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AN INDUCIBLE ACETATE TRANSPORT SYSTEM IN *NEUROSPORA CRASSA* CONIDIA

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Summary

Neurospora crassa conidia possess an active transport system for the uptake of acetate. This system was characterized as: (a) energy dependent; (b) taking place against a concentration gradient; (c) saturating at higher substrate concentrations and (d) competitively inhibited by propionate.

Activity of the acetate transport system can be further enhanced by preincubating conidia in 1 mM acetate medium for 180 min (the inducible transport system). The conidial system and the inducible system have similar properties. The development of the inducible transport was dependent on RNA and protein synthesis. A genetic control of this system was further confirmed by isolating a mutant (*acp*$^{-i}$ acetate permease, inducible) that fails to develop the inducible transport system.

Introduction

Several microorganisms use carboxylic acids as the sole carbon source. These compounds are not only oxidized to derive metabolic energy but are also required for the synthesis of various cellular components.

Transport of carboxylic acids represents various interesting features. In eukaryotes the transport has to occur at two levels: one at that of cytoplasmic membrane for the uptake of the metabolite and the other at the level of mitochondrial membrane for its subsequent metabolism. Another interesting feature about carboxylic acid transport is that many bacteria [1,2] do not possess transport activity in whole cells, while their cell free extracts exhibit enzyme activities for their oxidation. When these organisms are grown on carboxylic

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acids as the main carbon source, they adapt to the new carbon source by developing a new transport system (adaptive permeation).

Existence of well defined carboxylic acid transport systems which were apparently under genetic control were reported in *Escherichia coli* [3], *Bacillus subtilis* [4], *Azotobacter vinelandii* [5], *Streptococcus faecalis* [6], *Aerobacter aerogenes* [7], and *Pseudomonas* [8]. However, evidence for the existence of such systems in eukaryotes was very scarce. Lanier [9] has reported acetate transport mutants in *Aspergillus* that would not grow on acetate as the sole carbon source. Wolfinbarger and Kay [10] have reported a dicarboxylic acid transport system (*dct*) in *Neurospora crassa*. Flavell and Fincham [11,12] have reported acetate non-utilizer mutants in *N. crassa* suggesting the ability to transport exogenous acetate. We investigated the problem of acetate transport and would like to report here biochemical characterization and genetic control of an inducible acetate transport system in *N. crassa* conidia.

Materials and Methods

Strains employed. A wild type stock (74^A, OR-23 1A) was obtained from the Fungal Genetics Stock Center, Arcata, California. It was grown on Vogel's medium N [15] supplemented with 2% sucrose and 2% agar (DIFCO - Bacto agar). Seven-day-old conidia were used in all these experiments.

Transport studies. Uptake of [^{14}C]acetate was undertaken essentially as described previously [13]. The basic uptake medium contained Vogel's medium N, conidia (0.1 mg/ml), [^{14}C]acetate (0.01 μCi/0.1 μmol per ml) and glass distilled water to make the desired volume. 5-ml aliquots were removed at specified time intervals, filtered through millipore membrane filters (type AA, 0.8 μm and 25 mm), washed with ice-cold water, glued to an aluminum planchett and counted in a Beckman low-beta counter. Short-term transport assays were made for a period of 8 min to determine kinetic constants. Transport velocities were determined from the slope values of a best fit straight line using a digital (link-8) computer.

Identification of intracellular acetate. Conidia that were preincubated in [^{14}C]acetate (0.02 μCi/0.2 μmol per ml) for 60 min were extracted with boiling distilled water. The extract was chromatographed on a Whatman No. 1 chromatography paper using an ethanol/ammonia/water (5 : 2 : 3 by vol.) solvent system. [^{14}C]Acetate was run parallel as a standard. 0.5 cm pieces of paper were cut and counted in a Beckman low-beta counter.

Isolation of the acetate permease mutant. An acetate transport mutant was isolated by selecting for acetate non-utilizer mutants, using a modified version of the Catcheside's mutant enrichment technique (Catcheside, D.E.A., personal communication).

