Glucose uptake in germinating Aspergillus nidulans conidia: involvement of the creA and sorA genes

Andrew P. MacCabe,¹ Pilar Miró,¹ Luisa Ventura¹ and Daniel Ramón¹,²

¹Instituto de Agroquímica y Tecnología de Alimentos, CSIC, Apartado de Correos 73, Burjassot, 46100 Valencia, Spain
²Departamento de Medicina Preventiva y Salud Pública, Bromatología, Toxicología y Medicina Legal, Facultad de Farmacia, Universitat de València, Avenida Vicente Andrés Estellés s/n, 46100-Burjassot, Valencia, Spain

D-Glucose uptake in germinating wild-type Aspergillus nidulans conidia is an energy-requiring process mediated by at least two transport systems of differing affinities for glucose: a low-affinity system (Km ~ 1.4 mM) and a high-affinity system (Km ~ 16 μM). The low-affinity system is inducible by glucose; the high-affinity system is subject to glucose repression effected by the carbon catabolite repressor CreA and is absent in sorA3 mutant conidia, which exhibit resistance to L-sorbose toxicity. An intermediate-affinity system (Km ~ 400 μM) is present in sorA3 conidia germinating in derepressing conditions. creA derepressed mutants show enhanced sensitivity to L-sorbose. The high-affinity uptake system appears to be responsible for the uptake of this toxic sugar.

Apart from being a principal source of carbon and energy, glucose exerts important regulatory effects on cellular metabolism, and previous studies have identified several genetic loci implicated in the regulation of carbon catabolite repression (CCR) of metabolic processes in A. nidulans (Felenbok & Kelly, 1996). The physiology and genetics of glucose uptake is however largely uncharacterized in this hyphal fungus. Earlier studies provided evidence for acetate- and pyruvate-repressible (Romano & Kornberg, 1968, 1969; Desai & Modi, 1977), energy-dependent D-glucose uptake by A. nidulans mycelia distinct from the bacterial phosphoenolpyruvate sugar phosphotransferase system in that hexose uptake is not dependent on phosphorylation of the transported sugar (Brown & Romano, 1969; Mark & Romano, 1971). Since then little direct work on sugar transport in this micro-organism has been conducted.

As part of an effort to investigate sugar transport in A. nidulans we have initiated studies on glucose uptake. We report here an initial characterization of the kinetics of glucose uptake by germinating conidia. Evidence is presented for the existence of at least two kinetically distinct glucose uptake systems and roles for the products of the creA and sorA genes.

METHODS

Fungal strains and culture conditions. Additional genetic markers carried by the strains used in this study are as follows:

- Wild-type CECT2544 (biA1); sorA3 (pabaA1, fwA1); creA30 (biA1); V003 (pabaA1, yA2); creA3 (pabaA1); creA2 (pabaA1, fwA1);
- For sorA3 mutants: pabaA1, fwA1 and yA2.
- The following alleles were used: biA1, sorA3, creA30, pabaA1, fwA1, and yA2.

INTRODUCTION

Aspergillus nidulans constitutes an attractive and manageable system for the analysis of eukaryotic metabolic pathways and their regulation. The broad spectrum of metabolic activities it possesses, as reflected in its ability to grow on very diverse substrates (Arst & Scagazzocchio, 1985; Peñalva, 2001), and the relative ease of genetic analysis in this micro-organism (Clutterbuck, 1973), has resulted in considerable progress in the elucidation of the regulatory mechanisms involved in the control of the expression of enzymes required for the utilization of carbon and nitrogen sources and the response to environmental pH (reviewed by: Arst & Scagazzocchio, 1985; Felenbok & Kelly, 1996; MacCabe et al., 2001; Peñalva & Arst, 2002). Despite this, however, and in contrast to the recent advances in the analysis of hexose transport in Saccharomyces cerevisiae (Kruckeburg, 1996; Boles & Hollenberg, 1997; Ciriacy & Reifenberger, 1997; Kruckeburg et al., 1998; Johnston, 1999; Özcan & Johnston, 1999; Rolland et al., 2002), little is known of the mechanisms and control of sensing, signalling and cellular uptake of basic nutrients in A. nidulans. Given the wide range of both simple and complex compounds that A. nidulans can utilize as substrates for growth, this organism is likely to be endowed with an extensive array of uptake systems and associated regulatory mechanisms by virtue of which its metabolism can adjust to the nutrient availability of its environmental conditions.

