The Subcellular Localization and Properties of the Ferrochelatase of Etiolated Barley

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The etioplast fraction prepared from dark-grown barley contained the enzyme ferrochelatase. A mitochondrial fraction prepared from the same dark-grown tissue also contained ferrochelatase. After density-gradient centrifugation an etioplast band was collected that was free from detectable mitochondrial marker enzymes and yet retained ferrochelatase activity. A membrane band that was enriched in mitochondria also contained ferrochelatase. The ferrochelatase in these two bands had different pH optima, but appeared very similar in their porphyrin specificity and their inhibition by metalloporphyrins.

During the greening of etiolated plants there is a very active synthesis of chlorophyll and the development of functional photosynthetic electron-transport systems in newly formed chloroplasts. These changes are not accompanied by significant increases in the concentration of the leaf haemoproteins (Gregory & Bradbeer, 1973; Castelfranco & Jones, 1975) and so the rate of synthesis of haem must be closely regulated during this period of adaptation. The enzyme ferrochelatase functions at a branch-point of tetrapyrrole synthesis. Its substrate, protoporphyrin, is almost certainly a common intermediate in the formation of haems and chlorophylls; Fe^{2+} insertion into protoporphyrin by ferrochelatase yields protohaem, but this does not accumulate during the period of active Mg^{2+} insertion into protoporphyrin for chlorophyll formation. In non-photosynthetic eukaryotes ferrochelatase is localized exclusively in the mitochondrial membranes (Jones & Jones, 1969). If this were true in plants, regulation would be simplified. We have therefore re-examined the distribution of ferrochelatase in higher plants, using etiolated barley as experimental tissue.

Previous studies on the localization of this enzyme in green plants have used relatively crude preparations of organelles and do not exclude the possibility of cross-contamination of plastids by mitochondria or of mitochondria by plastid fragments (Jones, 1967; Porra & Lascelles, 1968).

Our results show that ferrochelatase is localized in more than one membrane fraction. It is found in an etioplast fraction, shown to be free from cytochrome oxidase or succinate dehydrogenase, used as markers for mitochondrial contamination. Ferrochelatase is also found in a membrane fraction that is enriched in mitochondria. The two enzymes have somewhat different properties.

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Methods and Materials

Porphyrins

Haemin was obtained from Sigma Chemical Co., Kingston-upon-Thames KT2 7BH, Surrey, U.K., and deuteroporphyrin IX dimethyl ester, mesoporphyrin IX and haematoporphyrin IX were from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Dimethyl protoporphyrin IX and dimethyl zinc protoporphyrin IX were prepared as described by Falk (1964) and dimethyl magnesium protoporphyrin was a gift from Professor G. Kenner, Department of Chemistry, University of Liverpool Liverpool, U.K. Protoporphyrin dimethyl ester was hydrolysed in 27% (w/w) HCl at 20°C for 3 h, zinc protoporphyrin dimethyl ester in 1% (w/w) methanolic KOH at 20°C overnight and magnesium protoporphyrin dimethyl ester in 30% (w/w) methanolic KOH at 40°C for 15 min. The free porphyrins and metalloporphyrins were dissolved in 0.1 M-KOH, which was then diluted 10–20-fold to give approx. 1 mM solutions of porphyrin in 0.1% (v/v) Tween 80. Concentrations of the porphyrins were calculated from the intensity of the Soret maximum in acid solution (Falk, 1964). Both zinc and magnesium porphyrins were converted by acid into the corresponding porphyrin and their concentrations similarly determined. The concentration of iron protoporphyrin (haemin) was determined by the alkaline pyridine haemochromogen method (Falk, 1964).