Wild type conidia were irradiated with ultraviolet light and incubated in Vogel's medium N containing 40 mM acetate as the sole carbon source. Conidia that were able to grow in acetate medium were filtered using a conidial filter, every eight hours. Fresh medium was supplemented after every twenty-four hours. After three days of incubation, conidia that failed to grow in acetate medium were plated on Vogel's medium N containing 1.5% sorbose, 0.1% glucose and 2% agar. Colonies that grew on these plates were transferred to slants

containing 2% sucrose medium and tested in a liquid growth test. Acetate permease activity was analyzed in a regular [^{14}C]acetate uptake experiment.

Chemicals. [^{14}C]Acetate (sodium salt) was purchased from Amersham/Searle Corporation, Arlington Heights, Illinois. The non-radioactive chemicals were purchased either from Sigma Chemical Company, St. Louis, Missouri or Calbiochem, LaJolla, California.

Results

Neurospora crassa was reported to use acetate as the sole carbon source, suggesting an ability to transport exogenously supplied acetate. Fig. 1 illustrates the existence of such a system in wild type conidia. Uptake of [^{14}C]acetate was found to be highly sensitive to the energy uncoupling agents such as sodium azide (NaN_3) or 2,4-dinitrophenol (DNP). Addition of these compounds to the basic uptake medium after initiation of [^{14}C]acetate accumulation, inhibited further accumulation. However, the label that was already accumulated was retained by the conidia.

Examination of a radiochromatogram of hot water extract obtained from conidia that were preincubated in [^{14}C]acetate (200 nmol/ml, external concentration) for 60 min indicates a single peak corresponding to the peak obtained with the [^{14}C]acetate standard (Fig. 2). This suggests that acetate was retained by the cells in the same molecular form. Calculation of the internal concentration of acetate was based on a conidial wet volume of 5.3 μl/10 mg dry wt. of conidia [14]. Counts attributed to [^{14}C]acetate indicated an internal concentration of 374 nmol/μl of intracellular water, resulting in a 1870-fold concentration of acetate inside the conidia.

Acetate transport activity saturated at higher substrate concentrations (Fig. 3) indicating involvement of a carrier in this process. The transport system

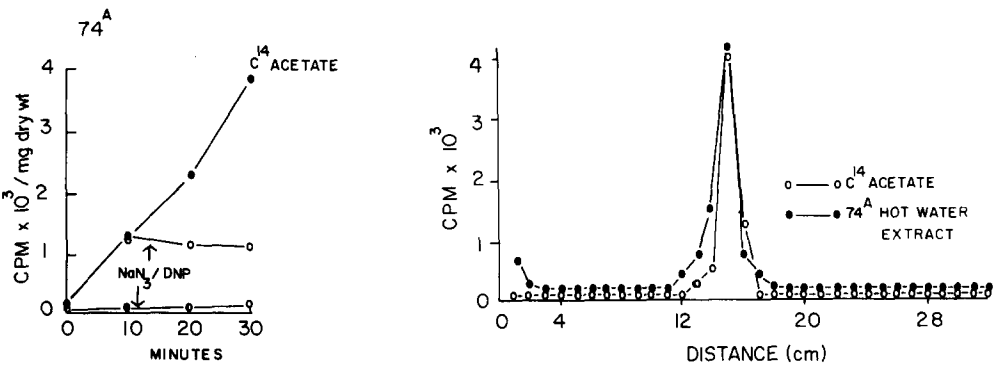

Fig. 1. Acetate transport in wild type (74^A) conidia and the effect of energy uncoupling agents. Transport of [^{14}C]acetate (0.01 μCi/0.1 μmol/ml) was initiated by the addition of 0.1 mg/ml conidia to the basic uptake medium. Sodium azide (NaN_3) or 2,4-dinitrophenol was added to the basic uptake medium at a concentration of 10 ug/ml.

Fig. 2. Radiochromatogram of hot water extract. Wild type (74^A) conidia were incubated in [^{14}C]acetate (0.02 μCi/0.2 μmol/ml) for 60 min and extracted with hot water. The extract was run parallel to a standard [^{14}C]acetate on a Whatman No. 1 chromatography paper using water/ammonia/ethanol (3 : 2 : 5 by vol.) solvent system. 0.5 cm pieces of paper were cut and counted in a low-beta counter.