Abbreviations: CCR, carbon catabolite repression; CM, complete medium; GM, germination medium; SGM, supplemented GM.
creAΔ4 (ya2, pabaA1, creAΔ4::argB); creAΔ25 (pabaA1, fwA1).
Conidia were harvested in 0-005% (v/v) Tween 80 from nitrate-
lacking complete medium (CM) plates (Cove, 1966) made to 0-5 M
by addition of solid NaH₂PO₄ (enhances conidiation in certain
mutant strains) after autoclaving. If not used immediately upon
harvesting, conidal suspensions were maintained at 4°C and inocu-
lated into liquid media within 12 h. Conidial titre was determined
by counting dilutions in a haemocytometer; conidal viability was
determined by spreading dilutions on NaH₂PO₄-supplemented CM
plates. The medium used for germination (GM) comprised, per
litre: 20 ml salt solution plus trace elements (Cove, 1966), 0-3 g
di-ammonium tartrate, and the carbon source at 1% (w/v); pH 6-8.
Supplemented germination medium (SGM) was prepared by addi-
tion of the appropriate auxotrophic supplements to GM.

Obtention of germinating conidia. Defined numbers of conidia
were inoculated into 300 ml SGM containing 0-1% (w/v) yeast
extract and 1% (w/v) carbon source (added from filter-sterilized
stocks as specified in Results) in 1 l Erlenmeyer flasks. Incubation
was carried out for 4 h at 37°C on an orbital shaker at 200 r.p.m.
Germinating conidia (prior to emergence of the germ tube) were
recovered by low-speed centrifugation (3200 g) for 6 min and
washed serially (five or six times) with ice-cold SGM lacking a
carbon source to eliminate traces of the growth substrate. Washed
conidia were then resuspended by gentle vortexing in ice-cold SGM
until a homogeneous suspension was obtained. Aliquots (250 µl)
containing a known number of conidia (within the range 5 × 10⁶–
5 × 10⁷ depending on the strain analysed) were dispensed into 2 ml
screw-capped Eppendorf tubes. Aliquots were maintained on ice
until required.

Glucose uptake measurements. Glucose uptake rates were
measured by assaying the incorporation of D-[U-¹⁴C]glucose
(11-655 GBq mmol⁻¹; American Radiolabeled Chemicals) into
conidia at various D-glucose concentrations in the range 0-1 μM
to 10 mM. Aliquots of germinating conidia (250 µl) were pre-incubated
at 37°C for 10 min in a shaking thermostirmer (Eppendorf) prior to
addition of an equal volume of warmed (37°C) substrate solution
containing a twofold concentration of radiolabelled glucose of
known specific activity. Agitation and incubation were continued
for periods of 5, 30, 60 and 90 s and uptake subsequently quenched
by rapid addition of 1-5 ml ice-cold 200 mM unlabelled glucose
in SGM and filtration over nitrocellulose filters (HAWP02500;
Millipore) mounted in a vacuum manifold, followed immediately
by two consecutive washes of 1-5 ml with the same ice-cold solu-
tion. Filters were subsequently lightly blotted on filter paper to
remove excess moisture and immersed in 3 ml OptiPhase ’Hisafe’ 3
(Wallac) for liquid scintillation counting. The amount of glucose
(nmol) retained on the filters was calculated from the specific
activities of the substrate solutions and plotted vs period of uptake.
The rate of glucose uptake was determined from the gradient of the
slope. Data analysis was done using SigmaPlot v 4.01.

To investigate the energy requirement of glucose uptake 4-nitrophenol
was added to conidal aliquots immediately prior to the start of the
10 min pre-incubation period.

