The structure and expression of the pyruvatekinase gene of *Aspergillus nidulans* and *Aspergillus niger*

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr.H.C. van der Plas,
in het openbaar te verdedigen
op woensdag 19 april 1989
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

Stellingen:

1. De conclusie van Devchand en Kapoor dat er twee vormen van pyruvaatkinase mRNA in Neurospora crassa voorkomen, volgt niet uit de door hen gepresenteerde resultaten.
   Devchand, M.R. and Kapoor, M., Can. J. Microbiol., 33 (1987), 322-326.

2. De stelling dat bij transformatie van schimmels zoals Aspergilli integratie van het DNA in het genoom via niet-homologe recombinatie veeleer regel dan uitzondering is, is onjuist.
   Wernars, K., proefschift Landbouwuniversiteit Wageningen, 1986.

3. De invloed die intron sequenties kunnen hebben op het transcriptieniveau wordt vaak onderschat.
   Bornstein et al., Proc. Natl. Acad. Sci. USA, 84 (1987), 8869-8873;
   Brinster et al., Proc. Natl. Acad. Sci. USA, 85 (1988), 836-840.

4. De uitspraak dat in het pyruvaatkinase gen uit Saccharomyces cerevisiae een downstream activating sequence voorkomt, wordt onvoldoende door experimentele resultaten onderbouwd.
   Purvis et al., Nucl. Acids Res., 15 (1987), 7963-7974.

5. Functionele promoter analyse via deletie studies geven slechts een indicatie van functionele gebieden betrokken bij de regulatie van het transcriptie niveau.

6. Het feit dat het niet-allostere M_1 type pyruvaatkinase en het allostere M_2 type pyruvaat kinase uit rat onderling slechts verschillen in de contact regio's $C\alpha 1$ en $C\alpha 2$, houdt niet in dat er geen andere gebieden in het eiwit betrokken zijn bij de allostere activatie door fructose 1,6-bifosfaat.
   Dit proefschrift.

7. Het groeiend aantal artikelen op het gebied van de in vivo NMR spectroscopie aan microbiële systemen is geen waarborg voor de fysiologische relevantie van deze studies.

Stellingen behorende bij het proefschrift "Structure and expression of the pyruvatekinase gene of Aspergillus nidulans and Aspergillus niger."

Wageningen, 19 april 1989

L.H. de Graaff

CONTENTS

CHAPTER 1

Scope of the investigation

Scope of the investigation

The physiology and genetics of carbon metabolism in *Aspergillus* has been studied intensively over the last ten years. The recent developments in recombinant DNA methodology for *Aspergillus*, combined with well known genetics, in particular the genetics of *A.nidulans*, provide powerful tools to study gene structure and gene expression in relation to this area of fungal metabolism.

Transformation of *Aspergillus* only occurs by integration of the transforming DNA into the genome. This leads to relatively low transformation frequencies, compared to yeast, where autonomously replicating plasmids are found. The direct cloning of genes by complementation was initially hampered by the low transformation frequencies found. The first *Aspergillus* genes were cloned by complementation in *E.coli* or yeast. However, this is only possible for genes which give functional expression in those organisms, and several other strategies have been applied to clone fungal genes (see Chapter 2 for an overview and examples).

As the result of many studies over recent years, transformation methods have been developed and considerably improved (reviewed by Rambosek and Leach, 1987), leading to much higher transformation frequencies, up to several thousands per microgram of transforming DNA for *A.nidulans* as well as for *A.niger*. Most of these transformation systems were primarily developed for *A.nidulans* (*e.g.* see Wernars, 1986) for which, due to its well known genetics, a large number of auxotrophic mutants is available. Some of these systems were later adapted for *A.niger* e.g. *AmdS* (Kelly and Hynes, 1985) and *pyrA* (Goosen *et al.*, 1987; van Hartingsveldt *et al.*, 1987). Studies on gene structure and expression in *Aspergillus* have been initiated only recently, since such studies depend on suitable transformation systems. Research in a large number of laboratories, has lead to a rapid development of our knowledge about the gene organization and regulation of expression in *Aspergillus* and other

fungi.

The aim of the investigation described in this thesis was to study gene structure and gene expression in *A.nidulans* and *A.niger* in relation to carbon metabolism. Eventually such studies may lead to a situation where one is able to alter carbon fluxes in primary metabolism by genetic manipulation.

The utilization rate of glucose in glycolysis can be controlled;

- by glucose uptake;.
- by phosphorylation of glucose by hexokinase;
- by phosphofructokinase which converts fructose 6-phosphate to fructose 1,6-biphosphate;
- by pyruvate kinase, which converts phosphoenolpyruvate and ADP to pyruvate and ATP; this reaction plays a role in balancing glycolysis and gluconeogenesis, and is therefore a potential control point between those metabolic pathways.

The gene encoding pyruvate kinase (*pki*) was chosen in this study because of the potential role of pyruvate kinase as a control point in glycolysis. The gene is highly expressed, indicating that transcription is under the control of a strong promoter, whereas the level of expression varies depending on the carbon source used for growth. This makes it interesting to study which sequences are involved in regulating the expression of this gene. Moreover, a study of this gene provides the opportunity to investigate whether it is regulated both by specific control and general control mechanisms, in response to changes in physiological conditions.

There are three other advantageous factors which make this system attractive;

- Glycolytic genes are quite well conserved. Therefore the yeast gene, which was cloned before, can be used as a probe;

- A number of different *A.nidulans pki* mutants are available, one of them completely lacking the pyruvate kinase gene product (Uitzetter, 1982). This provides the possibility to study the effect of *in vitro* mutations on the regulation of the gene expression or on the catalytic activity of the enzyme;
- Although the biochemistry of the *Aspergillus* pyruvate kinase is known only to some extent, the enzyme from other sources is studied in great detail.

The *Aspergillus* pyruvate kinase gene was cloned by heterologous hybridization, using the yeast pyruvate kinase gene (Burke *et al.*, 1983) as a probe. In *A.nidulans* a transformation system, based on a *pkiA*$_5$ mutant (WG231) lacking the pyruvate kinase gene product, was developed to study the identity and the expression of the cloned gene, as described in Chapter 2. The *A.nidulans* pyruvate kinase was purified from the wild type strain and from one of the transformants overproducing the enzyme. Using this purified enzyme some of its properties were determined, as described in Chapter 3. The gene structures of the *A.nidulans* (Chapter 4) and of the *A.niger* (Chapter 5) pyruvate kinase gene have been determined, and compared with the gene structures encoding pyruvate kinase in other organisms. In addition, in Chapter 5 the expression of the cloned *A.niger* pyruvate kinase gene in *A.niger* is described. Finally, in Chapter 6, the three dimensional modeling of the Aspergillus pyruvate kinase structure is described, based on the crystallographic data of the cat muscle pyruvate kinase. This provides information about the regions in the protein involved in the allosteric regulation by fructose 1,6-biphosphate.

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CHAPTER 2

Isolation and expression of the *Aspergillus nidulans* pyruvate kinase gene

Part of this Chapter has been published

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Curr. Genet. 13, 315–321, 1988

Summary

The Aspergillus nidulans pyruvate kinase gene was isolated by heterologous hybridization using the corresponding yeast gene as a probe. A 2.9 kb EcoRI/BamHI fragment which exclusively hybridized to the yeast gene, was subcloned in pBR322. This clone was used to transform an A.nidulans pkiA deletion mutant to PKI$^+$. Analysis of transformants with respect to the kind of integration revealed in about 80% homologous integration, 55% by a double cross-over event (type III integration), 25% by a single cross-over event (type I integration). Type II transformants (20%) which arise by non-homologous integration have not been further characterized with respect to the sites of integration.

A direct correlation between the number of copies of the gene integrated into the genome and the measured pyruvate kinase activity was found after growth on a glycolytic carbon source. From this it was concluded that the 2.9 kb EcoRI/BamHI fragment contains the complete pyruvate kinase structural gene, including the promoter region.

However, after growth on a gluconeogenic carbon source the regulation of gene expression was found to be disturbed. On acetate an increase in activity per gene copy (0.2 IU) was found in the transformants, as compared to wild type levels. It is suggested that the pyruvate kinase gene is regulated by negative control, and that some sequences involved in this regulation are missing in the cloned fragment.

Introduction

The study of genes and their regulation in *Aspergilli* is greatly enhanced by the development of efficient transformation systems during the last few years (*e.g.* Tilburn *et al.*, 1983; Yelton *et al.*, 1984; Ballance and Turner, 1985; Buxton *et al.*, 1985; Johnstone, 1985; Kelly and Hynes, 1985; Wernars *et al.*, 1985).

Although there are several reports on the cloning of genes by complementation in *Aspergilli* (Johnstone *et al.*, 1985; Yelton *et al.*, 1985; Ballance and Turner, 1986) these systems need to be elaborated more extensively, before they can be routinely used in the straightforward cloning of genes in this organism. To overcome difficulties in cloning by complementation several alternative strategies have been followed, *viz.* cDNA cloning after mRNA enrichment and differential hybridization *e.g.* in the case of the genes encoding *A.nidulans* alcohol dehydrogenase I(*alcA*) and aldehyde dehydrogenase (*aldA*) (Lockington *et al.*, 1985), and by cDNA cloning in an *E.coli* expression vector followed by immunochemical screening (de Graaff, unpublished results). Genes have also been isolated by heterologous complementation or expression as in the case of the *A.nidulans trpC* gene in *E.coli* (Yelton *et al.*, 1983), and the *A.niger* β-glucosidase gene in yeast (Penttilä *et al.*, 1984) or by heterologous hybridization with the corresponding gene of a related organism like the *A.nidulans* 3-phosphoglyceratekinase (*pgk*) with the yeast gene (Clements and Roberts 1985), and the *A.niger* orotidine-5'-phosphate decarboxylase (*pyrA*) gene with the *N.crassa pyr4* gene (Goosen *et al.*, 1987).

Like other glycolytic genes such as the glyceraldehyde-3-phosphate dehydrogenase (Stone *et al.*, 1985; Tso *et al.*, 1985[a,b]), and the 3-phosphoglycerate kinase gene (Hitzemann *et al.*, 1982; Clements and Roberts, 1986), the gene encoding pyruvate kinase is highly conserved in a broad range of organisms (Burke *et al.*, 1983; Lonberg and Gilbert, 1983; 1985). We have used this high degree of conservation to isolate the

pyruvate kinase (*pki*) gene of A.nidulans. The corresponding yeast gene, which was isolated by complementation (Kawasaki and Fraenkel, 1982), was used as a probe to screen the *A.nidulans* genomic library by heterologous hybridization. A similar approach was recently used by Clements and Roberts (1985) to isolate the *A.nidulans* 3-phosphoglycerate kinase gene.

Pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) is one of the key regulatory enzymes in glycolysis. It catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate, thereby generating ATP. The isolation of this enzyme has been described for several fungi viz. *Mucor rouxii* (Terenzi *et al.*, 1971), *Neurospora crassa* (Kapoor and Tronsgaard, 1972), and *A.nidulans* (Uitzetter, 1982) whereas partial purification has been obtained for the enzyme originating from *A.niger* (Meixner-Monori *et al.*, 1984). The *A.nidulans* enzyme is a homotetramer with a reported subunit molecular weight of 60 kDa (Uitzetter, 1982).

The enzyme activity is usually allosterically controlled both by substrate (PEP) and product (ATP) and in addition by the allosteric effector fructose 1,6-biphosphate (see *e.g.* Kapoor, 1975; Meixner-Monori *et al.*, 1984).

The *in vivo* activity of pyruvate kinase in *A.nidulans* is influenced by the carbon source used when culturing the organism (Payton and Roberts, 1976; Uitzetter, 1982). Using a glycolytic carbon source, a 20-fold increase in enzyme activity is found as compared to the level found when grown on a gluconeogenic carbon source. This reflects a regulation of the pyruvate kinase activity level required in order to maintain the balance between gluconeogenesis and glycolysis under gluconeogenic growth conditions (Uitzetter, 1982).

Pyruvate kinase genes of several organisms have been isolated and from genomic or cDNA nucleotide sequence analysis, structures are known for the genes originating from yeast (Burke *et al.*, 1983) and chicken (Lonberg and Gilbert, 1983; 1985). These studies reveal a strong conservation in the predicted amino acid sequence of the enzyme structure during its evolution. However, thus far not much is known in the various cases about the regulation of the gene and about the nucleotide sequences involved in gene regulation.

In this paper the isolation of the *A.nidulans pki* gene, the subsequent transformation of a pyruvate kinase mutant with the cloned gene, and an analysis of the transformants are described.

Materials and methods

Strains

The following strains were used: A.nidulans WG096 (yA_2; pabaA1) A.nidulans WG231 (yA_2; $pkiA_5$; pabaA1).

Chemicals and enzymes

Restriction enzymes and DNA polymerase I were obtained from Pharmacia and Promega; DNA ligase from BRL and α-^{32}P-dATP from New England Nuclear. All other chemicals used were of analytical grade. Schleicher and Schüll BA85 nitrocellulose was used in Southern blotting; Millipore HATF85 nitrocellulose filters for plaque lifts.

Media and growth conditions

For the preparation of conidiospores A.nidulans was grown on complete medium (Pontecorvo et al., 1953), solidified with 1.2% agar using either 25 mM sucrose (strain WG096) or 100 mM acetate (strain WG231) as carbon source. Mycelium was grown by inoculating 10^6 spores ml^{-1} in minimal medium (Pontecorvo et al., 1953), supplemented with 2µg ml^{-1} p-aminobenzoic acid and with the appropriate carbon source. The mycelium was grown for 18-22 hr at 37°C in a Gallenkamp orbital shaker (200 rpm), using 1 l flasks containing 300 ml minimal medium.

Preparation and transformation of A.nidulans protoplasts

Mycelium of WG231 was grown on cellophane according to Ballance and Turner (1985). Protoplasts were prepared from the mycelium by an overnight incubation with Novozyme 234 (5 mg ml^{-1}) in 0.6 M KCl. After purification by filtration and centrifugation the protoplasts were transformed essentially as described by Wernars et al. (1985).

Isolation of nucleic acids

Plasmid DNA was propagated and purified by standard methods (Maniatis et al., 1982).

Extraction of nucleic acids from A.nidulans

Fungal DNA was isolated by a slightly modified procedure used to isolate plant RNA (Slater, 1985). Mycelium, which was grown overnight in liquid minimal medium, was harvested, washed with cold saline, frozen in liquid nitrogen and stored at -80 °C. Nucleic acids were isolated by disrupting 0.5 g frozen mycelium using an microdismembrator (Braun). The mycelial powder obtained was extracted with freshly prepared extraction buffer.

The extraction buffer was prepared as follows: 1 ml tri-isopropyl-naphtalene sulfonic acid (TNS) (20 mg/ml) was thoroughly mixed with 1 ml p-aminosalicylic acid (PAS) (120 mg/ml) and 0.5 ml 5 x RNB buffer was added (5 x RNB contains 121.10 g Tris, 73.04 g NaCl and 95.10 g EGTA in 1 l, pH 8.5). After the addition of 1.5 ml phenol, the extraction buffer was equilibrated for 10 min. at 55 °C. The warm buffer was then added to the mycelial powder, and the suspension was thoroughly mixed for 1 min. using a vortex mixer. After addition of 1 ml chloroform the suspension was remixed for 1 min. After centrifugation at 10^4 x g for 10 min. using a Sorvall high speed centrifuge, the aqueous phase was extracted once more with an equal volume of phenol/chloroform (1:1) and then twice with chloroform. DNA or RNA was isolated from the aqueous phase using the following procedures.

Isolation of DNA

After extraction with chloroform the DNA was immediately precipitated from the aqueous phase with 2 vol. ethanol at room temperature. It was subsequently collected by centrifugation using a Sorvall high speed centrifuge at 10^4 x g for 10 min. and washed twice by redissolving the DNA in distilled, sterile water and precipitating it again with ethanol. RNA was removed by adding RNase A (20 $\mu g\ ml^{-1}$) to the final solution.

In our hands, this procedure results in the isolation of high molecular weight DNA (100-200 kb) with a yield of about 300 µg DNA per g mycelium.

Isolation of RNA

The RNA was precipitated from the aqueous phase after chloroform extraction by adding 1/3 vol. 8 M LiCl. The solution was thoroughly mixed, and incubated overnight at 0 °C. The precipitated RNA was collected by centrifugation using a MSE Super-Minor centrifuge, at 3000 rpm for 30 min. Then the precipitated RNA was washed once with cold (-20 °C) 2 M LiCl and once with cold (-20 °C) 96% ethanol.

Finally, the RNA was dissolved in distilled water at a concentration of about 1 mg/ml. This results in RNA preparations which are essentially free of DNA with a yield of 1-2 mg RNA per g of mycelium.

Heterologous hybridization and plaque hybridization

Heterologous hybridization conditions were determined by hybridizing chromosomal DNA digests of A.nidulans at different temperatures and under different washing conditions. The final conditions chosen to screen the genomic library were; hybridization at 56 °C and washing at 56 °C with 5x SSC, 0.5% SDS followed by a final washing step using 2 x SSC at 56 °C.

Plaque hybridization of the genomic library in bacteriophage lambda charon 4A was performed as described by Benton and Davis (1977) under the experimental conditions just mentioned.

Results

Isolation and physical map of the gene

The *A.nidulans pki* gene was isolated by heterologous hybridization with a 1.8 kb *Eco*RI fragment of the corresponding yeast gene (Burke *et al.*, 1983). This 1.8 kb *Eco*RI fragment contains the 5' coding region and comprises about 90% of the gene. Digestion of *A.nidulans* genomic DNA with *Eco*RI and subsequent Southern blotting and hybridization with the 1.8 kb *Eco*RI fragment of the yeast gene revealed a single hybridizing band, 9.5 kb in length.

Screening of about 5.10^4 recombinant plaques of the lambda charon 4A genomic library of *A.nidulans* with this probe, resulted in the isolation of 24 positive clones. Four clones with the strongest hybridization signal were selected for further restriction and hybridization analysis. All four contained the 9.5 kb *Eco*RI hybridizing fragment. The resulting restriction map is shown in Fig. 1. The smallest single hybridizing fragment, a 2.9 kb *Eco*RI/*Bam*HI fragment as well as a 5.0 kb *Bam*HI fragment were subcloned in pBR322 giving pGW402 and pGW403, respectively. The 2.9 kb *Eco*RI/*Bam*HI fragment was also subcloned in M13 mp 18/19 for sequence analysis of the gene.

The polarity of the gene on the cloned fragment was determined by digestion with the appropriate enzymes, followed by Southern analysis. Fragments containing the 5' and 3' end of the yeast gene, a 1.0 kb *Eco*RI/*Bgl*II and a 0.88 kb *Eco*RI/*Bgl*II fragment respectively, were used as probes. The orientation of the gene is indicated in Fig. 1.

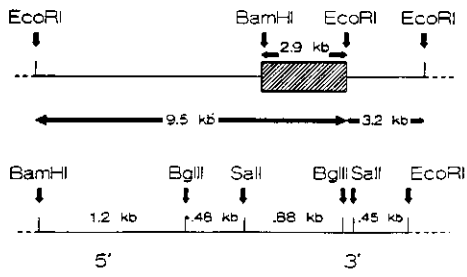

Fig. 1 *Restriction map of the pyruvate kinase gene of A.nidulans and its flanking regions on the chromosome. The hatched box represents the subcloned 2.9 kb EcoRI/BamHI fragment. The lower bar represents a more detailed restriction map of the cloned fragment.*

Transformation of A.nidulans

In order to prove that the cloned fragment contains the complete gene, including its regulatory sequences, *A.nidulans* was transformed from PKI$^-$ to PKI$^+$. The recipient strain in these experiments, WG231, is a *pki* mutant which was originally selected as glucose-negative. This particular mutant was shown to lack the pyruvate kinase protein (Uitzetter, 1982). Southern analysis of *Eco*R1/*Bam*H1 digested DNA originating from WG231 revealed that the *pki*A gene in this mutant contains a deletion of about 150 bp (results not shown). The presence of a deletion in WG231 makes this mutant very suitable as a recipient in transformation experiments, since no spontaneous reversion events can occur.

