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INTRACELLULAR LOCALISATION OF HEXOKINASE IN *CUSCUTA REFLEXA*

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Key Word Index—*Cuscuta reflexa*; Convolvulaceae; angiospermic parasite; hexokinase; phosphotransferase.

Abstract—In *Cuscuta reflexa* 16% of the hexokinase activity was associated with the particulate fraction and the rest in the 105000 *g*, 1 hr supernatant. In a sucrose gradient, hexokinase activity banded with an organelle at a mean density of 1.20 g cm^{-3}, coinciding with the mitochondrial marker, cytochrome *c* oxidase. Fractionation of isolated mitochondria by digitonin showed the presence of the enzyme in the outer membrane along with its marker rotenone-insensitive NADH cytochrome *c* reductase. No latent form of hexokinase was detected.

INTRODUCTION

The distribution of hexokinase within the plant cell is not clear. Most of the enzyme in maize endosperm was located in the 40000 *g* supernatant and less than 5% was precipitated from the crude homogenate [1]. On the other hand, the enzyme was found associated exclusively with mitochondria in potatoes [2]. The enzyme occurs in the particulate as well as in the soluble form in pea seeds [3], castor bean [4], lentil roots [5], *Lillium longiflorum* pollen [6] and pea stem [7]. About one third of the enzyme activity in *Hordeum distichum* grain was in the amyloplast fraction, the rest in the soluble fraction [8]. In sugar beet, hexokinase existed in three compartments; 12–20% in mitochondria, about 10% in microsomes, and the remainder in the soluble fraction [9]. In view of the varied localisation pattern of hexokinase in plant tissues, the compartmentation of the enzyme in *Cuscuta reflexa* was undertaken to understand the regulation of carbohydrate metabolism in angiosperm parasites.

RESULTS

Distribution of chlorophyll, protein, cytochrome c oxidase and hexokinase in subcellular fractions

Most (78%) of the chlorophyll of the homogenate was present in the 15000 *g* fraction; about 11 and 8% was present in 100 *g* and 105000 *g* fractions resp (Table 1). Over 55% of the total protein of the homogenate passed into the supernatant fraction, the rest was distributed among the particulate fractions. Cytochrome *c* oxidase activity was concentrated in 15000 *g* fraction with 7.5-fold enrichment.

The major part (81%) of hexokinase activity was present in the 105000 *g* supernatant. About 16% of the activity of the homogenate was associated with 15000 *g* fraction which contained chloroplasts and mitochondria, as is evident by the chlorophyll content and cytochrome *c* oxidase activity of the fraction. The enzyme was enriched 1.4- and 1.5-fold resp in the 105000 *g* supernatant and 15000 *g* residue. No activity was detected in

Table 1. Distribution of chlorophyll, protein, cytochrome *c* oxidase and hexokinase in subcellular fractions from *C. reflexa* by differential centrifugation

Fraction	Chlorophyll (mg)	Protein (mg)	Hexokinase		Cytochrome *c* oxidase	
			Activity unit	Sp. act. unit/mg protein	Activity unit	Sp. act. unit/mg protein
Homogenate	0.037 (100)	8.50 (100)	0.085 (100)	0.010	0.12 (100)	0.014
100 *g* pellet	0.004 (11)	0.96 (11)	0 (0)	0	0 (0)	0
15000 *g* pellet	0.029 (78)	0.95 (11)	0.014 (16)	0.015	0.10 (83)	0.105
105000 *g* pellet	0.003 (8)	0.77 (9)	0 (0)	0	0 (0)	0
105000 *g* supernatant	0 (0)	4.80 (55)	0.069 (81)	0.014	0 (0)	0

40 *g Cuscuta* filaments were employed. Enzyme units refer to μmol substrate reacted/min. Values within parentheses represent the relative distribution.

the 100 *g* fraction which consisted of cell debris, starch and whole chloroplasts or in the 105000 *g* pellet, presumably consisting of microsomes. The distribution of hexokinase remained unaltered on washing the particulate fractions two times instead of once. Also the change in pH of Pi buffer from 7 to 8, replacement of Pi buffer with Tris–HCl buffer, or incorporation of PVP (MW 40000) in the isolation medium had no effect on the distribution pattern of hexokinase. Enzyme activity could not be demonstrated in the absence of cysteine in the isolation medium. The optimum concentration of cysteine was 50 mM.

Density gradient centrifugation

The data on distribution of chlorophyll, protein, hexokinase, cytochrome *c* oxidase, and catalase among various fractions obtained from discontinuous sucrose density gradient separation of the 15000 *g* pellet are given in Fig. 1.

