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PURIFICATION OF AN NADH CYTOCHROME c REDUCTASE FROM CELL-FREE EXTRACTS OF BARLEY

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Key Word Index—*Hordeum vulgare*, Gramineae, barley, cytochrome c reductase species, purification

Abstract—The major cytochrome c reductase species present in cell-free extracts from 7-day old barley shoots was purified 848-fold by ammonium sulphate fractionation (30–60%), gel filtration on Biogel A1 5m and affinity chromatography on blue-dextran Sepharose Both cytochrome c reductase activity and nitroblue tetrazolium (NBT)-reductase activity of the preparation possessed a sedimentation coefficient of 3 1S. The single protein species seen after electrophoresis in non-denaturing gels comigrated with NBT-reductase activity SDS gel electrophoresis also showed that the preparation was homogeneous and had a MW of 38 000 The species could not be stained for haem in non-denaturing gels under conditions which stained both a purified barley nitrate reductase sample and equine heart cytochrome c This species is the 3 1S cytochrome c reductase species, with calculated MW of 40 000, which appears under conditions which allow loss of NADH nitrate reductase activity

INTRODUCTION

The assimilatory NADH nitrate reductase (NADH nitrate oxidoreductase, EC 1 6 6 1) of higher plants is a haemomolybdoflavoprotein which catalyses the two electron reduction of nitrate to nitrite This enzyme is unstable in cell-free extracts from a variety of plant species unless BSA or casein is included in the extraction buffer [1–7], and in barley, loss of nitrate reductase activity (and its associated cytochrome c reductase activity [8]) is accompanied by the appearance of smaller cytochrome c reductase species of 3 1S and 3 8S [9, 10] Although these species were first described in barley [8], apparently equivalent species have subsequently been identified in cell-free extracts from rice [11], spinach [12], maize [13] and tobacco [14, 15]

In this paper we describe the purification and partial characterisation of the 3 1S cytochrome c reductase species from extracts of 7-day old barley shoots A preliminary account of this work has been presented [16]

RESULTS

Purification of cytochrome c reductase

Seven-day old shoots were extracted as described in Experimental and after streptomycin sulphate treatment, the 30–60% ammonium sulphate fraction was applied to a Biogel A 1.5 m column The major peak of cytochrome c reductase activity (Fig 1A) was pooled, and after concentration by ammonium sulphate fractionation (60%), applied to a blue-dextran Sepharose column After washing off unadsorbed protein, cytochrome c reductase activity was eluted with buffer containing 5 μM NADH (Fig 1B) and concentrated with an Amicon thin channel concentrator (PM 10 membrane) The overall purification was 848-fold (Table 1)

Sucrose density gradient analysis of cytochrome c reductase

Sucrose density gradient analysis of the blue-dextran Sepharose eluted cytochrome c reductase species showed that it had a sedimentation coefficient of 3 1S (Fig 2) The preparation was able to catalyse the NADH dependent reduction of nitroblue tetrazolium (NBT) and a single band of NBT-reductase activity, which cosedimented with NADH cytochrome c reductase activity at 3 1S, was found after sucrose density gradient analysis (Fig 2)

Assessment of purity, MW and haem content of cytochrome c reductase

Electrophoresis in non-denaturing gels and subsequent gel staining with Coomassie blue showed the presence of a single protein species in the preparation which comigrated with NBT-reductase activity (Fig 3) This species could not be stained for the presence of haem under conditions which stained 30 μg of horse heart cytochrome c and 30 μg of purified barley nitrate reductase (Fig 3) SDS gel electrophoresis of the fragment showed that it was homogeneous (Fig 3) and had a MW of 38 000 (Fig 4)

DISCUSSION

Nitrate reductase is relatively stable in cell-free extracts from 4-day old barley shoots and these extracts contain only small amounts of cytochrome c reductase species of 3 1S and 3 8S, which possess MWs (calculated from the sedimentation coefficient and Stokes' radius by the method of Siegel and Monty [17]) of 40 000 and 61 000 [9] The enzyme is much more unstable in cell-free