Since pyruvate kinase mutants do not grow on glycolytic carbon sources as *e.g.* sucrose, we used a glycolytic carbon source to select directly for PKI$^+$ in our transformation experiments. Following this strategy WG231 was transformed with the plasmids pGW402 and pGW403 to PKI$^+$, with a frequency of 10-20 transformants per μg of plasmid DNA.

Analysis of transformants

The resulting transformants were analyzed with respect to the type of integration into the genome and with respect to the expression of the cloned gene.

a. Genomic analysis

Thirteen transformants were randomly selected for analysis by Southern blotting after digestion of the chromosomal DNA with the appropriate restriction enzymes. Digestion with *Eco*R1, which cuts only once in pGW402 and pGW403 and Southern analysis with the 2.9 kb *Eco*R1/*Bam*H1 fragment as a probe, showed in five transformants the presence of an additionally hybridizing fragment (Fig. 2A). The size of this fragment, 6.9 kb (indicated by an arrow), corresponds with the length of the

transforming plasmid, suggesting a tandem integration at the homologous locus (Fig. 3A and B) or a multicopy tandem integration at another site in the genome. In principle the two possibilities can be discriminated by appropriate digestions of the flanking sequences. Differences in intensity of the signal of this 6.9 kb fragment reflect differences in the number of copies integrated within the genome.

In three other transformants (4/402/1; 8/402; 9/402) no such straightforward hybridization patterns were found. In the case of 4/402/1 (lane 5) and of 8/402 (lane 9) a complex pattern of hybridizing fragments resulted after digesting the chromosomal DNA with *Eco*RI, suggesting integration at other sites than the homologous locus. Moreover, it is also possible that this event is still followed by internal rearrangements. Surprisingly the patterns are identical, an observation for which we do not have an appropriate explanation. In the case of 9/402 (lane 10) the pattern in Fig. 2A seems in accordance with a type I integration event. However, Figs. 2B (lane 10) and C (lane 1) provide the evidence that this is a type II integration. In the case of transformant 2/403 (Fig. 2C, lane 6) the hybridization pattern is more complex due to the fact that in pGW403, in contrast to pGW402, the *Hind*III restriction site of pBR322 is still present. The size of the fragments obtained corresponds well with a type I integration.

In six transformants no other hybridizing fragments were found besides the 9.5 kb *Eco*RI fragment. Hybridization of *Eco*RI digests using pBR322 as a probe, confirmed that no vector sequences were integrated into the genome in these cases (Fig. 2B). As the recipient strain WG231 carries a deletion, these transformants are most likely the result of a type III integration, and they are certainly not the result of reversion of the mutation in the recipient strain.

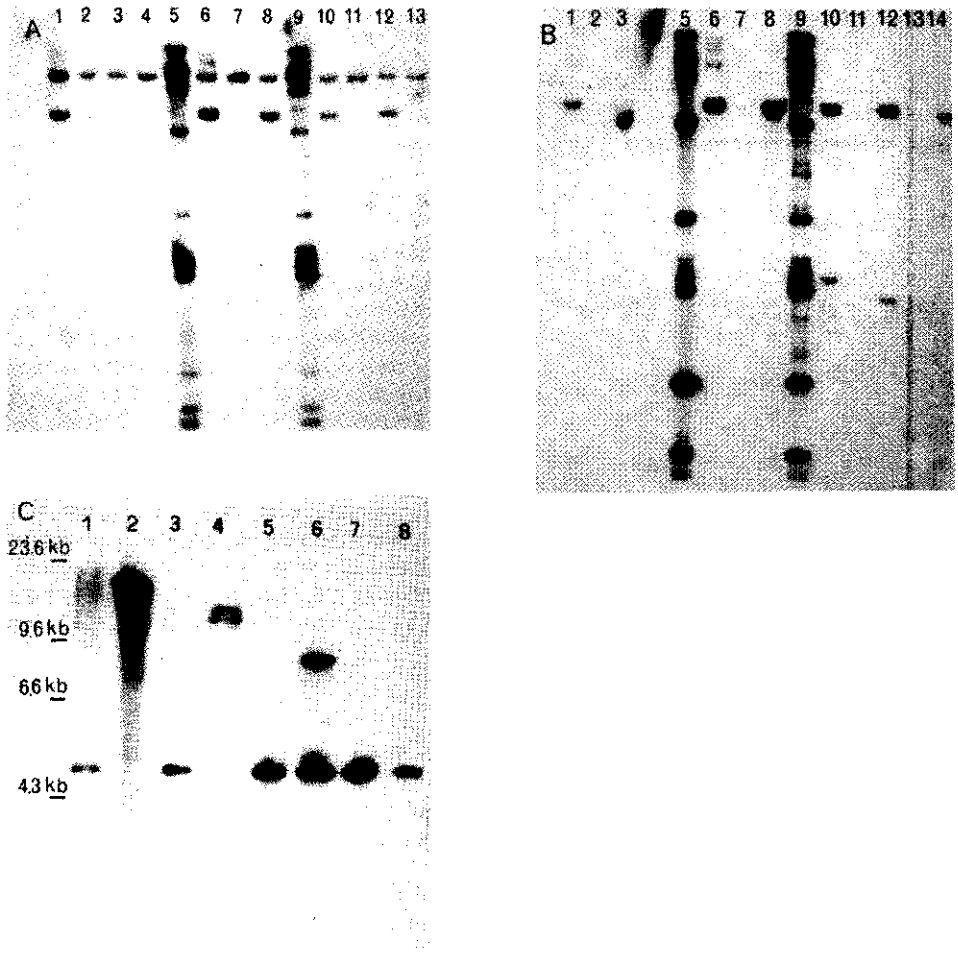

Fig. 2 *Analysis of genomic DNA from PKI^{+} transformants.*
DNA (1-2 µg) was digested with either EcoRI (panel A and B) or HindIII (panel C). After fractionation on an agarose gel and transfer to nitrocellulose the blots were probed with ^{32}P-labelled 2.9 kb EcoRI/BamHI fragment (panel A and C) or with ^{32}P-labelled pBR322 (panel B).

2A, B: lane 1: 1/402; 2: 2/402; 3: 3/402; 4: 4/402; 5: 4/402/1;
6: 5/402; 7: 6/402; 8: 7/402; 9: 8/402; 10: 9/402;
11: 1/403; 12: 2/403; 13: WG096; 14: pGW402

2C: lane 1: 9/402; 2: 5/402; 3: 6/402; 4: 1/402; 5: 1/403;
6: 2/403; 7: WG096; 8: WG231

The number of copies integrated at the homologous locus can be determined after digestion with *Hind*III. This enzyme does not cut within the gene, as shown in Fig. 1, whereas in the plasmid pGW402 itself there is also no restriction site available. For every copy of pGW402 integrated into the genome the 4.7 kb *Hind*III fragment is therefore enlarged with 6.9 kb (Fig. 3C and D). The values predicted this way are 11.6, 18.5 and 24.4 kb respectively for 1, 2 and 3 copies integrated.

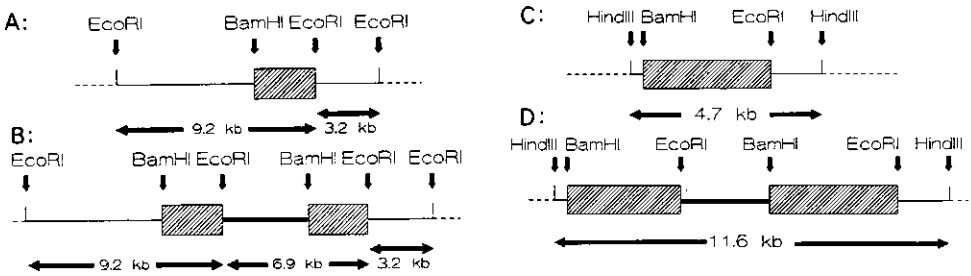

Fig. 3 Integration of pGW402 DNA by homologous recombination.
The structure of the pki region after a single homologous recombination event with pGW402 DNA is shown. The hybridizing fragments after digestion with EcoRI or HindIII in the wild type (A, C) and a single copy transformation (B, D) are shown.

In two transformants (1/402 (Fig. 2C, lane 4) and 7/402) a fragment of 12kb corresponding to the predicted length upon integration of a single copy is found. In another transformant 5/402 (Fig. 2C, lane 2) a fragment of 19 kb is found, corresponding with two copies integrated into the genome. Non-homologous integration tends to lead to multiple copy transformants as shown in Table I. However, the exact copy number has not been determined at the DNA level.

b. Analysis of pyruvate kinase activity

The expression of the *pkiA* gene in the transformants was studied by growing these transformants on MM containing 25 mM sucrose as the sole carbon source. The pyruvate kinase activity measured in mycelial extracts was compared with the activity found in the wild type strain (Table I). The activities found in these transformants ranged from 60% of the wild type activity to 450%. On the basis of these results the transformants can be classified into two classes: one group of transformants with a pyruvate kinase activity level lower than or comparable to that found in the wild type, and another group with an increased level of activity suggesting single and multicopy integration respectively.

Table I *Pyruvate kinase activities in wild type A.nidulans and PKI*$^{+}$ transformants after growth on minimal medium with 25 Mm sucrose as a carbon source.

Strain	Activity (IU/mg protein)	Relative activity (%)	Type of integration*	Copy number
WG096	0.8	100		1
1/402	0.6	75	I	1
2/402	0.4	50	III	1
3/402	0.5	63	III	1
4/402	0.5	63	III	1
4/402/1	1.5	190	II	?
5/402	1.8	225	I	2
6/402	0.9	110	III	1
7/402	0.6	75	I	1
8/402	2.8	350	II	?
9/402	3.5	440	II	?
1/403	0.9	110	III	1
2/403	0.6	75	I	1
3/403	0.8	100	III	1

*According to the classification by Hinnen *et al.* (1978)
type I: single cross over at the homologous locus; II: integration into a nonhomologous locus; III: integration by a double cross over at the homologous locus

In addition we have studied the regulation of the pyruvate kinase activity in some transformants after growth on acetate which was given as gluconeogenic carbon source (Table II).

The level of activities found in the transformants after growth on acetate were significantly higher compared to those found in the wild type strain. Transformant 5/402 (type I integration) is useful in this respect since integration pattern and copy number are exactly known in this case. From this data it can be calculated that the pyruvate kinase activity on acetate in this transformant is 0.25 IU per mg per copy. It can also be calculated that the pyruvate kinase activity in the transformants is increased by about 0.2 IU per mg protein per copy integrated in crude extracts compared to the wild type strain. Correcting for wild type activity in transformant 9/402, which is a type II transformant, the activities correspond, both in the case of sucrose and acetate grown mycelium, with five additional active copies. In type III transformants (see 6/402 *e.g.*) the activity level, both on sucrose and on acetate is in the same order as in the wild type.

Table II *Pyruvate kinase activities (IU/mg protein) in the wild type (WG096) and some of the transformants after growth on different carbon sources.*

Strain:	WG096	6/402	5/402	9/402
Carbon source:				
sucrose	0.4	0.5	0.9	2.6
sucrose/acetate	0.2	0.2	0.7	2.0
acetate	0.06	0.06	0.5	1.4

Sucrose (50 mM), sucrose/acetate (5 mM/100 mM) and acetate (100 mM) were used as a carbon source.

Discussion

The *A.nidulans* pyruvate kinase gene was cloned from a genomic library by heterologous hybridization with the homologous yeast gene. Hybridization conditions were established on the basis of a restriction enzyme analysis of chromosomal DNA using the yeast gene as a probe and they were found to be quite stringent. Subsequently the genomic library was screened under identical conditions. After Southern analysis of the positive clones, a 2.9 kb *Eco*RI/*Bam*HI fragment and a 5.0 kb *Bam*HI fragment were subcloned. Both fragments were shown to contain the pyruvate kinase gene of *A.nidulans*. The identity of the gene was confirmed by transformation. Both subcloned fragments were able to complement the *pki* mutation in a *pki*A deletion mutant.

The pyruvate kinase gene itself is potentially a selection marker, which can be used in transformation and cotransformation experiments. Direct selection for this marker is possible by using a glycolytic carbon source, like glucose or sucrose. However, the transformation frequencies obtained are still low, 10-20 transformants per μg DNA, compared to 500 per μg DNA in the case of *arg*B (Johnstone, 1985) and 3-5.10^3 per μg DNA in the case of *pyr*4 (Ballance and Turner, 1985). However, since no attempts were made to optimize this transformation system, it may well be possible to increase the transformation frequency by improving the formation and stabilization of protoplasts from mycelium grown on acetate as a carbon source.

Genomic analysis of the transformants reveals a strong preference for integration at the homologous locus. About 60% (7/13) of the transformants analyzed were the result of a double cross-over event at the homologous locus (type III integration according to the classification of Hinnen *et al.*, 1978), while about 25% (3/13) were the result of a single cross-over event at the homologous locus (type I integration). In the latter case, multicopy integration was found with up to three copies. Only in three of

the transformants tested had integration occurred at a locus unlinked to the *pki* locus (type II integration), resulting in multicopy integration in all cases.

These results are in strong contrast to those found after transformation with the *amd*S marker in *A.nidulans* (Kelly and Hynes, 1985; Wernars *et al.*, 1985), but correspond with the results found for the *trp*C (Yelton *et al.*, 1983) and the *arg*B marker (John and Peberdy, 1984). The high ratio of non-homologous integration found in the *amd*S transformation system (Wernars *et al.*, 1985) is possibly the result of additional selective pressure due to the addition of CsCl to the medium although effects of the genetic background cannot be excluded.

Since transformation, as phenomenon, was not primarily the objective of this study no further attempts were made to investigate the mitotic and meiotic stability of the transformants. However, mitotic instability was observed in the case of one of the type II multicopy transformants, which was estimated originally to contain 5 copies of the gene. The intensity of the plasmid band decreased during a period of extensive subculturing, and in parallel a decrease in pyruvate kinase activity was found.
This instability was also reflected by morphological differences in progeny colonies. The instability may be due to the fact that tandem multicopy integration has occurred at a non-homologous locus, which is followed by a process of recombination in which the number of integrated copies is reduced. A reflection of this recombination process is found in Southern blots. In addition to the hybridizing 9.5 kb *Eco*RI fragment, the 6.9 kb plasmid band is found in this transformant, and its intensity decreases during subculturing. Besides these discrete hybridizing bands, non-discrete hybridization is found in the high molecular weight area of the blot. This non-discrete hybridization pattern must thus be the result of different stages of the recombination process.

Both the 2.9 kb *Eco*RI/*Bam*HI fragment and the 5.0 kb *Bam*HI fragment

contain the whole structural pyruvate kinase gene including the regulatory sequences, as can be concluded from the results presented in Table I. Integration of a single copy of the cloned fragment leads to a pyruvate kinase activity in the recipient strain, which varies but can reach approximately the same level as in the wild type. Since these transformants represented also type III integrations restoring the wild type genome, we have no appropriate explanation for the variation in activity observed (50-110%). In those transformants, in which more than one copy of the fragment is integrated into the genome, a good correlation is found between the number of copies integrated and the pyruvate kinase activity measured under glycolytic growth conditions.

However, the regulation of the pyruvate kinase activity in the transformants thus obtained is disturbed which becomes obvious upon growth on a gluconeogenic carbon source. The pyruvate kinase activity which is measured, has a proportional increase in activity compared to the wild type activity (Table II). The extent to which this occurs turns out to be about 0.2 IU per mg of protein per copy integrated. As mentioned before, the regulation of the pyruvate kinase activity is rather complex. The activity is regulated by gene expression, by allosteric effectors and by the turnover of the protein. In the case of the wild type strain, a 7-fold decrease in activity is found under gluconeogenic growth conditions compared to glycolytic growth conditions.

One can speculate that the differences in the pyruvate kinase activity level in these transformants grown on acetate, might be due to titration of regulating factors (see Kelly and Hynes, 1985), lack of regulatory sequences in the transforming DNA, or an increased stability of the enzyme due to a higher enzyme concentration, thus leading to a higher proportion of the functional tetramer.

If titration of regulatory factors or a higher stability of the enzyme is involved, one does not expect the degree to which the activity

per gene copy integrated increases, to be constant. However, this is actually observed. Besides, in these cases one would not expect such drastic effects by just raising the gene copy number from one to two. It is more likely that the changes in regulation occur due to the lack of regulating sequences in the 2.9 kb *Eco*RI/*Bam*HI fragment. Since the pyruvate kinase gene is fully expressed in transformants under glycolytic conditions, this would imply that regulation of the pyruvate kinase gene expression under gluconeogenic conditions is under control of a negative regulatory element.

The validity of this conclusion is subject of a further study on the pyruvate kinase gene expression, in which the effects of sequences located further upstream of the 2.9 kb *Eco*RI/*Bam*HI cloned fragment will be investigated.

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CHAPTER 3

A rapid purification procedure for pyruvate kinase from the hyphal fungus *Aspergillus nidulans*

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Summary

Pyruvate kinase was purified from the filamentous fungus Aspergillus nidulans *with a 45-55% yield. The procedure involves dye-affinity chromatography and Fast Protein Liquid Chromatography (FPLC), which results in a highly active and pure enzyme in mg quantities within two days time. The purified enzyme, a tetramer with a subunit molecular weight of 65 kDa and an isoelectric point of 4.7, was used to determine the amino acid composition.*

Introduction

Pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate, thereby generating adenosine-5'-tri-phosphate (ATP). Fungal pyruvate kinases have been isolated from Mucor rouxii (Terenzi *et al.*, 1971) and *Neurospora crassa* (Kapoor, 1975). Partial purification has been obtained for pyruvate kinase originating from *Aspergillus niger* (Meixner-Monori *et al.*, 1984).

Investigators attempting to use low-pressure chromatographic techniques experienced the instability of pyruvate kinase at various stages of purification, especially in dilute solutions the enzyme activity was rapidly lost. For the enzyme isolated from *A.niger*, Meixner-Monori *et al.* (1977) demonstrated that addition of glycerol and fructose 1,6-biphosphate (FBP) was essential to stabilize the enzyme. Even then the enzyme was purified with a very low recovery.

Heat treatment and ammonium sulphate precipitation have been used in other protocols as initial steps to purify pyruvate kinase from fungal origin (Kapoor and Tronsgaard, 1972). In the case of *Aspergillus nidulans* salt precipitation resulted in a considerable loss (50%) of the initial activity. Heat treatment was determined to be not appropriate as a purification step, because the procedure should also be sufficiently mild to allow the isolation of pyruvate kinase from *Aspergillus* pyruvate kinase mutant (*pkiA*) strains (Payton and Roberts, 1976; Bos *et al.*, 1981). Therefore, we developed a method using an affinity chromatographic step.

Studies with Blue Dextran and its chromophore, Cibacron Blue 3G-A, have shown that these ligands can be used to prepare affinity adsorbants specific for enzymes whose nucleoside phosphate binding sites are composed with an dinucleotide fold (Stellwagen *et al.*, 1975; Thompson *et al.*, 1975). This supersecondary structure is found to be present in a number of kinases and dehydrogenases. Pyruvate kinase of *N.crassa* (Kapoor and O'Brien, 1980) also was found to interact with this triazine dye.

The application of Cibacron Blue and other reactive dyes in affinity chromatography is well documented (Dean and Watson, 1979; Lowe *et al.*, 1981). The rapid purification method to be presented here for the pyruvate kinase of *A.nidulans*, is also based on the application of a dye affinity column as an initial purification step. Final purification was achieved by using pre-packed small particle columns at high flow rates. The molecular cloning of the pyruvate kinase gene of A.nidulans and subsequent transformation of this fungus with the homologous gene resulted in strains with increased enzyme levels as described in Chapter 2 (de Graaff *et al.*, 1988. Such strains further improve the enzyme yield obtained per gram of fungal biomass.