Peroxisomes as indicated by the marker enzyme, catalase, were well separated from the mitochondria and chloroplasts and banded at sucrose density of 1.23 $g\,cm^{-3}$. Although the mitochondrial fraction, characterized by the content of cytochrome *c* oxidase, contained also chlorophyll, the peak of mitochondria could be well distinguished from the chloroplasts. Two peaks of chlorophyll were observed, a small peak at density of 1.22 $g\,cm^{-3}$, presumably representing intact chloroplasts and a major peak at density of 1.18–1.19 $g\,cm^{-3}$ representing broken chloroplasts. Hexokinase activity was found in fractions 9–11, which banded at density of 1.19–1.21 $g\,cm^{-3}$ and overlapped cytochrome *c* oxidase activity. Since hexokinase activity was not present in other fractions containing chlorophyll, it is unlikely that hexokinase was localised in chloroplasts. It is also significant that both hexokinase and cytochrome *c* oxidase activities in fractions 9–11 were enriched 3 to 5-fold from the 15000 *g* pellet.

Effect of detergents on mitochondrial enzyme activities

Preincubation of the mitochondrial fraction with 0.1, 0.2 and 0.5 % Triton X-100 for 30 min in cold did not result in increased hexokinase activity. On the other hand, 35 % lower activity was observed with the 0.5 % Triton treated preparation. Treatment of the mitochondrial fraction with digitonin (digitonin–protein = 5:12) did not alter the hexokinase activity, but the activity of succinate cytochrome *c* reductase, another mitochondrial enzyme, was enhanced 6-fold.

Fractionation of mitochondrial membranes

Hexokinase activity was concentrated in the 105000 *g* pellet with about 5-fold enrichment (Table 2). This fraction appears to be the outer membrane, since it contained practically all rotenone-insensitive NADH cytochrome *c* reductase with about 5-fold enrichment, was devoid of rotenone-sensitive NADH cytochrome *c* reductase, and was very poor in succinate cytochrome *c* reductase and malate dehydrogenase. Hexokinase activity was not found in 8500 *g* pellet, which consisted largely of the inner membrane, being enriched in rotenone sensitive NADH cytochrome *c* reductase, and possessing high succinate cytochrome *c* reductase and malate dehydrogenase activities. The 105000 *g* supernatant, containing the major part of the malate dehydrogenase activity with about 8-fold enrichment, and no succinate cytochrome *c* reductase and NADH cytochrome *c* reductase, thus representing the matrix, also had no hexokinase activity.

DISCUSSION

Hexokinase activity in *Cuscuta reflexa* filaments was found partially associated with the particulate fraction (15000 *g*, 45 min), and the rest was present in the soluble form. The presence of the enzyme in the particulate fraction was not due to adherence of the soluble enzyme

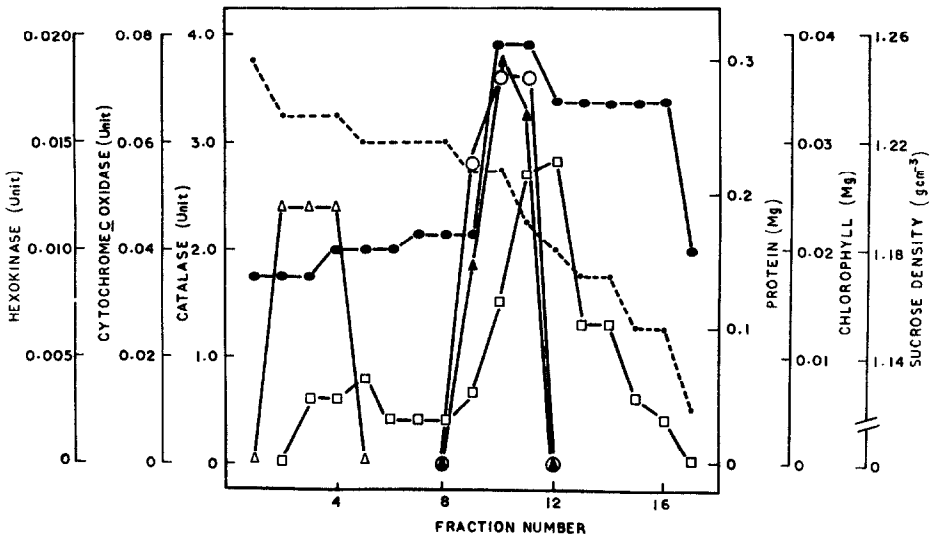

Fig. 1. Sucrose density gradient profile of 15000 *g*, 45 min pellet from *C. reflexa*. Hexokinase (○———○), Cytochrome *c* oxidase (▲———▲), Catalase (△———△), Protein (●———●), Chlorophyll (□———), Sucrose density (●-----●).

