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Extracellular arabinases in *Aspergillus nidulans*: the effect of different *cre* mutations on enzyme levels

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Abstract The regulation of the syntheses of two arabinan-degrading extracellular enzymes and several intracellular L-arabinose catabolic enzymes was examined in wild-type and carbon catabolite derepressed mutants of *Aspergillus nidulans*. α-L-Arabinofuranosidase B, endo-arabinase, L-arabinose reductase, L-arabitol dehydrogenase, xylitol dehydrogenase, and L-xylulose reductase were all inducible to varying degrees by L-arabinose and L-arabitol and subject to carbon catabolite repression by D-glucose. With the exception of L-xylulose reductase, all were clearly under the control of *cre*A, a negative-acting wide domain regulatory gene mediating carbon catabolite repression. Measurements of intracellular enzyme activities and of intracellular concentrations of arabitol and xylitol in mycelia grown on D-glucose in the presence of inducer indicated that carbon catabolite repression diminishes, but does not prevent uptake of inducer. Mutations in *cre*A resulted in an apparently, in some instances very marked, elevated inducibility, perhaps reflecting an element of "self" catabolite repression by the inducing substrate. *cre*A mutations also resulted in carbon catabolite derepression to varying degrees. The regulatory effects of a mutation in *cre*B and in *cre*C, two genes whose roles are unclear, but likely to be indirect, were, when observable, more modest. As with previous data showing the effect of *cre*A mutations on structural gene expression, there were striking instances of phenotypic variation amongst *cre*A mutant alleles and this variation followed no discernible pattern, i.e. it was non-hierarchical. This further supports molecular data obtained elsewhere, indicating a direct role for *cre*A in regulating structural gene expression, and extends the range of activities under *cre*A control.

Key words *Aspergillus nidulans* · Carbon catabolite repression · Arabinases · Induction

Abbreviations *PNPA* *p*-Nitrophenyl-α-L-arabinofuranoside · *ABFB* α-L-Arabinofuranosidase B *ABNA* Endo-arabinase

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Introduction

L-Arabinose is a major constituent of a variety of complex structural plant cell wall polysaccharides. In nature, hyphal fungi play an important role in the degradation of these complex polysaccharides into monomeric sugars, such as L-arabinose, which are then subsequently catabolized. A major form of gene regulation affecting catabolism of L-arabinose and other sugars released from polysaccharides is carbon catabolite repression (Arst et al. 1990).

Amongst eukaryotes, carbon catabolite repression has been analyzed in detail in *Saccharomyces cerevisiae* and *Aspergillus nidulans*. In yeast, the SUC2 gene encoding invertase and the GAL genes required for the utilisation of galactose, have been particularly useful in identifying mutations resulting in altered responses to carbon catabolite repression, as reviewed by Carlson (1987) and Gancedo (1992). MIG1 (Nehlin and Ronne 1990) is one of the few genes involved in carbon catabolite repression in yeast that has been shown to have a direct regulatory effect on gene expression. Strategies to obtain mutations in *A. nidulans* leading to carbon catabolite derepression have been devised by Arst and Cove (1973) and Bailey and Arst (1975), which resulted in the identification of several *cre* mutant strains. From the *cre*A gene thus identified, a number of mutant alleles have been selected which, when tested, are recessive to wild-type and display non-hierarchical heterogeneity of mutant phenotypes, indicative of a direct role in regulating gene expression (Arst and Bailey

1977). Arst and MacDonald (1975) and Arst and Bailey (1977) postulated the *cre*A product to be a negatively acting wide-domain regulator.

Dowzer and Kelly (1989,1991) and Drysdale et al. (1993) cloned *cre*A from both *A. nidulans* and *Aspergillus niger* and found it to encode a zinc finger DNA-binding protein of the Cys_2 His_2 type, having considerable sequence similarity in the DNA-binding region to MIG1. A fusion protein containing the zinc finger of *A. nidulans* CREA shows sequence-specific binding to DNA and experiments at the molecular level confirm the negative-acting nature of this regulatory protein (Kulmburg et al. 1993; Cubero and Scazzocchio 1994). Hynes and Kelly (1977) using a similar selection strategy as Arst and Cove (1973), obtained some additional *cre*A alleles as well as mutations in two other genes, designated *cre*B and *cre*C, which probably do not have a direct role in carbon catabolite repression. Mutations in *cre*B had been obtained previously by selection for resistance to molybdate toxicity and designated *mol*B alleles [Arst and Cove 1970; Arst et al. 1970; C.R. Bailey as cited in Arst (1981)]. *cre*B (= *mol*B) and *cre*C mutations affect toxicities and/or utilisation of a wide range of compounds and probably play an indirect metabolic role, for example, in affecting intracellular metabolite concentrations and/or their subcellular distribution (Arst 1981). Another *cre* mutation (*cre*-34) had been found as a spontaneous suppressor of the effects of the *cre*C mutation and is thought to encode a protein that interacts with the *cre*A gene product (Kelly 1994).