Preparation of particulate fractions

Barley seed (*Hordeum vulgare* L. var. Proctor) was soaked overnight in tap water and germinated in the dark in water-saturated peat soil at 25°C. The etiolated leaves were harvested and stored at 5°C in the dark until used. During subsequent operations all experimental material was kept as close to 0°C as possible

and protected from undue exposure to light. The barley leaves were cut into short segments and macerated in a mortar for 90 s with twice their weight of the extraction medium of Brunton & Palmer (1973). The extract, after filtration through four layers of muslin and four layers of Miracloth, supplied by Calbiochem, San Diego, CA 92112, U.S.A., was placed in an MSE 18 centrifuge and accelerated to 9000 rev./min (12500g). The rotor was then rapidly stopped and the etioplasts were resuspended in extraction medium lacking EGTA [ethanedioxybis(ethylamine)tetraacetic acid]. This suspension was filtered through Miracloth and further clarified by centrifugation at 300g for 90 s. The fraction termed 'washed etioplasts' was sedimented from the resulting supernatant at 4000g for 90 s and then resuspended in 0.5 M-sucrose. Mitochondrial fractions were separated either from the supernatant from the washed-etoplast preparation or the supernatants of the original plant extract which had first been centrifuged at 1000g for 10 min. Such supernatants were further centrifuged at 15000g for 10 min and the resulting crude mitochondrial fraction was resuspended in the extraction medium lacking EGTA. Large particulate matter was removed from such suspensions by centrifugation at 1000g for 10 min and smaller particulate matter recovered by centrifugation at 15000g. This latter fraction was resuspended in 0.5 M-sucrose and in this paper is termed 'washed mitochondria'.

Equilibrium sucrose-density centrifugation

Linear and/or discontinuous sucrose gradients with a final volume of 18 ml were prepared with sucrose solutions containing 10 mM-Mops [3-(*N*-morpholino)-propanesulphonic acid], pH 7.0. Samples were centrifuged for 3 h in a MSE Automatic Superspeed 50 centrifuge at 27000 rev./min (85000g_{av.}) in a 3 × 25 ml swing-out rotor. Fractions (30 drops each) were collected from the bottom of the tubes for analysis.

Enzyme assays

Except when noted, ferrochelatase was measured at 20°C by the pyridine haemochromogen method of Porra *et al.* (1967). This reaction mixture contained (per ml): 68 μmol of Tris/HCl buffer, pH 7.5; 0.7 mg of Tween 80; approx. 30 nmol of deuteroporphyrin; 4 μmol of reduced glutathione; and 80 μmol of FeSO₄. An iron concentration twice that used by Porra *et al.* (1967) was found necessary for optimal activity. One explanation for this excessive iron requirement is that EGTA was in part irreversibly bound to particulate matter and thereby decreased the effective iron concentration in the assay mixtures. Deuteroporphyrin was used as substrate unless otherwise indicated. For studies of the inhibition of ferrochelatase by iron protoporphyrin the dual-wavelength assay described by Jones & Jones (1969)

was used. In this assay the reaction mixture contained (per ml): 82 μmol of Tris/HCl buffer (pH 7.5); 0.82 mg of Tween 80; 30 nmol of deuteroporphyrin; and 40 μmol of CoSO₄.

Cytochrome oxidase activity was measured by the initial rate of cytochrome *c* oxidation. In this assay the *E*₅₃₀ compared with the *E*₅₄₀ was measured in a double-beam spectrophotometer. The reaction mixture contained (per ml) 50 μmol of Tris/HCl buffer, pH 7.5, and 10 μmol of reduced cytochrome *c*. In the calculations the value $\Delta\epsilon_{\text{mm}}$ (550 nm–540 nm) = 18.9 was used (Margoliash & Frohwirt, 1959).

Succinate dehydrogenase was determined spectrophotometrically from the rate of reduction of phenazine methosulphate as described by King (1967).

Protein concentration was measured by the method of Lowry *et al.* (1951), with crystalline bovine albumin as standard.

Results and Discussion

Washed etioplasts showed sedimentation behaviour characteristic of etioplasts during differential centrifugation. This fraction contained both protochlorophyllide and the NADPH-dependent protochlorophyllide-activating system reported by Griffiths (1974) to occur in etioplasts.

