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FERROCHELATASE: ISOLATION AND PURIFICATION VIA AFFINITY CHROMATOGRAPHY

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SUMMARY: **Ferrochelatase (protoheme ferro-lyase, EC 4.99.1.1) from rat liver has been purified by affinity chromatography. Synthesis of the affinity column ligand and a preliminary investigation of enzyme properties are reported. The SDS gel-electrophoresis molecular weight was 63,000 daltons; purified ferrochelatase ferro-lyase activity had a $t_{1/2}$ (4^{o} or -20^{o}) of approximately 12 hours in the presence of beta-mercaptoethanol. Addition of $CuCl_2$ to the purified enzyme resulted in a decrease in activity contrary to the previously reported increase in activity with crude enzyme.**

Heme (iron protoporphyrin) is produced *in vivo* by a sequence of enzymatic reactions that begins with the condensation of glycine and succinyl-CoA and ends upon the insertion of iron into protoporphyrin (1).

Defects in the heme biosynthetic pathway lead to toxic accumulations of porphyrins and their precursors in a set of pathological conditions called porphyrias (2). Both erythropoetic protoporphyria and porphyria variegata are characterised by increased levels of free protoporphyrin and it is postulated that these pathological conditions are due to defects in the enzymatic step in which iron is incorporated into

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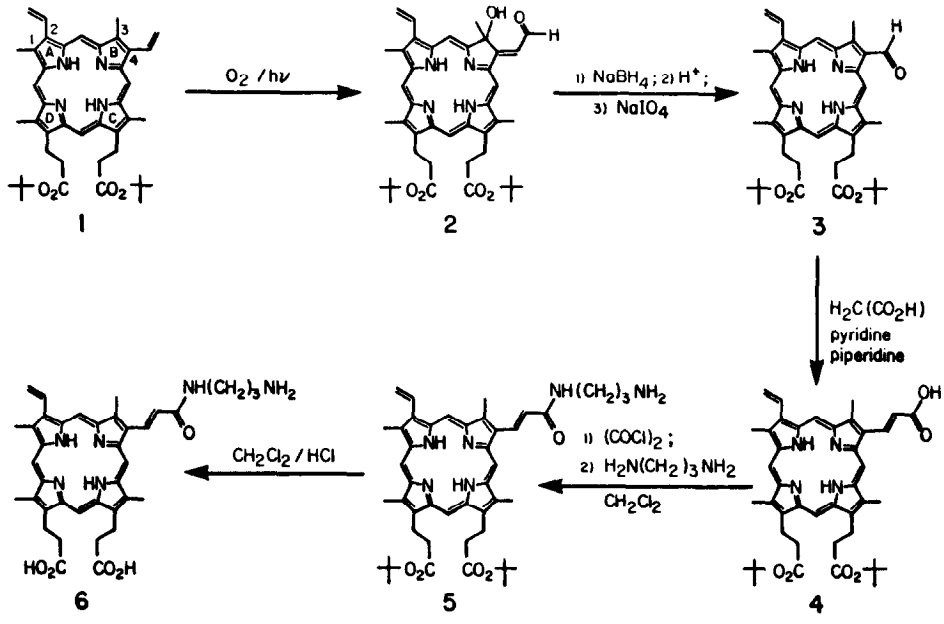
protoporphyrin (3). The enzyme which mediates this step is ferrochelatase and recent evidence suggests that this enzyme also plays an important regulatory function in heme biosynthesis (4).

Previous attempts to purify this enzyme to homogeneity have never resulted in a preparation containing less than twenty different proteins (5). Until a homogeneous enzyme is available the mechanism of action of ferrochelatase in both normal and pathological conditions, and the control which the enzyme exerts on heme biosynthesis, will remain obscure.

Affinity chromatography has been shown in recent years to be a powerful tool for protein purification (6). Here we report the synthesis (7) of an affinity ligand 2-vinyl-4-(3'-(N-3"-aminopropyl)acrylamidodeuteroporphyrin (**6**), its immobilisation on agarose, and the use of the resulting affinity column for the single step purification of rat liver ferrochelatase to a homogeneous protein which runs as a single band in SDS disc gel electrophoresis.