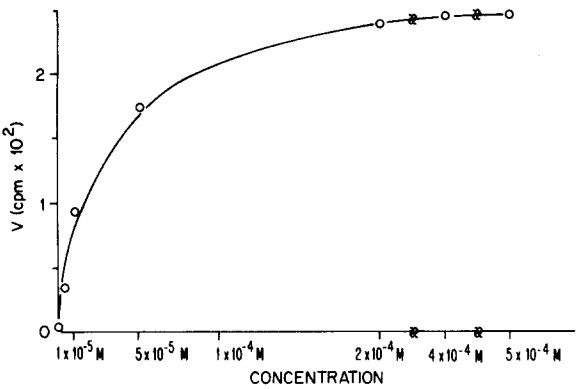

Fig. 3. Acetate transport as a function of external concentration. Transport velocities (counts/min per mg) at various substrate concentrations were calculated from short-term (8 min) uptake experiments. ○———○, 74A.

saturates at a concentration of 10^{-4} M and this concentration was used in most of the transport experiments. A K_m value of $2.5 \cdot 10^{-5}$ M and a V value of 3.5 nmol/min per mg were determined using Hofstee transformations ($V : V/S$) on a digital computer.

Various structural analogues such as chloroacetate and acetamide were used to compete for the acetate transport system but failed to show any competition. Propionate was able to compete with acetate for the acetate transport system (Fig. 4A). Kinetic analysis of propionate competition by adding a constant amount of propionate to various concentrations of [^{14}C]acetate (Fig. 4B) resulted in a shift in the K_m value, while the V value remained unchanged. This was a characteristic feature of competitive inhibition indicating that both propionate and acetate compete for the same binding site on the carrier molecule.

The acetate transport system can be amplified several times in its activity by prior incubation of conidia in a medium containing 1 mM acetate. Fig. 5 illustrates that this inducible activity was sensitive to both RNA or protein synthesis inhibitors (actinomycin-D or actidione). Conidia that were pre-incubated

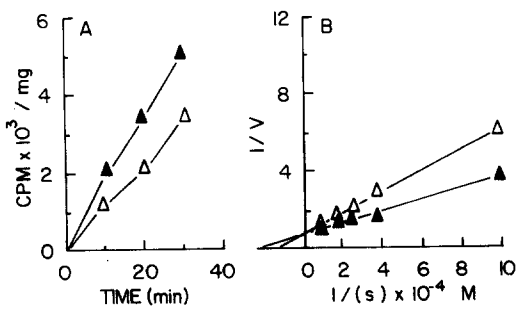

Fig. 4. Competition for acetate transport system. A. Sodium propionate (1 μmol/ml) was added to the basic uptake medium containing [^{14}C]acetate (0.01 μCi/0.1 μmol/ml). B. Transport velocities (counts/min per mg) for [^{14}C]acetate transport with and without the inhibitor (10^{-3} M propionate) were determined from short-term uptake experiments. Double reciprocals of substrate concentration and transport velocities were plotted. Intercepts of x and y axis represent $-1/K_m$ and V respectively. ▲———▲, control; △———△, 10^{-3} M propionate.

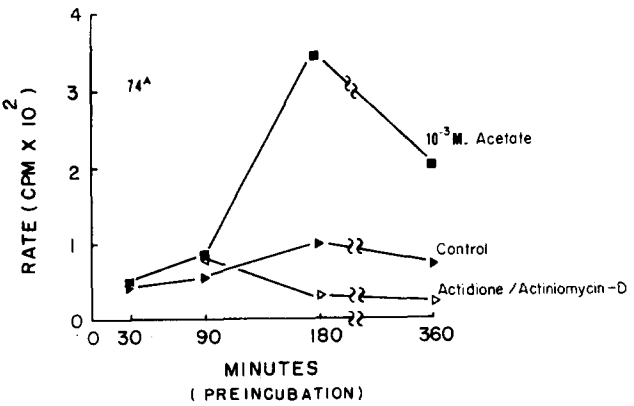

Fig. 5. Induction of acetate transport. Wild type (74^A) conidia were incubated for the specified amounts of time in Vogel's medium N containing 1 mM acetate as the sole carbon source. Incubation in Vogel's medium N with no carbon source was used as a control. The cells were filtered, washed and resuspended in a fresh uptake medium containing [^{14}C]acetate (0.01 μCi/0.1 μmol/ml). Velocity of transport (counts/min per mg) was calculated from the slope value of an 8 min uptake experiment. Actidione or actinomycin-D was added at a concentration of 10 μg/ml.

in Vogel's medium without any carbon source did not show a significant change in their transport rates.

