NONENZYMIC PROTOHEME FORMATION

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The final step in heme biosynthesis, i.e. the chelation of iron into protoporphyrin ring, seems to be catalyzed by the enzyme in liver mitochondria and in the particles from hemolyzed chicken or duck erythrocytes (Labbe, Hubbard and Caughey (1963); Porra and Jones (1963); Yoneyama, Tamai, Yasuda and Yoshikawa (1965)). These authors, however, demonstrated that the nonenzymic protoheme formation from protoporphyrin was negligible compared to the case of the enzymic reaction. On the other hand, Heikel, Lockwood and Rimington (1958) reported that protoheme was not formed nonenzymically from protoporphyrin and ferrous iron but when native globin or teepol was added in order to solubilize the porphyrin there was an appreciable formation of protoheme. Nevertheless, they reported that a high degree of reproducibility was not possible. Granick and Mauzerall (1958) also reported that protoporphyrin and ferrous iron would readily combine without enzymes under appropriate conditions but no supporting data was presented.

In this communication we wish to present a definite evidence of highly reproducible nonenzymic protoheme formation from protoporphyrin and ferrous iron in the presence of sodium dithionite under physiological condition and also describe the presence of some active ferrous iron compound containing sulfur which might be considered to play an important role on the chelation of iron into protoporphyrin ring.

EXPERIMENTAL METHOD The aqueous solution of protoporphyrin was incubated with ferrous iron at 38° C under strictly anaerobic conditions and the protoheme formed was estimated as pyridine hemochromogen. Protoporphyrin (2 mg) was dissolved in 5 ml of 1 M Tris-HCl buffer, pH 8.2, at room temperature, then the solution was centrifuged at 15,000 r.p.m. for 20 min to remove the insoluble protoporphyrin. The supernatant was used as a stock solution and diluted before the use to the desired concentration with the same buffer. The concentration of protoporphyrin was determined spectrophotometrically at 408 mμ in 1 N HCl (ε 0.1 mM = 24.0). The stock solution should be used within three days. Protoporphyrin solution with a different concentration and pH values was prepared in a similar way.

The incubation was carried out anaerobically in Thunberg tube with mechanical shaking at 38° C in the dark. Protoporphyrin (0.15 μmole) in 3 ml of 1 M Tris-HCl buffer, pH 8.2, was placed in the main arm of Thumberg tube, and ferrous ammonium sulfate (5.25 μmoles) and sodium dithionite (7.50 μmoles) dissolved in 1 ml of the same buffer was placed in the side arm. Oxygen was removed by two or three cycles of freezing and thawing under vacuum (< 0.02 mm Hg) with an intermittent flushing with nitrogen. Then the contents of both arms were mixed and incubated by the following two different methods; Method-1, the contents in both arms were mixed at 38° C and incubated; Method-2, the tube was incubated at 38° C for 60 to 90 min, then mixed, followed by incubation.

After the incubation, the reaction was stopped by the addition of 40 ml of the mixture of ethyl acetate-glacial acetic acid (3:1, v/v) and the mixture was transferred to a separatory funnel, washed with distilled water. Protoporphyrin was extracted from ethyl acetate with each 5 ml of 2.5 N HCl until no fluorescence remained in ethyl acetate layer. The remaining ethyl acetate layer containing protoheme was washed with distilled water and then evaporated to dryness under reduced pressure. The protoheme was dissolved

in pyridine-0.1 N NaOH solution (20:80, v/v) and estimated as pyridine hemochromogen.

RESULTS and DISCUSSION It was clearly demonstrated that protoheme was obtained nonenzymically by the mere incubation of protoporphyrin and ferrous ammonium sulfate in the presence of sodium dithionite. The formation of protoheme increased with time and reached to maximum after approximately 30 to 40 minutes (Fig. 1). Maximum formation of protoheme was constantly obtained in a yield of 30 to 35 % over the pH range 7.8 - 9.6 with a high degree of reproducibility under the same conditions (Fig. 1 and 2). Tris-HCl buffer (1 M, pH 8.2) was found to give the highest yield of protoheme, but Tris-HCl buffer (0.1 M, pH 8.2), maleate-NaOH buffer (0.05 M, pH 8.2) or barbital-HCl buffer (0.05 M, pH 8.2) yielded 20 %, 7 % and 3 % of protoheme in one hour, respectively.

