# SYNTHESIS OF CYTOCHROME C TYPE CORE FROM PROTOPORPHYRINOGEN*

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Sano and Granick (1961) first found that sulfhydryl compounds reacted readily with the vinyl side chains of protoporphyrinogen. Cysteine reacted with protoporphyrinogen to form a porphyrin c-type compound. Sano (1961) and Sano, Nanzyo and Rimington (1964) concluded that porphyrin c-type compound prepared from protoporphyrinogen and cysteine in either acetic acid or neutral condition is a protoporphyrin-dicysteine adduct linked covalently between two sulfhydryl groups of two cysteines and the $\alpha$-C of the vinyl groups. Popper and Tuppy (1963) also confirmed the formation of porphyrin c from protoporphyrinogen and cysteine. Here we wish to report a synthesis of a new hemopeptide of cytochrome c-type namely from protoporphyrinogen and L-cysteinyl-glycyl-glycyl-L-cysteine.

Preparation of protoporphyrinogen. A solution of protoporphyrin IX (22.4 mg, 40 μmoles) dissolved in 30 ml of 0.02 N KOH containing a few drops of alcohol was reduced with 50 g of freshly prepared sodium amalgam (3 %) at 80°C within two minutes under nitrogen in dim red light according to Sano and Granick (1961). The mixture was filtered through

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a sintered glass funnel, washed with 80 ml of distilled water, and the filtrate and washings were adjusted to pH 7.0 with 50 % $H_3PO_4$. The neutralized solution was immediately combined with the following peptide solution.

Preparation of cysteinyl peptide solution. Mercury sulfate of L-cysteinyl-glycyl-glycyl-L-cysteine was prepared from carbobenzoxy-S-benzyl-L-cysteinyl-glycyl-glycyl-S-benzyl-L-cysteine benzylester (Inoue, Sakakibara and Akabori, 1964) by reduction with sodium in liquid ammonia followed by treatment with mercuric sulfate in dilute sulfuric acid. A suspension of peptide mercury sulfate (40 μmoles) in 10 ml of cold 0.1 N HCl was treated with hydrogen sulfide, and the resulting mercuric sulfide was removed by centrifugation. After removing the excess hydrogen sulfide under reduced pressure, the peptide solution was used immediately for the coupling reaction.

Reaction of protoporphyrinogen with L-cysteinyl-glycyl-glycyl-L-cysteine. To the protoporphyrinogen solution was added the cysteinyl-glycyl-glycyl-cysteine solution and the mixture was brought to pH 6.0 with 50 % $H_3PO_4$, diluted up to 120 ml with distilled water, and incubated at 37°C in a shaking apparatus under nitrogen in the dark. After 5 hours, the mixture was poured onto a column of Amberlite CG 50 type 2 (2 x 30 cm) in acid form, followed by washing the column with 1,000 ml of 0.2 M pyridine-acetic acid, pH 5.0, until the effluent became negative in ninhydrin reaction. The coloured material was removed from the top of the column and the porphyrin was extracted from the resin with 30 % pyridine. The extract was adjusted to pH 3.5 with glacial acetic acid and a small amount of contaminated protoporphyrin and hematoporphyrin was removed from this solution by several extractions with the mixture of ether and ethyl acetate (1 : 1 V/V). The aqueous solution was evaporated to dryness under reduced pressure below 40°C. The residue was dissolved in a small amount of the upper phase of a freshly prepared mixture of n-butanol-glacial acetic acid-water (4 : 1 : 5 V/V) and the solution

was poured on the top of a Hyflo Supercel column prepared according to the procedure described by Tuppy and Bodo (1954) and chromatographed with the upper phase liquid. The effluent of the main band was collected and evaporated to dryness under reduced pressure. The yield of protoporphyrin-tetrapeptide was 14.1 mg (15.7 μmoles).

Identification of protoporphyrin-tetrapeptide. Maxima of absorption spectrum of protoporphyrin-tetrapeptide were at 406, 503, 539, 572, 624 mμ in butanol, and 407, 553, 596 mμ in 1 N HCl (Fig. 1).

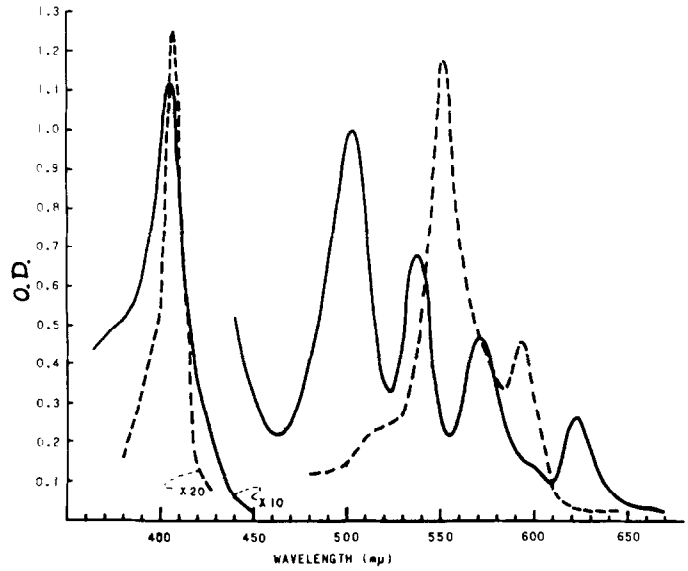


Fig. 1 Absorption spectra of protoporphyrin-tetrapeptide. (——) dissolved in the upper phase of n-butanol-glacial acetic acid-water (4 : 1 : 5 V/V), (---) dissolved in 1 N HCl.