Development of the inducible transport system was tested using various carbon sources. Incubation in sugars (1% glucose or 1% sucrose) failed to develop this activity indicating that starvation for a carbon source was not the reason for the development of the inducible transport system. Propionate (1 mM) was able to induce the acetate transport system but not as effectively as acetate.

The inducible transport system has the same properties as the conidial transport system. Fig. 6A illustrates that the inducible transport system was also sensitive to the energy uncoupling agents and that propionate exhibits competition for transport (Fig. 6B).

Kinetic analysis of the inducible transport system indicates it to be distinct from the conidial system. K_m and V values were determined using Hofstee transformations (with a link-8, digital computer). The inducible system (refer Table II) had a K_m value of $1.4 \cdot 10^{-4}$ M and a V value of 51 nmol/min per mg, which were higher than the conidial system ($K_m = 2.5 \cdot 10^{-5}$ M and $V = 3.5$ nmol/min per mg). These results indicate that the inducible system is a low affinity system with higher transport activity.

Isolation of the acetate permease mutant. Out of 56 colonies that were isolated and tested 8 colonies failed to grow on acetate (40 mM) as the sole carbon source. All the isolates grew normally on 2% sucrose medium. One out of these eight isolates (*acp*$^{-i}$, acetate permease, inducible) was noticed to be transport deficient and was subjected to further investigation.

Table I illustrates the growth pattern of *acp*$^{-i}$ compared to a wild type (74^A) and *acu*-3 (acetate non-utilizer, isolated by Flavell and Fincham [11,12]). Both *acp*$^{-i}$ and *acu*-3 failed to grow on acetate as the sole carbon source. Both these mutants, as well as the wild type, grew normally on a medium containing 0.5% sucrose and 20 mM acetate to indicate that both *acp*$^{-i}$ and *acu*-3 were not acetate-sensitive mutants.