Both protoporphyrin and mesoporphyrin exhibit high binding constants for ferrochelatase and coupling of either of these porphyrins to an insoluble support via their propionic acid side chains would be a simple matter. However, since the two carboxylic acid residues represent the only hydrophilic sites on the porphyrin we anticipated that their modification would result in dramatic changes in both the mode and magnitude of their binding to enzyme. Rather, we chose to extend a vinyl group of protoporphyrin to an acrylic acid side chain and couple the resultant porphyrin to an insoluble support at this position thereby leaving the two propionate side chains free. This has not only the advantage of generating an affinity ligand which is both constitutionally and electronically similar to the natural



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substrate, but the two unperturbed propionic acid residues confer water solubility on the final affinity ligand.

The synthesis of the affinity ligand is outlined in the scheme. Photolysis of protoporphyrin di-t-butyl ester (**1**) (8) in air generated singlet oxygen which via a Diels-Alder cyclisation gave photoprotoporphyrin (**2**). Reduction of **2** followed by periodate cleavage of the resulting diol gave the mono-vinyl-mono-formyldeuteroporphyrin (**3**) (9). Condensation of **3** with malonic acid under Knoevenagel conditions, followed by decarboxylation gave the mono-vinyl-mono-acrylate derivative (**4**) which was condensed, via the acid chloride, with 1,3-diaminopropane to give the amino amide **5** . Finally removal of the t-butyl esters with dry HCl gave the affinity ligand **6** .

Using a cholate-solubilised, beef liver ferrochelatase preparation (4) the affinity ligand **6** was enzymatically converted to the corresponding iron complex at just under half

the rate protoporphyrin was converted to heme by the same enzyme preparation. From these observations we concluded that the interaction of our ligand with the enzyme was similar to that of the natural substrate (protoporphyrin).

Commercially prepared cyanogen bromide activated Sepharose 4B was coupled to the affinity ligand **6** in 0.1M $NaHCO_3$ at pH 9.5 for 24 hr at 0°. Excess ligand was removed by filtration.

In vivo ferrochelatase is bound to the inner mitochrondrial membrane. Purification of this enzyme to the detergent solubilised form (i.e. material remaining in the supernatant solution at 176,000 x g) has been described by Simpson and Poulson (4). In this form it is a clear yellow solution which can be stored with 10-20% glycerol at -70° and subsequently defrosted with no significant loss in activity. Both defrosted solutions containing glycerol and fresh preparations, lacking glycerol, were used on the affinity column with similar results.

The cholate suspended enzyme mixture prepared from the livers of male Wistar rats was applied to the affinity column and incubated for 20-30 minutes at 37°. Unbound proteins were then removed by washing with 50mM tris.Cl/0.1 M KCl buffer at pH 7.5. Some elution of the bound ferrochelatase occurred when the column was eluted with a solution of 50mM tris.Cl/1% sodium cholate/0.8% KCl at pH 8.0. However, when a 0.1mM aqueous solution of mesoporphyrin (pH 8.0) was added to the column with incubation at 37° for 20 minutes followed by elution at 37° with the same tris/cholate buffer at pH 7.5 twice the recovery of ferrochelatase with a three times higher specific activity was obtained. These conditions do not remove all of the ferrochelatase from the column and additional protein could be removed by elution with a 0.1% solution of triton X-100. This material,

however, showed no ferrochelatase activity. The ferrochelatase eluted from the affinity column showed only one band in SDS gel electrophoresis. Visual estimation of the intensity of this band indicated that ferrochelatase comprised less than 10% of the material applied to the column. Considering the diversity of mitochondrial membrane-bound proteins this is perhaps not surprising.

