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DIFFERENTIAL INHIBITION OF HEPATIC FERROCHELATASE BY THE ISOMERS OF N-ETHYLPROTOPORPHYRIN IX

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Summary: The four isomers of N-ethylprotoporphyrin IX have been synthesized. The two isomers with the N-ethyl group on pyrrole rings A or B inhibit rat liver ferrochelatase as effectively as the corresponding N-methyl analogues, whereas those with the N-ethyl moiety on rings C or D are 30-100 times less effective. The ability of N-alkyl porphyrins to inhibit ferrochelatase thus depends not only on the size of the N-alkyl group but also on its precise location on the porphyrin face.

A green pigment isolated from mice treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) has been found to be a highly effective inhibitor of mitochondrial ferrochelatase (E.C. 4.99.1.1), the enzyme that inserts an iron atom into protoporphyrin IX in the final step of heme biosynthesis (1,2). The analogous green pigment formed in DDC-treated rats has been resolved in one of our laboratories into four components (3). The major component has been unambiguously identified as the isomer of N-methylprotoporphyrin IX in which the N-methyl group is on pyrrole ring A (3,4). Evidence has also been obtained which suggests that the three minor or trace components of the pigment are the other three isomers of N-methylprotoporphyrin IX (3). The four individual isomers of this porphyrin, obtained by chemical synthesis (4), have been shown to be equally potent inhibitors of the ferrochelatase enzymes from cultured chick embryo liver cells and from rat liver (5). The equal potency of the isomers suggests that they bind in a relatively nondiscriminating site because

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the isomers differ not only in the location of the methyl on the porphyrin face but also in the region of the molecule which is tilted out of the porphyrin plane by the N-alkyl group (4,6,7).

A restrictive binding site is suggested, in contrast, by the report that an unresolved mixture of N-ethylmesoporphyrin isomers inhibits ferrochelatase much more poorly than a mixture of the N-methyl analogues (8). Support for this view is provided by the finding that the N-alkylated porphyrins formed in the suicidal interaction of cytochrome P-450 with ethylene (9-12), ethinylcyclohexanol (13), and 2-isopropyl-4-pentenamide (14-17) are also, at best, weak inhibitors of ferrochelatase (8,18). These results have given rise to the conclusion that the binding site of ferrochelatase is too congested to accommodate N-alkyl groups larger than a methyl (8). Two ambiguities, however, compromise the validity of this conclusion. The first is that the detailed structure of the three biologically obtained porphyrins remains unknown except for our recent characterization of the ethylene-derived porphyrin (12), although we have established the stoichiometry of both the ethinylcyclohexanol (13) and 2-isopropyl-4-pentenamide (16) adducts by mass spectrometry. The second is that the isomeric composition of the biologically obtained porphyrins and of the N-ethylmesoporphyrin mixture utilized in the inhibition studies was not defined (8). In order to determine if the specific nitrogen alkylated in the porphyrin becomes important for alkyl groups larger than a methyl, we have synthesized and individually assayed the four isomers of N-ethylprotoporphyrin IX. These studies, reported here, establish that the identity of the nitrogen alkylated in protoporphyrin IX is a major determinant of the inhibitory activity.

Experimental Procedures

N-Ethylprotoporphyrin IX Isomers: The procedure used for the synthesis of the N-methyl isomers (3,4) was also used to prepare the N-ethyl structures, ethyl fluorosulphonate being used in place of the analogous methyl reagent. The isomers, separated by high pressure liquid chromatography (Figure 1), have been fully characterized by electronic absorption, mass spectrometric, and NMR techniques (19). Each isomer was shown by chromatography and NMR to be

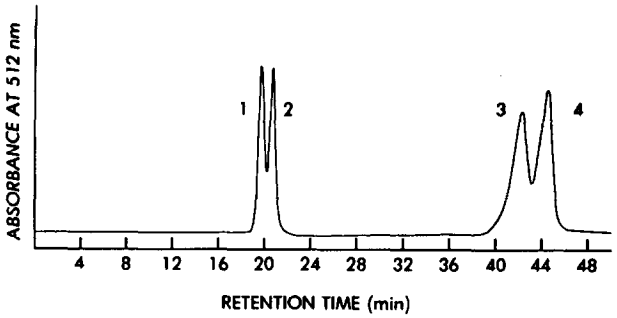

Figure 1. Separation by high pressure liquid chromatography of the four dimethyl esterified isomers of N-ethylprotoporphyrin IX. A 9.4 x 250 mm Partisil 10-PAC column, eluted with 97:97:6 (vol/vol) hexane: tetrahydrofuran: methanol, was used. The variable wavelength detector was set at 512 nm. The isomers are numbered in order of elution from the column.

free of the others before it was hydrolyzed to the free acid form by stirring in 10% aqueous HCl for 24 hr. The free acids were extracted with chloroform and were purified as already reported for the N-methyl isomers (5). The electronic absorption and NMR spectra of the hydrolyzed isomers were consistent with the assigned structures. No evidence was found in these spectra for significant hydration of the vinyl groups during hydrolysis of the ester functions.