The pathways in which catabolite repression in *A. nidulans* has been studied heretofore concern intracellular enzyme systems and permeases. Less is known about the regulation of carbon-regulated extracellular enzyme systems in *Aspergillus*. However, the regulatory mechanisms involved seem to follow the usual pattern of pathway-specific induction and carbon catabolite repression as observed, for example, with polygalacturonase and pectin esterase in *A. niger* (Maldonado et al. 1989) and cellulase in *A. nidulans* (Bagga et al. 1989). The low-molecular-weight inducers for these enzymes, however, await identification.

We are particulary interested in the arabinan-degrading extracellular enzyme spectra of *A. niger* and *A. nidulans*. From *A. niger* cultures grown on complex carbon sources, such as sugar beet pulp, three enzymes have been purified, viz. α-L-arabinofuranosidases A and B and endo-arabinase (Rombouts et al. 1988; van der Veen et al. 1991). *A. nidulans* secretes two such enzymes, viz. α-L-arabinofuranosidase B and endo-arabinase (Ramon et al. 1993). The arabinan-degrading enzymes in both fungi can also be induced by simple carbon sources such as L-arabinose, and particularly L-arabitol, but only in the absence of D-glucose (Ramon et al. 1993; van der Veen et al. 1993). Since L-arabitol arises as an intermediate of L-arabinose catabolism (Fig. 1), the regulation of the biosynthesis of extracellular arabinan-degrading enzymes also depends on the regulation of L-arabinose utilisation itself.

The availability of various carbon catabolite derepressed mutants in *A. nidulans* and of a well-defined set of co-inducers for extracellular arabinase biosynthesis enables analysis of the role of carbon catabolite repression in relation to the production of these enzymes.

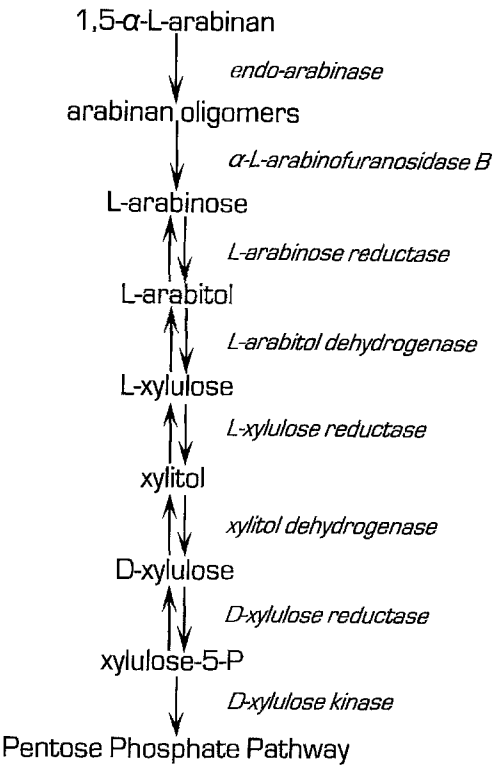

Fig. 1 L-Arabinose catabolic pathway in *Aspergillus nidulans*. (cf. Witteveen et al. 1989)

Materials and methods

Materials

All chemicals used were of analytical quality and obtained from Merck (Darmstadt, Germany). *p*-Nitrophenyl-α-L-arabinofuranoside (PNPA) was obtained from Sigma (St. Louis, Mo., USA). Arabinazyme tablets were obtained from Megazyme (Sydney, Australia).

Strains and growth conditions

The *Aspergillus nidulans* strain WG096 (*yA2 pabaA1*), originating from a strain of Glasgow origin (FGSC4), was used as wild-type strain.

