Purification and characterization of chicken erythrocyte ferrochelatase

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Ferrochelatase (EC 4.99.1.1) was purified 2000-fold to apparent homogeneity from isolated chicken erythrocyte mitochondria. The purified enzyme yields a single band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with an apparent M_r of 42000. The enzyme utilizes proto-, meso- and deuterio-porphyrin with K_m values of 37, 51 and 80 μM respectively. The disubstituted porphyrins 2,4-bisglycol deuterio-porphyrin and 2,4-disulphonic deuterio-porphyrin were not substrates. Mn^{2+} , Hg^{2+} , Pb^{2+} and Co^{2+} were strong inhibitors of the purified enzyme. Palmitic acid and oleic acid stimulated activity, whereas linoleic acid inhibited and phospholipids had variable effects. Chicken ferrochelatase was inhibited by *N*-ethylmaleimide and iodoacetamide. Inhibition by iodoacetamide was pseudo-first-order, but inhibition by *N*-ethylmaleimide appeared to be biphasic in nature with an initial high rate followed by a much lower rate of inactivation. The characteristics of the chicken erythrocyte enzyme are compared with those previously reported for mammalian liver ferrochelatase.

The terminal enzyme of the haem-biosynthetic pathway, ferrochelatase (protohaem ferro-lyase, EC 4.99.1.1), catalyses the insertion of ferrous iron into protoporphyrin IX to form protohaem. This enzyme, which is an intrinsic membrane protein bound to the mitochondrial inner membrane (Jones & Jones, 1969), has been purified to homogeneity from rat and bovine liver mitochondria (Taketani & Tokunaga, 1981, 1982; Dailey & Fleming, 1983) and from the facultative photosynthetic bacterium *Rhodospseudomonas sphaeroides* (Dailey, 1982). The availability of pure enzyme has allowed the characterization of both physical and kinetic properties (Dailey & Fleming, 1983; Dailey, 1984). To date, however, ferrochelatase has not been purified from either an erythropoietic cell type or a non-mammalian eukaryotic organism.

In the present study we have purified and characterized ferrochelatase from chicken erythrocytes. Characterization of the enzyme from this source is of importance for two major reasons. First, the haem-biosynthetic pathway in chickens has been shown to be particularly sensitive to a variety of drugs, hormones and chemicals (Granick *et al.*, 1975; Sassa & Kappas, 1977). Secondly, ferrochelatase has never been purified from erythropoietic cells. Findings obtained with differ-

entiating Friend erythroleukaemia cell cultures strongly suggest that control of the pathway in erythropoietic cells differs from that found in hepatic cells and that ferrochelatase may be the rate-limiting enzyme during development (Rutherford *et al.*, 1980; Sassa, 1980).

Experimental

Materials

Chicken blood was obtained from Central Soya, Athens, GA, U.S.A. Porphyrin substrates were obtained from Porphyrin Products, Logan, UT, U.S.A. Chromatography materials, including Blue Sepharose CL-6B and Sephacryl S-300, were obtained from Pharmacia Fine Chemicals. Fatty acids (oleic acid, palmitic acid and linoleic acid) and phospholipids (cardiolipin and L-1-phosphatidylethanolamine) were obtained from Sigma Chemical Co. Blue Dextran and the marker proteins, ferritin, catalase, fructose biphosphate aldolase and bovine serum albumin, were also obtained from the Sigma Chemical Co., as well as *N*-ethylmaleimide and phenylmethanesulphonyl fluoride. All other chemicals used were of analytical grade.

Isolation of mitochondria

Chicken blood was obtained from freshly killed broilers at Central Soya Processing Plant. During

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collection of the blood, sodium citrate was added at a concentration of 30g/l as an anticoagulant, and the blood was kept on ice. Whole blood was centrifuged at 2000g for 10min and the plasma discarded. The cells were then washed twice by resuspension to the original volume in 100mM-NaCl and centrifuging at 2000g for 10min.

