Porphyrin Specificity of Ferro:protoporphyrin Chelatase from Rat Liver*

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To aid in understanding the reaction mechanism of ferro:protoporphyrin chelatase prepared from rat liver, an investigation of the porphyrin specificity was carried out. The results showed that the vinyl groups in the 2 and 4 positions, as in protoporphyrin, were not essential for the porphyrin to be utilized as a substrate by the enzyme. Deuteroporphyrin and mesoporphyrin were converted to their corresponding hemins at three times and one and one-half times the rate of protoporphyrin, respectively. The introduction of electron-withdrawing substituents, as in 2,4-dibromo-deuteroporphyrin and 2,4-diacetyldeuteroporphyrin, or of a bulky propionic acid group, as in coproporphyrin, diminished or completely prevented utilization of the porphyrin. Free carboxyl groups on the propionic acid side-chains in positions 6 and 7 were essential for the porphyrin to serve as substrate. In chelating iron, it was unlikely that the porphyrins were first converted to a reduced state such as a porphyrinogen.

The last reaction in heme biosynthesis, that is, the chelation of iron by protoporphyrin, is catalyzed by an enzyme referred to here as ferro:protoporphyrin chelatase. This enzyme has been prepared from rat liver mitochondria, and a number of its properties have been described (Minakami, 1958; Nishida and Labbe, 1959; Labbe, 1959; Labbe and Hubbard, 1960, 1961). The enzyme is activated by several reducing substances (for example, reduced glutathione, ascorbic acid), but no essential co-factors have as yet been found. Sulfhydryl groups are required for activity, and evidence obtained with inhibitors indicates that these groups may be involved with porphyrin binding to the enzyme. A high degree of specificity for the metal is shown. Besides iron, cobalt is the only other metal which can be utilized to any significant extent by the enzyme. Apparently these metals are utilized as the ferrous or cobaltous ion and indiscriminately *via* the same enzymatic mechanism. To aid in further understanding the mode of action of the enzyme, a porphyrin specificity study has been carried out and is described.

EXPERIMENTAL

Ferro:protoporphyrin chelatase was prepared from rat liver mitochondria as described previously (Labbe and Hubbard, 1960). The enzyme was extracted from thoroughly washed mitochondria with either 0.1 M Tris buffer, *p*H 8.2, or 0.1 M $KHCO_3$ containing up to 10 mg/ml of Tween-20. Repeated attempts at purification have met with little success, and therefore the enzyme was not purified further for the following studies. Enzymatic activity had been assayed previously by measuring either Fe^{59} uptake into hemin, which was isolated with carrier, or protoporphyrin remaining after deproteinization. However, to facilitate the evaluation of various types of porphyrins, a new assay was developed. A reaction mixture consisting of 2–5 mμmoles porphyrin, 15 mμmoles $FeSO_4$, 100 μmoles Tris buffer, 40 μmoles glutathione, 4 mg Tween-20, 0.20 ml enzyme (about 1 mg protein), and water to 1.00 ml was incubated in air at 37°. The rate of porphyrin disappearance was measured at its Soret peak during the course of the reaction. The ferrous porphyrin (heme) was the product of the enzymatic reaction; however, this heme rapidly oxidized to the ferric porphyrin (hemin) under the experimental conditions (Labbe and Hubbard, 1961). Although a perfectly linear reaction rate could not be obtained, the initial rate was sufficiently linear to be useful in measuring the utilization rates of the various porphyrins. The ϵ_{mM} values given in Table I were used in calculating the rates of utilization of the different porphyrins. A correction for hemin absorption was not introduced. The ratio of hemin-porphyrin absorption was judged from the spectra to be approximately the same in each reaction, and therefore the relative reaction rates should not be altered significantly by omission of this correction. In a typical assay protoporphyrin was utilized at the rate of 3 mμmoles per hour. Spectra which show the simultaneous protoporphyrin disappearance and heme formation are illustrated elsewhere (Labbe and Hubbard, 1961). In the absence of enzyme there was no observable chelation by any of the porphyrins under the assay conditions.

Protoporphyrin was prepared from hemin by refluxing with formic acid containing iron powder (Shemin, 1957). Uroporphyrin III and coproporphyrin III were isolated from the urine of a patient with acute intermittent porphyria. Hematoporphyrin IX and the azide, hydrazide, and urethane derivatives were generous gifts of Dr. Samuel Schwartz, Department of Medicine, University of Minnesota. Hematoporphyrin was also obtained from Mann Research Laboratories, New York. Deuteroporphyrin IX, mesoporphyrin IX, and the dibromo-, diacetyl-, and bis(2-carboxycyclopropyl)-deuteroporphyrins IX were in each case prepared by hydrolysis of the respective analytically pure esters in 25% aqueous hydrochloric acid. The preparation of these compounds and their corresponding esters, including the dimethyl and diethyl esters of protoporphyrin IX, will be described separately.[1] Porphyrinogens were prepared by reducing porphyrins either with potassium borohydride (Nishida and Labbe, 1959) or with sodium amalgam (Mauzerall and Granick, 1958). The porphyrins with free carboxyl groups were dissolved in 0.15 M sodium bicarbonate containing up to 1 mg/ml Tween-20. These solutions were diluted before use in 0.1 M Tris buffer, *p*H 8.2. The several porphyrins lacking free carboxyl groups were dissolved in propylene

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[1] W. S. Caughey and co-workers, manuscripts in preparation.

glycol or triturated in Tween-20 and then diluted with Tris buffer. Concentrations of propylene glycol to 0.24 M and of Tween-20 to 10 mg/ml did not interfere with the enzymatic reaction.