Materials and methods

Chemicals

All chemicals used were standard analytical grade and provided by Merck, Darmstadt, Germany, except PEP (monocyclohexylammonium salt), ADP (monopotassium salt), NADH (disodium salt), FBP (trisodium salt), ATP (free acid) and pig heart lactate dehydrogenase (EC 1.1.1.27) which were purchased from Boehringer, Mannheim, Germany. Sepharose Cl-4B, Sepharose 4B and the MONO Q column were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Mikacion Brilliant Yellow 6GS was supplied by Nippon Kayaku Co Ltd., Tokyo, Japan. Procion dyes were obtained from ICI Ltd., Manchester, England and Cibacron Blue 3G-A from Ciba Geigy AG, Basel, Switzerland. The gel permeation chromatography column G 3000 SW (7.5 x 300 mm) was purchased from LKB, Bromma, Sweden.

Strains and growth conditions

A.nidulans wild type strain WG 096 (yA2, paba A1) and the A.nidulans transformant 9/402, a multi copy transformant produced as described in Chapter 2, were used to isolate the pyruvate kinase enzyme. For preparation of conidiospores, A.nidulans was grown on complete medium according to Pontecorvo et al. (1953) solidified with 1.2% agar and supplemented with 25 mM sucrose as carbon source. To obtain large quantities of mycelial biomass for the preparation of cell free extracts, A.nidulans was cultured in a 7 litre batch of minimal medium supplemented with 2 µg/l p-aminobenzoic acid and 90 mM sucrose as described previously (Dijkema et al.,

1986). After a growth period of 16-18 h at 37 °C the mycelium was harvested by filtration, washed with cold saline and squeezed to remove excess of liquid.

Preparation of cell free extract

Freshly harvested mycelium was frozen with liquid nitrogen and homogenized in a Waring Blender with liquid nitrogen for 8 min. at 100 Volt. After evaporation of the liquid nitrogen, extraction buffer was added to the powder (3 ml per gram initial mycelium). The extraction buffer consisted of 0.05 M sodium phosphate buffer, pH 7.0, 0.5 mM EDTA, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and in the case of an optimized purification also 5% (v/v) ethylene glycol. Extraction was performed for 1 h at 4 °C, with gentle stirring of the suspension. The cell debris was removed by centrifugation for 20 min at 40,000 x g and the supernatant was used as the crude extract in further purification.

Enzyme assay and protein determination

Pyruvate kinase activity was determined by measuring the decrease in absorption of NADH at 340 nm using a continuous lactate dehydrogenase coupled assay as described by Bergmeyer (1974).

Protein was determined by the micro-Biuret method (Itzhaki and Gill, 1964) with bovine serum albumin as standard. Although this method is not extremely sensitive, it is simple and has high reproducibility and therefore has been recommended for protein determinations in mycelial extracts (Jernejc et al., 1986).

Electrophoresis and isoelectric focussing

Electrophoresis in 10% polyacrylamide gels containing 0.1% SDS was performed according to Laemmli (1970). The pyruvate kinase subunit molecular weight was determined after electrophoresis in a 10% SDS-containing gel calibrated with the following protein standards: Carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (68 kDa) and phosphorylase B (92.5 kDa).

Analytical thin-layer gel isoelectric focussing was performed in the pH range 3-7 on the FBE-3000 apparatus (Pharmacia, Uppsala, Sweden) using the focussing and staining conditions supplied by the manufacturer.

Preparation of affinity matrices

The method used was a modification of that described before by Dean and Watson (1979). Sepharose Cl-4B was washed extensively with deionized

water and filtrated with suction to remove excess water. The packed beads (3 g) were resuspended in 10 ml of water and the reactive dye (15-60 mg) was dissolved by gently shaking. After the addition of 2 ml 3 M NaCl the suspension was allowed to stand at room temperature for 15 min. The reaction was initiated by elevating the pH to 10-11 by adding 1 M Na_2CO_3. The reaction mixture was incubated for 1 h at 55 °C. The suspension was washed with water, 2 M NaCl, water, 4 M urea and water successively. The matrices were stored at 4 °C in 0.05 M sodium phosphate buffer pH 7.0 containing 0.02% sodium azide.

Preparation of large quantities of a particular matrix (30-90 g of packed beads) followed the same procedure used in small coupling experiments, with only adjustments made to maintain the same volume and weight ratios.

The Blue Dextran 2000 conjugate was prepared by cyanogen bromide activation of Sepharose 4B according to March et al.(1974) using 50 mg of dextran per gram of agarose.

Determination of ligand concentrations

The amount of Cibacron Blue 3G-A (14 μmoles/g dry weight) bound to Sepharose 4B, in the case of the Blue Dextran 2000 matrix was determined spectrophotometrically after its release by acid hydrolysis (Chambers, 1977). The concentration of Mikacion Brilliant Yellow 6GS (colour index no 18971, Reactive Yellow 1) was determined by elemental analysis of the lyophilized matrix using Sepharose Cl-4B as a control.

Amino acid composition determination

Amino acid analysis was performed according to the method described by Bidlingmeyer et al. (1984). The protein was reduced, S-carboxymethylated using the method of Crestfield et al. (1963). The protein was hydrolyzed by gas-phase hydrolysis with 6 N hydrochloric acid for 20, 24, 48 and 72 hrs at 110 °C. Tryptophan was measured by the spectroscopic method of Edelhoch (1967).

Results

Screening of reactive dyes for pyruvate kinase affinity

Samples (0.5-1.0 U of pyruvate kinase) of a mycelial extract from strain WG 096 were applied at 4 °C to small columns (0.5 x 5 cm) packed with different dye-Sepharose conjugates which were eluted with extraction buffer. The following dyes were tested: Procion Red P3BN, Procion Yellow M-4G, Mikacion Brilliant Yellow 6 GS, Procion Yellow H-5G, Procion Turquoise MX-G, Procion Navy MX-RB, Cibacron Blue 3G-A, Procion Brown MX-GRN, Blue Dextran 2000, Procion Orange MX-G and Procion Yellow MX-4G. The last two matrices and unsubstituted Sepharose Cl-4B showed no binding of pyruvate kinase under these conditions.

Elution by the addition of 0.5 M NaCl to the eluent resulted in very low recoveries (<10 %) of pyruvate kinase activity, indicating enzyme inactivation during its interaction with the various dyes or very tight binding. Higher recoveries (35-100 %) were achieved by the addition of ethylene glycol (5-10 %,v/v) to the crude extract and to the eluent used. In most cases, however, the activity of pyruvate kinase now appeared in the void volume except for matrices using Procion Red P3BN, Mikacion Brilliant Yellow 6GS, Procion Yellow H-5G or Blue Dextran 2000 as the ligand. Both non specific elution by increasing salt concentration and biospecific elution by PEP, FBP or ATP were further analyzed.

The biospecific interactions between pyruvate kinase and Mikacion Brilliant Yellow 6 GS (30 μmoles/g dry weight) and Cibacron Blue 3G-A (14 μmoles/g dry weight), which is the actual ligand in the case of the Blue Dextran matrix, were analyzed in particular and found to be different. With Cibacron Blue 3G-A both PEP and FDP interfered with the enzyme binding, whereas ATP was not effective in releasing the bound enzyme. In the case of Mikacion Brilliant Yellow ATP and PEP were effective to elute the bound enzyme, whereas FBP was not. Based on total recoveries and the increase in specific activity, Mikacion Brilliant Yellow 6 GS was finally

chosen as the appropriate ligand when used in combination with a salt elution. The ligand density was optimized by coupling different amounts of dye per gram of Sepharose Cl-4B. A linear relationship was found between the amount of dye added during the coupling reaction (tested up to 80 mg/g suction dried beads) and the actual amount of dye bound determined by nitrogen content. A concentration of 30 μmoles/g dry weight was found to be optimal, providing 80-85 % recovery, using the standard loading of 3 ml of mycelial extract per ml bed volume. Lower ligand densities, 10-15 μmoles/g dry weight resulted in a low binding capacity of the matrix, only part of a standard loading of 3 ml of crude extract per ml bedvolume was bound. Higher ligand densities 50-60 mole/g dry weight) however, resulted in a 15-20 % reduction in enzyme recovery.

Although the differences in interaction between pyruvate kinase and the different dye matrices could be exploited in further purification steps, our final protocol involves ionic exchange and gel permeation chromatography.

Pyruvate kinase purification

All steps in the purification were carried out at 4 °C except for the FPLC purification steps which were preformed at room temperature. Freshly harvested mycelium (10 g) was used to prepare a cell free extract containing 5% (v/v) ethylene glycol as described in earlier. The supernatant was loaded on a 15 ml (0 2.6 cm) Mikacion Brilliant Yellow 6GS Sepharose CL-4B column (30 μmoles/ g of dry Sepharose). Non-binding proteins were eluted with extraction buffer until the absorbance at 280 nm of the eluent was less then 0.1. Pyruvate kinase activity was recovered by elution with extraction buffer containing 0.5 M NaCl (Fig. 1A). The pyruvate kinase containing fractions were pooled and overnight dialyzed at 4 °C against buffer A (identical to extraction buffer except that 20 mM sodium phosphate is used instead of 50 mM).

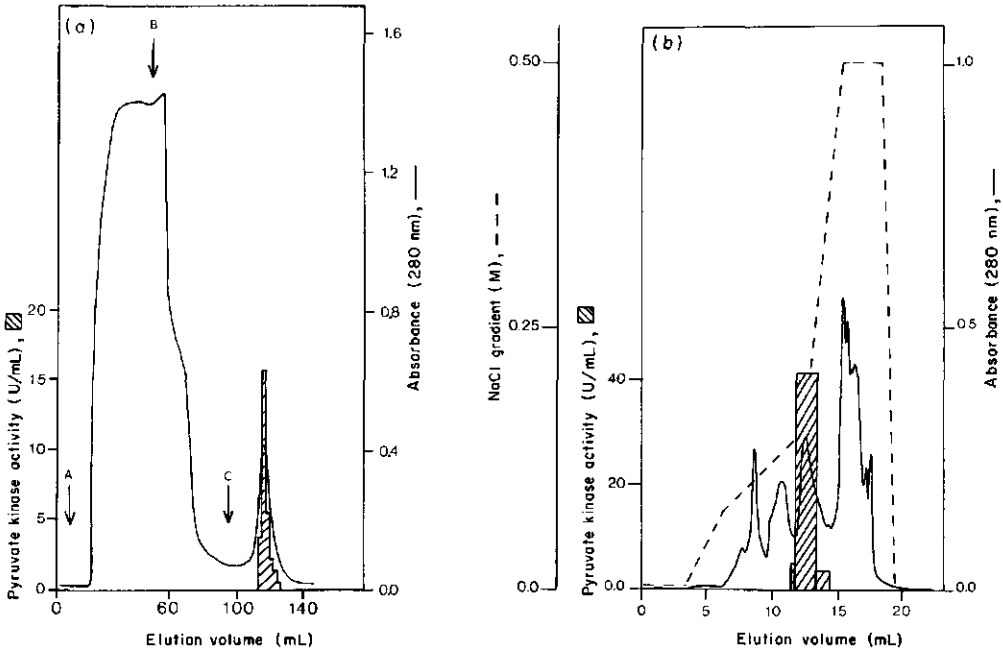

Fig. 1A Elution of pyruvate kinase from Mikacron Brilliant Yellow 6Gs Sepharose Cl-4B (Ø 2.6 cm, 15 ml bedvolume).
42 ml of cell-free extract was loaded (arrow A) and non bound proteins were eluted with extraction buffer (arrow B) Pyruvate kinase activity was eluted with extraction buffer + 0.5 M NaCl (arrow C). Fraction of 2 ml were collected.
——— Absorbance at 280 nm.
The hatched area represents the pyruvate kinase activity.

Fig. 1B Elution profile of pyruvate kinase on Mono Q HR 5/5 column.
17.5 ml enzyme solution was loaded at room temperature at a flow rate of 1.5 ml/min. Proteins were eluted with a stepwise linear sodium chloride gradient. The maximum peakfraction size was 2 ml.
——— protein absorbance at 280 nm.
----- NaCl gradient.
The hatched area represents the pyruvate kinase activity.

The following day the enzyme was loaded on a MONO Q HR 5/5 column pre-equilibrated with buffer A. Stepwise gradient elution with buffer A + 0.5 M NaCl was found to separate the enzyme from the bulk of contaminating proteins (Fig. 1B). The fractions containing the enzyme were diluted 5 fold in buffer A. Further purification was achieved by rechromatography with the MONO Q HR 5/5 column. The final purification step was immediately done as the enzyme was eluted from the 2nd Mono Q column. Small aliquots (0.5 ml) of the eluted enzyme were loaded on a TSK G 3000 SW gel permeation column. Using this protocol, the results are summarized for the wild type strain WG 096 in Table I and Fig. 2. Starting with 10-40 g of mycelium and following this protocol, highly active pyruvate kinase was obtained in only 2 days. With the multi copy *pki* transformant 9/402, containing approx. 4-fold more of the pyruvate kinase per g of mycelium, similar high yields were also obtained.

Table 1 Purification of pyruvate kinase from A.nidulans wild type strain WG 096.

Step	volume (ml)	total protein (mg)	total activity (Units)	specific activity (U/mg)	yield (%)
Crude extract	42.0	96.2	104.6	1.1	100
Pooled and dialyzed MBY Sepharose fractions	17.5	10.3	123.5	12.0	118
After rechromatography on MONO Q HR 5/5 column	2.0	0.88	69.4	78.9	66
After gel permeation chromatography	3.5	0.44	58.6	133.2	56

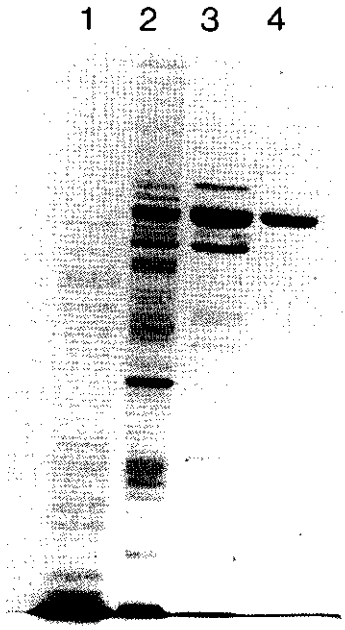

Fig. 2 SDS-polyacrylamide gel electrophoresis of pyruvate kinase from A.nidulans wild type strain WG 096 at different stages of purification:
lane 1: crude extract;
lane 2: active fraction after elution of MBY 6GS Sepharose Cl-4B column;
lane 3: active fraction after chromatography on MONO Q HR 5/5 column;
lane 4: final preparation after TSK 3000 SW column.

Physiochemical properties and amino acid composition

From the SDS-polyacrylamide gel electrophoretic pattern, a subunit molecular weight of 65 kDa was determined. Gel permeation chromatography with the native enzyme indicated a tetrameric structure which was expected. The isoelectric point was found to have a pI value of 4.7. The amino acid composition of the purified enzyme from transformant 9/402 is given in Table 2.

Table 2 Amino acid composition of pyruvate kinase from A.nidulans transformant 9/402.

Amino acid	Number of residues[a]	per cent of total
asx[b]	68	11.4
glx[b]	59	9.9
ser[c]	36	6.1
gly	46	7.7
his	11	1.9
thr	31	5.2
ala	46	7.7
arg	39	6.6
pro	27	4.5
tyr	9	1.5
val	51	8.6
met	16	2.7
cys[d]	10	1.7
ile	49	8.2
leu	43	7.2
phe	15	2.5
trp	6	1.0
lys	33	5.6

[a]Number determined based on a subunit molecular weight of 65 kDa.
[b]Sum of acid and amide forms.
[c]Value extrapolated to zero time hydrolysis.
[d]Determined as S-carboxymethylcysteine.

Discussion

The instability of pyruvate kinase, especially at low concentrations in the various stages of purification, is a well-known problem when isolating this enzyme from eukaryotes like Saccharomyces cerevisiae (Haeckel *et al.*, 1968) or from filamentous fungi like *N.crassa* (Kapoor, 1975) and *A.niger* (Meixner-Monori *et al.*, 1984). In the latter case, the instability persisted to such an extent as to prevent final purification. Except for the purification procedure of Hunsley and Suelter (1969), who in the case of Baker's yeast reported a 45% yield, most protocols have relatively low yields (5-20%) . The purification procedure for *A.nidulans*, described here, uses the immobilized dye Mikacion Brilliant Yellow 6GS as affinity ligand as a very efficient first purification step (see Table I). The yield of this first step usually varies between 80% and 120%, the

reason for this is not clear.

Although further steps based on affinity chromatography with other dyes and gel permeation chromatography also result in a pure enzyme, the final recovery was always low (3-8% yield), particularly because gel filtration is a major bottleneck. Inactivation of the enzyme is likely to involve dissociation and subsequent unfolding of the tetrameric active form of the pyruvate kinase, as can be concluded from the extensive studies by Kapoor and Tronsgaard (1972) with the purified pyruvate kinase of *N.crassa*. By applying high pressure ionic exchange and gel permeation chromatography as described here, we demonstrate that the instability problem during the purification can be largely reduced and a pure enzyme is obtainable with much higher yields (45-55%). The mycelium used to isolate the enzyme is grown on sucrose since nutritional conditions known to stimulate glycolysis lead to a 15-20 fold increase in specific activity of the pyruvate kinase in fungus (Uitzetter, 1982). The level of pyruvate kinase expression has been further increased by 4 fold with genetic manipulation (Chapter 2) and correspondingly, the enzyme was also isolated from the transformed strain. The purified enzyme from the wild type strain was used to compare some physio-chemical properties with those obtained for other fungi and yeasts. The subunit molecular weight of 65 kDa as determined by SDS polyacrylamide gel electrophoresis is higher than those determined for the enzyme of *A.niger* (Meixner-Monori *et al.*, 1984) and for the enzyme of Baker's yeast (Fell *et al.*, 1974) with an approximate values of 55-60 kDa. The *N.crassa* pyruvate kinase has a slightly lower subunit molecular weight (62 kDa) as was reported by Kapoor (1975), In the case of *Mucor rouxii*, a dimorphic phycomycete, in which pyruvate kinase isoenzymes occur, a subunit molecular weight of 45.5 kDa was reported (Friedenthal *et al.*, 1973). In the latter case, analogous to earlier observations with the Baker's yeast enzyme, this may be due to limited proteolysis.

The isoelectric point found for the *A.nidulans* pyruvate kinase, in the presence of FBP (pI = 4.7), is lower when compared to the value of 6.4 reported for the unliganded *N.crassa* enzyme (Kapoor *et al.*, 1976). The isoelectric point of the *N.crassa* enzyme is lowered to 5.5 by FBP binding. In *A.nidulans* some micro-heterogeneity of the enzyme is observed, which has not been further investigated but which might be related to FBP binding. The amino acid composition of pyruvate kinase from *A.nidulans*, calculated on a molar basis, shows similarity with that of the yeast enzyme (Yun *et al.*, 1976) except for the number of tryptophan residues which is five times higher in the *A.nidulans* enzyme.

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CHAPTER 4

Structure of the *Aspergillus nidulans* pyruvate kinase gene

Part of this Chapter is accepted for publication;

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Curr. Genet. 14, in press

Summary

The complete nucleotide sequence of the Aspergillus nidulans pyruvate kinase gene, including its flanking sequences is presented. The gene has a coding sequence of 1578 bp, which encodes a protein of 526 amino acids and is strongly homologous with the corresponding Saccharomyces cerevisiae (66%) and mammalian (53%) proteins.