After the washing, the cells were lysed in a hypo-osmotic buffer containing 25mM-Tris/acetate, pH8.2, 1mM-dithiothreitol and 1mM-MgCl₂. The cells were resuspended to 2.5 times their original volume and vigorously stirred for 3h at 4°C. This solution was then centrifuged at 1000g for 10min to remove whole cells and intact nuclei. The supernatant was saved, and the pelleted nuclei, large membrane fragments and whole cells were discarded. The mitochondria were collected by centrifuging the supernatant at 8000g for 25min.

The supernatant was discarded, and the mitochondrial pellets were collected and washed repeatedly to remove haemoglobin. This procedure was usually performed four or five times by resuspension in 500ml centrifuge bottles containing 25mM-Tris/acetate, pH8.2, and then centrifuging at 8000g for 25min until the pellets were a light-brown colour. In the final wash, dithiothreitol was added to a concentration of 1mM and glycerol to a concentration of 20% (v/v). The mitochondria were then stored at -20°C until needed.

Purification of ferrochelatase

Whole frozen mitochondria (approx. 20mg/ml) were thawed and sonicated at 10°C in a Heat Systems Cell Disruptor set at 60Hz for five 1min intervals. The membranes were then collected by centrifugation at 100000g for 2h. The pellet was resuspended to a concentration of 15mg of protein/ml in 25mM-Tris/acetate, pH8.2, containing 1mM-dithiothreitol and 20% glycerol. Sodium cholate was added to yield a final concentration of 1% (w/v), and the membranes were sonicated for 30s. Membranes were centrifuged at 100000g for 2h, and the supernatant, which contained solubilized enzyme, was collected.

Granular (NH₄)₂SO₄ was added over 5min with continuous stirring at 4°C until 30% saturation was achieved. This mixture was stirred for 1h at this temperature and then centrifuged at 12000g for 15min. After centrifugation the supernatant was decanted off, and the pellet was discarded. Additional granular (NH₄)₂SO₄ was added until 55% saturation was reached. The pH was maintained by addition of NaOH when necessary. This mixture was stirred for 1h at 4°C and centrifuged at 12000g for 15min. After centrifugation, the precipitate was resuspended in buffer containing

25mM-Tris/acetate, pH8.2, containing 1mM-dithiothreitol and 20% glycerol.

The resuspended precipitate was applied to a Sepharose Reactive Blue CL-6B column (2.5cm × 20cm) that had been previously equilibrated with a buffer containing 25mM-Tris/acetate, pH8.2, 20% glycerol, 0.5M-KCl, 1% Triton X-100, 1mM-dithiothreitol and 1μg of phenylmethanesulphonyl fluoride/ml. The column was washed with 1 litre of the equilibration buffer at a flow rate of 80ml/h and then with 250ml of a buffer containing 25mM-Tris/acetate, pH8.2, 20% glycerol, 1.0M-KCl, 0.25% sodium cholate, 1% Triton X-100, 1mM-dithiothreitol and 1μg of phenylmethanesulphonyl fluoride/ml at a flow rate of 80ml/h. The enzyme was eluted with a buffer containing 25mM-Tris/acetate, pH8.2, 20% glycerol, 1.5M-KCl, 1% sodium cholate, 1% Triton X-100, 1mM-dithiothreitol and 1μg of phenylmethanesulphonyl fluoride/ml. The peak fractions were pooled and concentrated in an Amicon ultrafiltration apparatus with a YM-30 filter.

Gel filtration was performed on a Sephacryl S-300 column (2.5cm × 80cm) previously equilibrated with the eluent buffer containing 25mM-Tris/acetate, pH8.2, 20% glycerol, 1.5M-KCl, 1% Triton X-100, 1mM-dithiothreitol and 1μg of phenylmethanesulphonyl fluoride/ml. A 5ml portion of the concentrated fractions containing 0.2mg of concentrated protein/ml was applied to the column and eluted with the same buffer at a flow rate of 40ml/h.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970) in the presence of 0.1% sodium dodecyl sulphate. The gels were stained by using the silver staining procedure of Merrill *et al.* (1981).

