PROTEIN-BOUND PORPHYRINS ASSOCIATED WITH PROTOPORPHYRIN BIOSYNTHESIS

R.J. Porra [1] and J.E. Falk

Division of Plant Industry, C.S.I.R.O., Canberra.

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As shown in this paper, protoporphyrinogen IX (PROTOGEN) is formed from coproporphyrinogen III (COPROGEN III) by the oxidative decarboxylation of two propionic acid side-chains to form vinyl groups. We call the enzyme responsible for this conversion "coproporphyrinogen oxidative decarboxylase" (COD). Protoporphyrin IX (PROTO) is subsequently formed by the oxidative removal of six hydrogen atoms from the porphyrinogen nucleus; if an oxidase is involved, we suggest that it be called "protoporphyrinogen oxidase".

COD is a particulate enzyme system (Dresel, 1955; Granick & Mauzerall, 1958; Sano, 1958) and is inhibited by anaerobic conditions (Falk, Dresel & Rimington, 1953; Dresel & Falk, 1956). Presumed intermediates have been detected by Bogorad & Granick (1953) and Falk et al. (1956). COD has been obtained in soluble form from cell particles of Euglena and chicken erythrocytes (Granick & Mauzerall, 1958) and from acetone-dried powders of liver mitochondria (Sano, 1958).

The results reported here provide evidence that an intermediate in the conversion of COPROGEN III to PROTOGEN is, or can become, bound covalently to protein.

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[1] Australian National University Ph.D. student.

METHODS

Enzyme Extract.- Fresh bovine liver mitochondria were prepared by the method of Mahler, Wakil & Boch (1953). An acetone-dried powder was prepared, by the method of Morton (1955), from the mitochondrial paste obtained from the barrel of the Sharples centrifuge. The acetone-dried powder (10 g) was then extracted for 6 min with 50 ml of chilled 0.04 M-potassium phosphate buffer, pH 7.4, in an ice-cooled Servall Omni-mixer, using a 50 ml chamber, operating at 83% of line voltage. Two further extractions (20 ml each) were required to extract all the enzymic activity. The extract was centrifuged at 105,000 x g for 60 min and then dialysed against 10 volumes of the same buffer at 4°C overnight. From 10 g of powder approximately 90 ml of extract were obtained. The protein concentration was approximately 12 mg per ml (Lowry et al. (1951)).

Assay for COD activity.- 0.4 ml of enzyme extract was incubated in air for 30 min at 37°C, in the dark, with 10 mμ moles of COPROGEN III, 50 μ moles of reduced sodium glutathione and 100 μ moles of potassium phosphate in a final volume of 3.9 ml and at pH 7.4. COPROGEN III was prepared from coproporphyrin III (COPRO III) by the sodium amalgam method of Mauzerall and Granick (1958); yield ca. 95%. At the end of the incubations, I_2 was added to oxidize all porphyrinogens present, and excess free I_2 was removed by adding cysteine. Sufficient 11.6 N-HCl was then added to make the final HCl concentration 1.37 N. The protein precipitate was centrifuged down and washed free of soluble porphyrins with 1.37 N-HCl. The supernatant and washings were combined and the optical densities read at 401.5 mμ and 407 mμ. The concentrations of COPRO III and PROTO were then determined by solving two simultaneous equations, where the four constants are the ε_{mM} of COPRO III and PROTO at 401.5 mμ and 407 mμ.

By this method the porphyrinogens and the porphyrins formed from them by autoxidation during incubation are estimated together as the corresponding

porphyrins. The assay, as described, does not distinguish between COD and protoporphyrinogen oxidase activities. Recovery experiments with porphyrins need no I_2 treatment.

RESULTS

Under the stated assay conditions PROTO formation increases linearly with time up to 60 min and, in 60 min incubations, increases linearly with enzyme extract concentration up to 0.8 ml; the activity of extracts varies from batch to batch. PROTO formation was inhibited completely by boiling the enzyme and by anaerobiosis, but was not affected by dialysis.

In short aerobic incubations with COPROGEN III the total recovery of porphyrin was between 50% and 70%. The lost porphyrin was found to be bound to the protein precipitate. Surprisingly, this "protein-bound" porphyrin did not fluoresce in ultra-violet light when suspended in 1 N-HCl. On the addition of 1 N-NaOH so that the pH rose above 2.5, the usual pink fluorescence was observed, and was abolished on reacidifying.

The loss of porphyrin onto the protein occurs with COPROGEN III only. COPRO III, COPRO I, COPROGEN I and PROTO (approx. 10 mμ mole each) were recovered completely after 30 min incubation with the enzyme extract. Complete recoveries of PROTO were obtained also after incubation in the presence of Fe^{++}, demonstrating the absence of ferrochelatase (Nishida & Labbe, 1959; Labbe, 1959).

When COPROGEN III was added to the enzyme extract there was no loss of porphyrin onto the protein at zero-time, or after 30 min incubation in anaerobic conditions, or after 30 min aerobic incubations with boiled enzyme extract. An aerobic enzyme reaction thus appears to be necessary for the formation of the "protein-bound" porphyrin. Since COPROGEN III is recovered completely under these conditions, the binding cannot be due to simple adsorption, or to reactions of COPROGEN III with I_2 or to the subsequent HCl treatment in the presence of protein.

