Oxidation of protoporphyrinogen to protoporphyrin, a step in chlorophyll and haem biosynthesis

Purification and partial characterization of the enzyme from barley organelles

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The protoporphyrinogen-oxidizing enzyme from Triton X-100 extracts of the mitochondrial and etioplast fractions of etiolated barley was purified by using ion-exchange and hydroxyapatite chromatography. The purified enzyme from both organelle fractions exhibited a K_m of $5 \mu M$ and was labile to mild heat and acidification. The pH optimum (5–6) and the substrate-specificity (mesoporphyrinogen was oxidized as rapidly as protoporphyrinogen) revealed properties very different from the protoporphyrinogen-oxidizing enzyme of rat liver or yeast mitochondria, which is specific for protoporphyrinogen as substrate. The purest fractions showed a polypeptide band corresponding to an M_r of approx. 36000 on SDS/polyacrylamide-gel electrophoresis. This is the first purification and characterization of the enzyme from a plant, and indicates no readily detectable differences between the enzyme isolated from mitochondrial or etioplast fractions, although only the latter organelle has the capacity for both haem and chlorophyll synthesis.

INTRODUCTION

Protoporphyrinogen, an intermediate common to the haem and chlorophyll synthesis pathways (Scheme 1), can be enzymically oxidized to protoporphyrin in mammalian, yeast, bacterial and plant cells [1–20]. High levels of this activity are found in the membrane fraction of barley mitochondria and etioplasts [17,18]. Since etioplasts are capable of rapid chlorophyll synthesis [21–25], a role for this enzyme in chlorophyll synthesis is likely. Although the enzyme has been partially purified and characterized from mammalian and yeast mitochondria [3,4,26], no obvious cofactors were found and the mechanism of oxidation is unknown. Our previous studies with bacterial systems [10,12] have indicated involvement of quinones and other electron-transport components as intermediary carriers in anaerobic protoporphyrinogen oxidation.

The enzyme has not hitherto been purified from a plant source, although preliminary studies in crude extracts of

barley and spinach organelles suggested properties different from those of mammalian and bacterial systems [18,27]. An important question is whether this enzyme differs in cells capable of both chlorophyll and haem synthesis as compared with cells only capable of haem synthesis. The present paper reports the purification and partial characterization of the enzyme from barley mitochondrial and etioplast fractions. It was of interest to compare the enzyme from both organelles, since only the latter has the capacity for chlorophyll synthesis.

MATERIALS AND METHODS

Preparation and analysis of enzyme

Mitochondria from about 2000 g or etioplasts from about 6000 g of etiolated barley shoots were isolated by differential centrifugation as described previously [18] and stored at $-70^\circ C$ before use. Organelles were extracted with Triton X-100 as described previously [18], except that the buffer was 20 mM-Tris/HCl buffer, pH 7.6, containing dithiothreitol ($9 \mu g/ml$). The organelle concentration was adjusted to 12–16 mg of protein/ml. After ultracentrifugation [18], the supernatant (crude Triton extract) contained about 1.5 times the enzyme units found in the original organelles and the specific activity increased 1.5–3-fold (see the Results section). The crude Triton extract (15–20 ml) was desalted on a Sephadex G-25 column ($2.5 \text{ cm} \times 24 \text{ cm}$) equilibrated and eluted with buffer A [20 mM-Bis-Tris/HCl buffer, pH 6.8, containing 20% (v/v) glycerol and 0.1% (v/v) Triton X-100]. Enzyme activity was retained by this step, but no significant purification was achieved. The fractions with enzyme activity were pooled and placed on a DEAE-Bio-Gel A column ($16 \text{ cm} \times 2.5 \text{ cm}$) equilibrated with buffer A, and 4 ml fractions were collected. Some activity was eluted with 2 column volumes of buffer A (see the Results section). Further activity

Scheme 1. Pathway for haem and chlorophyll synthesis

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis.