The *A. nidulans cre*A mutant strains used carried: *cre*A^d-1 (*pabaA1*), *cre*A^d-2 (*pabaA1*), *cre*A^d-3 (*pabaA1*), *cre*A^d-4 (*biA1*), or *cre*A^d-30 (*biA1*) (Arst and Cove 1973; Bailey and Arst 1975; Arst et al. 1990). Strains carrying *cre*B35 (= *mol*B35) (*biA1*) (Arst et al. 1970) or *cre*C302 (*pabaA1*) [C.R. Bailey as cited in Arst (1981)] were also used. See Clutterbuck (1993) for definition of gene symbols.

The strains were grown on minimal medium as described previously (Pontecorvo et al. 1953). The vitamin *p*-aminobenzoic acid was added to a final concentration of 2 mg/l and biotin to a final concentration of 4 μg/l to all cultures. In the transfer experiments, all strains were grown before transfer at 37° C in 1-l Erlenmeyer flasks containing 300 ml minimal medium, 2% (w/v) D-glucose, and 0.05% (w/v) yeast extract. After 24 h, the mycelia were har-

vested and washed with saline, and aliquots of mycelium (1 g wet weight) were transferred to 100-ml Erlenmeyer flasks containing 30 ml minimal medium and a carbon source at a final concentration of 1% (w/v) and were incubated for another 6 h. Mixed carbon sources were each added at 1% (w/v). Mycelia were harvested, washed with saline, and frozen using liquid nitrogen. Culture filtrates were used directly for enzyme assays.

Preparation of cell-free extracts and protein determination

The preparation of cell-free extracts was carried out as described by Witteveen et al. (1989). Protein concentrations were measured using the Biorad BCA method as described previously (van der Veen et al. 1991).

Determination of enzyme activities

α-L-Arabinofuranosidase activities were determined as described previously (van der Veen et al. 1991). Endo-arabinase was measured using Arabinazyme tablets according to the supplier's instructions. The activities of L-arabinose reductase, D-xylose reductase, L-arabitol dehydrogenase, and xylitol dehydrogenase were measured as described by Witteveen et al. (1989). Both arabinases were measured from two different cultures in duplicate; the intracellular enzymes were measured from one culture in duplicate. Within one experiment, the activities found did not vary more than 5–8% (measured in duplicate). The variations found between different experiments were higher and varied between 15 and 25%. The tendency in activity changes between the various strains and growth conditions was found to be the same in the different experiments.

Polyol extraction and determination

The extraction of intracellular polyols was carried out as described by Witteveen et al. (1993). Polyol concentrations were measured on a HPAEC (Dionex) with a CarboPac MA1 column using isocratic elution with 0.48 M NaOH (Witteveen et al. 1993). The polyol concentrations were measured once, but simultaneously with arabinase activities.

Results

Induction characteristics of arabinase activity in various carbon catabolite derepressed mutant strains

The induction levels between some of the *cre*A mutant strains and the wild-type differed markedly (Fig. 2). Although strains carrying *cre*A^d-1, *cre*A^d-2, *cre*A^d-3, or *cre*B35 differed relatively little from the wild-type, strains carrying *cre*A^d-4, *cre*A^d-30, and *cre*C302 showed much higher induction levels, the difference reaching nearly an order of magnitude. *cre*A^d-30 showed a particularly rapid induction of PNPA-hydrolysing activity even after as little as 3 h. A late decline of activity in the *cre*A^d-30 mutant was not further investigated, but might have resulted from degradation by proteases.

The most rapid induction was observed in most of the strains between 6 and 12 h after transfer. In further experiments, an induction time of 6 h was chosen to ensure that the D-glucose concentration in mixed carbon source cultures remained sufficiently high (6–8 mg/ml) to exert full carbon catabolite repression.