Determinations

The ferrochelatase assay was performed as described previously with deuteroporphyrin as substrate (Dailey, 1977). Porphyrin concentrations were determined spectrally as described by Falk (1964). Protein determination was performed by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

Purification and physical properties

The purification scheme described above is detailed in Table 1. One unusual feature encountered with the avian enzyme was the nature of the pellet formed during (NH₄)₂SO₄ precipitation. The enzyme precipitate does not pellet out on

Table 1. Purification of chicken erythrocyte ferrochelatase

For full experimental details see the text. One unit of ferrochelatase activity represents 1 nmol of deuterohaem formed/min.

Fraction	Total activity (units)	Protein (mg/ml)	Sp. activity (units/mg)	Yield (%)	Purification
Mitochondrial membranes	66.8	14	0.012	100	1
Solubilized enzyme	47.2	0.45	0.326	70	27
(NH ₄) ₂ SO ₄ fractionation:					
30–55% pellet	36.8	0.152	0.44	55	36
Blue Sepharose (CL-6B)	24.4	0.05	24.4	36	2033
Sephacryl S-300	10.6	0.03	17.6	16	1467

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of purified chicken erythrocyte ferrochelatase. Lanes 1 and 3 contain standards; lane 2 contains 5 µg of purified chicken ferrochelatase. The *M_r* values of the standards are (from top to bottom) 94000, 67000, 43000, 30000, 20100 and 14400.

centrifugation, but instead floats on the surface and adheres to the walls of the centrifuge bottle. With careful handling, however, it is possible to recover most of the precipitate. The Sephacryl S-300 column was necessary in some preparations to eliminate minor contaminations that occur with repeated use of the dye-column matrix. The enzyme fraction from the Sephacryl column contained only one band on SDS/polyacrylamide gels, but always had a lower specific activity than the

Table 2. Apparent *K_m* and *V_{max}* values for purified ferrochelatase

Ferrochelatase activity was assayed as described in the Experimental section.

Substrate	<i>K_m</i> (µM)	<i>V_{max}</i> (nmol of haem/min per mg)
Ferrous citrate	166	—*
Deuteroporphyrin	80	192
Mesoporphyrin	51	122
Protoporphyrin	37	83

* The *V_{max}* for iron was found to be dependent on the porphyrin substrate.

preceding step. It was found that inclusion of 1.5 M-KCl dramatically improved enzyme recovery from the Sephacryl S-300 column, but even the best preparations had lower specific activities. The purified protein consists of a single band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 1). The estimated *M_r* was 42000 and was independent of acrylamide concentration between 7.5 and 12.5%. The absorption spectrum has a peak at 276nm, with no evidence for any chromophoric cofactors.

Kinetic properties

The *K_m* and the *V_{max}* for porphyrins and iron were calculated from Lineweaver–Burk plots. The apparent *K_m* was found to be 80, 51 and 37 µM for deuter-, meso- and proto-porphyrin respectively. The apparent *V_{max}* was 192, 122 and 83 nmol of haem formed/min per mg of protein respectively (Table 2). The *K_m* obtained for ferrous iron was 166 µM. To ascertain the limits of substrate specificity, substituted porphyrins were employed as possible substrates for ferrochelatase. Those tested were 2,4-bisglycol deuteroporphyrin IX and 2,4-disulphonic deuteroporphyrin IX. Both disubstituted porphyrins had less than 5% of the enzymic activity of unsubstituted deuteroporphyrin. The pH optima for proto-, meso- and deuteroporphyrin were found to be 8.25, 8.35 and 8.45 respectively.

