Definitive Identification of Enzymatically Formed Zinc Protoporphyrin*

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ABSTRACT: The synthesis of zinc protoporphyrin by an enzyme present in chromatophores of *Rhodopseudomonas spheroides* has been proved by demonstrating stoichiometric amounts of zinc by atomic absorption spectroscopy in enzymatically produced metalloprotoporphyrin. The analytical method provides the operational means of identifying the presence of naturally occurring zinc porphyrin.

Chromatophores from *Rhodopseudomonas spheroides* and mitochondria from a number of animal tissues catalyze the formation of zinc protoporphyrin from Zn^{2+} ions and protoporphyrin (Neuberger and Tait, 1964). The rate of Zn^{2+} incorporation in an aqueous-ether emulsion was found to be greater than in a completely aqueous system. While the amount of zinc protoporphyrin formed was calculated from the absorption of the extracted porphyrins in the Soret region of the spectrum it must be recognized that zinc, calcium, and magnesium protoporphyrins all have similar spectral properties, and cannot be distinguished by this method alone. Hence the identification of the product as zinc protoporphyrin depended on the correlation of spectral with isotope incorporation data. Although in the absence of Zn^{2+} or when Zn^{2+} was replaced by Ca^{2+} or Mg^{2+} there were no appreciable increases in absorbance denoting the formation of these metal protoporphyrins, it is conceivable, in principle, that Ca^{2+} or Mg^{2+} present in the crude enzyme sources might form their respective protoporphyrins even in the presence of Zn^{2+}. Experiments with $^{65}Zn^{2+}$ showed that the specific activity of the metal protoporphyrin isolated was considerably lower than that of the $^{65}Zn^{2+}$ added (Neuberger and Tait, 1964). This, in turn, could be owing to the formation of a metal protoporphyrin complex other than zinc protoporphyrin under these experimental conditions or alternatively to the contamination of the reaction mixture and reagents with Zn^{2+}.

The present experiments were performed to demonstrate the formation of zinc protoporphyrin by measuring the stoichiometry of stable zinc in the enzymatically synthesized metal porphyrin, thereby eliminating uncertainty. Hence the *total* amount of zinc incorporated into protoporphyrin has been determined directly by atomic absorption spectroscopy and compared with the amount of metal protoporphyrin formed as measured spectrophotometrically, providing the only operationally satisfactory means of establishing the existence of zinc protoporphyrin with certainty. Since zinc protoporphyrin has not been demonstrated to exist in nature, the results of this study provide the means of exploring the possibility that it does occur as a constituent of cells.

Methods and Materials

Chromatophores prepared from *R. spheroides* as described by Gibson *et al.* (1963) were used as the enzyme source. Protoporphyrin was prepared from blood as described by Grinstein (1947). Ether was dried over Na_2SO_4 before use. All glassware was cleaned in acid before use, to ensure against metal contamination (Thiers, 1957). Enzyme assays were performed as follows: Tris, *p*H 8.4 (100 μmoles), protoporphyrin (54 mμmoles), $ZnSO_4$ (50 mμmoles), and chromatophores were mixed in a volume of 0.5 ml of water. Ether (0.2 ml) was added and after thorough mixing the tubes were incubated at 37°. After incubation the porphyrins were extracted as described by Neuberger and Tait (1964). The final ether solution of porphyrins was washed with ion-free water and aliquots were taken for measurement of metal protoporphyrin (Neuberger and Tait, 1964) and for estimation of zinc by atomic absorption (Fuwa *et al.*, 1964).

Results

In one set of experiments different amounts of chromatophores were incubated with 50 mμmoles of Zn^{2+} for the same period of time (Figure 1A). In another set this same concentration of zinc and identical aliquots of chromatophores were incubated for different time intervals (Figure 1B). In both instances the resultant metal protoporphyrin was isolated and its zinc content and absorbance were measured. Enzymatic activity as

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TABLE I: Zinc Chelatase Activity of Chromatophores.[a]

Experiment[b]	Metal Protoporphyrin[c] (mμmoles/aliquot)	Zinc[d] (mμatoms/aliquot)
1	13.3	9.7
2	12.5	14.0
3	11.9	11.9
4	9.9	10.0
5	11.4	9.5
6	11.6	12.0
Mean	11.8	11.2
Standard deviation	±1.2	±1.76
Coefficient of variation	10%	15%

[a] Comparison of metal porphyrin produced with zinc incorporated into protoporphyrin. [b] In all experiments 2.28 mg of chromatophore protein was incubated for 60 minutes. [c] Measured spectrophotometrically. [d] By atomic absorption.