Results

The conversion of porphyrins to their corresponding hemins was followed by measuring the rate of decrease in absorbtivity at the Soret peak of the porphyrin. Reactions were also followed by spectral scanning of the reaction between 350 and 500 mμ periodically on a ratio-recording spectrophotometer (Beckman DK-2). When all of the porphyrin had been utilized, solid sodium hydrosulfite was added to reduce the hemins and locate the heme-protein peaks. The absorption peaks before and after incubation and on reduction with hydrosulfite are given in Table I. These data indicate that the porphyrins were converted to a hemin corresponding to the porphyrin used as substrate and not to protohemin. In Figure 1 are illustrated the spectra of protoporphyrin, of hemin after the incubation period, and of heme formed on reduction with hydrosulfite. These are typical of the spectra obtained for the other porphyrins and their iron chelates.

Table I

Spectroscopic Properties of Reactants and Products

Hemin was the final reaction product under the incubation conditions (Labbe and Hubbard, 1961). Sodium hydrosulfite was added to convert this to heme. ϵ_{mM} was determined in 0.1 M Tris, *p*H 8.2, containing 1 mg Tween-20 per ml.

Porphyrin Substrate	Wave Length at Soret Peak (mμ)			Extinction Coefficient Porphyrin ϵ_{mM}
	Porphyrin	Hemin	Heme	
Proto-	401	403	422	504
Deutero-	394	408	410	588
Meso-	398	407	410	718
Hemato-	390	394	400	1250
2,4-Dibromo-deutero-	396	412	412	423

Table II lists the relative rates of utilization of several porphyrins. Deutero- was utilized at three times and meso- at one and one-half times the rate of protoporphyrin. 2,4 - Dibromodeuteroporphyrin was converted to hemin at 60% the rate of proto-. Hemato- utilization was one-third that of proto-, but this may not be a true value since the crystalline hemato- was only about 85% pure.[2] Hematoporphyrin obtained from both sources behaved similarly. Of the porphyrins examined, hemato- would be most likely to form protohemin by passing through proto-. Absorption peaks shown in Table I indicate that this was not the case but rather that hematohemin was the reaction product.

Porphyrins that were altered only in the 2 and 4 positions included also copro-, 2,4-diacetyldeutero-, and 2,4-bis(2-carboxycyclopropyl)deutero-. None of these porphyrins was utilized by the enzyme. As would be predicted, uro- likewise was not converted to hemin. The five porphyrins with blocked carboxyl groups on the propionic acid side-chains in positions 6 and 7 were not utilized when observed by spectrophotometric assay. Protoprophyrin dimethyl ester was re-examined with Fe^{59} uptake used as an assay. In this case the proto- ester incorporated up to 10% of the isotope as compared with the same amount of hydrolyzed ester.

[2] Personal communication, Dr. Samuel Schwartz.

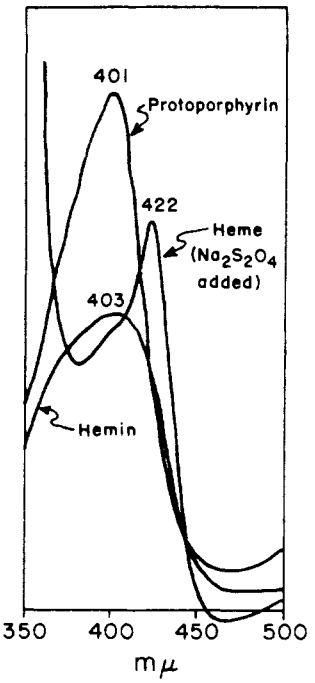

Fig. 1.—Spectra of protoporphyrin before incubation, hemin formed during incubation, and heme produced by further addition of sodium hydrosulfite, all in incubation medium.

Table II

Porphyrin Substrates

Porphyrin	Groups Altered	Relative Reaction Rate	pK_a[a]
Deutero-	Vinyl	290	5.63[b]
Meso-	Vinyl	150	5.94[b]
Proto-	None	100	4.89[b]
2,4-Dibromodeutero-	Vinyl	60	3.0[c]
Hemato-	Vinyl	30(?)	
Copro-	Vinyl	0	5.58[b]
2,4-Diacetyldeutero-	Vinyl	0	3.50[b]
2,4-Bis(2-carboxycyclopropyl) deutero-	Vinyl	0	4.8[c]
Uro-	Vinyl and methyl	0	
Proto- dimethyl ester	Carboxyl	0	
Proto- diethyl ester	Carboxyl	0	
Hemato- diazide	Carboxyl	0	
Hemato- dihydrazide	Carboxyl	0	
Hemato- diurethane	Carboxyl	0	
Porphyrinogens	Porphyrin rings	0(?)	