The washed etioplasts appeared to be fairly homogeneous. Fig. 1 indicates that mitochondrial contamination was small, since the cytochrome *a*+*a*₃ content of such preparations, as estimated from the intensity of the *E*₆₀₀ in the spectrum of reduced-minus-oxidized material measured at 77 K, was negligible. Density-gradient centrifugation of washed etioplasts in a 30–60% (w/w) sucrose gradient resolved these preparations into two visible bands. The particulate band which sedimented with the higher density (1.26 g/ml) was considered to contain intact etioplasts (Fig. 2). This band of high-density particles was absent when washed etioplasts were lysed with water before centrifugation. The lysed preparation showed a single visible band at a density of 1.16 g/ml, which corresponded in density to that observed for the lighter band observed with our washed etioplasts and which is identified therefore as being due to etioplast fragments.

Washed etioplasts consistently possessed ferrochelatase activity. For seven different preparations the specific activity of this enzyme was 9.5 ± 4.7 nmol of haem/h per mg of protein. Assay of sucrose density gradients of washed etioplasts showed that ferrochelatase activity sedimented predominantly as a single band of activity at a density of 1.22–1.24 g/ml. That this band of ferrochelatase activity is due to etioplastic rather than mitochondrial membranes is concluded from the observation that washed etioplasts showed only weak cytochrome oxidase activity

Fig. 1. Absorption spectra of washed etioplasts and washed mitochondria prepared from etiolated barley

Spectra were recorded at the temperature of liquid N_2 as difference spectra of dithionite-reduced-minus-oxidized sample. Curve E is for washed etioplasts (0.83 mg of protein/ml) and curve M is for washed mitochondria (1.8 mg of protein/ml).

Fig. 2. Distribution of ferrochelataase activity after sucrose-density-gradient centrifugation of washed etioplasts from etiolated barley

Washed etioplasts were prepared and centrifuged in a linear 30–60% (w/w) sucrose gradient, as described in the Methods and Materials section. Ferrochelataase activity was determined at pH 7.5 by the pyridine haemochromogen method. No cytochrome oxidase activity could be detected in any fraction. (a) Sucrose density (g/ml); (b) protein distribution (mg/ml); (c) ferrochelataase (nmol of deuterohaem formed/s).

(0.32 ± 0.20 nmol/s per mg of protein), and such activity was not found to be measurable in any fraction after density-gradient centrifugation of the washed etioplasts.

The washed mitochondrial fraction showed sedimentation behaviour characteristic of mitochondria during differential centrifugation and appeared to include mitochondrial membranes. Fig. 1 indicates that the reduced-minus-oxidized spectrum measured at 77 K of washed mitochondria had a strong absorbance at 600 nm. It is concluded therefore that this fraction contains significant amounts of cytochrome $a+a_3$. The specific activity of cytochrome oxidase in this fraction was 1.06 ± 0.54 nmol/s per mg of protein.

All washed mitochondrial preparations exhibited ferrochelataase activity comparable with that of washed etioplasts. The average specific activity of the ferrochelataase activity for seven such preparations was 10.5 ± 2.8 nmol of haem/h per mg of protein.

Further fractionation of washed mitochondria by sucrose-density-gradient centrifugation indicated that the ferrochelataase activity was associated predominantly with particles sedimenting with two

different densities. As indicated in Fig. 3, one peak of ferrochelataase activity was found at a density of 1.18–1.19 g/ml (band 1) and a second peak was found at a density of 1.22–1.24 g/ml (band 2).

Band 1 seemed to be enriched with mitochondrial membranes. Fig. 3 shows that the cytochrome oxidase activity of washed mitochondrial preparations sedimented as a single peak of activity having the same density as band 1. The specific activity of cytochrome oxidase in this band was 4–5-fold greater than that of either the unfractionated washed mitochondria or of band 2. The single peak of succinate dehydrogenase activity which was observed in these sucrose gradients coincided with that of ferrochelataase and cytochrome oxidase activity. On this basis it is concluded that the ferrochelataase activity of band 1 may be of mitochondrial origin.

