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# Formation of Porphyrin *c* and Porphyrin-Protein from Reduced Protoporphyrin

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Cysteine and protoporphyrin reduced with sodium amalgam or titanous chloride have been found to react under mild conditions to give porphyrin *c*.

It has also been shown that reduced protoporphyrin combines covalently with bovine serum albumin, which was used as a model sulfhydryl-containing protein. Proteolytic digestion of the resulting porphyrin-protein yielded peptides containing bound porphyrin.

As shown by Theorell[1] in 1938, the covalent linkages between protein and prosthetic group in cytochrome *c* are due to the addition of sulfhydryl groups of cysteine residues in the protein moiety across the vinyl groups of a protoheme molecule. He succeeded in isolating porphyrin *c*, the compound formally derived from one protoporphyrin and two cysteine molecules, from cytochrome *c* and in synthetizing it by heating protoporphyrin with cysteine in the presence of mineral acid[2]. Other methods [3,4] of preparing this compound have also involved rather harsh conditions.

Sano and Granick[5] observed that when protoporphyrinogen and cysteine in phosphoric acid were illuminated with white light, a porphyrin *c*-type compound was formed, which, however, was not further investigated.

In this laboratory in the course of an investigation of the formation of thioethers from reduced protoporphyrin, the reaction of the latter with cysteine and with bovine serum albumin was studied in detail.

## MATERIALS AND METHODS

For the *preparation of protoporphyrin* the method of Ramsey[6] was used. Pure protoporphyrin was prepared by hydrolysis of recrystallized protoporphyrin dimethylester. Commercial preparations of crystalline bovine serum albumin (Sigma lots A 70B–088, 090) were used without further purification. Pepsin was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, and titanous chloride from Riedel-de Haën AG, Seelze, Hannover, as a 15 % solution in hydrochloric acid. Sephadex G-50 was purchased from Pharmacia, Uppsala, Sweden. The hydrochloric acid used for hydrolysis was distilled over $SnCl_2$.

All spectrophotometric measurements were made in a Beckman Model DU spectrophotometer.

*Reduction of protoporphyrin with sodium amalgam.* 5.5 mg (10 μmoles) of protoporphyrin in 10 ml of 0.1 N $NH_4OH$ was heated at 80°C for 10 min with 25 g of freshly prepared 3 % sodium amalgam. The brownish solution was filtered and adjusted to pH 6.5–7.5. All these operations were carried out in an atmosphere of nitrogen. The neutralized solution was immediately used for further reactions.

*Reaction with cysteine.* 157.5 mg (1 mmole) of L-cysteine hydrochloride in 10 ml of water was adjusted to pH 7, and quickly added to the above prepared solution of reduced protoporphyrin. The solution was thoroughly mixed and allowed to stand at 20°C for 2 h under an atmosphere of nitrogen. The solution was concentrated *in vacuo* to 6 ml and adjusted to pH 1.6, when most of the protoporphyrin, contaminated by lesser amounts of porphyrin *c*, precipitated and was removed by centrifugation. The red supernatant was brought to pH 3.35 and the precipitate, which contained the bulk of porphyrin *c*, was separated by centrifugation and dried (1.7 mg).

*Purification of the crude porphyrin c by partition chromatography.* To 5 g of carefully washed[7] and dried Hyflo Supercel 1.8 g of the lower layer of a freshly prepared mixture of butanol, glacial acetic acid and water (4 : 1 : 5) was added. After thorough mixing, the Hyflo Supercel was suspended in a suitable volume of the upper phase of the solvent system so as to form a slurry, and was poured into a chromatography column 1.2 cm in diameter. After settling, the Hyflo Supercel formed a column 13.2 cm high. On the top of this column was placed 1.7 mg of the above-described precipitate dissolved in the smallest possible amount of the upper phase. The main component formed a narrow, reddish-brown band which moved down the column with an $R_F$ value of approx. 0.8, while a small amount of colored material stayed on the top. The effluent containing the main band was collected and evaporated to dryness under reduced pressure.

*Identification of porphyrin c.* An aliquot of the main fraction dissolved in 1 N HCl showed absorption maxima at 406, 552.5 and 594 mμ (*Ref.* 4, 406, 553 mμ). Paper chromatography was carried out by the method of Eriksen[8]. Descending chromatograms were run with Schleicher and Schüll paper 2043b in a 2,6-lutidine-water system at 21°C under an atmosphere of ammonia. The following $R_F$ values were observed: protoporphyrin 0.66; porphyrin *c*[4] 0.21; and porphyrin *c* obtained above 0.22.

