (pH7.0) having 1.3 wt/vol% liposomes of phosphatidylcholine was about 3 hr.

This liposome-enzyme system with reducing ability is interesting for further developement.

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