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was subsequently eluted with a linear gradient of 0–0.1 M-NaCl in buffer A. More concentrated gradients resulted in no further elution of activity (see the Results section). All fractions were assayed for both activity and protein. To obtain the purest fractions, several fractions with the highest specific activity from the DEAE-Bio-Gel A column were pooled and concentrated to a volume of 2–5 ml by dialysis against buffer B [10 mM-potassium phosphate buffer, pH 6.8, containing 20% (v/v) glycerol] containing 30% (v/v) poly(ethylene glycol). Additional contaminating protein was removed from the concentrated DEAE-Bio-Gel A fractions by absorption with 0.7 vol. of packed hydroxyapatite that had been previously washed twice by centrifugation in buffer B. The mixture was stirred for 20 min, and the gel was then removed by centrifugation. The supernatant contained the enzyme. Equivalent removal of contaminating protein was achieved by passage of 2 ml of concentrated DEAE-Bio-Gel-A-purified enzyme over a hydroxyapatite column (2 cm × 2.5 cm) equilibrated with buffer B containing 0.1% Triton X-100. Fractions containing active enzyme did not adsorb on the column and were concentrated as above.

Purified fractions were analysed for polypeptide content by SDS/PAGE [28,29]. A 15% (1.5 mm-thick) slab gel was subjected to electrophoresis at 4 °C at constant current (29 mA) and stained with silver [30]. Enzymic activity and enzyme units (nmol of protoporphyrin formed/h) were determined as previously described, except that the non-enzymic rate of protoporphyrin formation was determined in the presence of buffer A, since this was found equivalent to the rate with heated enzyme [18,31].

Other methods

Routine protein analysis was by the method of Bradford [32]. Samples were diluted to less than 0.005% detergent, where interference by detergent was insignificant. Accuracy of this method was confirmed by analysing several crude and purified fractions for protein by a method involving prior removal of detergent [33] or a method that is independent of detergent (Bulletin 23225, BCA protein assay reagent; Pierce Chemical Co., Rockford, IL, U.S.A.). Triton was measured colorimetrically [34]. Absorption spectra were obtained with a Cary model 14 recording spectrophotometer, with buffer B in the reference cuvette. Reduced-minus-oxidized difference spectra were obtained with enzyme added to both the sample and reference cuvettes and a few crystals of $\text{Na}_2\text{S}_2\text{O}_4$ added to the sample cuvette.

Materials

Aquacide [poly(ethylene glycol)] was purchased from Calbiochem-Behring Corp., DEAE-Bio-Gel A, hydroxyapatite (Bio-Gel HT) and Bio-Rad protein assay reagent were from Bio-Rad Laboratories, and Sephadex G-25 and Sephacryl S-300 were from Pharmacia Fine Chemicals. M_r markers were from Bio-Rad Laboratories or Sigma Chemical Co.

RESULTS

Enzyme purification

For enzyme from both organelle fractions, chromatography on DEAE-Bio-Gel A achieved extensive purification. The elution pattern for a typical mitochon-

Fig. 1. Elution pattern of barley mitochondrial fraction on DEAE-Bio-Gel A

For experimental details see the text. ●··●, Protoporphyrinogen oxidase activity; △—△, protein.

drial fraction (Fig. 1) shows some activity being eluted before the 0.1–0.2 M-NaCl gradient but the bulk of the activity being eluted at the start of the gradient (fractions 59–78). The best fractions (fractions 64–68) from this latter peak were 50-fold purified over the original mitochondrial fraction (Table 1). (There was about a 2-fold variation in the specific activities of different batches of the original organelle fractions.)

When etioplast extract was applied to the DEAE-Bio-Gel A column, a similar pattern of elution was obtained except that more activity was eluted before application of the salt gradient (Table 2). These fractions had the highest specific activity (Table 2) and appeared the purest on SDS/PAGE (see below). A large amount of activity was also eluted from the DEAE-Bio-Gel A column at the start of the salt gradient (Table 2), as had occurred with the mitochondrial fraction.

The next step in purification of the DEAE-Bio-Gel A fractions from both organelles was treatment with hydroxyapatite (see the Materials and methods section). This usually resulted in a 2-fold increase in specific activity (see Tables 1 and 2) and the removal of minor amounts of several contaminating proteins that were apparent as contaminating bands on SDS/PAGE (see below).

Enzyme fractions eluted from the DEAE-Bio-Gel A column before and after the 0–0.1 M-NaCl gradient were compared, but we found no evidence that this difference in elution pattern might reflect different enzymes. For instance, no difference was found in pH optima and both fractions showed a similar pattern of the most prominent polypeptide bands on SDS/PAGE. In addition, when the major mitochondrial fraction that was eluted after the start of the salt gradient was dialysed and rechromatographed on a second DEAE-Bio-Gel A column (1.6 cm × 2.0 cm) prepared as described above, most of the enzyme units were now eluted in the fraction before the salt gradient. This procedure resulted in the purest fractions of mitochondrial enzyme as revealed by SDS/PAGE (see below).