Table 2. Fractionation of mitochondrial membranes from *C. reflexa*

Fraction	Protein mg	Hexokinase		NADH cytochrome c reductase				Succinate cytochrome c reductase		Malate dehydrogenase	
				Rotenone sensitive		Rotenone insensitive					
		Total units	unit/mg protein	Total units	unit/mg protein	Total units	unit/mg protein	Total units	unit/mg protein	Total units	unit/mg protein
Control	23.8 (100)	0.50 (100)	0.021	1.80 (100)	0.075	0.98 (100)	0.041	0.20 (100)	0.008	5.75 (100)	0.24
8500 *g* pellet	14.7 (62)	0	0	1.26 (70)	0.085	0	0	0.17 (85)	0.011	2.17 (38)	0.14
105000 *g* pellet	4.4 (18)	0.44 (88)	0.100	0	0	0.89 (91)	0.202	0.01 (5)	0.002	0.90 (16)	0.20
105000 *g* supernatant	1.5 (6)	0	0	0	0	0	0	0	0	2.70 (47)	1.80

Fractionation with digitonin was carried out employing 7 ml of the mitochondrial preparation, keeping digitonin–protein ratio of 5:12. Enzyme units refer to μmol substrate reacted/min. Values within parentheses represent recovery from unfractionated mitochondrial preparation

to the organelle, since no hexokinase activity was detected in the second washing.

In contrast to microsomal localisation of hexokinase in sugar beet (9), no hexokinase activity was found in microsomes or peroxisomes of *Cuscuta* filaments. The 15000 *g* fraction consisted of chloroplasts and mitochondria, as was evident from the chlorophyll content and cytochrome *c* oxidase activity. Since hexokinase activity coincided with cytochrome *c* oxidase activity on a sucrose gradient and cytochrome *c* oxidase is an established marker of the mitochondria, it is concluded that the particulate hexokinase is associated with mitochondria. Although chlorophyll was present in fractions containing hexokinase and cytochrome *c* oxidase activities, there appears to be less possibility of the association of hexokinase with chloroplasts, since the chlorophyll peaks did not coincide with the hexokinase activity.

It is also concluded that *Cuscuta* hexokinase was localised in the outer membrane of mitochondria, since it was associated with the fraction enriched in rotenone-insensitive NADH cytochrome c reductase, an outer membrane marker [10–12]. Also no hexokinase activity was detected in the inner membrane fraction, identified by the marker succinate cytochrome *c* reductase [10], or the matrix fraction, enriched in malate dehydrogenase activity [10]. The rat brain hexokinase is reported to be present in the outer membrane of mitochondria [13, 14], but Wilson [15] and Vallejo [16] observed its association with the inner membrane fragments. The lentil root hexokinase is believed to be present in the outer membrane of mitochondria [5]. The integrity of mitochondria isolated from *Cuscuta* is evident by a low succinate cytochrome *c* reductase activity in the untreated mitochondrial fraction and over 6-fold increased activity on digitonin treatment. However, no disruption of the mitochondrial membrane was essential to assay the hexokinase activity, since digitonin known to disrupt mitochondrial membrane [17] had no effect on assayable hexokinase activity. This could be considered as an additional evidence in favour of localisation of the *Cuscuta* hexokinase on the outer membrane, since low MW substrates may be freely permeable through the outer mitochondrial membrane [18].

The dual localisation of hexokinase in cytosol and mitochondria suggests a special role of the enzyme in *Cuscuta* filaments. Whereas, the cytosol enzyme may be involved in glycolysis and starch synthesis, the mitochondrial enzyme may play an important role in cellular energy economy. Also hexokinase activity could be regulated by compartmentation of the enzyme between the mitochondria and cytosol.

EXPERIMENTAL

Plant. Cuscuta reflexa Roxb. growing on *Lantana camara* Linn. was collected from the University campus.

Preparation of homogenate. 40 g *Cuscuta* filaments, collected from the haustorial region, were ground in a medium consisting of 0.5 M sucrose, 50 mM freshly neutralised cysteine–HCl and 50 mM Pi buffer, pH 7.5, in a chilled glass mortar, and after straining through 2 layers of muslin the vol. was made up to 100 ml.