Effects of metal ions on ferrochelatase activity

The effects of a variety of metal and non-metal ions were tested on ferrochelatase activity (Table 3). NH_4^+ and Ni^+ showed small increases in activity, whereas Pb^{2+} , Co^{2+} and Mn^{2+} inhibited at a concentration of $10\text{ }\mu\text{M}$. Since Co^{2+} has previously been shown to be a substrate for ferrochelatase, the inhibition found with it is probably due to competitive inhibition with respect to iron. Pb^{2+} and Mn^{2+} are not substrates for chicken ferrochelatase (results not shown).

Effects of fatty acids and phospholipids

It had been previously reported (Poulson & Polglase, 1975; Taketani & Tokunaga, 1981) that fatty acids and phospholipids stimulate the activity of ferrochelatase in crude extracts and in purified preparations. The effects of increasing concentrations of three fatty acids and two phospholipids were tested on ferrochelatase activity. The results of these experiments (Fig. 2) indicated that ferrochelatase was stimulated by the addition of palmitic acid or oleic acid and inhibited by linoleic acid. Palmitic acid stimulated ferrochelatase activity at a concentration of $50\text{ }\mu\text{M}$ by 100%. Concentrations above this caused a decrease in enzymic activity.

The effects of phospholipids on ferrochelatase activity were similar to fatty acids in their variability. Increasing concentrations of cardiolipin caused a decrease in activity. L-1-Phosphatidylethanolamine, on the other hand, doubled ferrochelatase activity at a concentration of $100\text{ }\mu\text{M}$.

Effects of thiol-blocking reagents

To test the possible role that thiol groups might have in the enzyme activity, chicken ferrochelatase was treated with the thiol-blocking reagents

Table 3. Effects of metal ions on ferrochelatase activity

Assays were performed as described in the Experimental section. All salt solutions were freshly prepared the day of the experiment. The results are given as means $\pm$ S.D. for three determinations.

Salt (final concn. $10\text{ }\mu\text{M}$)	Activity (% of control)
MnCl_2	41 ± 6
NiCl_2	102 ± 8
HgCl_2	35 ± 3
PbNO_3	45 ± 15
CoCl_2	25 ± 7
MgCl_2	95 ± 2
KCl	98 ± 1
NaCl	98 ± 1
NH_4Cl	105 ± 16
CuSO_4	95 ± 2

N-ethylmaleimide and iodoacetamide (Fig. 3). Ferrochelatase was inhibited by both reagents. Other experiments were performed with ferrous iron and deuteroporphyrin to test for their ability

Fig. 2. Effects of fatty acids and phospholipids on chicken ferrochelatase activity

(a) Effects of fatty acids: ●, oleic acid; □, palmitic acid; ▲, linoleic acid. (b) Effects of phospholipids: ●, phosphatidylethanolamine; Δ, cardiolipin. All fatty acids and phospholipids were prepared by sonication of an N_2 -dried film into 50 mM -Tris/acetate, pH 8.1, immediately before use. All manipulations and storage were under N_2 . Results shown are the averages of three determinations.

Fig. 3. Effects of thiol-blocking reagents on chicken ferrochelatase activity

(a) Inactivation by *N*-ethylmaleimide and protection by ferrous iron. A 50 μ g portion of purified enzyme in 1 ml of 1 M-Tris/acetate, pH 8.2, was incubated in the presence of 0.010 ml of 0.1 M-*N*-ethylmaleimide. A 2000 μ l portion of 4 mM- $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ was added to one of the two reaction tubes, and 0.1 ml samples were removed at 30 intervals for a period of 5 min. The reaction was terminated by the addition of 0.025 ml of 2.5 M-2-mercaptoethanol, and the samples were assayed for ferrochelatase activity. $\circ$, Reaction in the presence of ferrous iron; $\bullet$, reaction without ferrous iron. (b) Inactivation by iodoacetamide and protection by ferrous iron. A 50 μ g portion of purified enzyme in 1 ml of 0.1 M-Tris/acetate, pH 8.2, was incubated in the presence of 0.025 ml of 0.1 M-iodoacetamide. $\circ$, Reaction in the presence of ferrous iron; $\bullet$, reaction without ferrous iron. E_0 is enzyme activity at zero time and E_t was enzyme activity at time t after the addition of reagent.

able to inactivate the enzyme. Deuteroporphyrin was not effective in protecting the enzyme from inactivation by either *N*-ethylmaleimide or iodoacetamide.