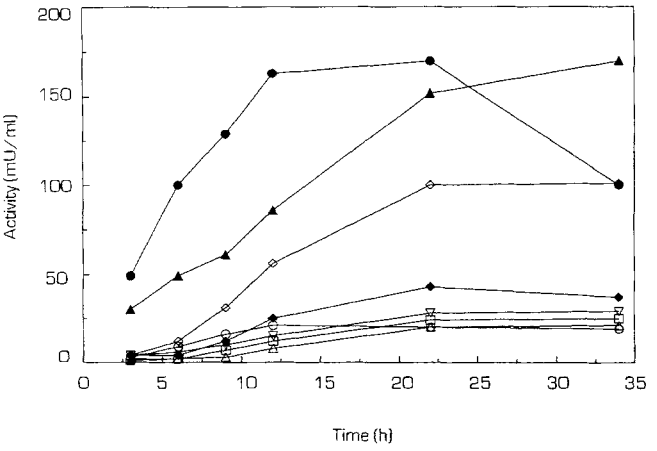

Fig. 2 PNPA-hydrolysing activities of *Aspergillus nidulans* strains pregrown on 2% (w/v) D-glucose and transferred to fresh media containing 1% (w/v) L-arabitol as an inducer. Samples of the culture fluid were taken at various time intervals. Activities are expressed in mU/ml culture filtrate. *Open squares* WG096, *open triangles* *cre*A^d-1, *open circles* *cre*A^d-2, *filled diamonds* *cre*A^d-3, *filled triangles* *cre*A^d-4, *filled circles* *cre*A^d-30, *inverted open triangles* *cre*B35, *open diamonds* *cre*C302

Induction and carbon catabolite repression of arabinase activities

To investigate induction and carbon catabolite repression of arabinase activities independently, the various *A. nidulans* strains were grown for 24 h on D-glucose and then transferred to fresh media containing different carbon sources. D-glucose is a strong repressing carbon source and glycerol is derepressing (Arst and Cove 1973; Bailey and Arst 1975). L-Arabinose and L-arabitol are carbon sources that induce the arabinases (van der Veen et al. 1993) and are carbon catabolite derepressing (Arst and Cove 1973; Bailey and Arst 1975), irrespective of *are*A mutations (unpublished results). The simultaneous effects of induction and repression were determined using a combination of D-glucose with L-arabinose or L-arabitol. All cultures were inoculated with mycelia (1 g wet weight) as stated in Materials and methods. We checked the biomass data for wild-type and the *cre*A^d-30 strain that had the most outspoken growth problems. Within the 6 h transfer period, the two strains show an equal increase in biomass (dry weight) on the same carbon sources. The differences in biomass between the different carbon sources were found to vary within 10–20% (1.1–1.2 g wet weight). D-Glucose is an exception, as in both strains the biomass increased by 50% (1.5 g wet weight).

α-L-Arabinofuranosidase B and endo-arabinase activities were measured in culture filtrates. As we did not measure the actual dry weights for all strains, we present the activities in mU/ml culture filtrate (Table 1). In the wild-type, activities of α-L-arabinofuranosidase B (ABFB; Table 1) were low and comparable to repressing D-glucose and derepressing glycerol. L-Arabinose and L-arabitol induce, but repression by D-glucose negated the effects of induction.

Table 1 Activities of α-L-arabinofuranosidase B (*section I*) and endo-arabinase (*section II*) in culture filtrates of *Aspergillus nidulans* wild-type and *cre*A, *cre*B35, and *cre*C302 mutant strains under various growth conditions. All carbon sources were used at a concentration of 1% (w/v). Activities are expressed in mU/ml culture filtrate

	Carbon source	Strains							
		WG096	*cre*A^d-1	*cre*A^d-2	*cre*A^d-3	*cre*A^d-4	*cre*A^d-30	*cre*B35	*cre*C302
I	D-Glucose	2.6	3.0	2.0	1.8	2.5	10.6	2.7	10.2
	L-Arabinose	8.2	10.1	8.5	9.0	70.1	147.3	14.2	24.2
	L-Arabitol	15.2	26.6	11.7	25.9	119.5	157.2	11.7	31.7
	L-Arabinose/ D-glucose	2.8	2.6	2.5	2.2	11.2	17.9	4.0	2.4
	L-Arabitol/ D-glucose	2.0	2.4	2.2	2.2	29.9	27.4	5.0	8.1
	Glycerol	2.9	2.3	1.8	1.8	2.8	4.2	3.4	2.4
II	D-Glucose	3.1	1.5	4.3	0.1	1.4	6.7	4.4	4.6
	L-Arabinose	6.5	9.4	17.3	15.8	43.8	63.4	16.3	8.9
	L-Arabitol	15.2	9.7	23.6	30.3	86.3	90.9	35.4	20.7
	L-Arabinose/ D-glucose	4.7	4.6	10.0	3.3	4.1	9.8	7.4	4.8
	L-Arabitol/ D-glucose	4.7	4.5	7.3	7.9	3.6	7.6	6.3	4.8
	Glycerol	5.1	3.5	3.4	4.0	1.2	4.2	6.3	3.9