Table 1. Purification of protoporphyrinogen oxidase from barley mitochondrial fraction

Details of purification procedure are described in the Materials and methods and Results sections. DEAE-Bio-Gel A fraction 59–78 represents the total activity in fractions 59–78 (see Fig. 2). DEAE-Bio-Gel A fraction 64–68 was the purest fraction from within this peak. Fractions were combined and concentrated as described in the text.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Sonicated mitochondria	22	352	4130	12
Crude Triton extract	16	192	6080	32
Sephadex G-25 fraction	39	183	6708	36
DEAE-Bio-Gel A fraction 59–78	10.4	8	3852	481
DEAE-Bio-Gel A fraction 64–68	3.5	1.7	1008	609
Hydroxyapatite fraction of DEAE-Bio-Gel A fraction 64–68	2.4	0.5	456	912

Table 2. Purification of protoporphyrinogen oxidase from barley etioplast fraction

The DEAE-Bio-Gel A fractions were concentrated from the active fractions that were eluted either before or at the beginning of the 0–0.1 M-NaCl gradient (see the Materials and methods and Results section). These values are an average for two typical purifications of two etioplast preparations.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Sonicated etioplasts	21.5	238	5860	25
Crude Triton extract	21	176	7428	42
Sephadex G-25 fraction	38	149	8027	54
DEAE-Bio-Gel A fraction (before salt gradient)	4.5	1.1	1183	1075
Hydroxyapatite fraction of above fraction	2.1	0.17	388	2282
DEAE-Bio-Gel A fraction (after salt gradient)	4.0	2.4	1513	630
Hydroxyapatite fraction of above fraction	3.7	0.9	670	744

Fig. 2. K_m of protoporphyrinogen oxidase purified from barley mitochondrial fraction

The assay mixture containing 0.19 mg of DEAE-Bio-Gel-A-purified mitochondrial fraction/ml was as described in the Materials and methods section except that the phosphate buffer used gave a final pH of 6.2. Samples for assay of activity were removed at 1 min intervals during the initial 5 min period of the reaction.

Fig. 3. pH optimum of protoporphyrinogen oxidase purified from barley mitochondrial and etioplast fractions

The assay was conducted as described in the Materials and methods section except for the buffers used and the addition of protoporphyrinogen at 8.8 mM. The buffers used were: glycine/HCl below pH 3; sodium acetate at pH 3.7–5.4; potassium phosphate at pH 6.2–7; Tris/HCl at pH 7.9–8.1. $\triangle$ — $\triangle$, DEAE-Bio-Gel-A-purified mitochondrial enzyme, 0.17 mg/ml of assay mixture; $\bullet$ — $\bullet$, DEAE-Bio-Gel-A-purified etioplast enzyme, 0.07 mg/ml of assay mixture.

Fig. 4. SDS/PAGE of stages in the purification of protoporphyrinogen oxidase from barley etioplast and mitochondrial fractions

(a) SDS/PAGE of stages in the purification of the enzyme from the etioplast fraction. Lane 1, crude etioplast extract (25 μ g of protein); lane 2, DEAE-Bio-Gel-A-purified enzyme (15 enzyme units, 8 μ g of protein); lane 3, most highly purified hydroxyapatite preparation (11 units, 4 μ g of protein); lane 4, M_r markers (92500, 66200, 45000, 31000, 21500 and 14400). (b) Stages in the purification of the enzyme from the mitochondrial fraction. Lane 1, M_r markers (66000, 45000, 36000, 29000, 24000, 20100 and 14200); lane 2, crude extract (20 μ g of protein); lane 3, DEAE-Bio-Gel-A-purified enzyme (6 units, 28 μ g of protein); lane 4, hydroxyapatite-purified enzyme re-purified on second DEAE-Bio-Gel A column (see the text) (1.2 units, 5 μ g of protein); lane 5, M_r markers (92000, 66200, 45000, 31000, 21500 and 14400).

pH optimum and K_m of purified enzyme

The pH-activity curve, showing an optimum at pH 5–6, was the same for the enzyme purified from both the barley mitochondrial and etioplast fractions (Fig. 3). The K_m value for protoporphyrinogen of the DEAE-Bio-Gel-A-purified enzyme from the mitochondrial fraction was approx. 5 μ M as determined by a double-reciprocal plot [35] (Fig. 2). The same value was obtained for the DEAE-Bio-Gel-A-purified etioplast enzyme (results not shown).