RESULTS

Glucose uptake by wild-type germinating conidia

The present study has been carried out using a method adapted from
that employed by Diallinas et al. (1995) to measure purine uptake by germinating A. nidulans
conidia prior to appearance of the germ tube. The use of
germinating conidia avoids the potential complication of
strain-related morphological effects of differential mycelial
growth on the measurement of uptake (i.e. filamentous
versus pellet-like growth: Torres et al., 1996). Preliminary

Fig. 1. Michaelis–Menten plot of glucose uptake rate vs
substrate concentration for wild-type conidia germinated in 1%

glucose medium. The data points are the means from six
independent experiments. The solid line represents the best fit
to the data and bars represent standard deviations. The inset
shows the Eadie–Hofstee plot of the data. The solid line shows
the theoretical curve obtained by applying the values for Km
and Vmax deduced from non-linear regression of the data to a
two-component Michaelis–Menten equation.

Measurement of glucose uptake (see Methods for details)
by wild-type conidia germinating in the presence of glucose
showed typical Michaelis–Menten saturation kinetics (Fig. 1). The
Km (~1·3 mM) and Vmax (~0·5 nmol glucose s⁻¹ per
5 × 10⁸ conidia) values for overall glucose uptake were
obtained directly by non-linear regression analysis. Apart
from the direct plot (v₀ vs [S]), the data were transformed to
the Eadie–Hofstee representation (v₀ vs v₀ [S]⁻¹). This
transformation yielded a biphasic plot indicative of the
presence of at least two kinetically distinct modes of uptake
(Fig. 1 inset). The form of the plot reveals a major con-
tribution to overall glucose transport by a low-affinity
uptake system and a minor contribution by a system of
much greater glucose affinity. Given that extrapolation of
represents more than 95% of the uptake (Fig. 3) by conidia germinated for 4 h in GM containing 1% glycerol (http://mic.sgmjournals.org 2131 min by wild-type conidia harvested from plates containing either glucose or glycerol as sole carbon source. After 4 h of germination conidia were washed extensively to remove traces of carbon source and glucose uptake rates were measured. The forms of the Michaelis–Menten plots (Fig. 2) show that glucose uptake by glycerol- and glucose-germinated conidia is effected by a system of much higher affinity than that present in conidia germinated in glucose, and Eadie–Hofstee representation of the data (not shown) was indicative of the presence of a single kinetic system. Non-linear regression analysis for overall glucose uptake ($V_0$ vs $[S]$) by glycerol- and glucose-germinated conidia yields $K_m$ values of $\sim 23 \mu M$ and $\sim 1.5 \mu M$, and $V_{max}$ values of $\sim 0.37$ and $\sim 0.45$ nmol glucose s$^{-1}$ per conidium, respectively.

To investigate possible effects of the plate source of conidia on glucose transport, the amount of glucose taken up in 1 min by wild-type conidia harvested from plates containing two commonly used growth substrates and incubated for different periods of time was measured at four glucose uptake assay substrate concentrations: 25 µM, 125 µM, 500 µM and 2 mM. The similarity of the amounts of glucose taken up (Fig. 3) by conidia germinated for 4 h in GM containing glucose, irrespective of their plate origin, indicates little or no influence of the different parameters tested in the kinetic properties of glucose uptake. However, germination of conidia in GM containing glycerol as carbon source resulted in much greater uptake at the lower glucose concentrations, consistent with the presence of a kinetic system of much higher affinity than that observed for conidia germinating in glucose medium and hence in agreement with the previous analysis.

With regard to possible energy requirements, glucose uptake by wild-type conidia germinated in the presence of glucose or glycerol was assayed in the presence of the uncoupling agent 4-nitrophenol (1 mM) (Schleissner et al., 1994). Uptake was found to be considerably reduced across the whole range of glucose concentrations tested (Fig. 4). In addition, comparison by parallel assay of the initial uptake rates of glucose-germinated conidia in the presence and absence of the uncoupler showed greatly reduced rates in conidia assayed in the presence of 4-nitrophenol (data not shown). These data indicate that glucose uptake by both the high- and low-affinity systems is an energy-requiring process in A. nidulans.