The combined column fractions lose activity rapidly; $t_{1/2}$ was approximately 12 hours when samples were stored with 20mM beta-mercaptoethanol (at 4^{o} or -20^{o}). In the absence of beta-mercaptoethanol enzyme activity was lost completely within 12 hours. Wagner and Tephly (10) reported an increase in the activity of a crude ferrochelatase preparation, stored in glutathione, when treated with $CuCl_2$. This suggested that perhaps ferrochelatase was a copper containing enzyme. However, we found that addition of $CuCl_2$ to the purified enzyme decreases or eliminated activity at 6.75 μM. It seems likely that the initial observations result from a modification of the glutathione (11) rather than any interaction between copper ion and ferrochelatase. On several occasions the addition of E. Coli lipids to column fractions enhanced the activity approximately 10%. Addition of the lipids did not, however, increase the stability of the enzyme.

Ferrochelatase activity was assayed aerobically by the pyridine hemochrome procedure described by Porra and Ross (12). Protein concentrations were determined using the coomassie blue dye binding method (13). The table shows ferrochelatase activity in various fractions of rat liver mitochondria, and of ferrochelatase purified via affinity chromatography. It is apparent that while the specific activity of the homogeneous

Table I

Material	Total protein (mg)	Protein concentration (mg/ml)	specific[a] activity
RL mitochondria	1440	120	3.4
Sub-mitochondrial particles	480	5.5	4.9
RL solubilized ferrochelatase	148	14	8.3
Column eluate	2.5	0.34	37

a) The assay mixture contained 50mM tris.Cl, 1mM cysteine, 0.1 mM $FeSO_4$, 0.1 mM mesoporphyrin, and 0.4 ml of enzyme preparation (pH = 7.5) in a total volume of 1.6 ml. Enzyme and substrate were incubated for one hour at 43°. The concentration of heme produced was measured on a Cary 15 spectrophotometer using the heme beta-band (515 nm) and a value of ϵ_β = 20.7 mM^{-1}. Specific activity is expressed as units per milligram protein. One unit of ferrochelatase activity is defined as the amount which catalyses the formation of one nmole of heme in one hour under standard conditions.

ferrochelatase has increased after chromatography the recovery of the enzyme is low. This is a problem frequently encountered in affinity chromatography, but it is a situation which can be improved by more efficient removal of the bound protein from the affinity column. The obvious solution to this problem would be to incubate the protein bound column with ferrous iron which the enzyme would incorporate into the affinity ligand. The exceptionally low binding constant of ferrochelatase for heme, indicated by the work of Koller and Romslo (14), suggests that the protein would be released at this stage. However, this would prevent reuse of an affinity column (which at this time has been used for more than 10 consecutive runs), since the iron could not be removed from the porphyrin without destroying the affinity column. We have found the rat liver ferrochelatase to have a molecular weight of 63,000 daltons (15) which under ideal conditions, would allow 1 g of affinity ligand to yield 100 g of protein. The synthesis of 1 g of affinity ligand is readily achievable so that isolation of the enzyme by irreversibly

destroying the affinity column with ferrous iron can, in the future, be expected to make available sufficient amounts of ferrochelatase for detailed studies.

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7. In this sequence photoprotoporphyrin **2** was formed as a mixture of two isomers where the initial 1,4-singlet oxygen addition took place at either ring A or B. The two isomers were separated by chromatography on silica gel using methylene dichloride:ether (20:1), and the affinity ligand was prepared separately from both isomers. However, the affinity ligand used in these studies was prepared from the mixture of photoprotoporphyrin isomers. All compounds in each of the three series (ring A, ring B, rings A and B) gave satisfactory elemental analysis and exhibited spectroscopic properties (ms, ir, uv-vis, nmr) consistent with their designated structures.
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15. Ferrochelatase exhibited an electrophoretic mobility, on SDS gel electrophoresis, between that of catalase (60,000 daltons) and bovine serum albumin (66,000 daltons).

Mobilities were determined at different gel concentrations; the Ferguson plot for ferrochelatase was linear and gave a retardation coefficient (K_R) of 0.09. Using the published data of Neville which relate log K_R to log molecular weight, we determined the molecular weight of ferrochelatase to be 63,000 daltons.

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