The gene is interrupted by seven introns, three of which are in a conserved position compared to those present in the mammalian pyruvate kinase genes thus far sequenced. A fourth intron within the mononucleotide binding fold domain, is found in a conserved position with respect to the position of an intron within the NAD^+ binding region of maize ADH I.

The transcription start site has been determined; a major site of transcription was found 80 bp before the translation initiation codon. The promoter region of the A.nidulans pyruvate kinase gene contains no direct homologies with TATA or CCAAT sequences in the expected region (30-70 bp) before the transcription initiation site. However, extended CT enriched regions are found in the promoter region, similar to genes which are highly expressed in S.cerevisiae and filamentous fungi.

Introduction

In *Aspergillus nidulans*, pyruvate kinase (pyruvate phosphotransferase, EC 2.7.1.40), a homotetramer that catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate, is encoded by the *pki* gene. In both, in *Saccharomyces cerevisiae* and *A.nidulans* only one form of the enzyme exists, whereas in *Escherichia coli* (Valentini *et al.*, 1979) and *Mucor rouxii* (Friedenthal *et al.*, 1973) two and three forms of the enzyme are found, respectively. In higher eukaryotes usually four isozymes are found, termed the L, M_1, M_2, and R type pyruvate kinases. These forms are tissue specific and differ in size and kinetic behaviour (for review see Engström *et al.*, 1987).

Regulation of pyruvate kinase activity is required in order to prevent substrate cycling between PEP and pyruvate during gluconeogenesis. Regulation of the activity is achieved by allosteric modulation of the enzyme by its substrate PEP and its product ATP, but also by the allosteric effector fructose 1,6-biphosphate (Kapoor, 1976; Meixner-Monori *et al.*, 1984). In addition to this allosteric modulation, there might still be another level of control analogous to the mammalian L type pyruvate kinase. Besides being allosterically regulated, the L type is also regulated by cAMP-dependent phosphorylation, with the site of phosphorylation being a serine located near the N-terminus of the protein (Humble, 1980). The other pyruvate kinase isozymes found in higher eukaryotes have shorter N-terminal sequences and, consequently, lack the specific serine residue which is phosphorylated in the L type isozyme. In addition, enzyme activity is also regulated by the rate of transcription as was shown for yeast (Burke *et al.*, 1983) and the rat L type pyruvate kinase (Cognet *et al.*, 1987).

Regulation of the pyruvate kinase activity in *A.nidulans* is reflected by the enzyme activities measured after growth on different carbon sources. The activity after growth on sucrose, as a glycolytic carbon

source, is 10-20 fold higher compared to the activity after growth on acetate, as a gluconeogenic carbon source (Chapter 2; Uitzetter, 1982; de Graaff *et al.*, 1988).

The primary structure of the *S.cerevisiae* pyruvate kinase gene (Burke *et al.*, 1983) and of the cDNA clones of chicken M type (Lonberg and Gilbert, 1983), rat L type pyruvate kinase (Lone *et al.*, 1986; Inoue *et al.*, 1986),the rat M type (Noguchi *et al.*, 1986), human L type (Tani *et al.*, 1988) have been determined. A comparison of the primary structures of these genes reveals a strong homology between the pyruvate kinases of these organisms, especially at those positions which are involved in catalysis or in the binding of substrate and ions (Johnson *et al.*, 1979; Stuart *et al.*, 1979; Muirhead *et al.*, 1986).

Besides, the gene structure is also known for both the rat L type gene (Cognet *et al.*, 1987) and the chicken M type gene (Lonberg and Gilbert, 1985). From these studies it has been concluded that the four isozymes in rat are encoded by two genes, the L and M type. The L and R (L') isozymes are produced from the L-type gene by the use of different promoters and of different translational start sites. As a result, the isozymes differ in size and in the N-terminal amino acid sequence (Marie *et al.*, 1981; Noguchi *et al.*, 1987). The M_1 and M_2 isozymes are the result of differential splicing of a single primary transcript of the M-type gene. The M_1 isozyme is not allosterically regulated, while the other isozymes have allosteric properties. As a result of the differential splicing differences in amino acid sequence are found in the C domain of both proteins (Noguchi *et al.*, 1986). This domain is involved in subunit interaction (Stuart *et al.*, 1979; Muirhead *et al.*, 1986), and since the two isozymes are identical except for a region located within this C domain, the differences in the kinetic behaviour of the two enzymes must be the result of these differences. Noguchi *et al.* (1986) conclude that the region responsible for the allosteric behaviour of the pyruvate kinase isozymes

is located within this region of the C domain.

The three-dimensional structure of cat muscle pyruvate kinase has been determined (Stuart *et al.*, 1979; Muirhead *et al.*, 1986), and was used to predict the structure of the chicken M type pyruvate kinase. Comparison of the chicken pyruvate kinase protein three dimensional structure with the corresponding gene structure, reveals a nonrandom distribution of the introns and a conservation of the position of the introns in the chicken and rat genes (Lonberg and Gilbert, 1985; Cognet *et al.*, 1987).

The *A.nidulans* pyruvate kinase (*pki*) gene was cloned by heterologous hybridization, using a fragment of the pyruvate kinase gene of S.cerevisiae (Chapter 2; de Graaff *et al.*, 1988). In this Chapter the primary structure of the *A.nidulans* pyruvate kinase gene is described and our findings are compared with those of other pyruvate kinases.

Materials and methods

Strains

The following strains were used: A.nidulans WG096 (yA2, paba A1) was used for the isolation of RNA and E.coli DH5α (endA1, hsdR17, [rk⁻, mk⁺], supE44, thi-1, λ⁻, recA1, gyrA, relAL, 80dlacZ M15, (lacZYA-argF), U169) and DH5αF' were used for the propagation, of plasmid DNA and M13 phages, respectively.

Chemicals and enzymes

Restriction enzymes, DNA polymerase I Klenow fragment, AMV Reverse Transcriptase and bacteriophage T_4 DNA ligase were obtained from BRL, bacteriophage T_7 DNA polymerase from Pharmacia and α-^{32}-P-dATP from New England Nuclear. Urea, acrylamide bisacrylamide and oligo dT cellulose were of Ultra Pure grade (BRL). Deoxynucleotidetriphosphates and dideoxynucleotdetriphosphates were from Boehringer. All other chemicals used were of analytical grade.

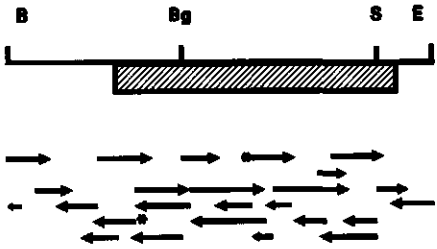
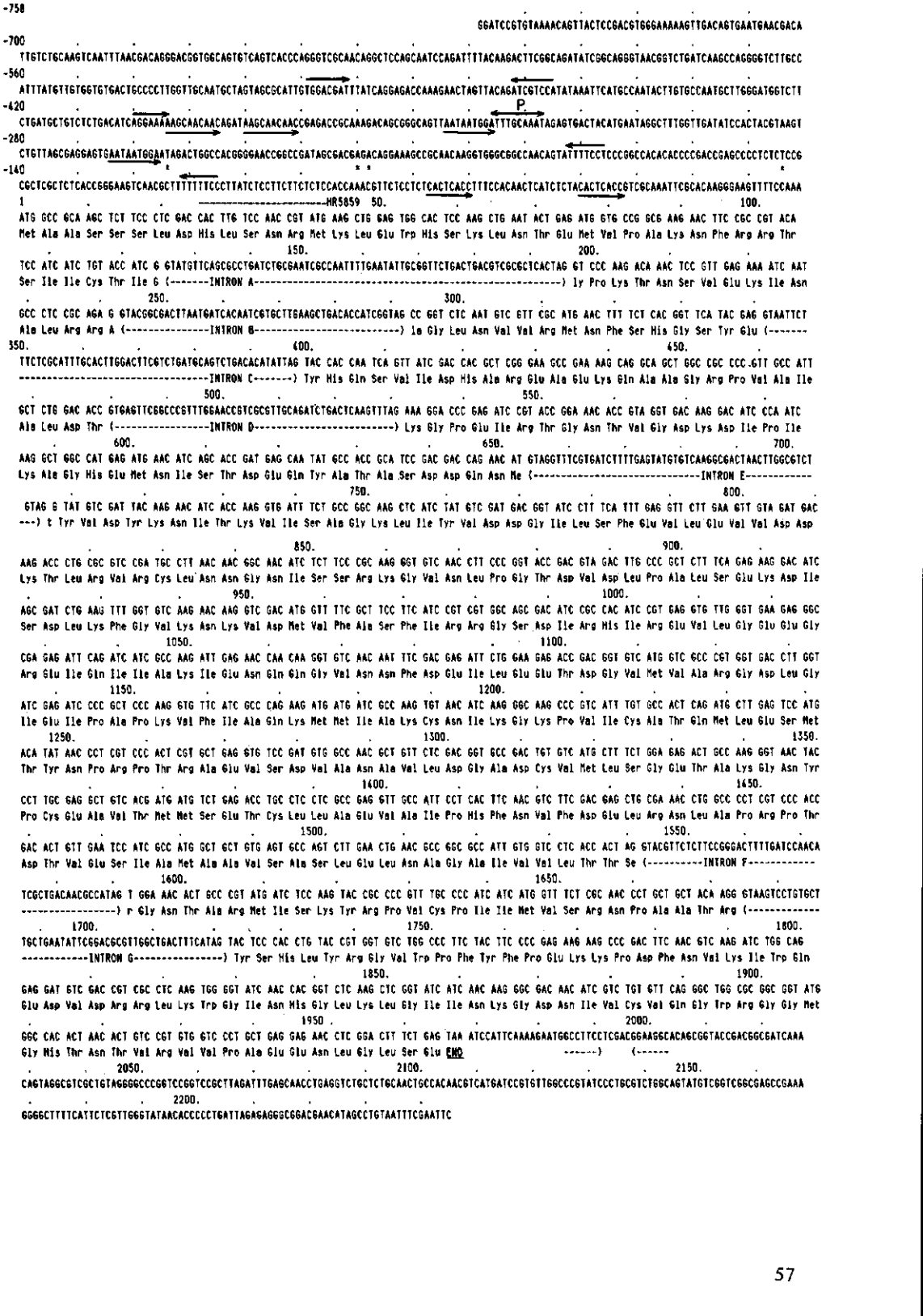


*Fig.1 Strategy employed to sequence the 3003 bp BamHI/EcoRI fragment. Only major restriction sites are indicated: B: BamHI, Bg: BglII, S: SalI and E: EcoRI. The arrows indicate the position, length and direction of sequencing from restriction enzyme site or oligonucleotide primer (*1:HR5859, *2:HR5858). The hatched box represents the coding region of the A.nidulans pki gene.*

The *pki* gene was found to have a coding region of 1578 bp and seven putative introns based on homology from the deduced amino acid sequence with other pyruvate kinases, and on the consensus sequence of the splice signals (Rambosek and Leach, 1987). Sevenhundred fiftyeight bp upstream of the start codon were determined, whereas 277 bp were determined beyond the stop codon. The entire gene, including noncoding regions, is presented in Fig. 2.

Fig. 2 Nucleotide sequence of the A.nidulans pyruvate kinase gene with its amino acid translation, the position of the introns is as indicated.

The position, and sequence, of the nucleotide sequence complementary to the synthetic oligonucleotide used in the primer extension experiments is overlined. The sites of transcription initiation are marked with an asterix. In the 5'non-coding region repeats are indicated by arrows under the sequence, and the inverted repeats by arrows above the sequence. P indicates a 10 bp palindromic sequence. The dashed arrow, under the sequence, in the 3' non-coding region, indicates one of the inverted repeats found in this region.



Nucleotide sequencing and analysis

The sequence was determined by subcloning fragments from the 2.9 kb BamHI/EcoRI fragment, containing the functional pki gene (de Graaff et al., 1988), into M13 mp18/19 (Messing, 1983; Norrander et al., 1983). Subclone sequences were determined by using the dideoxynucleotide chain-termination procedure (Sanger et al., 1977). A small part of the sequence was determined using specific oligonucleotide primers (HR5858 and HR5859) as indicated in Fig. 1. Almost all of the sequence (95%) was determined by sequencing both DNA strands, while all the sequences were determined at least twice. Computer analysis was done using the PC/GENE program.

Primer extension mapping

Mycelium was grown by inoculating 10^6 spores ml^{-1} in minimal medium (Pontecorvo et al., 1953), supplemented with 2 μg ml^{-1} p-aminobenzoic acid and 50 mM glucose as a carbon source (glycolytic growth conditions) or 100 mM acetate (gluconeogenic growth conditions).

Total A.nidulans RNA was isolated by a procedure as described (Chapter 2; de Graaff et al., 1988) and poly(A)$^+$ RNA was isolated by oligo dT cellulose chromatography as described by Maniatis et al. (1982).

The primer extension reaction was performed according to Calzone et al. (1987) with some smaller modifications, using the synthetic oligonucleotide HR5859. The primer extension reaction products were analyzed on a 6% polyacrylamide sequencing gels, using dideoxysequencing reaction products as a marker.

Results and discussion

Structure of the pki gene

The primary structure of the *A.nidulans* pyruvate kinase gene was determined using a sequencing strategy as shown in Fig. 1. Using this strategy the complete sequence of the 3003 bp *Bam*HI/*Eco*RI fragment, containing the functional *pki* gene, was determined.

The 5'-noncoding region

The site of transcription initiation was determined by primer extension experiments, using poly A^+ RNA isolated from mycelium grown under glycolytic conditions, and a *pki* specific oligonucleotide, HR5859, located at the 5' end of the gene (Fig. 2). Reverse transcription of the *pki* mRNA resulted in cDNA strands of different lengths. From repeated experiments, it was found that two major extension products terminated at positions -4 and -80 before the translation initiation site (Fig. 3), while two minor extension products terminated at positions -78 and -113 (results not shown).

We have investigated the possibility whether different transcription initiation sites play a role in the regulation of the *pki* gene expression, by using RNA isolated from mycelium grown on a medium with glycolytic carbon source and mycelium grown on a medium with a gluconeogenic carbon source. No detectable differences were found the primer extension experiments, regardless of the mRNA used.

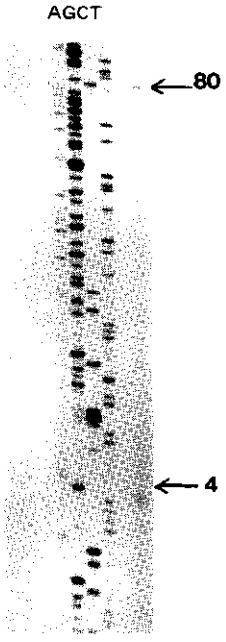


Fig. *3 Primer extension mapping of the transcription initiation site.*
The lanes A, G, C and T represent the sequencing reaction products. The position from the translation initiation site of the cDNA products obtained by the extension of the synthetic oligonucleotide primer hybridized with poly(A)+ RNA, are indicated with an arrow.

Thus, differential use of transcription initiation sites seems to play no significant role in the regulation of the *A.nidulans* pyruvate kinase gene expression.

Although one of the major primer extension products, ending at position -4, is located 43 bp from a second AUG codon, the latter is unlikely to function as a start for translation initiation. Two main arguments led us to the conclusion that only the first AUG codon is used as translation initiation codon:

1) The first AUG codon fits the consensus sequence for eukaryotic translation initiation sites, (CC(**A**/G)CCAUG(G)) (Kozak, 1984). The sequence found in the *A.nidulans pki* gene is CCAAAAUGG, the second AUG does not fit this sequence;
2) Complete amino acid sequence homology is observed in the open reading frame (ORF) between the two AUG codons, when the *A.nidulans* and *A.niger* pyruvate kinase sequence (Chapter 5) are compared, whereas the homology is lost immediately upstream of the AUG codon.

Initiation of transcription at position -4 can not be ruled out, but seems unlikely at such a short distance before the translation initiation site. It does not seem to play a role in differential transcription either, as no indications for differential transcription were found. Possibly, this fragment is the result of a premature stop during reverse transcription, caused by RNA structure.

The sequence at position -80, the major site of transcription, ccaA-acg (the nucleotide were transcription starts is in bold) shows a striking similarity with the sequences found at the transcription initiation site in several other fungal genes, *e.g. A.nidulans trpC* (ccaAcg) (Mullaney *et al.*, 1985), *A.nidulans alcA* (ccaAca) (Gwynne *et al.*, 1987), *aldA* (ccaAct) (Pickett *et al.*, 1987), and one of the transcription initiation sites found in the *A.nidulans argB* gene (ccaAgt) (Upshall *et al.*, 1986). However, this type of sequence is not found at the transcription initiati-

on sites of the *A.nidulans pgk* gene (gccCgt) (Clements and Roberts, 1986) and the *A.niger argB* gene (cgTgac/cgtGac) (Buxton *et al.*, 1987). Despite this variation, it is clear that a certain class of transcription initiation sites is formed around the sequence ccaAc/g.

The *A.nidulans* promoter contains a TATA box-like sequence at position -109, which is at a distance larger than normally found in eukaryotic genes. Whether this sequence, TATTTT, is functional is questionable. In the fungal genes containing TATA like sequences, they are found approximately 30-70 bp before the transcription initiation site (Rambosek and Leach, 1987). There are, however, several examples of fungal genes which lack a typical TATA sequence, *e.g. A.nidulans trpC* (Mullaney *et al.*, 1985), *argB* (Upshall *et al.*, 1986), *tpiA* (McKnight *et al.*, 1986). Functional mapping of these promoter sequences will be necessary to identify the sequences which play a role in setting the site of transcription initiation. In a deletion study done with the *A.nidulans trpC* promoter, Hamer and Timberlake (1987) found that deleting the sequence TTTATTT in the region -20 to -40 resulted in mRNAs with heterogeneous 5' ends. This indicates the possible involvement of this sequence in determining the site of transcription initiation. Interestingly, a similar T rich sequence, TTTTTTT located at position -27 to -33, is found in the *A.nidulans pki* gene.

A CCAAT box, which is usually found between 30-70 bp upstream of the TATA sequence in most eukaryotic promoters (Maniatis, 1987), is not found in the *A.nidulans pki* promoter. However, a CCAAT-like sequence (CCAAC) is found at position -114 before the transcription initiation site. Whether the CCAAC sequence could function like the CCAAT sequence, by increasing the level of transcription, is not clear since in other eukaryotic genes single base mutations in the CCAAT sequence leads to a loss in function, as reflected by a dramatic decrease in the level of transcription (Meyers *et al.*, 1986).

The *A.nidulans pki* sequence upstream of the transcription initiation site shares a typical feature with other glycolytic promoters in yeast and fungi, *viz.* a pyrimidine rich region (CT blocks) just before this site of transcription initiation. CT rich regions are found, for instance, in yeast *pgk* (Dobson *et al.*, 1982), yeast *pyk* (Burke *et al.*, 1983), *A.nidulans pgk* (Clements and Roberts, 1986), and *A.nidulans tpi* (McKnight *et al.*, 1986). Besides the CT blocks, the *A.nidulans pki* 5' noncoding region contains many direct and inverted repeats, some of which are indicated in Fig. 2. The functionality of these repeats is currently under investigation.

Comparison of the sequences in the 5' noncoding regions of the *A.nidulans* glycolytic genes which are known (*pgk* and *tpi*), shows that there is no obvious concerned sequence which might be related to a general control of the level of transcription glycolytic genes.