**Glucose uptake by CCR mutants**

Genetic analyses have previously identified the creA locus as that principally responsible for exercising CCR in A. nidulans (Arst & Cove, 1973; Bailey & Arst, 1975; Hynes & Kelly, 1977) and the creA gene has since been shown to encode the wide domain DNA-binding transcriptional repressor CreA (Dowzer & Kelly, 1989, 1991;...
Kulmburg et al., 1993). Derepressed mutants (creA<sup>4</sup>) exhibit various degrees of failure of CCR in response to the presence of repressing carbon sources such as glucose. Given the difference in glucose uptake kinetics between glycerol-germinated (derepressing conditions) and glucose-germinated (repressing conditions) wild-type conidia, the effects of creA mutations on glucose transport were examined. Parallel experiments measuring glucose uptake by conidia carrying the phenotypically severe creA<sup>30</sup> mutation (Arst et al., 1990) germinating in the presence of glucose or glycerol exhibited similar high-affinity uptake kinetics (Fig. 5). Non-linear regression analyses yielded $K_m$ values of $\sim 30$ mM for each curve. In both cases Eadie–Hofstee plots (not shown) suggest monophasic kinetics and hence the absence of the low-affinity system.

Given the pleiotropic nature of the creA<sup>30</sup> mutation and its influence on growth (Arst et al., 1990; Shroff et al., 1997), glucose uptake rates for conidia carrying either this or the less severe creA<sup>4</sup> mutation (Bailey & Arst, 1975; Shroff et al., 1997) were compared in parallel experiments with those of the wild-type strain and a yA<sub>2</sub>, pabaA<sub>1</sub> mutant at glucose concentrations of 25 μM, 125 μM, 500 μM and 2 mM. The latter strain was used to provide a comparison to the overall uptake kinetics of the wild-type strain since the yA<sub>2</sub> and pabaA<sub>1</sub> mutations are not expected to have any effect on glucose uptake. The shapes of the curves obtained (Fig. 6) for the creA<sup>4</sup> and creA<sup>30</sup> mutant conidia are very similar and quite distinct from those of the wild-type and the yA<sub>2</sub>, pabaA<sub>1</sub> mutant, thus indicating that the apparent derepression of the high-affinity system is due to mutation in the creA gene rather than a collateral effect on growth specific to the creA<sup>30</sup> allele. It is not possible to distinguish whether the difference in $V_{\text{max}}$ values reflects differing degrees of derepression of the high-affinity system between the two creA alleles or is simply due to intrinsic variation between experiments.

Analysis of the L-sorbose-resistant sorA3 mutant

The sorA locus has been previously suggested to be involved in the formation of an uptake system for L-sorbose on the basis of the resistance conferred by the sorA mutations to the toxic effects of this sugar and its greatly reduced uptake (Elorza & Arst, 1971). Whilst these mutations apparently have no effect on glucose utilization, overall D-glucose uptake appeared to be slightly reduced in mutant protoplasts and mycelia compared to wild-type (Elorza & Arst, 1971).

Overall glucose uptake kinetics by sorA3 mutant conidia germinated in glucose medium were found to be similar to those of wild-type (Fig. 7) except that Eadie–Hofstee representation is indicative of monophasic kinetics. Parallel analyses of sorA3 conidia germinating in the presence of glucose or glycerol indicate that uptake is effected by distinct monophasic kinetic systems (Fig. 7 inset) depending
on the carbon source used for germination. The estimated $K_m$ ($\sim 1.3$ mM) for uptake by glucose-germinated conidia corresponds to the low-affinity component present in wild-type conidia. However, in glycerol-germinated sorA3 conidia uptake is not effected by a system with a $K_m$ as low as that observed for the high-affinity component in glycerol-germinated wild-type conidia. Non-linear regression analysis yields a $K_m$ of $\sim 400$ $\mu$M for this component. The absence of the high-affinity uptake system in the glycerol-germinated sorA3 mutant indicates that the sorA gene encodes a function required for high-affinity glucose uptake.