Substrate specificity

We previously reported that mesoporphyrinogen was oxidized as rapidly as protoporphyrinogen by crude Triton extracts of barley etioplasts. Other porphyrinogens were oxidized more slowly or not at all [18]. Using the techniques described previously [18] we determined that this degree of substrate-specificity persisted throughout the purification procedure of both barley mitochondrial and etioplast fractions. For instance, a hydroxyapatite-purified barley mitochondrial fraction exhibited an average specific activity (nmol of porphyrinogen oxidized/h per mg of protein) of 1157 for mesoporphyrinogen and 1093 for protoporphyrinogen. A hydroxyapatite-purified barley etioplast fraction exhibited an average specific activity of 979 for mesoporphyrinogen and 973 for protoporphyrinogen. Uroporphyrinogen I and coproporphyrinogen I were oxidized at less than 11% of the rate of protoporphyrinogen by purified enzyme from both organelle fractions.

Analysis of purified fractions by SDS/PAGE and gel-filtration chromatography

The preparation with the highest specific activity was a hydroxyapatite-purified preparation of etioplasts exhibiting a specific activity of 2750, which represented a 110-fold purification over the original etioplasts. On SDS/PAGE, this fraction showed a polypeptide corresponding to an M_r near 36000, which was apparent on gels stained with silver (Fig. 4a). This fraction also showed diffuse bands near the bottom of the gel. These lower bands did not stain with Coomassie Blue, which stained the 36000- M_r band lightly (results not shown). The significance of the bands near the bottom of the gel is unclear, but they could represent small degradation polypeptides or mixed micelles or SDS and Triton.

The purified preparations of the mitochondrial fraction revealed a similar pattern on SDS/PAGE. Hydroxyapatite-purified preparations typically had specific activities near 600, and always showed the prominent band near M_r 36000. However, several additional bands were present. These additional bands were removed by rechromatography on a second DEAE-Bio-Gel A column (described above). All the enzyme units eluted from this DEAE-Bio-Gel A column were associated with a single polypeptide band near M_r 36000, although the specific activity of this fraction was somewhat diminished. This highly purified fraction, along with a crude extract and the DEAE-Bio-Gel-A-purified fraction, is shown in Fig. 4(b).

The hydroxyapatite-purified enzyme preparations were also analysed by gel-filtration chromatography on columns of Sephacryl S-300. Fig. 5, illustrating the elution

Fig. 5. Gel-filtration chromatography of purified barley etioplast protoporphyrinogen oxidase

Hydroxyapatite-purified etioplast enzyme (44 units, 90 µg of protein) was chromatographed on a Sephacryl S-300 column (30 cm × 0.9 cm) that was equilibrated and eluted with buffer B containing 0.1% Triton X-100. The fraction size was 0.9 ml. The arrow denotes elution of Blue Dextran marker. ●—●, Enzyme activity; △--△, protein. For estimation of M_r , marker proteins of M_r 232000 (catalase), 150000 (alcohol dehydrogenase) and 67000 (bovine serum albumin) were chromatographed separately on the same column.

pattern for etioplasts, indicates that all of the enzyme units and the protein were eluted in a single peak. Identical elution patterns were obtained for the purified enzyme from the mitochondrial fraction (63 units of enzyme and 140 µg of protein) (results not shown). These peaks corresponded to an M_r of approx. 210000 when plotted against the elution volume of standard marker proteins on the same Sephacryl column. A possible interpretation is that the individual polypeptides of M_r 36000 revealed by SDS/PAGE of these purified preparations are present as multiple copies in the undenatured preparations. The actual M_r of the native enzyme as determined by gel filtration remains in doubt owing to the presence of Triton micelles associated with the purified preparations (see below).

After passage of the purified enzyme from the mitochondrial fraction over the Sephacryl S-300 column, the enzyme activity exhibited a dependence on oleic acid for maximum activity. Addition of oleic acid (1 mg/ml) to the assay mixture caused a doubling in enzymic activity, as we previously reported [25] for other preparations of the barley enzyme after chromatography on Sephacryl S-200.

Reaction rate, stability and absorption spectra

The rate of enzyme activity was linear with time for approx. 10 min under standard assay conditions [18] and with DEAE-Bio-Gel-A-purified preparations. After 10 min the rate gradually declined, as shown previously for crude barley organelle preparations [18]. Purified preparations also showed approximately linear increases in activity with increase in enzyme concentration. However, at low enzyme concentrations somewhat higher-than-expected activities were observed, as previously illustrated for crude barley organelles [18].