The individual N-ethylprotoporphyrin IX isomers were dissolved in methanol and a 10 µl aliquot of each solution was diluted 300 fold with chloroform before 8 µl of triethylamine was added and the absorbance of the sample was measured at 416 nm. The concentration of the porphyrins in the original solutions was then calculated, using a value of 120,000 for the molar absorbance. The solutions of the isomers in methanol were stored at -20°C in the dark. Aliquots were withdrawn as required for biological assays and were diluted with 0.2 M tris HCl buffer (pH 8.2) as described below. A 1:9 (v/v) mixture of methanol in the same buffer was used in control experiments.

Rat Liver Ferrochelatase Inhibition: Solubilized rat liver ferrochelatase from male Sprague-Dawley rats (250-300g) was prepared as previously described (20). Aliquots of each of the four N-ethylprotoporphyrin IX isomer solutions, diluted with buffer to a volume of 45 µl, or an equal volume of the control solution were incubated with the solubilized enzyme preparation for 15 min in an oscillating bath at room temperature (5). The mixtures were then placed on ice and ferrochelatase activity was measured on aliquots of the mixtures containing 1.25 mg of protein using mesoporphyrin and iron as substrates (20). The formation of mesoheme, measured by the pyridine hemochromogen method, is expressed in Figure 2 as a percent of the activity in control incubations. The control ferrochelatase activity was 10.34 ± 1.97 nmol mesoheme/mg protein/8 min.

Results and Discussion

The four isomers of N-ethylprotoporphyrin IX (as the dimethyl esters) have been synthesized, separated by high pressure liquid chromatography (Figure 1), and individually characterized by complete spectroscopic analysis (19). The N-ethyl group is located, in order of elution of the isomers from the chroma-

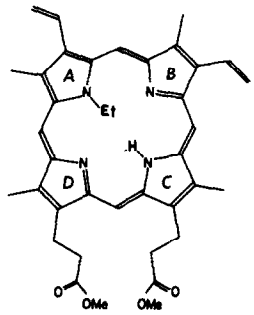

Figure 2. Isomer 2 of the dimethyl ester of N-ethylprotoporphyrin IX. The pyrrole rings are lettered for identification. Isomers 1, 3, and 4 have the N-ethyl group on pyrrole rings B, C, and D, respectively.

tography column (Figure 1), on pyrrole ring B (peak 1), pyrrole ring A (peak 2), pyrrole ring C (peak 3) and pyrrole ring D (peak 4) (Figure 2). Identification of the ring alkylated in each isomer (unpublished results) was accomplished by a variant of the procedure used to identify the four N-methyl isomers (4). Each of the four N-ethyl isomers, free of contamination from the others, was hydrolyzed to the corresponding free acid for biological experiments.

The inhibition of hepatic ferrochelatase as a function of the concentration of each of the four isomers is summarized in Figure 3. Isomers 1 and 2 are equally potent as inhibitors, decreasing the enzyme activity by 50% when present at a relative ratio of approximately 2.4×10^{-6} mg/mg protein. Isomers 3 and 4, on the other hand, are much weaker inhibitors. A 50% reduction in enzyme activity is only observed with isomers 3 and 4 at a ratio of $1\text{-}3 \times 10^{-4}$ mg/mg protein. Isomers 1 and 2 thus inhibit ferrochelatase as effectively as the previously studied N-methylprotoporphyrin IX isomers (5), whereas isomers 3 and 4 are 30-100 times less potent. The steric constraints on the N-alkyl group are, in sum, much more stringent when the group is located on the propionic acid substituted pyrrole rings (isomers 3 and 4) than when it resides on the vinyl substituted rings (isomers 1 and 2). The finding that the binding site does not discriminate between a methyl and an ethyl in the latter pair of isomers suggests that even larger groups may be compatible with inhibitory activity. The potency of N-alkylprotoporphyrin IX structures as ferroche-

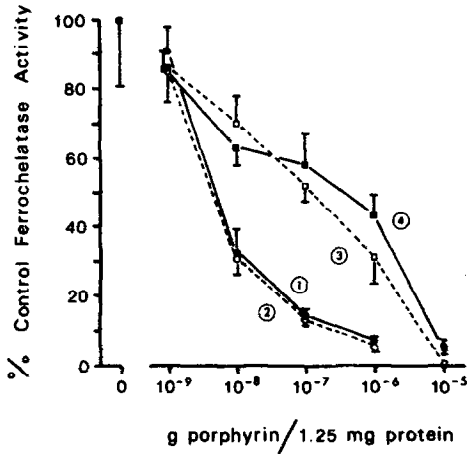

Figure 3. Inhibition of rat liver ferrochelatase by the four isomers of N-ethylprotoporphyrin IX. Each point represents the mean of four or five determinations (+ S.E.M.).

latase inhibitors is therefore determined not only by the size of the N-alkyl group, as previously suggested (8), but also by its specific location. The practical consequences of this are evident if one considers the green porphyrin isolated from ethylene treated rats. This porphyrin, which is reported to be a poor inhibitor of ferrochelatase (8), has recently been shown to be a single isomer of N-(2-hydroxyethyl)protoporphyrin IX (12). The N-alkyl group in the structure is exclusively located on one of the propionic acid substituted pyrrole rings (12). The inability of this porphyrin to inhibit ferrochelatase thus reflects location of the substituent on one of the nitrogens subject to steric restrictions. The present studies suggest that an isomer of the porphyrin with the 2-hydroxyethyl moiety on the nitrogen of ring A or B might be a much stronger inhibitor of ferrochelatase, although the steric constraints (if any) on N-alkyl groups on these rings remain to be defined.

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