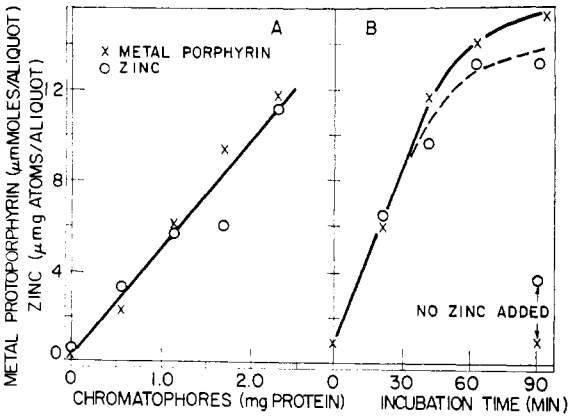

FIGURE 1: Enzymatic incorporation of zinc into protoporphyrin measured by the zinc protoporphyrin spectrum and by estimation of zinc using atomic absorption spectroscopy. (A) Incorporation as a function of enzyme concentration (chromatophores); time = 1 hour throughout. (B) Incorporation as a function of time. Controls incubated for 90 minutes without added zinc are shown. Enzyme = 2.15 mg chromatophore protein throughout.

measured by either method is virtually the same (Figure 1A, B). The results of six identical experiments are also closely similar (Table I). These data show the excellent repeatability of measuring zinc protoporphyrin by determination of absorbance and of metal content. Moreover, the results of the two independent procedures correspond remarkably well. It may be concluded, therefore, that under these assay conditions zinc protoporphyrin is the only metal porphyrin formed by chromatophores of *R. spheroides*.

Discussion

Neuberger and Tait (1964) have suggested that the enzyme catalyzing the formation of zinc protoporphyrin differs from another enzyme, present in the same source, which is responsible for the formation of heme. More recent experiments suggest however (A. Neuberger and G. Tait, unpublished work) that both of these syntheses are catalyzed by the same enzyme. Using isolated chromatophores or mitochondria under standard conditions, zinc protoporphyrin is formed as readily as heme. Rat liver mitochondria contain much more zinc than iron (Thiers and Vallee, 1957) and on this basis one might expect that zinc would be incorporated into protoporphyrin *in vivo*, though, in fact, zinc protoporphyrin has not been reported to occur in nature. If, indeed, none can be shown to exist it must be assumed that there is a mechanism which prevents zinc incorporation.

The nature of such a mechanism can only be speculated upon; however it should be noted that reduced glutathione or cysteine is required for the incorporation of iron into protoporphyrin in the absence of another reducing agent *in vitro*. However, both inhibit zinc incorporation (Neuberger and Tait, 1964). Such results are, of course, in keeping with the chemical characteristics of the two metals: GSH[1] serves to keep iron in the reduced state, a prerequisite for its enzymatic incorporation. Simultaneously GSH would be expected to inhibit zinc incorporation owing to the high stability constant of its zinc complexes, a specific example of the general preference of the zinc atom for the formation of complexes with sulfur ligands (Vallee *et al.*, 1961). Since ascorbic acid does not prevent zinc incorporation, reduction per se can be excluded as a cause of inhibition of zinc incorporation. GSH, which is apparently required to maintain hemoglobin in the reduced state in mature red cells, may serve a similar function in cells actively synthesizing heme (Stokinger and Mountain, 1963).

There are no known biologically active zinc porphyrins such as heme which contain iron, porphyrin derivatives such as chlorophyll containing magnesium, or compounds having a similar ring structure such as cobalamine which contain cobalt. Zinc uro- and coproporphyrins have been isolated from urine and bacterial culture media (Coulter and Stone, 1938; Kapp, 1939; Watson and Schwartz, 1941; Watson and Larson, 1947), though it is probable that they form secondarily by nonenzymatic means subsequent to their elimination (Neuberger and Tait, 1964). We have not been able to find evidence that a zinc protoporphyrin has been isolated from cells. In view of the similarity

[1] Abbreviations used in this work: GSH, reduced glutathione.

of the absorption spectra of Zn^{2+}, Ca^{2+}, and Mg^{2+} protoporphyrin, the joint determination of zinc and spectrophotometric characterization of the product as here presented provide the means of exploring this possibility. The existence of a zinc chelatase activity implies that under some circumstances zinc protoporphyrin might be formed. Indeed, the competition between zinc and iron for the same incorporating enzyme (Neuberger and Tait, 1964) could in fact result in an imbalance of the orderly formation of iron protoporphyrin with presumably deleterious effects on the organism.

Magnesium protoporphyrin is a precursor of chlorophyll and of bacteriochlorophyll (Granick, 1961), but it has not as yet proved possible to find an enzyme which catalyzes the synthesis of magnesium protoporphyrin in a cell-free system. It was thought possible that zinc protoporphyrin might be an intermediate in this process, being synthesized first, to be followed by an exchange of magnesium for zinc. However, attempts to demonstrate such a mechanism in a cell-free system have been unsuccessful (Neuberger and Tait, 1964). Furthermore, while zinc deficiency markedly affects the metabolism of *Euglena gracilis*, the chlorophyll content of the zinc deficient cells is unchanged (Wacker, 1962).

Further work is required in order to test the hypothesis that the ability of this enzyme to incorporate zinc into protoporphyrin is one of its normal functions and that zinc protoporphyrins might be normal constituents of biological systems.

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