Table 2 Intracellular specific activities of L-arabinose reductase under various growth conditions in *Aspergillus nidulans* wild-type and *cre*A, *cre*B35, and *cre*C302 mutant strains. All carbon sources were used at a concentration of 1% (w/v). Activities are expressed in mU/mg soluble protein in extracts

Carbon source	Strains							
	WG096	*cre*A^d-1	*cre*A^d-2	*cre*A^d-3	*cre*A^d-4	*cre*A^d-30	*cre*B35	*cre*C302
D-Glucose	40	27	71	75	97	28	35	75
L-Arabinose	301	384	616	378	557	479	362	322
L-Arabitol	252	283	431	465	919	545	161	354
L-Arabinose/ D-glucose	34	184	210	93	197	131	51	126
L-Arabitol/ D-glucose	90	198	161	152	96	107	100	88
Glycerol	54	10	58	39	100	47	25	71

All *cre* mutants had levels of ABFB activity on D-glucose similar to the wild-type, except for *cre*A^d-30 and *cre*C302, which had somewhat higher activities. Under inducing conditions, the *cre*A^d-30 mutant had strikingly more activity than the wild-type (18-fold on L-arabinose; 10-fold on L-arabitol). Of the other *cre*A mutants, only the *cre*A^d-4 mutant showed a markedly higher induced level of ABFB than the wild-type (8.5- and 7.9-fold, respectively). The other *cre*A mutants, as well as *cre*B35 and *cre*C302, had only slightly elevated ABFB activity under inducing conditions.

In the presence of D-glucose and an inducing carbon source, most *cre*A mutants, as well as *cre*B35 and *cre*C302, had ABFB activities comparable to that of the wild-type. Only the *cre*A^d-30 and *cre*A^d-4 strains showed markedly higher levels, albeit not nearly as high as in the absence of glucose. Under derepressing conditions (i.e. glycerol), low activities were found for all strains. With respect to these enzymes, one needs induction at the same time to observe differences between the effects of various carbon sources.

For the endo-arabinase (Table 1), a similar induction pattern was seen. Once again, L-arabitol was a more effective inducer than L-arabinose. In the presence of D-glucose and an inducing carbon source, ABNA activities were only slightly derepressed in some *cre* mutants, viz. *cre*A^d-30, *cre*A^d-2, and *cre*B35. In this case, *cre*A^d-4 had no marked effect.

Pentose catabolism and carbon catabolite repression

We established previously that in *A. niger* the catabolic pathway for L-arabinose plays an important role in the induction of the arabinases (van der Veen et al. 1993). Therefore, the intracellular activities of the relevant enzymes of this pathway were also measured.

L-Arabinose reductase (cf. Fig. 1) is the first enzyme involved in the degradation of L-arabinose. In the *cre*A^d-2, *cre*A^d-3, *cre*A^d-4, and *cre*C302 strains, activities higher than in the wild-type were found after growth on D-glucose (Table 2). Under derepressing conditions (i.e. glycerol as carbon source), the activities of these strains were comparable to those of the wild-type, except for the *cre*A^d-1, *cre*A^d-3, and *cre*B35 strains, which had lower activities, and for *cre*A^d-4, which had a two-fold higher activity. These enzyme data clearly illustrate a *cre*A allele-specific response of L-arabinose reductase expression, both on D-glucose and on glycerol. To explain these observations in a quantitative context would require further

Table 3 Intracellular specific activities of L-arabitol dehydrogenase (*section I*) and of NAD^+-dependent xylitol dehydrogenase (*section II*) under various growth conditions in *Aspergillus nidulans* wild-type and *cre*A, *cre*B35, and *cre*C302 mutant strains. All carbon sources were used at a concentration of 1% (w/v). Activities are expressed in mU/mg soluble protein in extracts