**Derepressed creA mutants are sensitive to L-sorbose**

The kinetics of glucose uptake in derepressed creA mutants (see Figs 5 and 6) could be considered to represent over-expression of the high-affinity glucose transport component. Given that the sorA3 mutation results in the absence of this system and also confers resistance to L-sorbose, creA mutants may be expected to exhibit enhanced sensitivity to the toxic effects of this sugar. Sensitivity to L-sorbose was tested by spotting similar numbers ($\sim 10^5$ conidia in 1.5 $\mu$l) of freshly harvested conidia on glycerol/urea plates containing different concentrations of this toxic sugar (31.25, 62.5, 125 and 250 $\mu$g ml$^{-1}$). Plates were incubated at 37°C and inspected after 12, 24 and 48 h. Across the concentration range 62.5 to 250 $\mu$g ml$^{-1}$ mutant strains carrying the creA$^{+}$I, -2, -4A, -2S and -30 alleles (Bailey & Arst, 1975; Arst & Bailey, 1977; Arst et al., 1990; Shroff et al., 1997) exhibited considerably less radial growth than the wild-type strain and the yA2, pabaA1 mutant. The sorA3 mutant showed the greatest radial growth of all the strains tested in the presence of L-sorbose. With the exception of the strains carrying the creAA4 and -30 mutant alleles, the growth of which was poorer than wild-type, all other strains showed practically identical radial growth on control glycerol/urea plates lacking L-sorbose. Table 1 summarizes the plate growth data.

**DISCUSSION**

The linearity of glucose uptake by germinating *A. nidulans* conidia with respect to time over the period 5–240 s under the conditions of assay used in the current work have allowed uptake rates across the substrate range 0.1–10 mM to be measured from the gradients of slopes generated from four independent measurements (5, 30, 60 and 90 s), hence reducing the errors intrinsic to single uptake measurements. Eadie–Hofstee representation of kinetic data, used as a diagnostic tool for multicomponent uptake kinetics in studies on both *S. cerevisiae* (Coons et al., 1995) and *Aspergillus niger* (Torres et al., 1996), was biphasic in form for glucose uptake by wild-type *A. nidulans* conidia germinating in the presence of glucose. Resolution of the uptake data by non-linear regression to a two-component Michaelis–Menten equation yielded a $K_m$ of $\sim 1.4$ mM for a low-affinity uptake system and a $K_m$ of $\sim 16$ $\mu$M for a high-affinity system. Monophasic glucose uptake of very similar $K_m$ to that of the high-affinity system is exhibited by wild-type conidia germinating in glycerol medium, and the carbon catabolite derepressed mutant strains creA$^{+}$30 and creA$^{+}$I also effect glucose uptake with similar high-affinity kinetics. These observations indicate that the high-affinity component involved in glucose uptake is subject to glucose repression mediated by the product of the creA gene. The regulatory effect of CreA on high-affinity uptake is determined by the conditions of germination and not by the conditions of conidial development (see Fig. 4). This means that the establishment of the high-affinity uptake system and the mechanism of its regulation must take place during the activation and isotropic growth phases of conidial germination (d’Enfert, 1997). In this regard, previous studies on proline uptake have shown regulated expression of the prnB gene during these growth phases (Tazebay et al., 1995). Interestingly, the early studies of...
Mark & Romano (1971) yielded a $K_m$ of $\sim 60 \mu M$ for glucose uptake by mycelia. The growth conditions used in that work (18–24 h of growth in 1% glucose medium) are likely to have resulted in depleted glucose concentrations tending towards derepressing conditions at the time of analysis of mycelial glucose uptake. The $K_m$ observed is reasonably similar to that noted in the present work for conidial glucose uptake under derepressing conditions.

The monophasic high-affinity kinetics of glucose uptake by glycerol-germinating wild-type conidia implies that low-affinity uptake is induced by the presence of glucose. Induction of this system is not however apparent in creA$^{30}$ mutant conidia germinating in the presence of glucose. Several speculative explanations for this observation are possible: (i) repression of the low-affinity uptake system by some property/component involved in the high-affinity uptake system; (ii) indirect or direct activation effects of CreA, i.e. via repression of a repressor of the low-affinity uptake system or an unidentified direct activation function of CreA exerted on the low-affinity system, respectively; or (iii) CreA involvement in glucose sensing either as a component of the sensing machinery or by regulation of the expression of a sensor such that in creA$^d$ mutants elevated levels of glucose fail to be detected. In the context of dual repressor/activator functions, the S. cerevisiae transcription factor Rgt1 has been shown to function as both repressor and activator of hexose transporters in response to glucose concentration (Özcan et al., 1996).