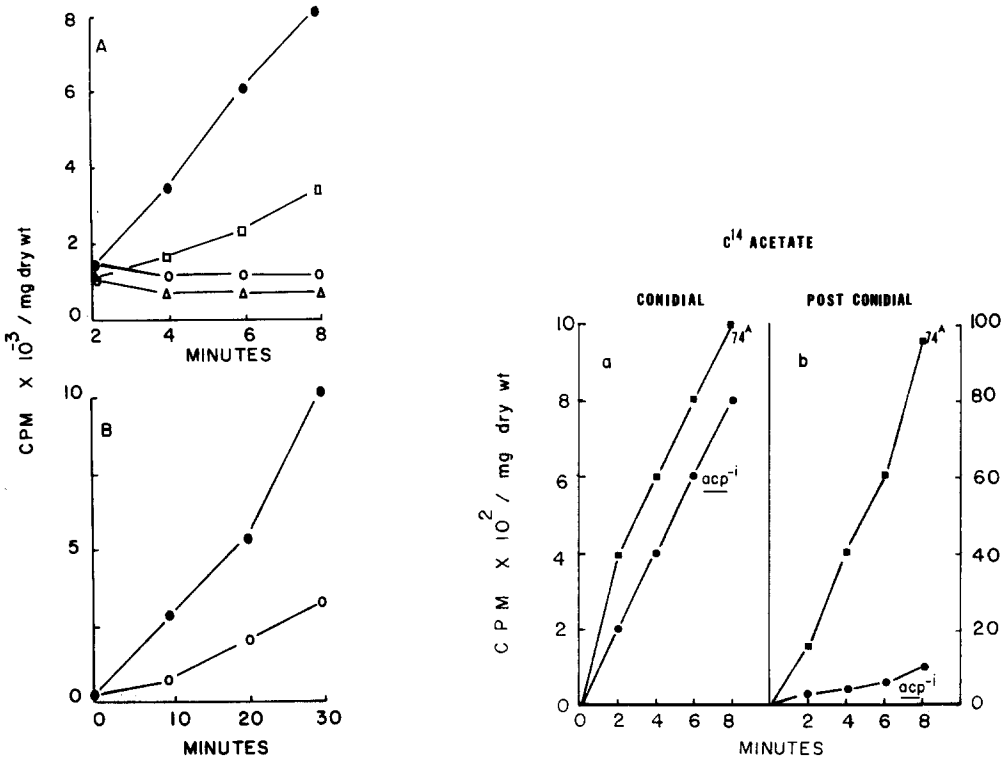

Fig. 6. Characteristics of the inducible acetate transport system. Wild type (74^A) conidia were used to compare the transport characteristics between uninduced (control) and acetate-induced (10^{-3} M) transport systems. After 180 min of preincubation, the cells were filtered, washed and resuspended in fresh uptake medium containing [^{14}C]acetate (0.01 μCi/0.1 μmol/ml). A. Sodium azide (10 μg/ml) was added to the uptake medium as a metabolic inhibitor. [^{14}C]Acetate accumulation in, control (□); control with sodium azide (△); acetate-induced (●) and acetate-induced with sodium azide (○). B. Competition with propionate for the inducible transport system. [^{14}C]Acetate accumulation in acetate-induced conidia (●); competition of [^{14}C]acetate transport by propionate (10^{-3} M) in acetate-induced conidia (○).

Fig. 7. Acetate transport in wild type (74^A) and *acp*$^{-i}$ conidia. (a) Transport of acetate in wild type (74^A) and *acp*$^{-i}$ conidia were compared. (b) Conidia obtained from wild type (74^A) and *acp*$^{-i}$ were preincubated in Vogel's medium N with 1 mM acetate for 180 min. Conidia were filtered, washed and resuspended in a fresh uptake medium.

TABLE I

FIVE DAY GROWTH TEST ON A MEDIUM SUPPLEMENTED WITH EITHER SUCROSE OR ACETATE

Vogel's medium N containing either acetate or sucrose as the sole carbon source was inoculated using a small wet inoculum. The cultures were incubated at 25°C on a reciprocal shaker for five days. Mycelial pads were removed, washed and dried overnight to determine the dry weights.

Strains used	1% Sucrose	40 mM Acetate	0.5% Sucrose + 20 mM acetate
Wild type (74^A)	89 mg	22 mg	48 mg
acp^{-i}	86 mg	0	38 mg
acu-3	78 mg	0	24 mg

TABLE II

KINETICS OF ACETATE TRANSPORT IN WILD TYPE (74^A) AND acp^{-i}

K_m and V values were determined using Hofstee transformations on a digital computer. Each point represents an average from five independent experiments.

Strain	Conidial		Preincubated for 180 min with 1 mM acetate	
	$K_m \pm$ S.D. 10^{-5} M	$V \pm$ S.D. nmol/min per mg	$K_m \pm$ S.D. 10^{-4} M	$V \pm$ S.D. nmol/min per mg
74^A	2.5 ± 0.22	3.5 ± 0.34	1.4 ± 0.21	51.1 ± 4.77
acp^{-i}	7.4 ± 0.82	4.7 ± 0.52	0.73 ± 0.06	5.4 ± 0.61

Complementation experiments were performed by forcing heterokaryons between acp^{-i} and the other *acu*-mutants and examining their ability to utilize acetate as the sole carbon source. acp^{-i} Complemented with all *acu* mutants (*acu*-3, *acu*-5, *acu*-6, and *acu*-7) as all the heterokaryons formed between acp^{-i} and *acu* mutants were able to grow on 40 mM acetate medium.

Examination of the transport characteristics of acp^{-i} indicate that the mutant has normal conidial transport activity (Fig. 7A) but completely lacks the inducible transport activity (Fig. 7B).