	Carbon source	Strains							
		WG096	*cre*A^d-1	*cre*A^d-2	*cre*A^d-3	*cre*A^d-4	*cre*A^d-30	*cre*B35	*cre*C302
I	D-Glucose	10	1	13	10	11	6	2	33
	L-Arabinose	70	171	335	188	392	308	131	122
	L-Arabitol	89	107	364	257	689	272	63	183
	L-Arabinose/D-glucose	24	88	270	109	78	70	10	73
	L-Arabitol/D-glucose	16	70	140	104	57	68	54	92
	Glycerol	11	15	11	27	39	75	20	27
II	D-Glucose	5	11	10	27	21	9	9	19
	L-Arabinose	111	182	348	218	188	308	149	131
	L-Arabitol	86	126	296	330	360	191	92	209
	L-Arabinose/D-glucose	17	84	100	100	50	62	17	61
	L-Arabitol/D-glucose	9	90	101	143	49	61	54	63
	Glycerol	22	24	24	45	55	50	30	51

molecular analysis of the interactions between the different CREA mutant proteins and the target gene. The inducing carbon sources L-arabinose and L-arabitol resulted in a strong increase in L-arabinose reductase activity in the wild-type strain (six- to seven-fold) compared to D-glucose. All *cre*A strains had elevated induced levels to varying degrees. The *cre*A alleles that on L-arabinose led to the highest levels of L-arabinose reductase were *cre*A^d-2 and *cre*A^d-4, whereas on L-arabitol these were *cre*A^d-4 and *cre*A^d-30. It is furthermore worth noting that L-arabitol was a more effective inducer in *cre*A^d-30 and *cre*A^d-3 strains, and particularly in *cre*A^d-4, whereas in the wild-type and other *cre*A mutants, L-arabinose was the more effective inducer. When induced with L-arabinose or L-arabitol in the presence of D-glucose as a repressing carbon source, levels decreased but tended to be higher than in the wild-type strain, particularly with L-arabinose as inducer.

For L-arabitol dehydrogenase, the second enzyme in the pathway (*cf* Fig. 1), the activities of the wild-type grown on D-glucose and glycerol were the same (Table 3, section I). Amongst *cre* strains, *cre*C302 showed elevated activity, and *cre*A^d-1 and *cre*B35 reduced activities on D-glucose. With growth on glycerol, both *cre*A^d-30 and *cre*A^d-4 had elevated L-arabitol dehydrogenase levels, as did slightly *cre*A^d-3 and *cre*C302. With L-arabinose or L-arabitol as the carbon source, a seven- to nine-fold increase in L-arabitol dehydrogenase was found in the wild-type. All *cre* mutants, with exception of *cre*B35, exhibited increased inducibility of L-arabitol dehydrogenase if grown on L-arabinose or L-arabitol. Relative to the wild-type, carbon catabolite derepression was evident in nearly every case of a *cre* strain grown with an inducer, particularly for *cre*A^d-2 and *cre*A^d-3. For both L-arabinose reductase (Table 2) and L-arabitol dehydrogenase (Table 3), carbon catabolite repression was observed in the *cre*B35 strain when L-arabinose, but not L-arabitol served as inducer.

The regulation of the synthesis of the fourth enzyme of the pathway, NAD^+-dependent xylitol dehydrogenase (Table 3, section II), was broadly similar to that of L-arabitol dehydrogenase. Activities were low when the strains were grown on D-glucose and somewhat higher on glycerol. Strong induction occurred with L-arabinose or L-arabitol in all cases. The *cre*A mutants had elevated induced levels compared to the wild-type. The highest induced levels were obtained with *cre*A^d-2 and *cre*A^d-30 on L-arabinose and with *cre*A^d-4 and *cre*A^d-3 on L-arabitol. When D-glucose was present together with one of the inducers, xylitol dehydrogenase activity was strongly repressed in the wild-type. The same *cre*A allele specificity towards carbon catabolite repression found for L-arabitol dehydrogenase was apparent, with *cre*A^d-3 and *cre*A^d-2 being the most derepressed.

L-Xylulose reductase (Table 4), the third enzyme of the pathway, might not be subject to *cre*A regulation, as even the usually phenotypically extreme *cre*A^d-30 mutation (Arst et al. 1990) scarcely affected enzyme levels apart from a possible modest effect on L-arabinose.