Heating the DEAE-Bio-Gel-A-purified enzyme from both organelle fractions in buffer A under the following conditions caused the percentage decrease in enzyme activity shown in parentheses: 55 °C, 60 min, (80%); 65 °C, 60 min, (97%); 85 °C, 20 min, (94%); 100 °C, 15 min,

(99%). When the DEAE-Bio-Gel-A-purified enzyme from both organelle fractions was adjusted to a pH below 1 with HCl and immediately neutralized, 85% of the activity was destroyed. Purified enzyme samples could be routinely stored at -70 °C in 20% (v/v) glycerol [18] for several weeks with minimal loss of activity. Incubation of the purified enzyme with trypsin had no effect on enzyme stability. This insensitivity to trypsin digestion was previously noted for the crude barley enzyme [18].

The absorption spectrum of an hydroxyapatite-purified mitochondrial preparation (381 units and 0.43 mg of protein/ml) showed no evidence for large amounts of chromophoric cofactors absorbing in the visible region (700 to 450 nm) either in direct spectra or in dithionite-reduced-minus-oxidized spectra (see the Materials and methods section). A dilution of this purified preparation to a protein concentration of 14 µg/ml showed a peak at 275 nm with a shoulder at 285 nm, which was nearly identical with the spectrum of a solution of Triton X-100 (200 µg/ml in buffer B). These findings suggest that the purified enzyme does not contain large amounts of chlorophyll-like pigments or cytochromes, which absorb in the visible region. However, even after extensive purification, large amounts of Triton remain associated with the enzyme. This was confirmed by chemical analysis of the DEAE-Bio-Gel-A-purified mitochondrial enzyme, which revealed 3.6 mg of Triton/0.24 mg of protein. Careful analysis for smaller amounts of respiratory pigments and metals must await preparation of larger amounts of pure enzyme.

DISCUSSION

Our previous studies demonstrating high activities of protoporphyrinogen oxidase in the membrane fraction of plant organelles capable of rapid chlorophyll synthesis suggest a role for this enzyme in chlorophyll as well as haem synthesis. However, the enzyme has not previously been purified or characterized from a plant source. In the present study, enzyme preparations purified more than 100-fold exhibited a prominent polypeptide band on SDS/PAGE equivalent to an M_r near 36000.

Several properties of the barley organelle enzyme are quite different from those of the mammalian or the yeast enzyme, which can only be involved in haem synthesis. The K_m value of the barley enzyme, 5 µM, was similar to those reported for the mammalian mitochondrial enzyme, 4 µM, and the yeast mitochondrial enzyme, 12 µM [3,4]. However, the pH optimum of the barley enzyme, pH 5-6, is considerably lower than reported for the mammalian or the yeast enzyme, at pH 8.5 and 7.5 respectively [3,4]. The substrate specificities of the mammalian and plant systems are remarkably different. The barley enzyme oxidized mesoporphyrinogen, (ethyl groups at positions 2 and 4 of the porphyrin ring) as rapidly as protoporphyrinogen (vinyl groups at these positions). In contrast, the rat liver enzyme is specific for protoporphyrinogen as substrate [3]. Although these differences in substrate-specificity may be characteristic of tissues that synthesize chlorophyll, a recent study has shown that the enzyme from human liver mitochondria also utilizes both protoporphyrinogen and mesoporphyrinogen as substrate [36].

A secondary objective of the present study was to compare the barley etioplast with the barley mito-

chondrial enzyme, since only the former organelle can make chlorophyll as well as haem. In a previous study, with organelles separated and purified by sucrose-gradient centrifugation, we demonstrated that this enzyme was present at high levels of activity in both barley etioplasts and mitochondria. Separation in sucrose gradients ensured the purity of the organelle fractions, and has been used by others to demonstrate the presence of the ferrochelatase enzyme in barley etioplasts and mitochondria [37]. Barley mitochondria prepared by simple differential centrifugation are contaminated with fragments of etioplasts [37]. In the present investigation, we used barley organelles prepared by simple differential centrifugation to obtain sufficient quantities of enzyme for purification. Using this technique, we found no evidence for readily detectable differences in either kinetic or physical properties between the enzymes isolated from the two organelle fractions.

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