The 3'-noncoding region

The 277 sequenced nucleotides extending beyond the *A.nidulans pki* coding region does not contain the consensus AATAAA sequence found in most eukaryotic genes in their 3' termination region (Proudfoot and Brownlee, 1976). This sequence is believed to be involved in polyadenylation, but is not generally a feature of fungal termination regions. The sequence ATCAAA, which is similar to the polyadenylation sequence and strikingly similar to the sequence AAATCAAAA, found in the *A.niger* argB terminating region (Buxton *et al.*, 1987), is located 55 bp downstream from the stop codon. This sequence is later followed by the sequence CGTGTTGG, 149 bp downstream from the stop codon, which fits perfectly with the consensus sequence YGTGTTYY identified by McLauchlan *et al.* (1985), for efficient and correct processing of the 3' end of the RNA. However, this GT rich region is normally found in about 30 bp downstream the AATAAA sequence.
In addition, two inverted repeats are found 18 and 154 bp downstream the

stop codon. These potential stem loop structures may function in efficient termination of transcription (Birnstiel *et al.*, 1985).

Intron-exon structure of the pyruvate kinase gene

In contrast to the *S.cerevisiae* pyruvate kinase gene the *A.nidulans* pyruvate kinase gene contains seven putative introns (A-G), which range in size from 50 to 72 bp. These introns were identified based of the fungal consensus sequence for splice junctions 5' GTRNGT--NRCTRAC--YAG 3' (Rambosek and Leach, 1987), and by comparison of the amino acid sequence with pyruvate kinase from *S.cerevisiae*, chicken M type and rat L type.

Table 1 *Intron sequences at the splice junctions in the Aspergillus nidulans pyruvate kinase gene.*

	5' sequence		lariat sequence		3'sequence	intron length
Consensus sequence[1]	GTRNGT		nrCTRAC		YAG	
Intron A	GTACGT	-45-	gaCTGACg	-10-	TAG	72 bp
Intron B	GTACGG	-27-	agCTGACa	- 7-	TAG	51 bp
Intron C	GTAATT	-36-	gtCTGACa	- 5-	TAG	58 bp
Intron D	GTGAGT	-29-	atCTGACt	- 6-	TAG	52 bp
Intron E	GTAGGT	-29-	gaCTAACt	- 9-	TAG	55 bp
Intron F	GTACGT	-27-	cgCTGACa	- 6-	TAG	50 bp
Intron G	GTAAGT	-30-	ggCTGACt	- 4-	TAG	51 bp

[1] Rambosek and Leach, 1987

Two of the seven introns have 5'splice junctions that lack the second GT (Table 1). Deviations from the 5'splice junction consensus sequence are found in other fungal genes too, *e.g.* in *A.nidulans adh*III (GTATTT) (McKnight *et al.*, 1985) and in the *A.nidulans amd*S gene (GTACAC) (Corrick *et al.*, 1987). In general, the seven introns interrupting the *pki* coding sequence resemble other fungal introns both in size and in their lariat

sequence.

In the three eukaryotic pyruvate kinase genes which have been completely sequenced thus far, *viz.* the *S.cerevisiae* pyruvate kinase gene (Burke *et al.*, 1983), the chicken M gene (Lonberg and Gilbert, 1985) and the rat L gene (Cognet *et al.*, 1987), the coding region contains respectively no introns, nine introns and ten introns. Alignment of these amino acid sequences and the intron positions of these pyruvate kinases genes, with the *A.nidulans* amino acid sequence and intron positions, reveals a conservation in the position of the introns A and C of the *A.nidulans pki* gene in all three intron containing genes, while the position of a third intron (F) has been shifted two codons.

As has been discussed by Lonberg and Gilbert (1985), the mononucleotide binding fold of pyruvate kinase can be aligned with the first mononucleotide binding fold of the alcohol dehydrogenase NAD^+ binding domain. The amino acid alignment in the mononucleotide binding fold of the pyruvate kinases of *A.nidulans*, *S.cerevisiae*, cat, chicken and rat with the mononucleotide binding fold of ADH I of horse and maize and ADH I (Gwynne *et al.* 1987) and III (McKnight *et al.1985)* of *A.nidulans* (Fig. 3) reveals only a weak sequence homology. However, in contrast to the chicken and rat *pki* gene, the second intron in the region coding the mononucleotide binding fold domain of *adh*I gene of maize (Dennis *et al.* 1984), is present in the *pki* gene of *A.nidulans* (intron G). This suggests that the mononucleotide binding fold in alcohol dehydrogenase and pyruvate kinase genes is the result of the evolution from a common ancestor rather than that of convergent evolution. The pyruvate kinase genes of rat and chicken possibly lack the second intron in the mononucleotide binding fold domain as the result of differential intron loss.

```
Cat              CLAAALIVLTESG RAHQVARYRPRAPIIAVTRNHQTARQAHLYRGIFPV V CKD
                             *
Chicken          CLAAALIVMTESG RAHLVSRYRPRAPIIAVTRNDQTARQAHLYRGVFPV L CKQ
                             *
Rat              CCAAAIIVLTKTG RAQILSQYRPRAAVIAVTRSAQAARQVHLSRGVFPL L YRE

S.cerevisiae     QKAKAIIVLSTSG TPRLVSKTRPNCPIILVTRCPRAARFSHLYRGVFPF F EKE
                           *                          *
A.nidulans       LNAGAIVVLTTSGNTARMISKYRPVCPIIMVSRNPAATRYSHLYRGVWPFYFPEKK
                             *                          *
Maize(ADH)       GSTVAVFGLGAVG AAAGGARIAGASRIIGVDLNPSRFEEARKFGCTEFVNPKDHN
                             *                          *
Horse(ADH)       GSTCAVFGLGGVGKSVIMGCKAAGAARIIGVDINKDKFAKAKEVGATECVNPQDYK
                                                        *
A.nid.(ADHI)     GQTVAIVGAGGGLGSLAQQYAKAMGIRVVAVDGGDGKRAMCESLGTGTYVDFTKSK
                                                        *
A.nid.(ADHIII)   GQTVAIVGAGGGLGSLAQQYAKAMGLRTIAIDSGDEKKAMCEQLGAEVFIDFSKSA
```

Fig. 3 Alignment of amino acid sequences in the mononucleotide binding fold of pyruvate kinase and alcohol dehydrogenase from different organisms. The position of an intron in the corresponding gene is indicated by an asterix. The introns marked are; chicken M type pki gene intron 8; rat L type pki gene intron I; A.nidulans pki gene introns F and G; maize adhI introns 5 and 6; A.nidulans adhI intron 1 and A.nidulans adhIII intron A.

Deduced protein structure

Removal of the putative intron sequences from the primary transcript results in a mRNA with an open reading frame of 1578 bp, corresponding to a protein of 526 amino acids with a molecular weight of 58.075 kDa. This is 2 kDa smaller than the molecular weight determined by SDS polyacrylamide gel electrophoresis (Uitzetter, 1982) and 7 kDa smaller than was reported in Chapter 3 of this thesis. The difference found between the molecular weight of the deduced protein, and the molecular weight, as it was determined experimentally, may be the result of secondary structure in the protein present even under denaturing conditions.

Table 2 Comparison of amino acid composition of A.nidulans pyruvate kinase.

Amino acid:	Number of residues:		Percent of total:	
	found	reported[1]	found	reported[1]
Ala	42	46	7.9	7.7
Arg	31	39	5.8	6.6
Asp/Asn	31/34	68[a]	12.6	11.4
Cys	9	10	1.7	1.7
Glu/Gln	35/11	59[a]	6.6	9.9
Gly	37	46	7.0	7.7
His	11	11	2.0	1.9
Ile	41	49	7.7	8.2
Leu	35	43	6.6	7.2
Lys	32	33	6.0	5.6
Met	19	16	3.6	2.7
Phe	13	15	2.4	2.5
Pro	23	27	4.3	4.5
Ser	32	36	6.0	6.1
Thr	27	31	5.1	5.2
Trp	5	6	.9	1.0
Tyr	12	9	2.2	1.5
Val	46	51	8.7	8.6

[1] Chapter 3.
[a] Sum of acid and amide forms

A very high correlation is found between the amino acid composition for the pyruvate kinase protein deduced from the nucleotide sequence, and the amino acid composition as determined (Chapter 3).The results are compared in Table 1.

The codon usage of the *A.nidulans pki* gene reveals a preference for certain codon usage and a bias in the third position of the codon. In only 7% of the codons used, is an A found in the third position, while 68% of the codons used have a C or G (46% C and 22% G) in the third position. This is a common feature of the fungal genes studied thus far (Rambosek and Leach, 1987).

The overall homology between *A.nidulans* pyruvate kinase and pyruvate kinases from other organisms is high, ranging between 53% homology with the rat L type to 66% with the *S.cerevisiae* pyruvate kinase. This high

degree of homology is one of the striking features of glycolytic enzymes, and a reflection of their central role in cellular metabolism (Fothergill-Gillmore, 1986). The amino acid residues of the protein, involved in the catalytic function (Johnson *et al.*, 1979; Muirhead *et al.*, 1986) are almost completely conserved (Fig. 4).

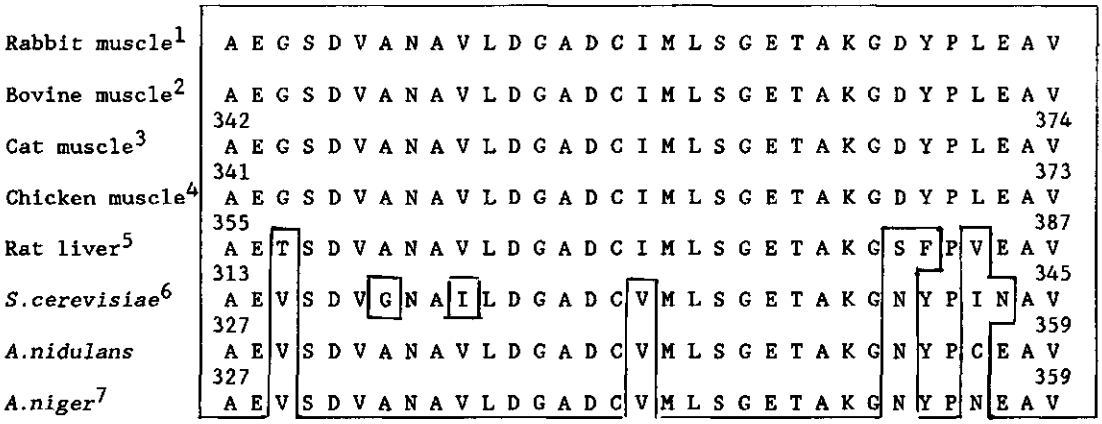


Fig. 4 Homology in pyruvate kinases from different sources in the peptide sequence related to the active site. The homologous residues are boxed. The numbers (above the sequences) indicate the residue number. The sequence of bovine and rabbit muscle was determined from a chemical modified tryptic peptide, their residue numbers are not known. Data from 1) Bezares et al.(1987), 2) Johnson et al. (1979), 3) Muirhead et al. (1986), 4) Lonberg and Gilbert (1983), 5) Inoue et al. (1986), Lone et al. (1986), 6) Burke et al. 1983, 7) Chapter 5.

Comparing the various pyruvate kinases, striking differences at the the N- and C-terminal end of *A.nidulans* the protein are found. Similar to the pyruvate kinases of higher eukaryotes, the *A.nidulans* pyruvate kinase contains additional amino acid residues at its N-terminus end in comparison to the *S.cerevisiae* pyruvate kinase. However, no sequence homology is found with either of the pyruvate kinase L and M types as shown in Fig. 5.

```
Rat L      MEGPAGYLRRASVAQLTQELGTAFFQQQQLPAAMADTFLEHLCLLD-IDSQPVAARSTSIIATIGP
Rat M                    MPLPDSEAGTAPIQTQQLH-AMADTFLEHMCRLD-IDSAPITARNTGIICTIGP
Chicken M                MSK-HHDAGTAFIQTQQLHAAMADTFLEHMCRLD-IDSEPTIARNTGIICTIGP
Cat M                    MSKPHSDVGTAFIQTQQLHAAMADTFLEHMCRLD-IDSPPITARNTGIICTIGP
Human L    MEGPAGYLRRASVAQLTQELGTAFFQQQQLPAAMADTFLEHLCLLD-IDSQPVAARSTSIIATIGP
S.cerevisiae                                MSRLERLTSLN-VVAGSDL-RRTSIIGTIGP
A.nidulans                        MAASSSLDHLSNRMKLEWHSKLNTEMVPAKNFRRTSIICTIGP
```

Fig. 5 Alignment of the amino acid sequences at the N-terminus of pyruvate kinases from rat liver, human liver, rat muscle, chicken muscle, cat muscle and yeast pyruvate kinase.

Presently, no experimental data exist on whether pyruvate kinase from yeast and/or *A.nidulans* is phosphorylated as a regulation mechanism of the enzyme activity. A phosphorylation site, ARG-ARG-ALA-SER (residues 9-12), found in porcine and rat L type pyruvate kinase, is lacking in the N-terminal region of *A.nidulans* pyruvate kinase. In *S.cerevisiae* pyruvate kinase the amino acid sequence ARG-ARG-THR-SER (residues 19-22) is found, which might be a potential site of phosphorylation, since this sequence is similar to the site of phosphorylation in the porcine and rat L type enzyme (Burke *et al.*, 1983). This sequence, ARG-ARG-THR-SER, found in the

yeast enzyme, is also found in the *A.nidulans* pyruvate kinase amino acid sequence (residue 33-36).

Determination of the N-terminal amino acid residue by Edmann degradation failed, indicating that the N-terminal amino acid is blocked. With the rabbit muscle enzyme (Cottam *et al.*, 1969) and the *S.cerevisiae* enzyme (Bornmann *et al.*, 1972) it was shown that the N-terminal amino acid is acetylated. No further attempts were been made to determine the modified N-terminal amino acid.

The C-terminal part of the *A.nidulans* pyruvate kinase shows amino acid homology to pyruvate kinases from other organisms, but contains seven additional residues. These additional amino acid residues are also found at the C-terminus of the *A.niger* pyruvate kinase, with six of these seven amino acids being conserved. Whether the additional amino acids at the N- and C-terminal end of the protein have a specific function remains to be investigated.

Conclusions

The promoter region of the *A.nidulans* pyruvate kinase gene does not contain the pattern of organization usually found in eukaryotic genes, a TATA sequence and a CCAAT sequence at a distinct distance from the transcription initiation site. The site where transcription starts is found 80 bp before the translation initiation codon. The sequence ccaAc/g is a motif found at several transcription initiation sites in genes of filamentous fungi. The promoter region, furthermore, is characterized by a series of CT blocks and repetitive sequences. CT blocks are found in a number of strongly expressed genes in yeast and *Aspergilli*, and are believed to facilitate a high level of transcription (Dobson *et al.*, 1982).

The deduced amino acid sequence is strongly homologous to that of other pyruvate kinase proteins already known. Striking differences however, are found at the N-and C-termini end of the protein. At the N-terminus, the protein is longer than the yeast enzyme but not as long as the mammalian enzymes. At the C-terminus the *Aspergillus* pyruvate kinase is longer than known pyruvate kinase. The additional amino acid sequence at the N-terminus show no homology with pyruvate kinases other than the *A.niger* enzyme (Chapter 5).

With respect to intron-exon structure, the *A.nidulans* pyruvate kinase represents a state between lower eukaryotes, such as *S.cerevisiae*, and the higher eukaryotes, like chicken and rat. This intermediate evolutionary position is also found for the *A.nidulans* genes encoding *pgk* and *tpi* and support the hypothesis presented by Gilbert (1986) that prokaryotes and lower eukaryotes, like *S.cerevisiae* and to a lesser extent *Aspergilli*, lost their introns during evolution. In the *A.nidulans pki* three out of seven introns (A, C and G) are conserved with respect to the intron positions in the genes of higher eukaryotes. In case of a fourth intron (F), situated within the mononucleotide binding fold domain, its position is conserved with respect to the NAD^+ binding domain of the maize *adh*I gene. The presence of this intron in the *A.nidulans pki*, which is lacking in corresponding genes of higher eukaryotes, illustrates the differential intron loss in the evolution of this gene.

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CHAPTER 5

Isolation and characterization of the *Aspergillus niger* pyruvate kinase gene

Part of this Chapter is submitted for publication;
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Summary

We have cloned the Aspergillus niger gene encoding pyruvate kinase, in order to study its structure and expression. The pyruvate kinase (pki) gene was cloned by heterologous hybridization using a fragment from the corresponding yeast gene as a probe.

Transformation experiments with the pyrA gene as a selection marker and the subcloned pki gene as the cotransforming marker led to increased levels of pyruvate kinase in eight out of eleven transformants analyzed. From these results, it was concluded that the subcloned fragment indeed encodes A.niger pyruvate kinase, and that it contains the whole functional gene.

Analysis of transformants with an elevated pyruvate kinase level, with respect to the type of integration, demonstrated that in none of these transformants integration at the pki locus had occurred. In the cases examined predominantly cointegration of the pyrA and the pki containing plasmids was found.

The structural part of the A.niger pki gene is 2054 bp long, and is interrupted by 7 putative introns. Removal of the intron sequences results in an open reading frame of 1578 bp, encoding a protein of 526 amino acid residues and a molecular weight of 58.130 kDa. Extensive homology is found with pyruvate kinase from A.nidulans, with differences occuring in the domains of the protein which are involved in subunit interaction.

Introduction

The rapid development of recombinant DNA methodology for filamentous fungi (for reviewe see Rambosek and Leach, 1987) has led to an increased interest in the possibilities of strain improvement of *Aspergillus niger* by genetic manipulation. Recently, transformation systems have been developed based on the *Aspergillus nidulans amdS* gene (Kelly and Hynes, 1985) and on the homologous *pyrA* gene (van Hartingsveldt *et al.*, 1987; Goosen *et al.*, 1987), which allows the introduction of homologous, as well as heterologous, genes in this economically important fungus. The implications of these developments are not only important for the industrial applications of *A.niger*, but also for fundamental studies on gene structure and gene regulation.

We have analyzed a strongly expressed glycolytic gene *viz.* the *A.niger* pyruvate kinase gene *(pki)*. Corresponding gene structures of the rat pyruvate kinase L type gene (Cognet *et al.*, 1987), the rat M type gene (Noguchi *et al.*, 1986), the chicken M type gene (Lonberg and Gilbert, 1985), the *Saccharomyces cerevisiae* gene (Burke *et al.*, 1983) and the *A.nidulans* gene (Chapter 4; de Graaff and Visser, 1989) are all known. These studies reveal that, like other glycolytic functions, the primary structure of the various pyruvate kinases is well conserved (Muirhead *et al.*, 1986). All of the pyruvate kinase genes mentioned, with the exception of the yeast gene, contain introns. Ten are found in the rat L type gene, 9 in the chicken M type and 7 in the *A.nidulans* gene. Whereas all nine introns of the coding region of the chicken M type gene are in a conserved position when compared to the rat L type gene (Cognet *et al.*, 1987), in the *A.nidulans* gene three of the seven introns are in a conserved position with respect to the L and M type genes (Chapter 4; de Graaff and Visser, 1989).

Pyruvate kinase is considered to be an important control enzyme in glycolysis and its properties have been subject of several review articles

(Kayne, 1973; Engström *et al.*, 1987; Muirhead, 1987). The enzyme (ATP: pyruvate phosphotransferase, EC 2.7.1.40) has been extensively studied in a broad range of organisms. In the case of the cat muscle enzyme the primary as well as the 3-D structure is known (Stuart *et al.*, 1979; Muirhead *et al.*, 1986). Of the pyruvate kinases with fungal origin, the *Neurospora crassa* enzyme has been most extensively studied (Tsao and Madley, 1975; Kapoor, 1976). The enzyme is activated by fructose 1,6-biphosphate (FBP) at micromolar concentrations, whereas it is inhibited by citrate, oxaloacetate and ATP in millimolar concentrations (Tsao and Madley, 1975). The enzyme of *A.niger* however, is insensitive to citrate and oxaloacetate (Meixner-Monori *et al.*, 1984). Similar results were found for the purified *A.nidulans* enzyme (H.C.M. Kester, unpublished results). The activation of the enzyme by FBP is due to the binding of this ligand to the enzyme, thereby converting the enzyme to the high affinity state for its substrate, phosphoenolpyruvate (Kapoor, 1976). Besides being an allosteric effector, FBP has also effects the stability of the enzyme, as noticed for the three fungal enzymes mentioned (O'Brien and Kapoor, 1980; Meixner-Monori *et al.*, 1984; H.C.M. Kester, unpublished results). Although regulation of the glycolytic flux at the pyruvate kinase level has been considered, Kubicek (1987) postulated, on the basis of experimental kinetic data and metabolite levels, that this is very unlikely.