Recovery experiments were carried out with PROTOGEN also. Our best preparations of PROTOGEN, made by reduction of PROTO with $NaBH_4$, showed only 30% reduction, but comparable PROTOGEN concentrations were maintained by reducing three times as much (30 mμ moles) PROTO. On oxidation, at the end of incubation, these high concentrations led to precipitation and loss of PROTO by adsorption onto the protein precipitate. This adsorbed PROTO had similar, but not identical fluorescence properties to the enzymically formed "protein-bound" porphyrin. The adsorbed PROTO could, however, be recovered completely from the protein by extraction with glacial acetic acid.

The "protein-bound" porphyrin cannot be split off by glacial acetic acid or by ethyl acetate-acetic acid, acetone-HCl, 5% w/v sodium dodecyl sulphate, 5% cetyltrimethylammonium bromide or 8 M-urea. Methods which will split this complex and yield ether-soluble porphyrin are: prolonged hydrolysis at 40°C for 60 hours in the dark with either 5 N-HCl or 1 N-NaOH, the silver salt method of Paul (1950), and treatment overnight at room temperature with 50% HBr in glacial acetic acid (Hill & Keilin, 1930). The porphyrin appears therefore to be bound covalently to the protein.

That the "protein-bound" porphyrin was not simply adsorbed PROTO was confirmed by paper chromatography of the porphyrins split from the protein by acid hydrolysis, using the 2:6-lutidine method of Falk et al. (1956). There were two spots: an intense spot corresponding to a tetracarboxylic porphyrin and a very faint spot corresponding to a dicarboxylic porphyrin.

When the acid-precipitated protein with its bound porphyrins was dissolved in equal parts of 1 N-NaOH, pyridine and water, a clear solution was obtained with a Soret absorption peak at 399 mμ. By assuming ε_{mM}=150 the results shown by the unshaded columns in figure 1 were obtained.

As shown in figure 1 the "protein-bound" porphyrins decrease on longer incubation with enzyme extract, while the PROTO increases.

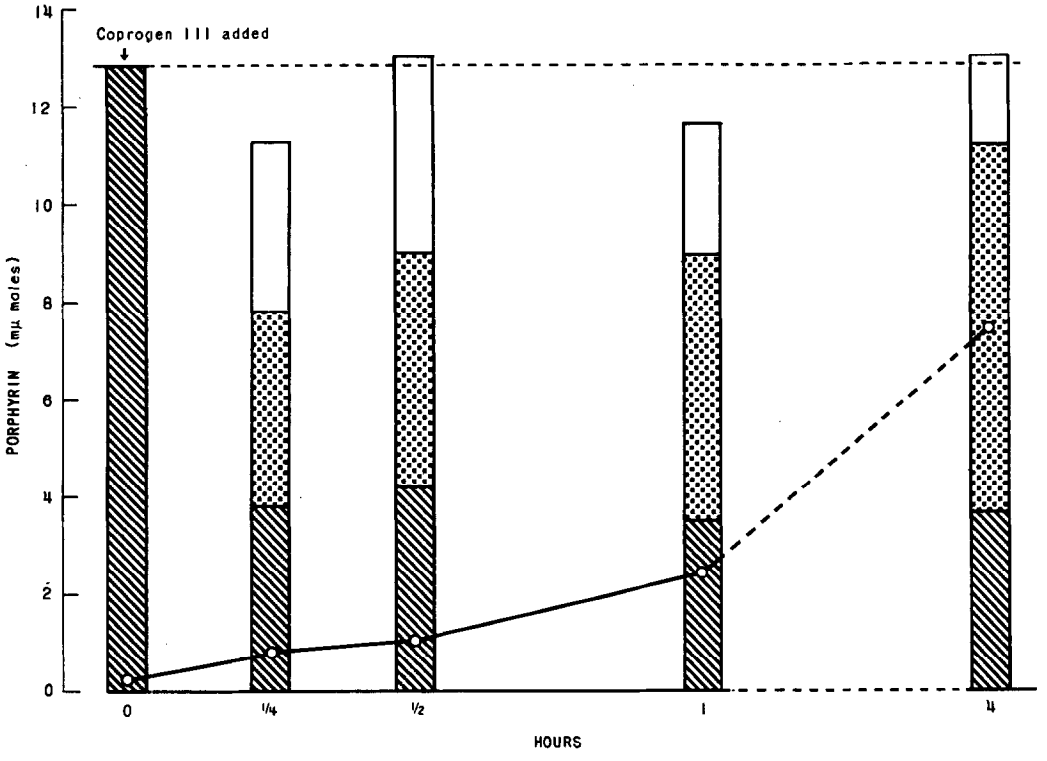

Fig. 1 Conversion of COPROGEN III by enzyme extract. Incubation conditions as in the text, except that 2.8 ml of enzyme extract were used. ▨, COPRO III; ▩, PROTO; □, "protein-bound" porphyrin estimated in alkaline pyridine as described in text. ○, COPROGEN autoxidation in the absence of enzyme extract. Porphyrinogens and porphyrins were estimated together as the corresponding porphyrins (see Text).

DISCUSSION

Evidence which will be presented elsewhere demonstrates that a considerable portion of the enzymically formed PROTO is present, before oxidation with I_2, in the form of PROTOGEN. It seems reasonable, therefore, to assume that all the intermediate steps catalysed by COD involve porphyrinogens rather than porphyrins.

The results show that an intermediate in the conversion of COPROGEN III to PROTOGEN becomes bound to protein. While neither COPROGEN III nor any

of the related compounds added became bound artefactually under the same conditions, it is possible that the true intermediate tetrapyrrole might do so. Alternatively the true intermediate may be a protein-porphyrinogen complex. The porphyrins obtained from these complexes by acid and alkaline hydrolysis have been studied and their properties will be reported elsewhere.

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