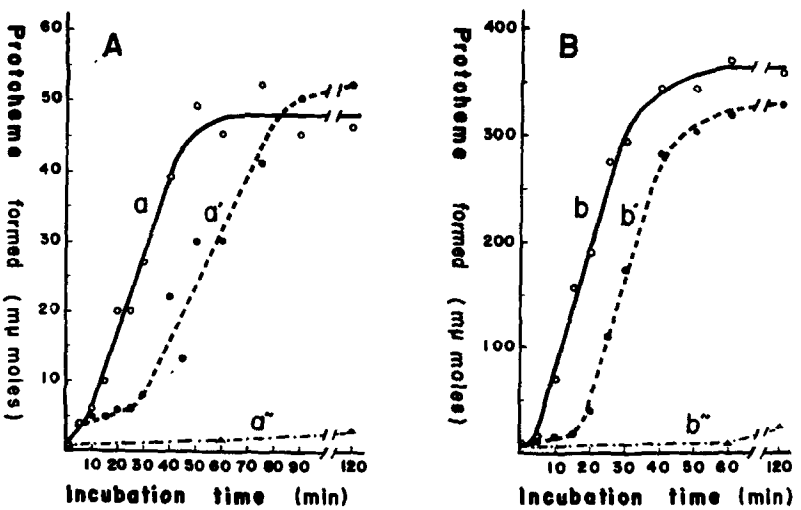

Fig. 1. Non-enzymic Protoheme Formation from Protoporphyrin.
A: Protoheme formation from low concentration of protoporphyrin. 0.15 μmole protoporphyrin, 5.25 μmoles ferrous iron, 7.5 μmoles $Na_2S_2O_4$.
B: Protoheme formation from high concentration of protoporphyrin. 1.0 μmole protoporphyrin, 35 μmoles ferrous iron, 50 μmoles $Na_2S_2O_4$.
Both A and B were incubated anaerobically in 4 ml of 1 M Tris-HCl buffer, pH 8.2.
a,b ——— with preincubation of ferrous iron and $Na_2S_2O_4$(Method-2).
a',b' ----- without preincubation of ferrous iron and $Na_2S_2O_4$ (Method-1).
a'',b'' -·-·- without $Na_2S_2O_4$ in Method-1.

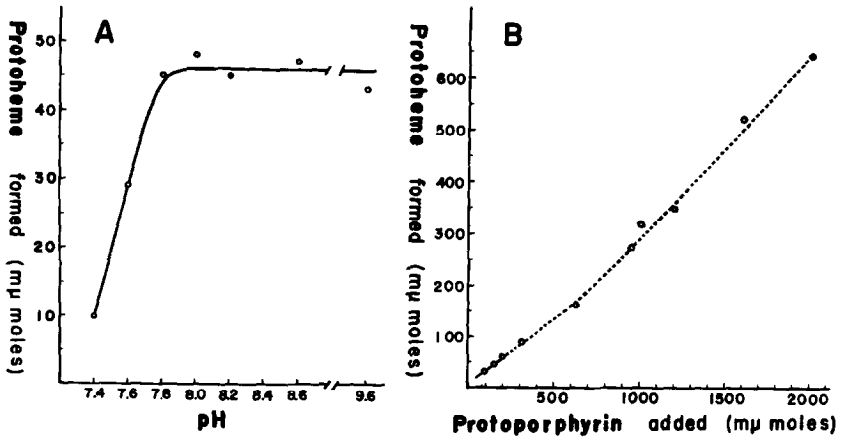

Fig. 2. A: Protoheme formation at 60 minutes incubation versus pH. The low concentration of protoporphyrin was used. B: Protoheme formation at pH 8.2 from various concentrations of protoporphyrin at 60 minutes incubation. ——— with preincubation (Method-2), ----- without preincubation (Method-1).

Three important evidences were found in nonenzymic protoheme formation. 1) The presence of sodium dithionite was essential for the reaction. No protoheme was obtained in the absence of sodium dithionite. However, other reducing agents such as cysteine, ascorbic acid, glutathione, sodium thiosulfate, sodium metabisulfite and sodium borohydride could not replace sodium dithionite. It appears thus that sodium dithionite is not acting as the reducing agent but it is essential in the formation of some complex with iron which is described below. Protoporphyrin was not reduced by sodium dithionite in the dark in this experiment.

2) A prominent formation of black precipitate always took place during the incubation. In the absence of sodium dithionite, neither black precipitate nor protoheme was observed (Fig. 1, A and B, curves a'' and b''). As clearly shown in Fig. 1, A and B, protoheme formation in Method-1 (curves a' and b') appeared with a lag phase (20 to 30 min). After that time, black precipitate appeared with the formation of protoheme. However, on the

preincubation experiment according to Method-2, the black precipitate was formed from the incubation of ferrous iron and sodium dithionite in one arm of Thunberg tube and, after the mixing, protoheme formation occurred promptly without the lag phase as shown in curves a and b.

These observations indicate that the condition when black precipitate was formed seemed to be absolutely essential for protoheme formation. No black precipitate was formed from ferrous iron and sodium thiosulfate or sodium metabisulfite. The black precipitate was found to contain sulfur (presumably labile sulfur) and ferrous iron. On addition of acid on the black precipitate, H_2S was liberated. Sodium borohydride, however, made a black precipitate but this was not incorporated into protoporphyrin. The finely ground ferrous sulfide or reduced iron powder in the presence of sodium dithionite reacted with protoporphyrin to give protoheme.

3) It is an important evidence for heme formation that protoporphyrin is involved by aggregation in such a reactive environment containing iron-sulfur compound. The failure of protoheme formation by the addition of detergents such as Tween 20 or Emasol 4130 could well be due to solubilization removing the porphyrin from the environment mentioned above. These findings seem to oppose the hypothesis that the solubilization of protoporphyrin is essential for iron incorporation. However, in the presence of sodium dodecyl sulfate, protoporphyrin was converted to the heme of which pyridine hemochromogen had an absorption maximum at 552 mμ in place of 557 mμ.

2,4-diformyl- and 2,4-diacetyl deuteroporphyrin behaved similarly to protoporphyrin as regards the absolute requirement of sodium dithionite. However, copro-, hemato-, deutero-, mesoporphyrin and porphyrin c were more readily converted to their corresponding hemes than protoporphyrin even in the absence of sodium dithionite. Therefore, the mechanism of iron incorporation into protoporphyrin may be different from that of other porphyrins and this may be due to the electrophilic character of double bonds in positions 2 and 4 of the porphyrin ring. The amounts of protoheme formed

from protoporphyrin in nonenzymic system were found to be in good agreement with those reported in enzymic system (Labbe, Hubbard and Caughey (1963); Porra and Jones (1963); Yoneyama, Tamai, Yasuda and Yoshikawa (1965)). Further investigation on ferrous sulfur compound found in this experiment seems to be more important than the discussion whether iron incorporation is nonenzymic or enzymic.

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