Table 4 Intracellular specific activities of NADPH-dependent L-xylulose reductase under various growth conditions in *Aspergillus nidulans* wild-type and *cre*A^d-30. All carbon sources were used at a concentration of 1% (w/v). Activities are expressed in mU/mg soluble protein in extracts

Carbon source	Strains	
	WG096	*cre*A^d-30
D-Glucose	5	2
L-Arabinose	79	151
L-Arabitol	172	179
L-Arabinose/D-glucose	30	34
L-Arabitol/D-glucose	18	22
Glycerol	4	4

Table 5 Intracellular xylitol and arabitol concentrations in wild-type *Aspergillus nidulans* and *cre*A strains under various growth conditions. All carbon sources are used at a concentration of 1% (w/v). Concentrations are expressed in μmol/g dry weight

Carbon source	Strains									
	WG096		*cre*A^d-1		*cre*A^d-2		*cre*A^d-4		*cre*A^d-30	
	Xylitol	Arabitol	Xylitol	Arabitol	Xylitol	Arabitol	Xylitol	Arabitol	Xylitol	Arabitol
D-Glucose	0	44	0	154	0	73	1	67	1	41
L-Arabinose	35	168	28	155	26	146	23	128	15	77
L-Arabinose/D-glucose	4	59	5	143	13	78	3	60	4	42

Intracellular arabitol and xylitol concentrations

When analysing the intracellular concentrations of xylitol and arabitol (Table 5), it is necessary to note that HPAEC cannot distinguish L-arabitol and D-arabitol. However, only L-arabitol acts as inducer for arabinase synthesis (van der Veen et al. 1993). Both isomers arise, but differently. The arabitol observed in all strains grown on D-glucose consists of D-arabitol, which is formed from the pentose phosphate pathway intermediate D-xylulose-5-phosphate. However, L-arabitol is formed as an intermediate of L-arabinose catabolism. In this case, the simultaneous appearance of xylitol (cf. Fig. 1) is diagnostic for the presence of L-arabitol. When wild-type and the various *cre*A mutants were grown on D-glucose, no appreciable xylitol was present (Table 5), whereas considerable arabitol was detected. Under inducing conditions (L-arabinose), large amounts of arabitol were detected in all strains. Xylitol was also present, but in lower amounts. On a mixture of D-glucose and L-arabinose, total arabitol was present at the same level as upon growth on D-glucose. However, in all strains, low levels of xylitol were also present, indicative of some L-arabinose uptake and subsequent catabolism under these conditions, even in the wild-type.

Discussion

Carbon catabolite repression by glucose and other favoured carbon sources prevents the synthesis of a large number of enzymes and permeases involved in the utilisation of less preferred substrates, including polysaccharides. In *Aspergillus nidulans*, carbon catabolite repression of many genes is mediated by the negative-acting wide domain regulatory gene *cre*A (Arst and MacDonald 1975; Bailey and Arst 1975). Arst and Bailey (1977) observed that various *cre*A mutant alleles show non-hierarchical heterogeneity in their effects on expression of different structural genes, indicating that *cre*A would encode a regulatory DNA-binding protein. The prediction has been confirmed by the presence of two zinc fingers of the Cys_2His_2 class in the derived CREA sequence (Dowzer and Kelly 1991) and by binding studies with a CREA fusion protein and wild-type and mutant cognate receptor sites having a hexanucleotide consensus 5′-G/CPyGG-PuG-3′ (Kulmburg et al. 1993; Cubero and Scazzocchio 1994). Two other genes, *cre*B35 and *cre*C302, in which mutations affect carbon catabolite repression, are more likely to act indirectly as their highly pleiotropic mutant phenotypes extend well beyond the realm of carbon catabolite repression [Arst and Cove 1970; Arst et al. 1970; Hynes and Kelly 1977; C. R. Bailey as cited in Arst (1981)].

In this study, we used five different *cre*A mutant alleles and a mutation in *cre*B35 and in *cre*C302 to investigate how the syntheses of α-L-arabinofuranosidase B and endoarabinase, and of the inducer L-arabitol, are influenced by carbon catabolite repression. Although a number of modest regulatory effects resulting from the *cre*B35 and *cre*C302 mutations were apparent, much more striking elevations of enzyme levels were seen with *cre*A mutations, perhaps in keeping with the more direct role of this gene.