Table II illustrates the kinetics of acetate transport in acp^{-i} and was compared with wild type (74^A) transport. acp^{-i} Possesses a slightly higher K_m value in conidia ($7.4 \cdot 10^{-5}$ M) with normal transport activity (4.7 nmol/min per mg). However, both the K_m and the V values remain unchanged under conditions of induction (preincubation in 1 mM acetate). This was unlike the wild type (74^A) conidia that showed a 10-fold increase in both K_m and V values under the conditions of induction. These observations suggest that acetate permease in acp^{-i} was altered, which prevents the development of the inducible transport system.

Discussion

Evidence has been presented here to confirm the conclusion that acetate is transported by a process of active transport in *N. crassa* conidia. An active transport process has several identifying features that serve as experimental criteria. They are: (1) the carrier-mediated processes are saturatable at higher substrate concentrations; (2) some structural analogues and derivatives show an affinity to bind with the carrier molecule and competitively inhibit the transport process; (3) active transport processes are sensitive to energy uncoupling agents and depend on metabolic energy and (4) the transport involves accumulation of the substrate against a concentration gradient.

The acetate transport system satisfies the above mentioned criteria of active transport process. When the velocity of acetate transport was plotted against the substrate concentration (Fig. 3) a hyperbolic curve was obtained indicating that the transport process saturates at higher substrate concentrations. The transport system for acetate was very specific for its substrate and various structural derivatives of acetate such as chloroacetate or acetamide failed to inhibit acetate transport. However, propionate was able to compete with acetate and this was shown to be due to the competition for the same binding

site on the carrier molecule by demonstrating inhibition kinetics. Acetate transport was energy dependent and energy uncoupling agents such as sodium azide or 2,4-dinitrophenol completely inhibited the acetate transport system. Most of the acetate that was transported by conidia was retained in the same molecular form during the first 60 min of incubation. The conidia were able to accumulate acetate by about 1870-fold concentration inside. All these characteristics indicate that acetate was transported in conidia by a process of active transport.

Several microorganisms were reported to possess inducible transport systems for the uptake of carboxylic acids [10]. Wolfinbarger and Kay have reported a dicarboxylic acid transport system (*dct*) in *Neurospora* conidia which was induced by preincubation in 40 mM acetate medium.

Unlike the other carboxylic acid transport systems, *N. crassa* conidia possess a constitutive acetate transport system for the uptake of acetate. However, the activity of this system was low with a maximal velocity of 3.5 nmol/min per mg of conidia. When conidia were grown on acetate as the sole carbon source (10^{-3} M, in pre-incubation medium), they develop a more efficient system for the influx of acetate. This high activity system (V = 51 nmol/min per mg) was kinetically distinct from the constitutive conidial system. Development of this system was noticed only when either acetate or propionate was used as the sole carbon source. The inducible transport system was sensitive to both RNA or protein synthesis inhibitors (actinomycin-D or actidione) suggesting a possible genetic control of this system.

Efforts to isolate acetate permease mutants resulted in the isolation of *acp*$^{-i}$ strain. *acp*$^{-i}$ conidia failed to grow on acetate (40 mM) as the sole carbon source (similar to the other acetate nonutilizer mutants isolated by Flavell and Fincham [11,12]). Even though *acp*$^{-i}$ possessed normal conidial transport activity, they failed to develop the inducible transport system (Fig. 7). This observation further confirms the earlier suggestion that the inducible transport system was under genetic control. Examination of acetate transport kinetics clearly indicates the presence of the conidial transport system in *acp*$^{-i}$ conidia. The K_m value was slightly higher than the wild type K_m (*acp*$^{-i}$ $K_m = 7.4 \cdot 10^{-5}$ M, and 74^A $K_m = 2.5 \cdot 10^{-5}$ M) value suggesting an alteration in the acetate permease molecules of *acp*$^{-i}$. Under conditions of induction (1 mM acetate preincubation) *acp*$^{-i}$ conidia failed to develop the higher activity transport system but possessed only the conidial transport activity ($K_m = 7.3 \cdot 10^{-5}$ M). It has been suggested that alteration in the carrier molecules of *acp*$^{-i}$ conidia prevents the development of the high activity transport system. Failure to develop the inducible transport system results in the inability to utilize acetate as the sole carbon source by *acp*$^{-i}$ conidia.

Thus, the acetate transport system serves as a model system in the study and understanding of the developmental transport systems and their relation to subsequent metabolism.

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