*Reduction of protoporphyrin with titanous chloride and reaction with cysteine.* 2.8 mg (5 μmoles) of protoporphyrin were stirred at 20°C, under an atmosphere of nitrogen, in 30 ml of a 1 : 1 : 1 mixture of 1 N HCl, ethanol and titanous chloride solution. After 30 min 79 mg (0.5 mmole) of L-cysteine hydrochloride in 5 ml water were added and the mixture was stirred for 1.5 h under nitrogen. The dark solution was brought to pH 1.5 and extracted successively with small portions of ethyl acetate and chloroform. The organic solutions were combined, washed with water, and evaporated to dryness *in vacuo*. The reddish-brown residue was subjected to partition chromatography as described above. A broad reddish-brown band with an $R_F$ value of approx. 0.85 was eluted. After evaporation of the solvent the residue was identified as porphyrin *c* by means of paper chromatography and absorption spectroscopy.

*Reaction with serum albumin.* 11.2 mg (20 μmoles) of protoporphyrin were reduced with sodium amalgam and neutralized as described above. To the resulting solution were added 650 mg (10 μmoles) of bovine serum albumin in 10 ml of water. The mixture was allowed to stand for 2 h at 20°C under nitrogen. The solution was then exposed to air, adjusted to pH 9.1, and 4 mg of sodium dodecyl sulfate were added. A gel-filtration column (30 × 4.2 cm) was prepared by suspending Sephadex G-50 in a 0.02 M sodium borate buffer (pH 9.1) containing 1.25 μmoles/ml of sodium dodecyl sulfate. The red solution was placed on the top of this column and the same buffered detergent solution was used for development. Whereas protoporphyrin stayed on the upper fourth of the column as a strong reddish-brown zone, another red band moved down rapidly with an $R_F$ value of approx. 0.95. This was collected in the smallest possible volume of eluent and had $\lambda_{max}$ 538 mμ, while protoporphyrin in the same solvent showed $\lambda_{max}$ 541 mμ. The eluate was concentrated *in vacuo* to 15 ml, mixed with 150 ml of ethanol and allowed to stand for a few h. The pink precipitate was removed by centrifugation, washed with ethanol and ether and dried.

*Enzymatic hydrolysis of the porphyrin-serum albumin adduct and isolation of porphyrin-containing peptides.* The protoporphyrin-serum albumin adduct (625 mg) obtained above was dissolved in 35 ml of 0.01 N HCl. A solution of 24 mg pepsin in 15 ml of 0.01 N HCl was added, the mixture was adjusted to pH 2.0 and incubated at 37°C for 16 h. Then the deep red solution was brought to pH 3.9, centrifuged, and the dark precipitate suspended in water

and dissolved by adjusting to pH 8.0. This procedure was repeated twice, the precipitate being finally taken up in a volume of 0.4 ml.

Talc was purified by the method of Witter and Tuppy[9], suspended in water and poured into a column 14 × 2.4 cm. The dark solution (0.4 ml, pH 8) obtained by dissolving the porphyrin-containing peptides was transferred to the column. The red peptides were adsorbed on the upper part of the column, which was then thoroughly washed with water. The red material was subsequently eluted from the talc with 50 % aqueous ethanol containing 2 % ammonia. This eluate was evaporated to dryness *in vacuo*. The dark residue was subjected to partition chromatography on 15 g of Hyflo Supercel as described above for the crude porphyrin *c*. When the chromatogram was developed with the upper phase of a freshly prepared mixture of butanol, glacial acetic acid and water (4 : 1 : 5) a broad band was observed to move downward with an $R_F$ value of approx. 0.7, while a rather narrow band stayed on the upper part of the column. The effluent containing the faster moving band was collected and evaporated to dryness *in vacuo*. The reddish-brown residue was hydrolyzed in 5.7 N HCl at 105°C for 16 h in a sealed capillary. The hydrolysate was divided into two parts, one of which was oxidized with performic acid; both were subjected to high voltage (1500–1600 V) ionophoresis on paper (Whatman 3MM, 24 × 40) using a pyridine-acetic acid buffer of pH 4.6. No basic amino acids were present. The following acidic amino acids were detected: glutamic, aspartic, and, in the oxidized sample, cysteic acid, the relative amounts being: Glu ⟩⟩ Asp ⟩ Cys-$SO_3H$. The neutral amino acids were eluted from the paper and subjected to descending paper chromatography, using the solvent system of butanol, glacial acetic acid and water (4 : 1 : 5). The following amino acids were detected: leucine, phenylalanine, proline and small amounts of serine and threonine.