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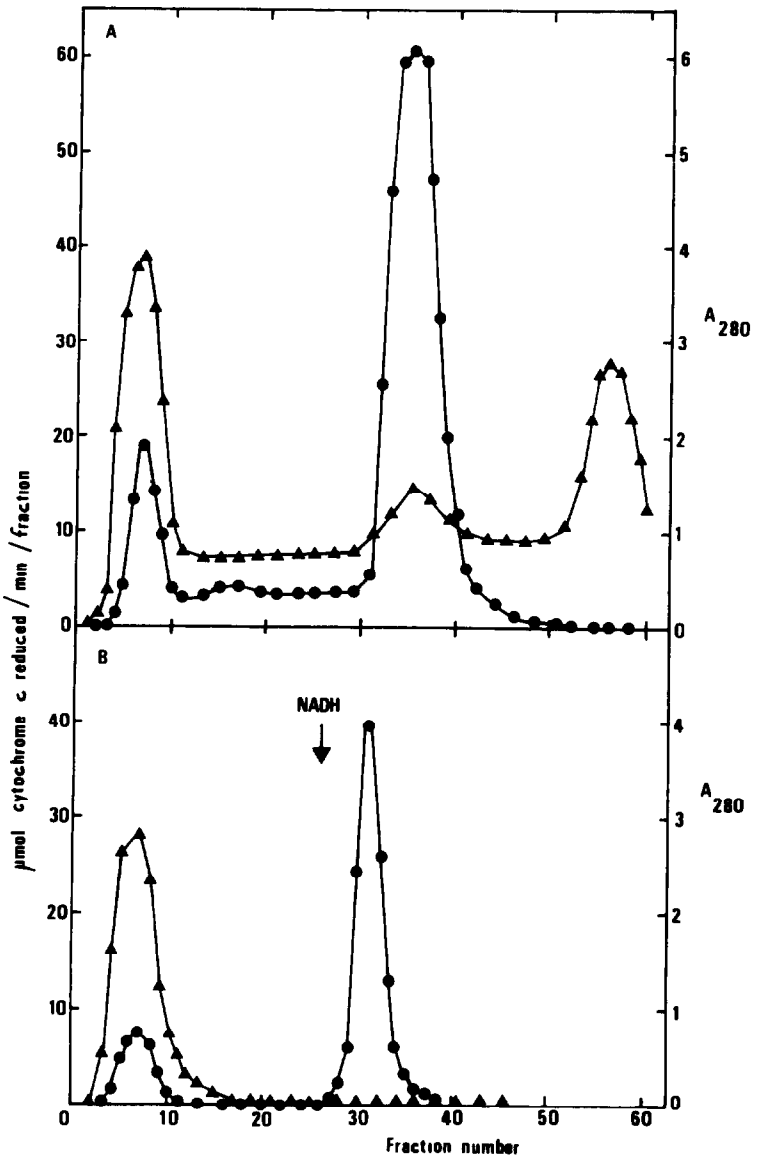

Fig 1 (A) Distribution of NADH cytochrome c reductase activity (●) and protein (▲) after Biogel A1 5m gel filtration (4 1 cm × 108 cm column) of a 30–60% $(NH_4)_2SO_4$ fraction derived from 168-hr old barley shoots Elution buffer was buffer I and 16 ml fractions were collected (B) Distribution of NADH cytochrome c reductase activity (●) and protein (▲) after blue dextran Sepharose affinity chromatography (2 × 9 cm column) of pooled fractions containing NADH cytochrome c reductase activity from Biogel A1 5m Elution buffer was buffer II (0 02 M potassium phosphate buffer, pH 7 5, containing 0 1 mM EDTA, 10 μm FAD and 1 mM cysteine) and 5 ml fractions were collected The arrow denotes start of elution of NADH cytochrome c reductase activity with buffer II containing 5 μm NADH

extracts from shoots older than 4 days and loss of nitrate reductase is accompanied by the appearance of increased amounts of these cytochrome c reductase species [10] We have taken advantage of this fact, and the likelihood that these cytochrome c reductase species, like nitrate reductase, possess a dinucleotide fold [18], to purify the major small cytochrome c reductase species from cell-free extracts of 7-day old barley shoots by a combination of fractional ammonium sulphate precipitation (30–60%), gel filtration on Biogel A1 5m and affinity chromatography on blue-dextran Sepharose (Table 1)

The preparation contained a single protein species which could transfer electrons from NADH to cytochrome c and nitroblue tetrazolium Since this species had a sedimentation coefficient of 3 1S it is equivalent to one of the cytochrome c reductase species seen previously in cell-free extracts [9, 10] The MW weight of 38 000 determined here by SDS gel electrophoresis (Fig 4) agrees well with the MW of 40 000 calculated previously from the sedimentation coefficient and Stokes radius [9] This MW 38 000 cytochrome c reductase species did not contain haem (Fig 3)

Several pieces of circumstantial evidence have led us to the suggestion that this 3 1S, MW 38 000 cytochrome c

Table 1 Purification of NADH cytochrome c reductase

Step	Volume (ml)	Protein (mg)	Activity* (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Supernatant from streptomycin sulphate (5 mg/ml) treated homogenate	803	3520	1120	0 32	100	—
30–60% $(NH_4)_2SO_4$ fraction	40	2680	739	0 28	66 2	—
Biogel peak (pooled and concentrated)	4	420	213	0 51	19 1	1 6
Blue dextran Sepharose peak (pooled and concentrated)	6 4	0 35	95	271 4	8 5	848