Apart from our interest in the pyruvate kinase gene itself, we considered the isolation of this gene also to be of importance for physiological studies. By transformation one can easily obtain strains with various levels of pyruvate kinase gene doses, which can be useful evaluating the level at which physiological control mechanisms operate. In this paper we describe the isolation, sequence and expression of the *A.niger* pyruvate kinase gene.

Materials and methods

Strains

A.niger N400 (wild type) was used for nucleic acid and protein extractions and A.niger N593 (cspA1, pyrA) was used as the recipient strain in transformation experiments. E.coli DH5α (endA1, hsdR17, [rk⁻, mk⁺], supE44, thi-1, λ⁻, recA1, gyrA, relAL, 80dlacZ M15, (lacZYA-argF), U169) and E.coli DH5αF' were used to propagate plasmids and M13 phages.

Chemicals and enzymes

Restriction enzymes, DNA polymerase I Klenow fragment and T_4 DNA ligase were obtained from BRL. Novozyme 234 was obtained from Novo Industries. Deoxynucleotidetriphosphates and dideoxynucleotidetriphosphates were from Boehringer. Schleicher and Schüll BA85 nitrocellulose was used in Southern blotting experiments. α-^{32}-P-dATP was from New England Nuclear. Urea, acrylamide and bisacrylamide were Ultra Pure grade (BRL), all other chemicals used were analytical grade.

Media and growth conditions

For the preparation of conidiospores A.niger was grown at 30°C for three days on complete medium, which was originally designed for A.nidulans (Pontecorvo et al., 1953), using 25 mM sucrose as a carbon source and solidified with 1.2% agar.

Transformation of A.niger N593

Protoplasts were prepared by growing A.niger N593 on minimal medium supplemented with 0.5% yeast extract, 0.2% casamino acids, 50 mM glucose and 10 mM uridine for 20 hrs at 30°C. The preparation of protoplasts of A.niger N593 and the transformation was performed as described before (Goosen et al., 1987).

Heterologous hybridization conditions

Heterologous hybridization conditions were determined and hybridization experiments were performed as previously described (Chapter 2; de Graaff et al., 1988).

Manipulation of nucleic acids.

A.niger DNA was extracted as published before (Chapter 2; de Graaff et al., 1988). Propagation of plasmid DNA and manipulation of DNA was by standard methods (Maniatis et al., 1982).

Results and discussion

Isolation and physical map of the gene

Screening of an ordered genomic library of *A.niger* N400 with a 1.8 kb *Eco*RI fragment of the *pki* gene of *S.cerevisiae* (Burke *et al.*, 1983), resulted in the isolation of 18 positive clones. From these, the 8 clones which gave the strongest hybridization signal were selected for further analysis. Southern analysis of clone 15B6, which had the strongest hybridization signal, was compared with a Southern analysis of *A.niger* genomic DNA, and the results suggested that the clone contained the complete *A.niger pki* gene (results not shown). From 15B6 the 5 kb *Bgl*II/*Hind*III fragment was subcloned in pBR322 producing pGW1100. The latter clone was further analyzed by restriction enzymes resulting in the restriction map shown in Fig. 1. The orientation of the gene was determined by hybridization using fragments containing the 5'- and 3'- end of the yeast gene, a 1.0 kb *Eco*RI/*Bgl*II and a 0.88 kb *Eco*RI/*Bgl*II fragment respectively, as probes.

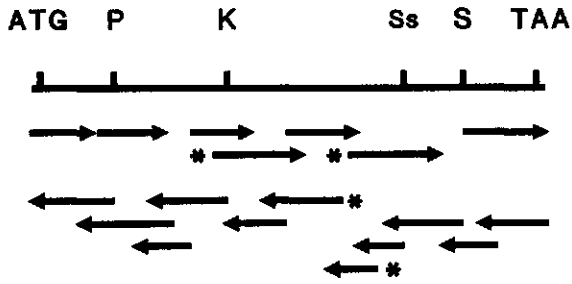


Fig. 1 Restriction map of the structural region of the pki gene and the sequencing strategy employed. Only major restriction sites are indicated: K: KpnI, P: PstI, S: SalI and Ss: SstI. Restriction fragments were subcloned in bacteriophage M13 mp18/19 vectors (Messing, 1983; Norrander et al., 1983), and nucleotide sequences were determined by using the dideoxynucleotide chain-termination procedure (Sanger et al., 1977). Part of the sequence was determined by using specific oligonucleotide primers as indicated (*). Computer analysis was done using the PC/GENE program.

Functional analysis by cotransformation

Since we have not been able to find any *pki* mutants in *A.niger*, unlike the case in *A.nidulans* (Uitzetter, 1982), transformation of *A.niger* by direct selection for a functional pyruvate kinase gene, as described for *A.nidulans* (Chapter 2; de Graaff *et al.*, 1988), was not possible. However, it is now well documented that genes which cannot be selected for, can be successfully introduced into the fungal genome by cotransformation. In such experiments *e.g.* *Amd*S (Kelly and Hynes, 1985; O'Connel and Kelly, 1988) or *pyr*A (Goosen *et al.*, 1987) is used as a selection marker, together with a second plasmid carrying the non-selectable marker. In some cases, up to 95% cotransformation has been achieved (Wernars *et al.*, 1987).

The identity and functionality of the *pki* gene isolated was confirmed in our case by transforming *A.niger* N593. In this experiment pGW613 containing the *pyr*A gene (Goosen *et al.*, 1987) was used as a selection marker and pGW1100 as the cotransforming plasmid. The molar ratio chosen between the two plasmids was 1:50, in order to favor the frequency of cotransformation. Transformants were obtained with a low frequency, about 15 transformants per μg pGW613 DNA. They were analyzed at the protein level and at the DNA level.

Pyruvate kinase levels

The transformants were analyzed by measuring the pyruvate kinase activity after growth on minimal medium (MM)(Pontecorvo *et al.*, 1953) containing sucrose as a carbon source. The results are presented in Table 1. As can be concluded from this Table, in 8 out of 11 transformants elevated levels of pyruvate kinase activity was found, indicating that about 73% of the *pyr*A transformants contain one or more copies of the cotransforming DNA, pGW1100. Based on the activity levels found, the number of functional copies integrated into the genome can be estimated to vary between one additional copy to about thirty extra copies.

Table 1 Pyruvate kinase activities in A.niger wild type (N400) and in transformants of N593.

Strain	Specific activity (IU/mg protein)	Activity ratio transformant*
N400	0.8	
3/1100	1.0	1
4/1100	4.2	5
5/1100	0.7	1
6/1100	5.5	7
7/1100	0.6	1
8/1100	1.8	2
9/1100	10.7	13
10/1100	5.6	7
11/1100	13.8	17
12/1100	23.1	29
13/1100	10.2	13

* round the nearest integer

When the pyruvate kinase activities measured (Table 1) and the amount of pyruvate kinase protein found in crude extracts as analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2) are compared, a correlation between activity and the amount of protein synthesized is found. Extreme levels of pyruvate kinase protein are found in transformants 9/1100, 11/1100, 12/1100 and 13/1100 and a fair correlation is found between the activity and the amount of protein present in the cell-free extract. Transformant 4/1100, which has a pyruvate kinase activity about 5 times higher than the wild type is the exception, since the amount of pyruvate kinase is dramatically more increased (Fig. 2). Based on a laser densitometric scanning analysis of the SDS-polyacrylamide gel electrophoresis pattern, at least 30% of the total protein content consists of pyruvate kinase. A possible explanation for the discrepancy found between the activity measured and the amount of protein found, is that in this particular case the extreme amount of protein synthesized within the cell, prevents the correct formation of active tetrameric enzyme.

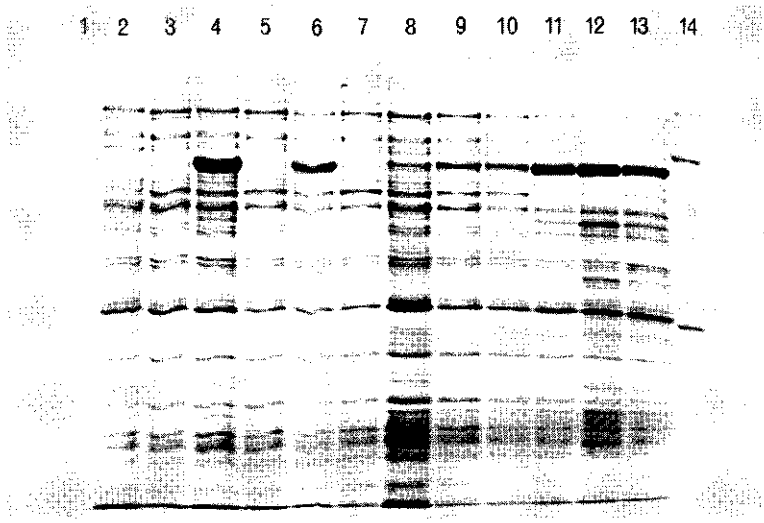


Fig. 2 Electrophoretic patterns of cell-free extracts of wild type and transformants after SDS-PAGE, and staining with Coomassie blue. The mycelium was grown, and the preparation of cell-free extract and SDS-PAGE was performed as described (Chapter 3; Kester et al. 1988).

Lane 1; purified pyruvate kinase, lane 2; N400, lane 3; 3/1100, lane 4; 4/1100, lane 5; 5/1100, lane 6; 9/1100, lane 7; 7/1100, lane 8; 8/1100, lane 9; 6/1100, lane 10; 10/1100, lane 11; 11/1100, lane 12; 12/1100, lane 13; 13/1100, lane 14; molecular weight markers; respectively 30 kDa, 46 kDa, 68 kDa and 92.5 kDa.

Genomic analysis

Transformants were analyzed by Southern analysis after digestion of the chromosomal DNA with the appropriate restriction enzymes. After digestion with *Bgl*II, which does not cut within the *pki* gene nor in the plasmid pGW1100, and subsequent hybridization with a fragment of the *pki* gene as a probe, the number of copies integrated at the *pki* locus can be determined. For every copy of plasmid integrated at this site, the length of the wild type hybridizing fragment is enlarged by the length of the

plasmid. Our results indicate, that in none of the transformants had integration occurred at the *pki* locus (Fig. 3A). This was a remarkable finding because with *A.nidulans* transformation experiments, with direct selection for the *pki* marker, the outcome of a similar analysis of 33 transformants is that homologous integration had occurred in 90% of the cases (Chapter 2, and unpublished results; de Graaff *et al.*, 1988).
Besides the wild type fragment, a 10 kb *Bgl*II fragment, an intense hybridizing fragment is found in all the transformants analyzed. This fragment is at least 20 kb in length.

Digestion with *Bam*HI (not shown), which cuts only once in pGW1100, and hybridization with a fragment of the *pki* gene, results in digestion patterns with a strong hybridizing fragment of 9.0 kb in the transformants, which is not present in the wild-type pattern. The length of this fragment corresponds to the length of the transforming plasmid, pGW1100. Differences in intensity of the signal from the fragment reflects differences in the number of copies integrated. Also, less pronounced bands of varying sizes are found. Transformants were also analyzed by digestion with *Xba*I, which does not cut in the plasmid pGW1100, and subsequent blotting and hybridization with a 3.8 kb *Xba*I fragment, containing the *pyr*A gene. In Fig. 3B, all the transformants showed the 3.8 kb *Xba*I fragment together with a high molecular weight fragment. Hybridization of a second blot, produced under the same conditions, and hybridized with a fragment of the *pki* gene, revealed a high molecular weight hybridizing band, found in an identical position as the one found after hybridization with the pyrA (Fig. 3C) fragment.

These results suggest the formation of a large concatamer containing the plasmid pGW1100 as well as the *pyr*A containing plasmid pGW613, before integration occurs or during the integration process. As a result of the concatamer formation, a tandem cointegrate of the plasmids pGW1100 and pGW613 is found. The much stronger hybridization signal with the *pki*

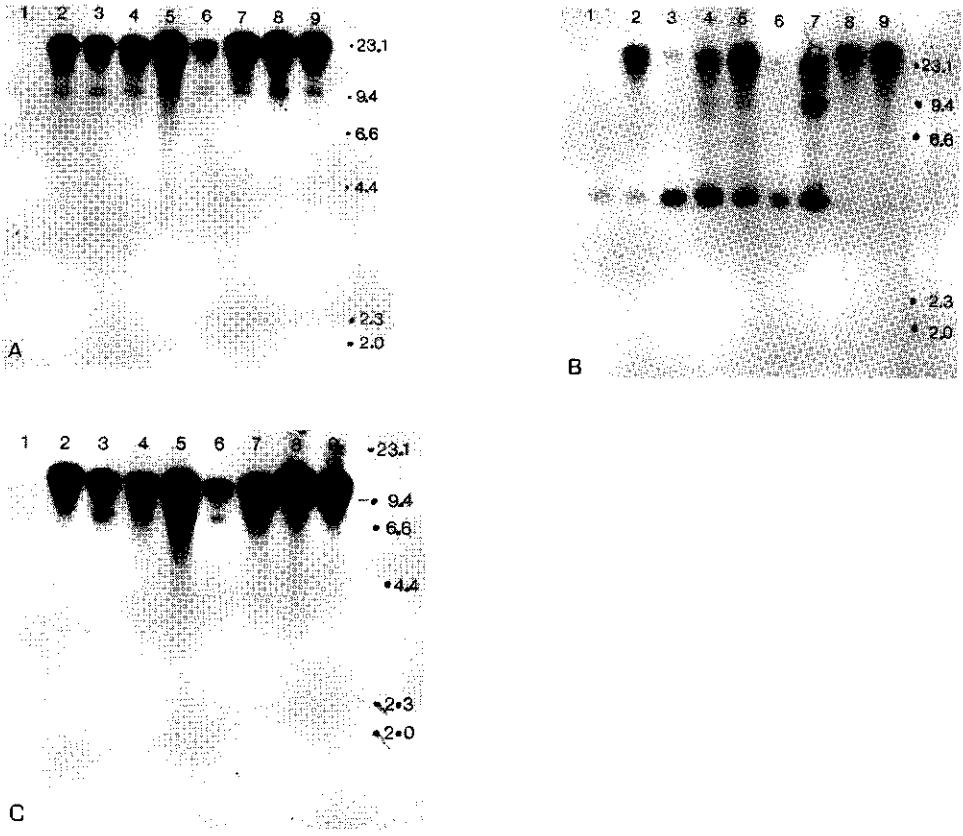


Fig. 3 Southern analysis of genomic DNA of transformants with an elevated level of pyruvate kinase activity.

DNA of independent transformants was digested with BglII and XbaI. After separation on a 0.6% agarose gel in TBE buffer the DNA was transferred to nitrocellulose and hybridized with a 1.8 kb SmaI fragment containing part of the pki gene (A and C) and with a 3.8 kb XbaI fragment containing the pyrA gene (B). The filters were washed in 0.2 x SSC for two hrs and exposed overnight to Konica X-ray film.

Transformant DNA was also analyzed by digestion with XbaI, and subsequent hybridization with a 3.5 kb XbaI fragment from the pyrA gene (Goosen et al., 1987).

Lane 1; N400, lane 2; 4/1100, lane 3; 6/1100; lane 4; 8/1100, lane 5; 9/1100, lane 6; 10/1100, lane 7; 11/1100, lane 8; 12/1100, lane 9; 13/1100.

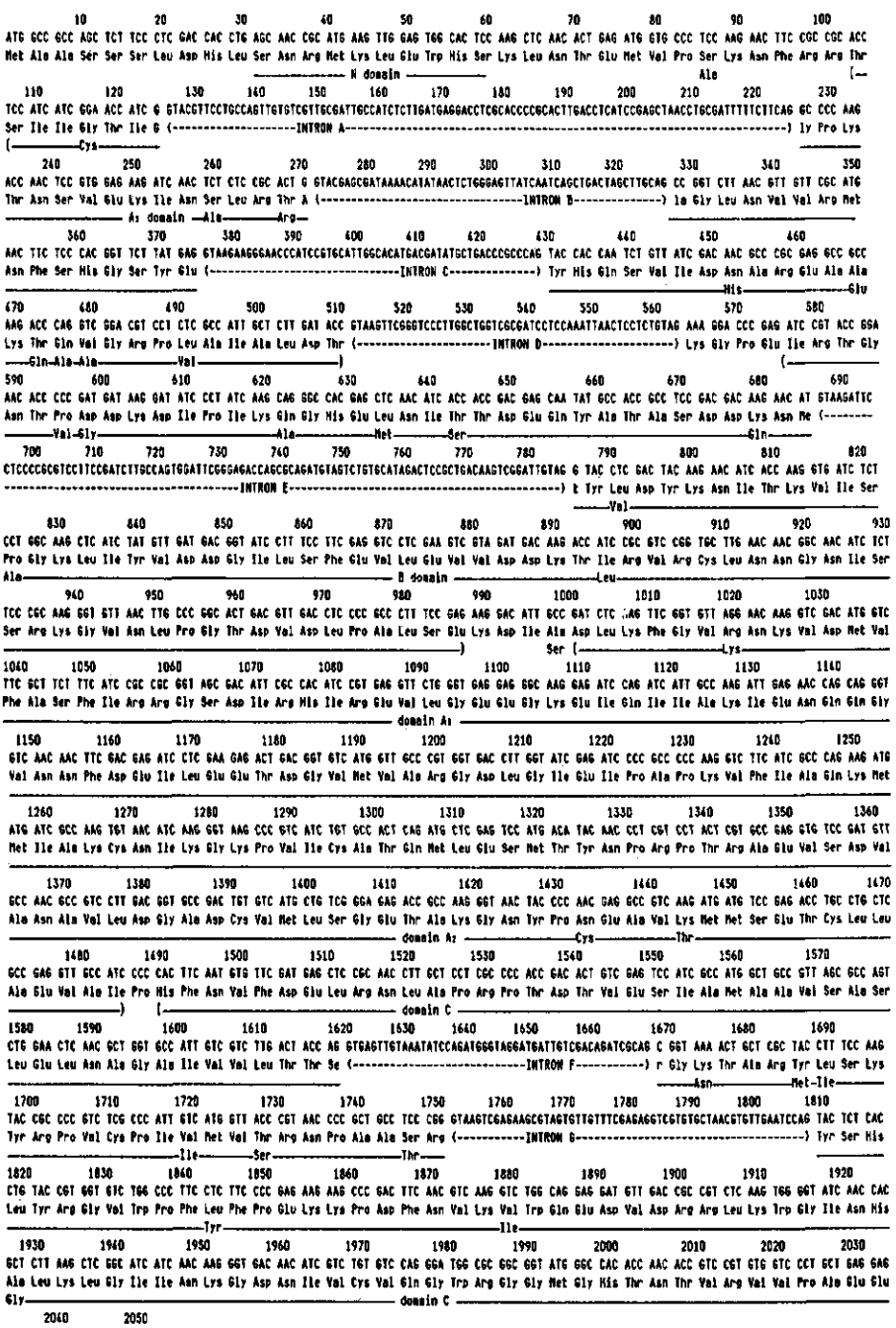
fragment as compared to the weaker signal seen with the *pyrA* fragment, is a reflection of the 50 fold excess of pGW1100 (*pki*). The formation of this concatamer would interfere with a recombination process, thus leading to a low frequency of integration at the *pki* locus. The high percentage of cotransformation can then be explained by assuming the concatamer integrates as a whole, with only minor internal recombination.