Cell fractionation. Homogenate was centrifuged at 100 *g* for 20 min in a refrigerated centrifuge. Supernatant was decanted; the sediment washed once with 10 ml isolation medium and centrifuged as above to give a fraction consisting of whole chloroplasts, starch grains and cell debris. Supernatants were combined and centrifuged at 15000 *g* for 45 min; Sediment was washed with 10 ml medium and resedimented at 15000 *g* for 45 min to give the crude mitochondrial fraction. The 15000 *g* supernatant together with the washing was centrifuged at 105000 *g* for 60 min in an ultracentrifuge and the supernatant decanted off. The sediment was washed once with 10 ml medium and recentrifuged at 105000 *g* for 60 min to give the microsomal fraction. The combined final supernatant was termed the soluble fraction. Each particulate fraction was resuspended in 5 ml of the isolation medium using a hand operated glass homogenizer. In one expt, the particulate fractions were washed 2× and the second washing analysed for hexokinase activity.

Separation of organelles by sucrose density gradient centrifugation. The 15000 *g*, 45 min pellet was obtained after discarding 100 *g*, 20 min particulate fraction and was suspended in 5 ml of the isolation medium. The gradient was prepared at 4° by pipetting in succession 0·9 ml of 2.5 M, 0.9 ml of 2.0 M, 0.9 ml of 1.8 M, 0.9 ml of 1.7 M and 0.9 ml of 1.5 M sucrose in 50 mM Pi buffer, pH 7.5, into a 5 ml polycarbonate tube and stored for 2 hr. 0.5 ml each of the pellet suspension was layered over the gradient contained in two centrifuge tubes, while the third tube received 0.5 ml of the suspending medium and served as a reference. The tubes were placed in 3 × 5 ml Swinging bucket

rotor (SW 39) of a Beckman ultracentrifuge, model L and spun at 100000 *g* for 2 hr. At the end of the run, fractions of 3 drops each were collected and the respective fraction numbers from the two experimental tubes were mixed together and the vol. made to 3 ml with H_2O. These fractions were analysed for protein, chlorophyll and enzymes. Fractions from the reference tube were analysed for sucrose.

Subfractionation of mitochondria. The technique of fractionation of membranes from cauliflower bud mitochondria [12] was slightly modified. Crude mitochondrial fraction from 40 g *Cuscuta* was washed with 0.3 M sucrose and suspended in 8 ml of 0.3 M sucrose. To 7 ml of the mitochondrial suspension, fresh digitonin soln (1% in 0.3 M sucrose) was added slowly with constant stirring. The digitonin concn of the final soln was adjusted to give a digitonin–protein ratio of 5:12. The suspension was incubated in cold for 30 min, diluted by adding 3 vol. cold 0.3 M sucrose, and gently homogenised. Homogenate was centrifuged at 8500 *g* for 20 min. The pellet obtained was washed with 0.3 M sucrose, recentrifuged for 20 min and suspended in 3 ml 0.3 M sucrose. The supernatant was centrifuged at 105000 *g* for 90 min and the pellet suspended in 3.5 ml 0.3 M sucrose. The final supernatant was retained.

Enzyme assay. The enzyme assays were carried out at room temp. (*ca* 30°) under conditions of linearity with respect to time and enzyme concn. Hexokinase was assayed according to the method of ref [19], with slight modifications. The assay system contained in a cell of 1 cm light path was comprised of the following in a total vol. of 3 ml: Tris–HCl buffer, pH 8.5, 30 μmol; $MgCl_2$, 5.6 μmol; glucose, 6 μmol; ATP, 1.4 μmol; NADP, 0.15 μmol; glucose-6-phosphate dehydrogenase, 0.1 unit, and the enzyme preparation. The reaction was started by the addition of ATP, and A measured at 340 nm from 0 to 10 min. A control run simultaneously, but without ATP showed A change of not more than 0.005 in 10 min. NaF was not incorporated in the assay system, since its inclusion did not alter the enzyme activity when used at a final concn of 5 to 10 mM. A unit of hexokinase catalysed the formation of 1 μmol of glucose-6-phosphate in 1 min under the experimental conditions. Cytochrome *c* oxidase was assayed according to ref [20]. Catalase was assayed at 240 nm as described in ref [21] and NADH cytochrome *c* reductase assayed according to ref [22]. Rotenone sensitive NADH cytochrome *c* reductase was assayed at a final concn of 0.4 μM rotenone. Initial rates of these reactions were calculated from the first order rate constants. Succinate cytochrome *c* reductase was determined by the method of ref [22]. Malate dehydrogenase was determined spectrophotometrically by the method of ref [23].

Protein determination. Protein was determined by the method of ref [24], as modified in ref [25]. *Chlorophyll* was determined according to the method of ref [26] and the concn calculated using the formula of ref [27].

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