Fig. 3. Distribution of enzymic activities after sucrose-density-gradient centrifugation of washed mitochondria from etiolated barley

A linear gradient was prepared by mixing 5 ml of 40% (w/w) sucrose and 5 ml of 60% (w/w) sucrose. On top of this was layered successively 3 ml of 36% (w/w) sucrose and 3 ml of 30% sucrose. Enzymic activities were determined as described in the Methods and Materials section, except that ferrochelatase activity was determined at pH 6.8 in 68 mM-Mops buffer. (a) ----, Density of sucrose in gradient (g/ml); ○, protein distribution (mg/ml); (b) ●, succinate dehydrogenase (nmol of succinate oxidized/s); ○, cytochrome oxidase (nmol of cytochrome *c* oxidized/s); (c) ○, ferrochelatase (nmol of deuterohaem formed/s).

The particles of band 2 appear to be of etioplasmic origin rather than mitochondrial origin. Fig. 3 indicates that this band, although containing ferrochelatase activity, is essentially devoid of cytochrome oxidase and succinate dehydrogenase activity. In addition, the ferrochelatase activity of the washed etioplast fraction was found to sediment with the same density as band 2. Samples of band 2 showed absorption maxima at 630 nm and 677 nm, characteristic of protochlorophyll(ide) and chlorophyll(ide). However, these pigments were not useful quantitative markers for etioplasmic membranes. Spectral observation of samples from a sucrose density gradient of washed mitochondria prepared from partially

greened (4 h light) barley showed that chlorophyll in such gradients occurred as a single broad peak between the densities of 1.18 and 1.24 g/ml as well as a slight residue of an apparently soluble form of chlorophyll at the top of the gradient. It is therefore apparent that a suitable marker for the quantitative assay of etioplasmic membranes must be found before the source of the ferrochelatase in band 2 can be unequivocally established, although the presence of cytochromes of the *b* and *c* type in these membranes, and the absence of cytochromes *a*+*a*₃, makes their etioplast origin highly likely.

Properties of ferrochelatase

Since the ferrochelatase of etiolated barley appeared to be associated with both etioplasts and mitochondrial membranes, studies were undertaken to determine whether this enzyme exhibited different properties depending on its intracellular location.

Effect of pH. Studies on the effect of pH on the ferrochelatase activity of various types of particulate preparations showed that the apparent pH optimum for ferrochelatase activity in etioplasts was lower than for that in the mitochondrial fractions. Fig. 4(a), for example, indicates that the ferrochelatase of washed etioplasts exhibited greatest activity at pH 7.3, whereas washed mitochondrial preparations showed greatest activity at pH 7.8. Since washed mitochondria were not homogeneous, the pH optimum of the ferrochelatase in the two major peaks was investigated. Ferrochelatase activity associated with the mitochondrial particles (band 1) showed an apparent optimal pH of 8.0 (Fig. 4b). Optimal activity for the ferrochelatase activity of particles sedimenting with the higher density (band 2) was observed to be, like that of the washed etioplasts, at a somewhat more acidic pH. This latter observation is in accord with the suggestion that the particulate components of this band are of etioplasmic origin.