It is not clear which factors determine the recombination process which leads to the integration of homologous DNA into the genome. The large amount of plasmid DNA and the extreme ratio between the two plasmids in the transformation experiment, may affect the character of the recombination process. The high concentration of plasmid DNA used perhaps stimulates the formation of the concatamers, while if used in a one to one ratio at a low concentration, this concatamer formation could be less pronounced.

Pyruvate kinase gene structure

Fragments containing the structural part of the *pki* gene, originating from the subclone pGW1100, were selected by hybridization with fragments from the yeast gene. These fragments were subcloned in M13mp18/19 vectors for sequencing of the gene. The strategy used to establish the nucleotide sequence of the *A.niger* pyruvate kinase gene was previously shown in Fig. 1. The resulting sequence of the pyruvate kinase structural gene can be found in Fig. 4.

***Fig. 4** Nucleotide sequence of the A.niger pyruvate kinase gene, and amino acid translation of the open reading frame.*
Those amino acids which are different in A.nidulans pyruvate kinase are given beneath the A.niger amino acid sequence. The position of the introns is as indicated. The stop codon is indicated by END. Sequencing was performed as described in Fig. 1.


N domain
INTRON A
INTRON B
A2 domain
INTRON C
INTRON D
INTRON E
domain A1
domain A2
domain C
INTRON F
INTRON G
EHD

The *A.niger pki* gene contains like the *A.nidulans pki* gene 7 introns. Although the lengths of the introns are different from those found in the *A.nidulans* pyruvate kinase gene (Chapter 4; de Graaff and Visser, 1989), all seven introns appear exactly at the same position in the nucleotide sequence, as those in the *A.nidulans pki* gene. The conserved intron sequences and their lengths are summarized in Table 2. With the exception of intron D, the introns contain the conserved sequences normally found in fungi (Rambosek and Leach, 1987). In intron D, the only intron not containg a conserved lariat sequence, a possible lariat sequence ATTAAC is found instead of the more general RCTRAC sequence, thus a C being replaced by a T.

Table 2 Intron sequences at the splice junctions in the Aspergillus niger pyruvate kinase gene.

	5'sequence		lariat sequence		3' sequence	intron length
Consensus sequence[1]	GTRNGT		RCTRAC		YAG	
Intron A	GTACGT	-72-	GCTAAC	-14-	CAG	101 bp
Intron B	GTACGA	-35-	GCTGAC	- 7-	CAG	57 bp
Intron C	GTAAGA	-35-	GCTGAC	- 5-	CAG	55 bp
Intron D	GTAAGT	-31-	ATTAAC(?)	- 7-	TAG	53 bp
Intron E	GTAAGA	-74-	GCTGAC	-11-	TAG	100 bp
Intron F	GTGAGT	-28-	GCTGAC	- 6-	CAG	49 bp
Intron G	GTAAGT	-33-	GCTAAC	-10-	CAG	58 bp

[1] Rambosek and Leach, 1987

The codon usage in the *A.niger* pki shows a preference for cytosine (53%) and a bias against adenosine (3%) in the third position, indicating that the codon bias of this gene is similar to that of the *A.nidulans* pki gene (46% and 7% respectively), and to fungal genes in general (Chapter 4; de Graaff and Visser, 1989 Rambosek and Leach, 1987).

The deduced pyruvate kinase proteins of *A.nidulans* and *A.niger* are strongly homologous, only 33 of the 526 amino acids are different between the enzymes of both species. These differences in residues are not randomly distributed over the protein, but are mainly found in the A_1 domain, in the B domain and in the C domain (Fig. 4). Those amino acid residues which are believed to be involved in the catalytic function of the protein and the binding of Mg^{2+} and K^+, are conserved (Bezares *et al.*, 1987; Muirhead *et al.*, 1986, 1987) with respect to the *A.nidulans* pyruvate kinase.

Differences in amino acid residues in both A domain and C domain may result in slight differences in stability and kinetic behaviour of both enzymes because both domains are involved in the subunit interaction of the tetrameric enzyme (Stuart *et al*, 1979). This subunit interaction plays an important role in the potentially allosteric modulation of the enzyme. Noguchi and coworkers (1986) have shown that the only difference between the M_1 and M_2 type isozymes from rat, which is the result of differential splicing, is found in the C domain. Both enzymes differ only 22 amino acids and as a result the M_2 type isozyme is allosterically regulated, whereas the M_1 type is not.

Conclusions

1. The fragment in pGW1100 contains the complete functional pyruvate kinase gene of *A.niger*. Transformation of *A.niger* by pGW1100 leads to an increase in pyruvate kinase activity in the transformants studied, varying with the number of copies integrated.

2. Although transformation of *A.niger* and *A.nidulans* using single selection markers like *pyrA* or *pki* leads predominantly to homologous integration, cotransformation of these markers results mainly in heterologous integration with respect to the *pki* locus.

3. Sequence analysis of the structural part of the *pki* gene, reveals a strong homology in the deduced primary structure of the *A.niger* pyruvate kinase with pyruvate kinase structures already known, especially when compared to the pyruvate kinase structure of *A.nidulans*. The primary structures of the *A.niger* and *A.nidulans* pyruvate kinases show no change in the amino acids believed to be involved in the catalytic function of the enzyme.

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CHAPTER 6

The structure of *Aspergillus* pyruvate kinase

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Introduction

Pyruvate kinase (EC 2.7.1.40) catalyzes the final reaction in the glycolytic pathway in which phosphoenolpyruvate (PEP) and ADP are converted to pyruvate and ATP, in an essentially irreversible reaction. The activity of pyruvate kinase is regulated, as part of the regulation of the balance between the glycolytic and gluconeogenic pathway. Regulation of the reaction catalyzed by pyruvate kinase is achieved at different levels:

By the level of transcription;

By allosteric modulation of the enzyme;

By phosphorylation/dephosphorylation of certain tissue specific types of isozymes in mammals (for review see Engström *et al.*, 1987);

By proteolytic breakdown of the protein.

Regulation of the amount of pyruvate kinase by the level of transcription, depending on either stimulation of glycolysis or gluconeogenesis, has been shown for the mammalian liver L type pyruvate kinase (Cladaras and Cottam, 1980; Noguchi *et al.*, 1982; Simon *et al.*, 1983), as well as for the *Neurospora crassa* pyruvate kinase (Devchand and Kapoor, 1987).

The allosteric regulation of pyruvate kinase from different origins is well studied. Pyruvate kinase has been purified from a large range of organisms, e.g. *Saccharomyces cerevisiae* (Yun *et al.*, 1976), *Neurospora crassa* (Kapoor and Tronsgaard, 1972), *Aspergillus nidulans* (Chapter 3), *Aspergillus niger* (Meixner-Monori *et al.*, 1984) and from different mammalian sources (for review see Kayne, 1973).

The enzyme is allosterically regulated by its substrate PEP and the product ATP, but also by the allosteric activator fructose 1,6-bisphosphate (FBP). Whereas in the absence of FBP the enzyme exhibits positive cooperativity with respect to binding of its substrate PEP, in the presence of micromolar quantities of FBP, the enzyme displays Michaelis-Menten kinetics toward its substrate PEP. This allosteric regulation allows a quick response to changes in physiological conditions.

In mammals four tissue specific forms of pyruvate kinase are found. The enzyme kinetic behaviour of the pyruvate kinase isozymes is related to the type of tissue in which they are expressed. In glycolytic tissues such as muscle and brain, where there is no need for allosteric regulation of the pyruvate kinase reaction, the non-allosteric M_1 type isozyme is found. The M_1 isozyme is not allosterically regulated, whereas the other three isozymes are.

In gluconeogenic tissues the reaction catalyzed by pyruvate kinase needs strict regulation, and thus in hepatocytes the allosterically regulated L type isozyme is found. In prokaryotes and lower eukaryotes, such as *S.cerevisiae* (Haeckel *et al.*, 1968) *N.crassa* (Tsao and Madley, 1975), *A.nidulans* (Chapter 3) and *A.niger* (Meixner-Monori *et al.*, 1984), generally one form of pyruvate kinase is found, which is allosterically regulated by FBP.

The binding of FBP to those pyruvate kinases which are allosterically regulated by this effector, induces changes in the enzyme conformation, as has been shown for pyruvate kinase originating from *N.crassa* (O'Brien and Kapoor, 1975) and for the rat L type pyruvate kinase (El-Maghrabi *et al.*, 1982). This change in conformation leads to a shift from a low affinity state (T state) to a high affinity state (R state) of the enzyme for its substrate PEP.

The cat muscle pyruvate kinase crystal structure was determined at a resolution of 2.6 Å (Stuart *et al.*, 1979). Each subunit of the tetrameric enzyme consists of four domains, N-terminal domain, the A domain, the B domain and the C domain. The central A domain consists of an eight stranded α/β structure, as was first described for triosephosphate isomerase (Banner *et al.*, 1975), which later was found to be a structure common to several proteins (see e.g. Chothia, 1988). The active site lies in a pocket formed by domains A and B.

After the primary structure of the enzyme was determined, a correlation was made between the protein three dimensional structure and its primary structure. From this model, and by comparison of the primary structures of other pyruvate kinases, a mechanism for catalysis by pyruvate kinase was proposed (Muirhead *et al.*, 1986; Muirhead, 1987).

The M_1 type pyruvate kinase is different from the other types of pyruvate kinases, since it is not allosterically activated by FBP. Thus, a comparison of the cat M_1 pyruvate kinase structure with pyruvate kinases which are activated by FBP, like the pyruvate kinases from *Aspergillus*, may be useful in deducing residues or regions involved in the allosteric activation of the enzyme.

This Chapter describes the model building of *Aspergillus* pyruvate kinase, based on the crystallographic data of cat M_1 pyruvate kinase and homology in the primary structures of both proteins. The modeling was done on an Evans and Sutherland PS300 graphics terminal, running the program Frodo version 3.2. The model was refined by repeated cycles of Hendrikson-Konnert restrained least squares refinement, to optimize the geometry of the protein model.

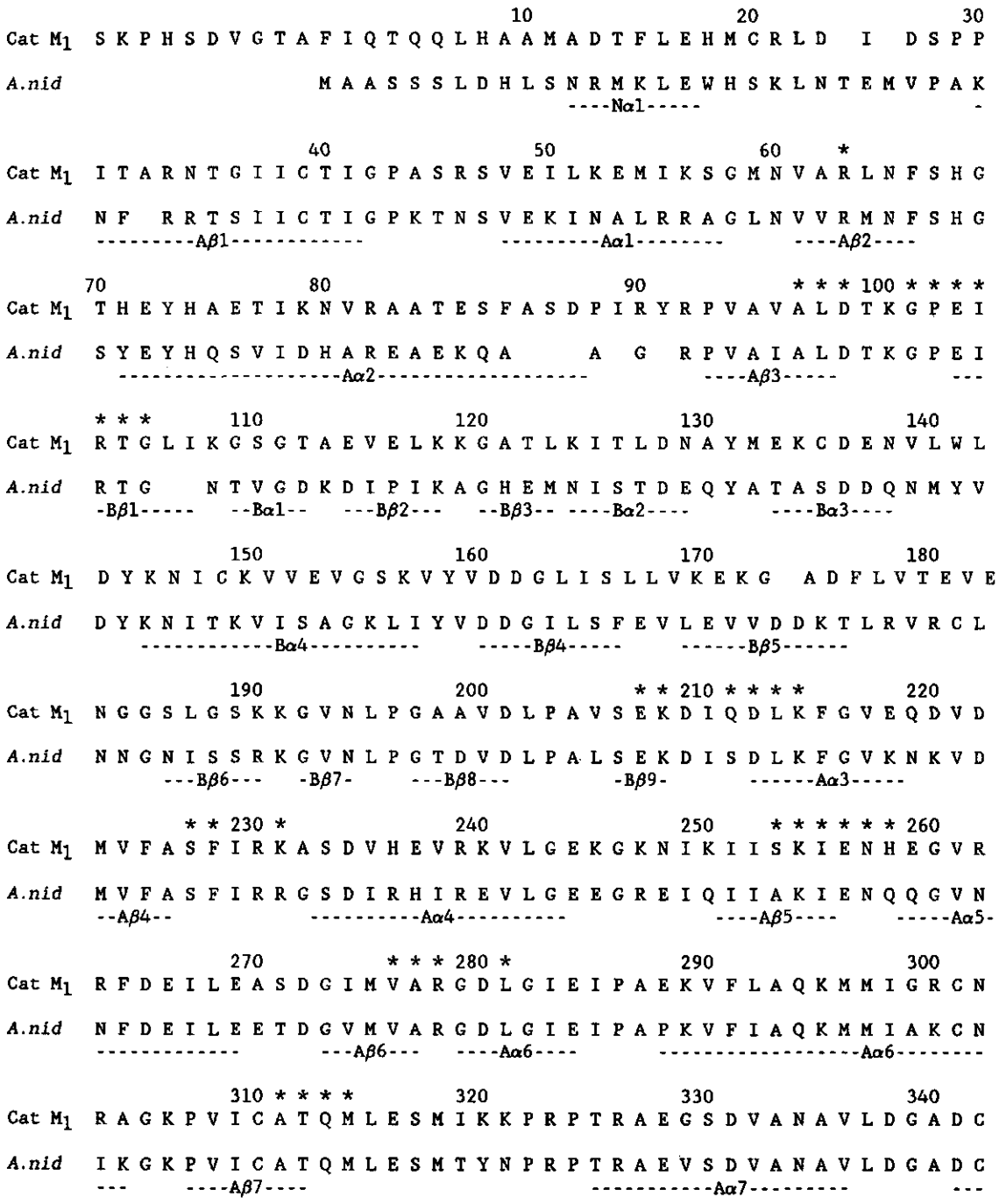
Results and Discussion

a. Aspergillus pyruvate kinase α-carbon backbone

Using the coordinates of the cat M_1 enzyme, and the alignment of the primary structures of both enzymes (Chapter 4 and 5) as shown in Fig. 1, the tertiary structure of *Aspergillus* pyruvate kinase was modeled. Using this alignment, and the known secondary and tertiary structure of the cat M_1 enzyme, the three-dimensional structure of the *A.nidulans* pyruvate kinase was modeled. In these calculations, the model was optimized for ideal bond lengths and geometry. As a result of this idealization, only major differences in both structures would be found. However the character and the orientation of amino acid residues at specific positions could be compared.

In Fig. 2 the predicted subunit tertiary structure of the *A.nidulans* pyruvate kinase is shown. The four domains (Stuart *et al.*, 1979) of the protein; N, A, B and C are indicated. The active site, located in the pocket between domains A and B (Muirhead *et al.*, 1986), is indicated by an arrow.

The alignment of the primary structures of both proteins shows significant homology between the fungal and the cat M_1 enzyme. According to this alignment 258 out of the 526 amino acids (49%) from the *A.nidulans* pyruvate kinase are identical to those in the cat M_1 enzyme. This homology is not randomly distributed over the protein, the most conserved region is located in the central A domain. Those residues involved in the catalytic function (Johnson *et al.*, 1979; Muirhead *et al.*, 1986; Bezares *et al.*, 1987) of the protein are almost completely identical (Fig. 4, Chapter 4).


Cat M1 S K P H S D V G T A F I Q T Q Q L H A A M A D T F L E H M C R L D I D S P P
A.nid M A A S S S L D H L S N R M K L E W H S K L N T E M V P A K
----Nα1-----
Cat M1 I T A R N T G I I C T I G P A S R S V E I L K E M I K S G M N V A R L N F S H G
A.nid N F R R T S I I C T I G P K T N S V E K I N A L R R A G L N V V R M N F S H G
---------Aβ1---------- ---------Aα1-------- ----Aβ2----
Cat M1 T H E Y H A E T I K N V R A A T E S F A S D P I R Y R P V A V A L D T K G P E I
A.nid S Y E Y H Q S V I D H A R E A E K Q A A G R P V A I A L D T K G P E I
----------Aα2---------- ----Aβ3----- ---
Cat M1 R T G L I K G S G T A E V E L K K G A T L K I T L D N A Y M E K C D E N V L W L
A.nid R T G N T V G D K D I P I K A G H E M N I S T D E Q Y A T A S D D Q N M Y V
-Bβ1----- --Bα1-- ---Bβ2--- --Bβ3-- ----Bα2---- ----Bα3----
Cat M1 D Y K N I C K V V E V G S K V Y V D D G L I S L L V K E K G A D F L V T E V E
A.nid D Y K N I T K V I S A G K L I Y V D D G I L S F E V L E V V D D K T L R V R C L
----------Bα4---------- -----Bβ4----- ------Bβ5------
Cat M1 N G G S L G S K K G V N L P G A A V D L P A V S E K D I Q D L K F G V E Q D V D
A.nid N N G N I S S R K G V N L P G T D V D L P A L S E K D I S D L K F G V K N K V D
---Bβ6--- -Bβ7- ---Bβ8--- -Bβ9- ------Aα3-----
Cat M1 M V F A S F I R K A S D V H E V R K V L G E K G K N I K I I S K I E N H E G V R
A.nid M V F A S F I R R G S D I R H I R E V L G E E G R E I Q I I A K I E N Q Q G V N
--Aβ4-- ----------Aα4---------- ----Aβ5---- -----Aα5-
Cat M1 R F D E I L E A S D G I M V A R G D L G I E I P A E K V F L A Q K M M I G R C N
A.nid N F D E I L E E T D G V M V A R G D L G I E I P A P K V F I A Q K M M I A K C N
---------- ---Aβ6--- ----Aα6---- ----------Aα6--------
Cat M1 R A G K P V I C A T Q M L E S M I K K P R P T R A E G S D V A N A V L D G A D C
A.nid I K G K P V I C A T Q M L E S M T Y N P R P T R A E V S D V A N A V L D G A D C
--- ----Aβ7---- ----------Aα7--------- ---

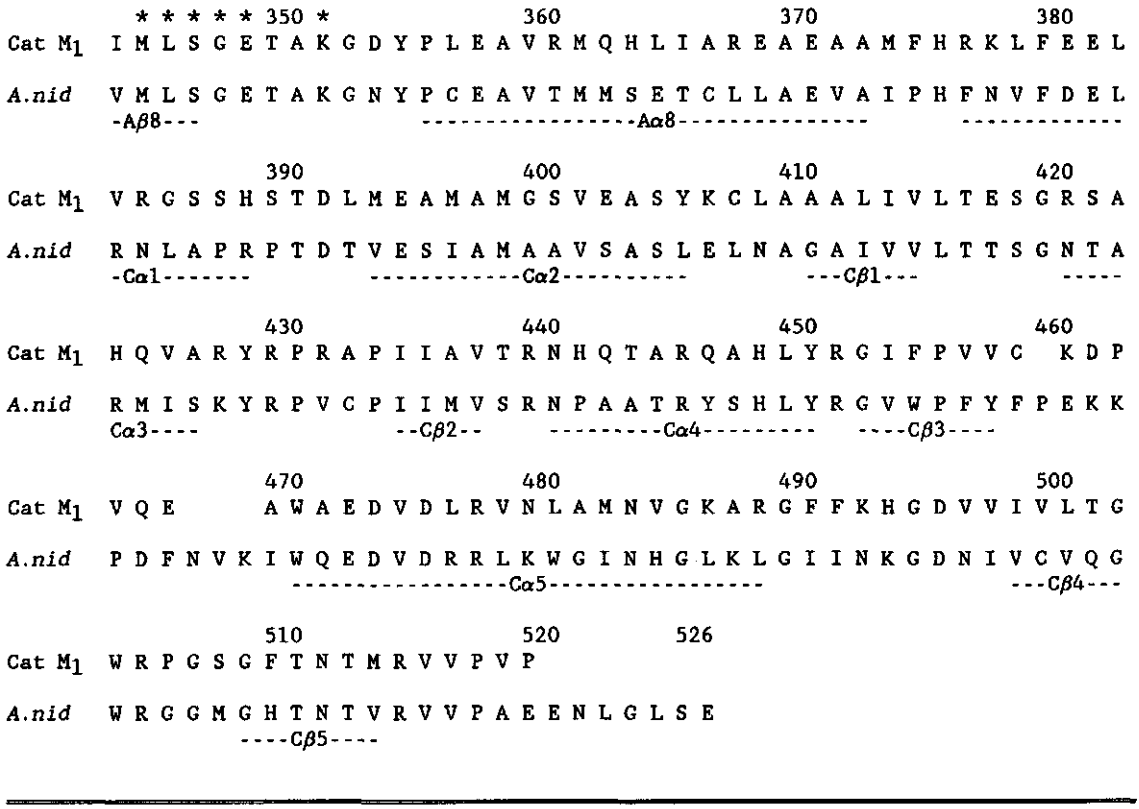


Fig.1 *Alignment of the primary structures of cat M_1 pyruvate kinase and* Aspergillus nidulans *pyruvate kinase. The residues close to the active site are indicated with an *. The elements of secondary structure, from the cat M_1 enzyme are indicated under the alignment of the primary structures of both proteins. Data are from Muirhead* et al., *1986. For comparison with the graphic model, the* Aspergillus nidulans *numbering is used.*

In Fig. 3 the predicted subunit tertiary structure of *A.nidulans* pyruvate kinase, is compared with the cat M_1 subunit tertiary structure. The *A.nidulans* structure closely resembles the cat M_1 subunit structure. Only a few differences in the tertiary structure of both proteins are found. The alignment shows that the cat M_1 pyruvate kinase contains ten additional amino acid residues at the N-terminal end, while the *A.nidulans* pyruvate kinase has seven additional residues at the C-terminal end of the protein. The prediction for the structure of these additional residues at the C-terminal end in the *A.nidulans* pyruvate kinase structure is ambiguous, since there is no crystallographic data available for this region. Four regions are found in which both proteins contain deletions or insertions with respect to each other. *A.nidulans* pyruvate kinase contains two regions with an insertion compared to the cat M_1 enzyme, *viz.* in the region 20-30 the residues 24 and 26, and in the region 458-470 the residues 459 and 466-468. Deletions compared to the cat M_1 enzyme are found in the regions 88-91 and 107-108 (Fig. 1). From Fig. 3 it is seen that the position of these insertions and deletions corresponds to regions connecting elements of secondary structure. For one of these regions this is shown in more detail in Fig. 4, the insertion is found in the loop connecting Cβ3 (B) and Cα5(A). The position of these regions within the tetrameric enzyme is illustrated in Fig. 5. They are located at the exterior of the tetrameric protein.