## RESULTS AND DISCUSSION

When protoporphyrin was reduced with sodium amalgam, subsequently acidified and illuminated with white light in 1 N $H_3PO_4$ and in the presence of a large excess of cysteine, porphyrin *c* was formed in small quantities. The yield of porphyrin *c* was remarkably increased by carrying out the reaction between pH 6.5 and 7.5. A further increase in the yield of porphyrin *c* was observed when reduced porphyrin and cysteine were allowed to react in the absence of air. The effect of illumination was negligible; the yields of porphyrin *c* did not change significantly when the reaction was performed under exclusion of light. The reaction time was varied between 1 and 3 h, a 2 h period generally being satisfactory.

Bovine serum albumin is known to have approx. 0.65 mole of free –SH groups/molecule[10] and was therefore used as a model for proteins containing available –SH groups. The reaction between serum albumin and protoporphyrin reduced by sodium amalgam was carried out under conditions similar to those in the case of cysteine, but only a slight molar excess of porphyrin over serum albumin was used. In neutral or almost neutral solutions, protoporphyrin is known to be strongly adsorbed on serum albumin[11]. The removal of adsorbed porphyrin from the protein was effected by gel-filtration on columns of Sephadex G-50 in a slightly basic (pH 9.1) borate buffer containing a small amount of sodium dodecyl sulfate detergent.

The reaction between reduced protoporphyrin and serum albumin resulted in a 3 mμ shift to lower wavelengths in the absorption maximum of band III of the reaction product relative to protoporphyrin. The formation of porphyrin *c* was characterized by a 5 mμ shift. The difference between the two changes can be explained by assuming that with excess cysteine both vinyl groups of the porphyrin reacted, while with serum albumin only one of the vinyl groups was involved.

The protein fraction obtained after the gel-filtration of the reaction product of reduced porphyrin and serum albumin contained, beside the porphyrin adduct of serum albumin, large amounts of unreacted protein, but was entirely free of adsorptively bound porphyrin. This mixture was subjected to digestion with pepsin in order to confirm the covalent nature of the protoporphyrin-protein linkage. Porphyrin-containing peptides were obtained and were separated from colorless peptides by adsorption on talc and partition chromatography. The fraction containing the colored peptides was hydrolyzed in 5.7 N hydrochloric acid. High voltage ionophoresis and paper chromatography of the hydrolysate showed the presence of glutamic acid, aspartic acid, cysteine, phenylalanine, leucine, proline, and, in minute quantities, serine and threonine. The six major amino acids are the same amino acids present in the yellow nonapeptide obtained by Witter and Tuppy[9] from serum albumin treated with N-(4-dimethylamino-3,5-dinitrophenyl)-maleimide (DDPM) and subjected to proteolytic digestion. A close examination of the peptide sequence or sequences attached to the porphyrin in the porphyrin-containing peptide fraction has yet to be carried out.

It has not been established which reduction stage of protoporphyrin is the molecular species most readily reacting with sulfhydryl compounds. Sodium amalgam has been shown to reduce porphyrins to porphyrinogens eventually, given sufficient time[12]. In the reductions carried out here, however, relatively short reaction periods were used in order to prevent reduction of the vinyl groups. It is possible that tetrahydro- or even dihydro-protoporphyrin present in the reduction-product may react with sulfhydryl compounds. It can be stated definitely that porphyrin reduced with titanous chloride, which according to Mauzerall[12] is the tetrahydro stage, combines readily with cysteine.

The rather facile reaction of cysteine or an —SH containing protein with reduced protoporphyrin under near-physiological conditions emphasizes the possibility suggested by Sano and Granick[5] that a reaction of the —SH groups of apocytochrome *c* with reduced protoporphyrin may be involved in the formation of cytochrome *c*.

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