*μmol cytochrome c reduced/min

Cytochrome c reductase was purified as described in Experimental from cell-free extracts of 7-day old barley shoots

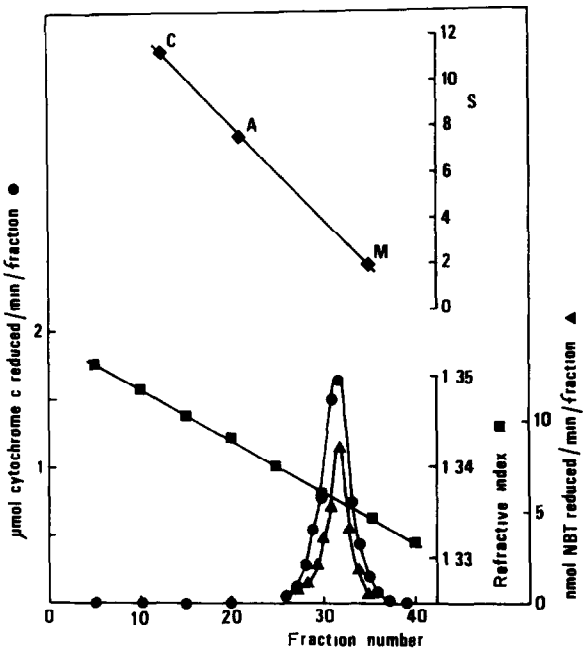

Fig 2 Distribution of NADH cytochrome c reductase (●) and nitroblue tetrazolium reductase (▲) activity after sucrose density gradient analysis of cytochrome c reductase purified as in Table 1 C, A and M denote the position of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation Conditions of centrifugation are described in the Experimental

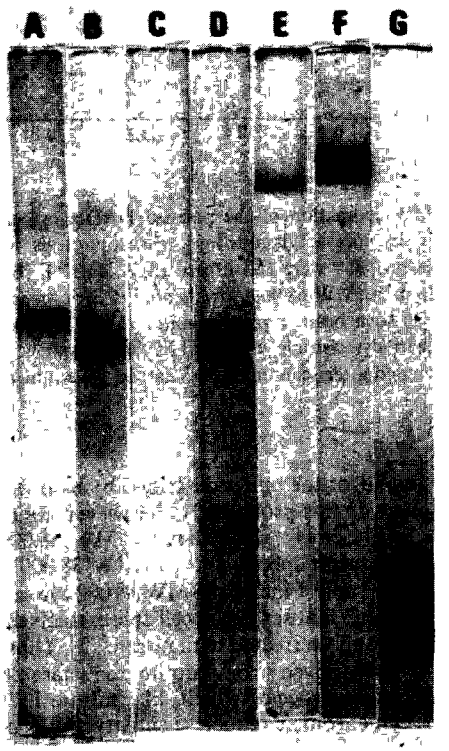

Fig 3 Polyacrylamide gel electrophoresis of 30 μg purified NADH cytochrome c reductase (CR) and 30 μg purified nitrate reductase (NR) Gels A–C and E–G are non-denaturing gels Gel D is an SDS gel After electrophoresis gels were specifically stained as follows (A) CR stained for protein with Coomassie blue, (B) CR stained for nitroblue tetrazolium reductase activity, (C) CR stained for haem, (D) CR stained for protein with Coomassie blue in an SDS gel, (E) NR stained for protein with Coomassie blue, (F) NR stained for haem according to ref [25] and (G) 30 μg equine heart cytochrome c stained for haem as in (F) The R_f of the band in (A) and (B) was 0 42 The R_f of the band in (E) and (F) was 0 23

reductase species is a degradation fragment of nitrate reductase [10, 19] Firstly, as indicated above, loss of the 7 7S nitrate reductase species (and its associated cytochrome c reductase activity), which occurs in cell-free extracts prepared from shoots older than 4 days, is accompanied by the appearance of increased amounts of the MW 38 000 cytochrome c reductase species [10] Secondly, inclusion of BSA in the extraction buffer not only prevents loss of the 7 7S nitrate reductase species (and its associated cytochrome c reductase activity), but also prevents formation of increased amounts of the MW 38 000 species [10] Thirdly, inclusion of leupeptin, a specific inhibitor of the major (cysteine) endoproteinase in barley [20], in the extraction buffer has the same effect as BSA [10] Fourthly, we have shown that the MW 103 000 subunit of the MW 205 000 barley nitrate reductase is susceptible to proteolytic nicking and can be degraded to smaller protein species, including one of MW 38 000 [21]