Discussion

Before the present investigation ferrochelatase has been purified from mammalian liver mitochondria (Taketani & Tokunaga, 1981, 1982; Dailey & Fleming, 1983), but had not been purified from avian or erythropoietic sources. Herein we have reported the 2000-fold purification of chicken erythrocyte ferrochelatase from isolated mitochondria. Chicken erythrocytes, rather than mammalian, were chosen, since circulating avian erythrocytes contain functional mitochondria, whereas it is necessary to obtain reticulocytes from mammalian sources. Chicken ferrochelatase shares similarities with both bovine and rat ferrochelatase, but is unlike the bacterial ferrochelatase described (Dailey, 1982). Its M_r is approx. 42000 and its K_m values are similar to those of the bovine enzymes. It is stimulated by some fatty acids and phospholipids, but not to the same extent as the rat enzyme (Taketani & Tokunaga, 1981).

One apparently major difference between chicken and bovine ferrochelatases is in their reactivities toward thiol-blocking reagents. The bovine enzyme is only slowly inactivated by iodoacetamide, but is rapidly inactivated by *N*-ethylmaleimide and other non-polar thiol-blocking reagents (Dailey, 1984). Chicken ferrochelatase is inactivated more slowly by iodoacetamide than by *N*-ethylmaleimide, but the difference in reactivities is approx. 3-fold as compared with the 50-fold difference found in the bovine enzyme. In addition, the inactivation by *N*-ethylmaleimide appears to be biphasic whereas the inactivation by iodoacetamide follows pseudo-first-order reaction kinetics. This is in sharp contrast with the bovine enzyme, where inactivation by all tested thiol-reactive reagents yields pseudo-first-order reaction kinetics. The cause for this difference is, at present, unknown, but it might result from either a different reaction mechanism or an altered active-site structure. Examination and comparison of putative active-site peptides containing the reactive thiol groups in ferrochelatase from these two animals may help to answer this question. Also, examination of the environment of the reactive thiol groups by using fluorescent probes may give clues to differences in the active sites of these two proteins.

The inherited disease protoporphyria results from decreased ferrochelatase activity. Although the activities of the enzyme are depressed in all cell types examined, it is the erythropoietic cells in

to prevent loss of enzyme activity by the action of either *N*-ethylmaleimide or iodoacetamide. The results from these experiments showed that the addition of ferrous iron did protect ferrochelatase from the *N*-ethylmaleimide but had only a slight effect on the extent to which iodoacetamide was

which the major accumulation of protoporphyrin occurs. Thus, unlike most other porphyrias, which are classified as hepatic, protoporphyria is an erythropoietic disorder. Two possible explanations for this distribution may be that a unique ferrochelatase with different properties from the hepatic enzyme is found in erythropoietic cells, or that the regulation of the haem-biosynthetic pathway is different in these two cell types. Findings obtained with Friend erythroleukaemia cells seem to support this second suggestion (Sassa, 1980). The data presented above show that the avian erythrocyte ferrochelatase is similar to the mammalian hepatic enzyme in many respects, but the difference in sensitivity to thiol-blocking reagents may be significant. Unfortunately, it is not possible to draw any conclusions from these comparisons, since the enzymes are from different species as well as different cell types. Further study of ferrochelatase in Friend cells and in erythropoietic compared with hepatic cell types from the same animal model will be required to answer these questions.

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