Unlike both wild-type and creA$^d$ mutants, germinating conidia of the L-sorbose resistant sorA3 mutant fail to show expression of the high-affinity glucose uptake system even under derepressing conditions. In contrast, creA$^d$ mutants take up glucose exclusively by high-affinity uptake and exhibit enhanced susceptibility to L-sorbose toxicity. These observations suggest that the high-affinity glucose uptake system is involved in L-sorbose uptake, which could explain the greatly reduced uptake of L-sorbose and slightly reduced D-glucose uptake previously observed for sorA mutants compared to wild-type (Elorza & Arst, 1971). Of particular interest in this regard are earlier studies in the filamentous fungus Neurospora crassa that showed L-sorbose uptake ($K_m \sim 4 \text{ mM}$) to be effected by an energy-requiring high-affinity glucose transporter ($K_m \sim 10 \mu M$) which is expressed under derepressing conditions (Scarborough, 1970; Schneider & Wiley, 1971). By analogy to the situation in N. crassa, the sorA gene is likely to be implicated in the high-affinity glucose uptake component of A. nidulans, possibly encoding either a glucose permease or a regulator of its expression. Under derepressing conditions sorA3 mutant conidia display two phenotypic variations compared to wild-type: (i) the absence of the high-affinity system, and (ii) the appearance of a glucose uptake activity of intermediate affinity ($K_m \sim 400 \mu M$). These two phenomena may be accounted for by a mutation in a structural gene encoding the high-affinity transporter which affects (increases) the $K_m$ of glucose (and L-sorbose) uptake. Hence, under derepressing conditions glucose uptake would be effected by a mutant high-affinity transporter having about tenfold less affinity for glucose. However, an alternative possibility of a role for the sorA gene product in the regulation of glucose uptake cannot be discounted. It is noteworthy in the latter context that the A. nidulans malA gene, previously identified in mutants affected in maltose utilization (Roberts, 1963), was ultimately revealed to encode a zinc finger transcription factor AmyR rather than an enzymic or permease activity (Tani et al., 2001).

Recognition of the multiplicity of hexose transporters and sensors resulting from the molecular cloning of genes involved in sugar transport in S. cerevisiae and the characterization of their protein products has greatly assisted in clarifying the data from earlier kinetic analyses of hexose uptake carried out in that micro-organism (Özcan & Johnston, 1999, and references therein). The suggestion that the variations observed in the kinetics of uptake could be brought about by alteration of the properties of a single transporter (Walsh et al., 1994) has been superseded by the concept of regulated expression of a range of transporters of differing kinetic characteristics. Evidence for multiplicity of sugar transport related proteins in A. nidulans (accession numbers AJ251561, AJ278285 and other unpublished data) suggests that $K_m$ values may be useful in identifying individual uptake systems. In that regard, the work reported here identifies three glucose uptake components. High- and low-affinity systems have been clearly identified from the analyses of wild-type and creA$^d$ germinating conidia. A third, intermediate-affinity system is detected in sorA3 conidia under derepressing conditions. However, the possibility that this system arises from alteration of another uptake activity cannot be excluded (see above).

The current study reveals certain differences between the system of hexose transport in A. nidulans and that of S. cerevisiae: whereas uptake in this yeast is by facilitated diffusion, the process in A. nidulans is energy requiring; $K_m$ values reveal the existence of glucose uptake systems of much greater affinity in A. nidulans than those observed in S. cerevisiae. These differences appear to reflect the differences in the niches occupied by these two microorganisms – the relatively sugar-rich environments favoured by S. cerevisiae and the wide variety of substrates which can be utilized by A. nidulans including those having little or no immediately available monosaccharidic carbon source. Further kinetic studies on mutants affected in sugar metabolism coupled with the identification, cloning and characterization of genes involved in sugar uptake will yield considerable insight into the nature and regulation of the process of uptake of basic nutrients by the model organism A. nidulans.

**ACKNOWLEDGEMENTS**

This work has been supported by grants from the European Union (BIO-4CT96-0535 and QLK3-CT99-00729). We are indebted to...
Dr José M. Sendra for help and advice with analysis of the kinetic data, Professor Herb Arst, Jr for kindly providing the creA and sorA mutant strains used in this work, and Dr Michel Flipphi for critical comments on the manuscript.

REFERENCES