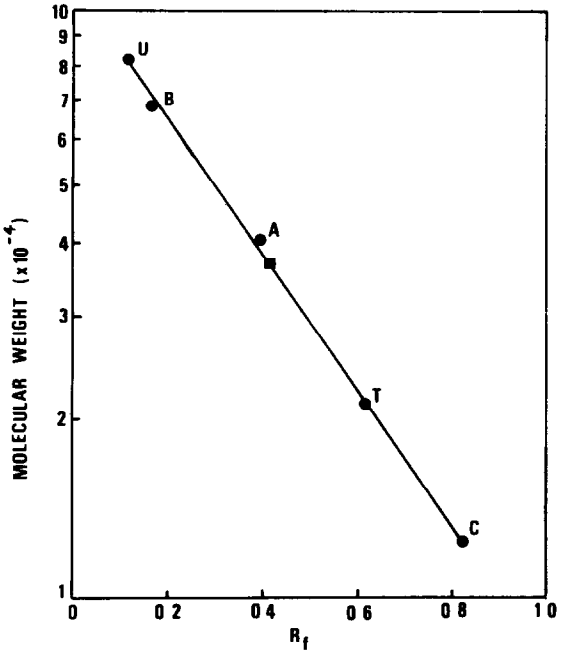

Fig. 4 MW estimation of the purified NADH cytochrome c reductase by polyacrylamide gel electrophoresis in SDS Reference proteins were (U) urease (MW 83 000), (B) bovine serum albumin (MW 68 000), (A) alcohol dehydrogenase (MW 41 000), (T) soya bean trypsin inhibitor (MW 21 000) and (C) equine heart cytochrome c (MW 12 500) R_f values were estimated after scanning in a Vitatron gel scanner

Leupeptin and/or the buffer described by Kuo *et al* [22] strongly retards this process [21] However, we have no evidence at present to show that the MW 38 000 cytochrome c reductase species and the MW 38 000 protein species are the same Finally, the MW 38 000 cytochrome c reductase species is absent from cell-free extracts of plants which have not been treated with nitrate [9] Thus, if this species is not derived from nitrate reductase it is an unrelated protein species which, like nitrate reductase, possesses cytochrome c reductase activity and is induced by nitrate

EXPERIMENTAL

Growth of plant material Plants of *Hordeum vulgare* cv Golden Promise were grown in trays of vermiculite as previously described [9, 10]

Purification of cytochrome c reductase 300 g 168-hr old barley shoots were ground in a chilled mortar with 0 05 M KPi buffer, pH 7 5, containing 0 1 mM EDTA and 10 μM FAD (buffer I) (3 ml buffer/g tissue) The brei was squeezed through muslin, streptomycin sulphate added (5 mg/g tissue) and after stirring at 4° for 5 min precipitated nucleic acid material was removed by centrifugation The supernatant was adjusted to 30% satn with solid $(NH_4)_2SO_4$ and precipitated protein, which includes most of the high MW cytochrome c reductase activity, was removed by centrifugation The supernatant was adjusted to 60% satn with $(NH_4)_2SO_4$, the precipitated protein was collected by centrifugation (the discarded supernatant contains the constitutive MW 27 800 cytochrome c reductase species [9]), dissolved in 20 ml of buffer I and applied to a column (4 1 × 108 cm) of Biogel A 1 5 m equilibrated in the same buffer 1 Fractions in the major peak of cytochrome c reductase activity were pooled, protein precipitated with $(NH_4)_2SO_4$, collected by centrifugation, resuspended in 25 ml of buffer I and glycerol to 40% (w/v) and stored at −70° overnight Protein was precipitated with $(NH_4)_2SO_4$ to 60% satn, dissolved in a minimum vol of 20 mM KPi buffer pH 7 5, containing 0 1 mM EDTA and 10 μM FAD (buffer II) and applied to a blue-dextran Sepharose column (2 × 9 cm) equilibrated in the same buffer The column was washed until the A_{280} of the eluate was less than 0 02 and cytochrome c reductase activity was eluted with 5 μM NADH in buffer II The peak fractions were pooled and concentrated to around 6 ml with an Amicon thin channel concentrator (PM 10 membrane)

Electrophoresis was carried out in 5% acrylamide gels prepared in 0 2 M Tris–HCl, pH 8 5 Running buffer was 0 08 M Tris–HCl, pH 8 5 Protein was detected after electrophoresis by staining with Coomassie blue, NBT reductase activity by the method of ref [23], methyl viologen nitrate reductase activity by the method of ref [24], except that nitrate was substituted for nitrite and haem by the method of ref [25] For SDS gel electrophoresis, samples and standard proteins were denatured by boiling in 0 02 M KPi buffer, pH 7, containing 1 25% SDS and 2 5% β-mercaptoethanol Electrophoresis was performed according to the method of ref [26]

Sucrose density gradient analysis was carried out as described previously [9, 10]

Nitrate reductase, cytochrome c reductase and NBT reductase were assayed as described previously [8, 23]

Protein was determined by the method of ref [27]

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