The data indicated a strong influence of *cre*A on the biosynthesis of both α-L-arabinofuranosidase B and endoarabinase, which were shown to be coordinately regulated (Ramon et al. 1993). It is possibly surprising that the effect of *cre*A mutations is most marked under inducing conditions. The induced enzyme levels in the *cre*A^d-30 and *cre*A^d-4 strains are six- to ten-fold higher than those reached in the wild-type. This suggests that there is considerable "self" repression in the wild-type, even under conditions of optimal induction. In the presence of D-glucose plus an inducer, certain *cre*A mutations, particularly *cre*A^d-30, resulted in carbon catabolite derepression. The *cre*A^d-30 mutant allele is the result of an inversion breakpoint within *cre*A that severs the region encoding the DNA-binding domain from most of the rest of the gene and results in a generally more extreme phenotype than seen in other fully viable *cre*A mutants characterised (Arst et al. 1990; Dowzer and Kelly 1991). The degrees of inducibility of α-L-arabinofuranosidase B (ABFB) and endoarabinase (ABNA) in the various *cre*A mutants give further indication of the non-hierarchical response of different activities in different mutants. For example, whereas *cre*A^d-2 exhibited elevated inducibility for ABNA and *cre*A^d-1 did not, the reverse was true for ABFB. This indicates a direct response of the two structural genes encoding ABFB and ABNA towards *cre*A. The *abf*B gene and *abn*A genes of *Aspergillus niger* (but not *A. nidulans*) have been cloned and sequenced (Flipphi et al. 1993a,b); their promoter regions contain several putative CREA binding sites (unpublished data).

The effects of various *cre* mutations on levels of the specific activities of enzymes involved in L-arabinose ca-

tabolism were also investigated. These levels determine the flux through this pathway and hence the intracellular levels of the inducers L-arabinose and L-arabitol (for arabinases as well as arabinose catabolic enzymes). Wild-type activity levels of L-arabinose reductase (Table 2), the first enzyme of the pathway, were higher than those of L-arabitol dehydrogenase (Table 3), xylitol dehydrogenase (Table 3) and L-xylulose reductase (Table 4), which leads to the formation of L-arabitol.

In contrast to the regulatory behaviour of the other three enzymes of arabinose catabolism, L-xylulose reductase levels were largely unaffected by *cre*A^{d}-30. It is therefore possible that the synthesis of this enzyme is not under *cre*A control, thus avoiding the risk of accumulating L-xylulose, which is more toxic to the cell than xylitol (Witteveen et al. 1994).

Further non-hierarchical behaviour is evident from the effects of the various *cre*A mutations on the regulation of the arabinose catabolic enzymes. For example, whereas L-arabitol was a better inducer than L-arabinose of L-arabinose reductase in the *cre*A^{d}-4 strain, the reverse was true for the *cre*A^{d}-2 strain. Also, whereas *cre*A^{d}-30 resulted in strong carbon catabolite derepression of ABFB and *cre*A^{d}-1 resulted in none, *cre*A^{d}-1 resulted on average in stronger carbon catabolite derepression than *cre*A^{d}-30 for all three arabinose catabolic enzymes examined.

When comparing the levels of the various enzymes measured, it should be noted that both L-arabitol dehydrogenase and xylitol dehydrogenase were assayed near their pH optima (pH 9.6). These activities at a physiologically more relevant pH value (e.g. pH 6.5) are probably at least five-fold lower, as shown for *A. niger* (Witteveen et al. 1994). The thermodynamic equilibrium of these alternating reduction and oxidation steps in L-arabinose catabolism leads towards polyol formation. This was also demonstrated in *A. niger* by ^{13}C NMR spectroscopy, leading to xylitol and especially L-arabitol accumulation to a ratio of approximately 1:3 (Witteveen el al. 1989).

From intracellular concentrations of arabitol and xylitol (Table 5), it is clear that under inducing conditions, substantial amounts of xylitol, and thus of the isomer L-arabitol, are present. On a mixed carbon source (D-glucose/L-arabinose), xylitol was also detected in all the strains, including the wild-type. Therefore, we conclude that L-arabitol is also present and that uptake of L-arabinose takes place even in the presence of D-glucose as repressing carbon source.

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