Hematoporphyrin IX was obtained from the protoporphyrin-tetrapeptide by treatment with mercuric sulfate in acetic acid at 80°C for 25 minutes in a yield of 75 %. The mercuric peptide in an aqueous layer was evaporated to dryness under reduced pressure and oxidized with performic acid at room temperature for 20 minutes. After that time the solution was evaporated to dryness under reduced pressure under 30°C. Three ml of 0.1

N HCl was added to the residue and hydrogen sulfide was bubbled thoroughly and the resulting mercuric sulfide was removed by centrifugation. The supernatant was evaporated to dryness under reduced pressure, then hydrolyzed in 5.6 N HCl at 110°C for 20 hours. Amino acids liberated were analyzed on the Hitachi automatic amino acid analyzer (KLA-type 2). The results were shown in Table 1.

Table 1

Amino acid composition of protoporphyrin-tetrapeptide

| protoporphyrin-tetrapeptide (μmoles) | cysteic acid (μmoles) | glycine (μmoles) |
|---|---|---|
| 0.63 | 1.14 | 1.22 |
| 0.53 | 0.86 | 1.04 |

This result justifies the conclusion that the compound is protoporphyrin-tetrapeptide adduct in the ratio of 1 : 1. Hematoporphyrin IX prepared above was converted to diacetyldeuteroporphyrin IX by chromic acid oxidation (Sano, 1961: Sano, Nanzyo and Rimington, 1964). From our previous observation (Sano, 1961: Sano, Nanzyo and Rimington, 1964), these findings support the idea that cysteinyl-glycyl-glycyl-cysteine is covalently linked by two sulfhydryl groups of the tetrapeptide to two vinyl groups of protoporphyrinogen at each $\alpha$-carbon.

Iron incorporation into protoporphyrin-tetrapeptide. Reduced iron powder (0.1 g) was boiled in 5 ml of 20 % acetic acid. The filtrate from this mixture was then added to a solution of protoporphyrin-tetrapeptide (1.0 mg) in 10 ml of 20 % acetic acid and the mixture kept anerobically at 70°C for 20 minutes until fluoresence had become very weak. The mixture was immediately passed through a column of Amberlite CG 50 type 2 (2 x 10 cm) in acid form. The column was washed

with 200 ml of 20 % acetic acid to remove ferrous acetate. The coloured resin was removed from the top of the column and hemopeptide was extracted with upper phase of n-butanol-glacial acetic acid-water. A small amount of protoporphyrin-tetrapeptide remaining with the hemopeptide was removed from the n-butanol layer by extraction with 1 N HCl. The upper layer was washed with distilled water, concentrated to a small amount and then again chromatographed on a Hyflo Supercel column as mentioned in the case of protoporphyrin-tetrapeptide. The main effluent of hemopeptide was collected and evaporated to dryness under reduced pressure. Yield was 55.7 %. The spectrum of pyridine hemochromogen had maxima at 413, 521 and 550 mμ (Fig. 2).

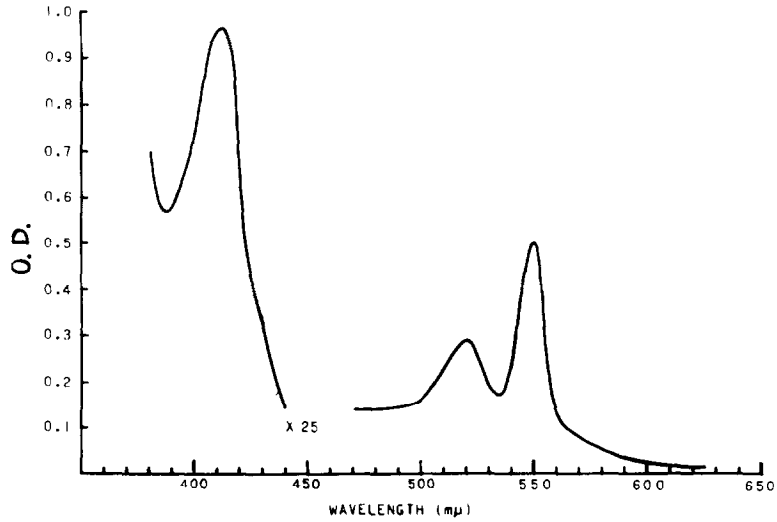


Fig. 2 Absorption spectrum of reduced hemo-tetrapeptide as pyridine hemochromogen in 0.1 N NaOH + 25 % pyridine + $Na_2S_2O_4$.

Beef heart cytochrome c has an amino acid sequence of ...-cysteinyl-alanyl-glutaminyl-cysteinyl-histidyl-... around heme, and, the cysteinyl residues are conjugated with the vinyl groups of heme. In our sample used here, the intracysteinyl amino acids were glycyl-glycine and the addition of cysteinyl-glycyl-glycyl-cysteine to protoporphyrinogen was

found to be as facile as the addition of cysteine itself. It is reasonable to expect that cysteinyl-alanyl-glutaminyl-cysteine will react with protoporphyrinogen in the same way. Synthesis of the natural hemopeptide is now being carried out in our laboratory and we hope to proceed with this stepwise synthesis until a biologically active preparation of cytochrome c will be obtained. Also studies of such a cytochrome c core will lead to various suggestions as to the basic groups in the protein that coordinate with the central iron atom present in heme.

Summary

L-cysteinyl-glycyl-glycyl-L-cysteine has been found to react with protoporphyrinogen to give protoporphyrin-tetrapeptide which has the covalent linkages between the two cysteines and protoporphyrinogen. A new hemopeptide was obtained from the protoporphyrin-tetrapeptide by insertion of iron.

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