[a] These data pertain to the equilibria between neutral porphyrins and monocations in sodium dodecyl sulfate. [b] At 20° (Phillips, 1960). [c] At 25° (J. N. Phillips and W. S. Caughey, unpublished observations).

An earlier report indicated that protoporphyrinogen was utilized at a much slower rate than protoporphyrin (Nishida and Labbe, 1959). In the current investigation, reduction products of proto-, meso-, and hemato- were prepared, and Fe^{59} incorporation was used as an enzyme assay to observe their utilization. However, because of the difficulty in obtaining reproducible preparations of the porphyrinogens, conclusive results could not be obtained. Nevertheless, the reoxidized porphyrinogen consistently took up more Fe^{59} than did the porphyrinogen itself. As another approach to

the question of reduced porphyrins being involved in iron chelation, proto- was incubated in an assay medium containing 64% deuterium oxide. The rates of proto-conversion to hemin were actually identical whether the reaction was carried out in water or in D_2O, a finding at least compatible with the lack of formation of a porphyrinogen.

Of the several porphyrins which were not utilized for hemin formation, none altered the rate of proto-utilization when both porphyrins were present in the same reaction mixture. It would have been interesting to examine some porphyrins of the type I isomer in which the propionate side-chain on position 7 would be transposed to position 8. However, none of the porphyrins which served as substrate was available as the type I isomer.

Discussion

It is evident that vinyl groups are not essential for a porphyrin to serve as a substrate for ferro:protoporphyrin chelatase. On the other hand, free propionic acid groups do appear to be necessary, since in no case where these groups were blocked did the compound serve as an effective substrate. The slight utilization of proto-ester could be an artifact for two reasons. First, there was a possibility of some hydrolysis during incubation. Second, some nonenzymatic Fe^{59} chelation undoubtedly occurred during hemin isolation. The possibility that the ester could have been contaminated with unesterified porphyrin to the extent of 10% is precluded by the chromatographic methods used in its preparation.

The effect of porphyrin ring substituents on utilization by the enzyme can be ascribed to electronic and/or steric effects. The electron-withdrawing or electron-donating character of peripheral substituents affects the basicity of the porphyrin nitrogen (Phillips, 1960; Caughey *et al.*, 1962) and can reasonably be expected also to influence the rate of iron incorporation. The pK_3 values, where known, are included in Table II. Among those compounds utilized by chelatase, the reaction rate in general decreases with decreasing pK_3. However, the relative rates for deutero- and meso- do not follow such a correlation. This could be rationalized in terms of an adverse steric effect of the ethyl groups of meso- compared with the smaller 2,4-hydrogens of deutero-. The lack of utilization of copro-, uro-, 2,4-diacetyldeutero-, and 2,4-bis(2-carboxycyclopropyl)deutero- can be attributed to steric effects which interfere with binding to the enzyme.

Blocking the propionic acid carboxyl groups may also have resulted in an inability of the porphyrin to attach to the enzyme. Another explanation, particularly for the proto- esters, might lie in their being so water-insoluble as to remain within a Tween-20 micelle, and therefore unavailable to the enzyme. The fact that some of these porphyrins were dissolved in propylene glycol and still were not utilized in this form might obviate the latter explanation.

Oyama *et al.* (1961) have shown that the duck erythrocyte enzyme, like rat liver chelatase, converts proto- to heme stoichiometrically. Using different experimental procedures, Yoneyama *et al.* (1962) have investigated the porphyrin specificity of the avian erythrocyte enzyme. With only one exception, hemato- being utilized to a greater extent than meso-, the results agree well with those described for rat liver chelatase.

References

Caughey, W. S., Deal, R. M., McLees, B. D., and Alben, J. O. (1962), *J. Am. Chem. Soc. 84*, 1735.

Labbe, R. F. (1959), *Biochim. Biophys. Acta 31*, 589.

Labbe, R. F., and Hubbard, N. (1960), *Biochim. Biophys. Acta 41*, 185; (1961), *ibid. 52*, 130.

Mauzerall, D., and Granick, S. (1958), *J. Biol. Chem. 232*, 1141.

Minakami, S. (1958), *J. Biochem. (Japan) 45*, 833.

Nishida, G., and Labbe, R. F. (1959), *Biochim. Biophys. Acta 31*, 519.

Oyama, H., Sugita, Y., Yoneyama, Y., and Yoshikawa, H. (1961), *Biochim. Biophys. Acta 47*, 413.

Phillips, J. N. (1960), *Rev. Pure Appl. Chem. 10*, 35.

Shemin, D. (1957), *in* Methods in Enzymology, Colowick, S. P., and Kaplan, N. O., editors, New York, Academic Press, Inc.

Yoneyama, Y., Oyama, H., Sugita, Y., and Yoshikawa, H. (1962), *Biochim. Biophys. Acta 62*, 261.