Inhibition by metal porphyrins. The ferrochelatase activity of both the washed etioplasts and of washed mitochondria was inhibited by metalloporphyrins (Table 1), although there was little distinction between the two preparations in their sensitivity to the inhibitors. Substantial inhibition of ferrochelatase was found at concentrations of Zn-, Mg- or Fe-protoporphyrins below 10 μ M, and this sensitivity could be important in the regulation of the biosynthesis of haemoproteins or chlorophylls. It is well established that in photosynthetic bacteria the required ratio of chlorophyll to cytochromes in the photosynthetic electron-transport system is maintained partly through the feedback inhibition by protohaem of ferrochelatase (Burnham & Lascelles, 1963). In these photosynthetic bacteria the insertion of Mg²⁺ into protoporphyrin is sensitive to oxygen (Gorchein, 1973) and this serves to inhibit bacteriochlorophyll

Fig. 4. Effect of pH on the activity of ferrochelatase of various particulate fractions from etiolated barley

Results are expressed as a percentage of the maximum value observed for a given type of particulate fraction. Ferrochelatase activity was determined as described in the Methods and Materials section, except that the buffer at pH 6.8 and 7.0 was 68 mM-Mops and the buffer at pH 7.3–8.3 was 68 mM-Tris/HCl. (a) Curve E is for washed etioplasts and curve M for washed mitochondria. (b) Data for fractions of washed mitochondria prepared by sucrose-density-gradient centrifugation. Curve M₂ is for particles sedimenting at a density of 1.170–1.186 g/ml and curve M₃ for particles sedimenting at a density of 1.215–1.230 g/ml.

Table 1. Inhibition by metal porphyrins of ferrochelatase in washed etioplasts and washed mitochondria

Activity of the inhibited sample is compared with that of the control sample to which no inhibitor was added. Inhibition of ferrochelatase by iron protoporphyrin was estimated by use of the dual-wavelength procedure described in the Methods and Materials section. The pyridine haemochromogen assay was used for studies of the inhibition by the other metal porphyrins.

Addition to assay mixture	Concentration in assay mixture (μM)	Percentage inhibition of ferrochelatase	
		Washed etioplasts	Washed mitochondria
Fe-protoporphyrin	3	67	53
	6	67	65
Mg-protoporphyrin	7	72	63
	21	80	88
Zn-protoporphyrin	4	87	85
	12	74	94

Table 2. Porphyrin specificity of ferrochelatase in washed etioplasts and washed mitochondria

Ferrochelatase was determined by the pyridine haemochromogen method as described in the Methods and Materials section except that the porphyrin substrate was varied as indicated. The final concentrations of the porphyrins were: deuteroporphyrin IX, 30 μM; protoporphyrin IX, 38 μM; mesoporphyrin, 20 μM.

Porphyrin	Specific activity of ferrochelatase (nmol of metal porphyrin/h per mg of protein)	
	Washed etioplasts	Washed mitochondria
Deuteroporphyrin	9.2	6.4
Mesoporphyrin	3.0	2.5
Protoporphyrin	0.50	0.73

synthesis in aerobic conditions. In higher plants regulation of chlorophyll synthesis by oxygen at the Mg²⁺-insertion step is extremely unlikely, but large

concentrations of magnesium porphyrins do not accumulate in the dark, nor do excessive amounts of protohaem accumulate during the period of rapid chlorophyll synthesis during the greening of etiolated tissue when there must be very considerable protoporphyrin synthesis. The sensitivity of the ferro-

chelatase reaction to inhibition by protohaem and magnesium protoporphyrin may be important in regulating the flux of protoporphyrin to cytochromes or chlorophylls.

Porphyrin specificity. As Table 2 indicates, the ferrochelatase of washed etioplasts and washed mitochondria exhibited very similar specificity for three different porphyrins. With these two particulate fractions greatest activity was observed with deuteroporphyrin IX. Activity with mesoporphyrin IX was about one-third as great, and both fractions showed very low activity with protoporphyrin IX. This ratio of specific activities with different porphyrin substrates closely resembles that reported for liver mitochondria (e.g. Porra & Jones, 1963) and for the photosynthetic bacterium *Rhodospseudomonas spheroides* (Jones & Jones, 1970). Clearly protoporphyrin, the presumed natural substrate, is not as good a substrate for ferrochelatase as are its synthetic derivatives deuteroporphyrin and mesoporphyrin. This effect may arise from the lower solubility of the substrate protoporphyrin or the protohaem product in aqueous media.

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