From these results it can be concluded that despite differences found in the primary structures, the tertiary structure and the overall quaternary structure of the *A.nidulans* pyruvate kinase closely resembles that of the cat M_1 subunit structure.

b. Potential phosphorylation site in A.nidulans pyruvate kinase

The role of phosphorylation in the regulation of the pyruvate kinase activity in mammalians has been studied extensively (for review see Engström *et al.*, 1987). The best studied is the liver L type enzyme which is phosphorylated by a cAMP-dependent protein kinase. Phosphorylation of the enzyme leads to a decreased affinity for PEP, allowing a fast response to changes in metabolic conditions.

Both for the pig liver enzyme and for the rat liver enzyme, the amino acid sequence involved in phosphorylation was determined (Humble, 1980). In both these enzymes the SER residue in the sequence ARG-ARG-ALA-SER is phosphorylated. This sequence is found to be phosphorylated in both pyruvate kinase enzymes as well as in synthetic peptides (Glass and Krebs, 1979; Engström *et al.*, 1987).

In contrast to the extensive experimental data available for the mammalian enzymes, no data exists in yeast, *N.crassa* or *Aspergillus*, neither from *in vivo* nor from *in vitro* experiments, determening whether phosphorylation is involved in the regulation of the pyruvate kinase activity. Based on similarity in amino acid sequence with the phosphorylation site in pig liver pyruvate kinase, Burke *et al.* (1983) suggested that the amino acid sequence LEU-ARG-ARG-THR-SER-ILE found in the N-terminal region of the protein, possibly represents a phosphorylation site in the yeast enzyme. In the amino acid sequence of *A.nidulans* and *A.niger* this sequence is conserved with respect to the yeast enzyme.

In this study we investigated the position of this potential phosphorylation site within the *Aspergillus* pyruvate kinase structure. From the predicted position of this residue within the modeled structure, it was deduced whether this serine residue is accesible for phosphorylation.

The serine (36) residue (Fig. 6A and B) of the potential phosphorylation site is found at the beginning of the first β strand of the α/β barrel of the protein in the A domain. However, the position of this residue within the three dimensional structure of the pyruvate kinase enzyme is such that it is very unlikely to function as a site of phosphorylation. The serine residue is buried within the protein structure, and seems not accessible for phosphorylating enzymes. This, in contrast to the L type isozyme, where the site of phosphorylation is located at the N-terminus of the enzyme (Cognet *et al.*, 1987), and which is removed by partial proteolysis by chymotrypsin and subtilisin. This gives rise to a fragment of about 25 residues, indicating that this fragment is located near the exterior of the enzyme (Simon *et al.*, 1982).

c. Allosteric activation by fructose 1,6-bisphosphate

The allosteric effect caused by the binding of FBP to pyruvate kinases from different sources is well studied, but little is known about the residues involved in the binding of this effector, and the conformational shifts induced by it.

The FBP binding site lies in a pocket formed by the domains A and C. In the M_1 structure this pocket contains four ARG residues, which could form part of the binding site for FBP, as was postulated by Muirhead *et al.*, 1987. Additionally the repulsion of the positively charged side chains of these residues would help to keep this pocket open, keeping the enzyme in the high affinity state for PEP.

One hypothesis is that this "regulatory" pocket, containing the FBP binding site, is closed in the T state and open in the R state. Thus if the pocket is open, either by FBP or by charge (as in the M_1 enzyme), it will stabilize the R quaternary structure. The effect of different residues in the inter-subunit contacts, in different pyruvate kinases, will work out as a difference in the equilibrium constants between the T

and R state structures (Muirhead *et al.*, 1987). Since the cat M_1 pyruvate kinase is in the R state, and no structural information exists for the T state quaternary structure, the model build for *Aspergillus* pyruvate kinase also describes the R state. For both the M_1 enzyme as well as for the *Aspergillus* enzyme, we have analyzed the structure at the FBP binding site and the inter-subunit contacts.

1. Analysis of the structure at the FBP binding site

Superposition of the FBP binding site of *A.niger* pyruvate kinase and the cat M_1 pyruvate kinase is shown in Fig. 7. The residues ARG(33), ARG(91) and HIS(448) are conserved in both proteins, and they are in essentially the same position in both proteins. The main differences are found at the entrance of the pocket. The M_1 enzyme has an ARG in position 90, a LYS in position 486 and an ARG in position 488, whereas in *Aspergillus* pyruvate kinase in position 90 a GLY is found, and one positively charged residue in position 487. This means that there exits a difference in both proteins of two positively charged side chains at the entrance of the FBP binding site.

The charged residues inside the FBP binding pocket of *Aspergillus* pyruvate kinase, are in an identical position as those in the cat M_1 enzyme (Fig. 7). These residues are conserved in the pyruvate kinase primary structures, as shown in Table I.

Table I *Amino acid comparison of residues at the FBP binding site of pyruvate kinase.*

Residue position:	33	34	90	91	448	470	486	487	488
Cat M_1[1]	R	N	R	R	H	W	K	A	R
Rat M_1[2]	R	N	L	R	H	W	K	A	R
Rat M_2[2]	R	N	L	R	H	W	K	A	R
Chicken M[1]	R	N	T	R	H	W	K	A	R
Rat L[1]	R	S	S	R	H	W	K	L	R
Yeast[1]	R	R	S	R	H	W	K	E	F
A.nidulans[3]	R	R	G	R	H	W	L	K	L
A.niger[3]	R	R	G	R	H	W	L	K	L

Data from: 1) Muirhead (1987); 2) Noguchi *et al.* (1987);
3) Chapter 4 and 5.

The conservation of these residues, ARG(33), ARG(91) and HIS(448), suggests that they form the actual binding site for the effector. The conformational change which occurs as a result of the binding of FBP, is paralleled by a change in the environment of a tryptophan residue nearby the site, as detected by an decrease in fluorescence (O'Brien and Kapoor, 1975; El-Maghrabi *et al.*, 1982). The tryptophan residue is probably the one found at position 470, in the loop connecting $C\beta3$ and $C\alpha5$, at the beginning of helix $C\alpha5$. The change in conformation observed may be a result of a shift of this loop, bringing the tryptophan residue in a different environment.

Differences in amino acid residues are found at the entrance of the FBP binding site, in *Aspergillus* the number of charged residues found is less (residues 486 and 488). In addition, a marked difference in the α carbon backbone at the entrance of this site is found (Fig. 5). The insertion in the region 458-470, and the deletion in the region 88-91 when compared to the M_1 enzyme, are both found at this entrance. The deletion in the region mentioned, which is also found when the yeast and the cat M_1 pyruvate kinase are aligned (Muirhead, 1987), may allow the pocket to

close more easily. Whether the insertion in the region 458-470, which is found in the *Aspergillus* enzymes, but not in pyruvate kinase from yeast and higher eukaryotes, affects the allosteric effect of FBP can not be deduced from this model. Nevertheless, the differences mentioned may affect the affinity for FBP and/or affect the equilibrium between the T and R states of the specific enzyme.

2. Analysis of the inter-subunit contacts

Inter-subunit contacts play a role in the conformational change induced by FBP, as has been shown for the *N.crassa* pyruvate kinase (Kapoor, 1976). The main regions of inter-subunit contacts involve the helices Aα6, Aα7, Aα8, Cα1, Cα2 and Cβ4 (Muirhead *et al.*, 1987). Differences in these contact regions were examined, in order to try to explain differences in allosteric behaviour of the cat M_1 and the *Aspergillus* enzymes with respect to the effect of FBP.

In three of these regions no clear differences in the subunit interactions were discovered. The residues involved in the contact region Aα7-A'α7, [THR(325), GLU(326), VAL(332), ALA(335) and ASP(338)] are conserved, not only in the cat M_1 pyruvate kinase and *Aspergillus* pyruvate kinases, but in all known pyruvate kinase structures. Also, no marked differences were found in the regions involved in subunit interaction Cα2 and Cβ4.

The interaction between Aα6-A'α8 in the *Aspergillus* enzymes, is a hydrophobic interaction, involving the residues PRO(288), ILE(292), ALA(293), LEU'(367) AND LEU)'(368). In the M_1 enzyme a potential electrostatic interaction may be present, between the residues GLU(288)-ARG'(367) and LYS(289)-GLU'(368) (Table II), the result of which may be a tighter inter-subunit contact in this region.

Table II *Amino acids involved in the Aα6-A'α8 inter-subunit contact.*

Residue position:	288	289	367	368
Cat M_1[1]	E	K	R	E
Rat M_1[2]	E	K	R	E
Rat M2[2]	E	K	R	E
Chicken M[1]	E	K	R	E
Rat L[1]	E	K	V	I
Yeast[1]	P	E	L	L
A.nidulans[3]	P	K	L	L
A.niger[3]	P	K	L	L

Data from: 1) Muirhead (1987); 2) Noguchi *et al.* (1987); 3) Chapter 4 and 5.

In the Cα1-C'α1 subunit interaction electrostatic interactions occur; in *Aspergillus* interactions between ASP(380)-ARG'(383) and ARG(383)-ASP'(380) are found, while in the M_1 enzyme these interactions are between GLU(380)-ARG'(384) and ARG(384)-GLU'(380) (Fig. 8)(Table III). In both enzymes the distance between these charged residues is about 5Å, as was determined from the graphic model. The orientation of the side chain of residue 381 (GLU) is such that it does not seem to be involved in this inter-subunit interaction.

Besides these charge interactions which are found in the subunit contact of both enzymes, the *Aspergillus* enzymes also exhibit hydrophobic interactions. The side-chain methylene residues of ARG(383) and ASN(384) and the residues PHE(376), PHE(379) and LEU(405) are involved in this. Suggesting that the Cα1 inter-subunit interaction in *Aspergillus* is more hydrophobic than in the cat M_1 enzyme.

Table III *Amino acid sequence in the Cα1 region.*

Residue position:	376				380			383	
Cat M_1[1]	R	K	L	F	E	E	L	V	R
Rat M_1[2]	R	L	L	F	E	E	L	A	R
Rat M_2[2]	L	Q	L	F	E	E	L	R	R
Chicken M[1]	R	Q	Q	F	E	E	I	L	R
Rat L[1]	R	Q	L	F	E	E	L	R	R
Yeast[1]	L	P	N	Y	D	D	-	M	R
A.nidulans[3]	F	N	V	F	D	E	L	R	N
A.niger[3]	F	N	V	F	D	E	L	R	N

Data from: 1) Muirhead (1987); 2) Noguchi *et al.* (1987);
3) Chapter 3 and 4.

Two possibly significant differences are found in the contact regions involved in subunit interaction, when the cat M_1 enzyme and the *Aspergillus* enzymes are compared. The first concerns the Aα6-A'α8 region, where a tighter subunit interaction is expected in the cat M_1 enzyme, because of the contribution of electrostatic interactions, when compared to the *Aspergillus* enzymes. This inter-subunit electrostatic interaction is found in all M type pyruvate kinases (Table II), but not in the inter-subunit contact region of the rat L type, yeast and *Aspergillus* pyruvate kinase. Comparison of the affinity of FBP for the rat M_2 type, the rat L type and the *A.niger* enzyme, shows that K_a for the rat M_2 is 0.1-0.4 μM, while for the latter two this parameter is 0.06-0.1 μM (Imamura *et al.*,1972; Meixner-Monori *et al.* 1984). This suggests that charge-charge interactions are important in determining the relative stabilities of the T and R quaternary structures.

A second region in which differences are found, is the Cα1 region. This region must be involved in the allosteric activation of pyruvate kinase, since the only difference between the rat M_1 and M_2 isozymes, is found in this region. Both enzymes are encoded by the same gene, the M gene, and are formed as a result of differential splicing (Noguchi *et al.* 1986). In an exon with a length of 45 amino acids, 22 different amino acids occur. As a result of these differences, the M_2 isozyme is allosterically activated by FBP, while the M_1 isozyme is not.

The Cα1 region contains conserved differences between the pyruvate kinases which are allosterically activated by FBP, and those which are not. The negative charge located at position 380 is conserved in both types of enzyme, while the allosteric type of pyruvate kinases have a positive charge at position 383, whereas the non-allosteric type (M_1) has this charge in position 384 (Table III). The deletion of one amino acid residue in the pyruvate kinase enzyme from yeast, may bring the Arg(384) in the position of Arg(383) as found in the *Aspergillus* enzymes.

The charged residues in position 380, 383 and 384 seem to be important for the allosteric behaviour of the enzyme, but from the model no marked differences between the subunit interactions of both enzymes were found. This suggests that a slight difference in subunit interaction between both types of enzymes, leads to the differences in allosteric behaviour.

Conclusions

The elucidation of the three dimensional structure of proteins is of importance in understanding enzyme catalysis, and in investigating the role particular amino acids residues have in this. For the fungal pyruvate kinase no crystal structures are available. Hence the protein structure has been modeled based on the crystallographic data and from homology in the primary structure of the cat M_1 pyruvate kinase isozyme. This situation can be considered to represent the introduction of a number of mutations at the same time. As a first approximation this provides useful structural information which, in combination with kinetic data, may help to determine future studies.

With respect to the α carbon backbone, the modeled structure of the *Aspergillus* enzyme shows minor differences compared to the cat M_1 isozyme. The similarity in tertiary structure, despite differences in primary structure, reflects the situation that during evolution only mutations were allowed which did not affect the overall tertiary structure. Both structure and function as an integral character of the protein have been conserved during evolution, and as a result, a conservation is found in those specific residues involved in catalysis.

The first conclusion which can be drawn from the structural comparison is, that it seems unlikely that the *Aspergillus* pyruvate kinases become phosphorylated like the L type isozyme. With respect to the binding and the allosteric effect of FBP, following conclusions have been made:

a. The residues ARG(33), ARG(91) and HIS(448) are very likely the residues involved in the binding of FBP. This can be concluded from the conservation of these residues in the three dimensional structure of both proteins. The strength of this conclusion is increased by the finding that those residues are conserved in all primary structures known. However, the model presented here, does not rule out the involvement of other residues.

b. Comparison of the subunit interactions suggests that the region Aα6-A'α8 is important in determing the enzyme's affinity for FBP, while the interaction in the region Cα1 is more important in determining whether the enzyme is allosterically regulated or not by FBP.

The validity of the model presented here, and the conclusions drawn from the comparison of the cat M_1 pyruvate kinase and the *Aspergillus* pyruvate kinases, need further investigations. The availability of an *A.nidulans pki* mutant, in which the enzyme is not activated by FBP, and seems to remain in the T state, will enable us to examine the validity of some of the conclusions drawn.

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CHAPTER 7

Concluding remarks

Concluding remarks

The project described in this thesis is part of the Industrial Biotechnology Programme of the Agricultural University in Wageningen. One of the aims of this programme is the generation of basic knowledge required to improve fungal strains of industrial importance. Strain breeding can be accomplished by classical genetics and by somatic and molecular genetic approaches. At the same time fundamental knowledge of the physiology and biochemistry of the system is required.

The investigations described in this thesis were focussed on gene structure and expression in relation to carbon metabolism in *Aspergillus nidulans* and *Aspergillus niger*. When this project was started the basic tools needed to study gene expression were not yet developed for *A.niger*, therefore the work was initiated with *A.nidulans*. The pyruvate kinase gene was chosen as the subject of this thesis for several reasons. First, the gene is strongly expressed, but its expression is modulated to some extent. Second, the gene codes for an enzyme which represents one of the control points in glycolysis.

The gene structure for four glycolytic genes in *A.nidulans* have been determined to date. These include the genes encoding triosephosphate isomerase (*tpi*)(McKnight *et al.*, 1986), 3-phosphoglycerate kinase (*pgk*) (Clements and Roberts, 1986), glyceraldehyde-3-phosphate dehydrogenase (*gpd*)(Punt *et al.*, 1988) and pyruvate kinase (*pki*) (Chapter 4).

The promoter regions of these four glycolytic genes from *A.nidulans*, all contain CT rich regions preceding the site of transcription initiation. The length of these CT rich regions has been correlated to their respective levels of transcription (Ballance, 1986). The typical TATA element is not generally found in fungal promoters, *e.g.* it is lacking in the *tpi* and *pki* promoter, whereas it is found in the fungal promoters of *pgk* and *gpd*. However, a TTTATTTT sequence is found 32 bp upstream the from 5' end of the *tpi* cDNA. Deletion of this same sequence in the

A.nidulans trpC promoter (also lacking the TATA element) results in mRNA's with heterogenous 5' ends (Hamer and Timberlake, 1987), indicating a similar function is served as by the TATA element. In the *pki* gene no such sequence is found within the 50 bp region upstream of the transcription initiation site. A TTTTTTT sequence is found 27 bp before the site of transcription initiation. As suggested in Chapter 4 this sequence might function as a TATA element, positioning the site where transcription starts.

The *A.nidulans pgk* and *gpd* 5' upstream sequences have a homologous sequence (16 bp in common in a sequence of 24 bp) in the region 500-600 bp before the transcription initiation site (Punt *et al.*, 1988), but which is not found within the 758 bp region preceding the *pki* gene.

The functional analysis of the 5'-upstream sequences of more fungal genes is required to reveal the role of regulating sequences in the regulation of gene expression. In most of the fungal genes analyzed thus far, no distinct TATA elements or CCAAT elements are found. Further analysis of other 5'-upstream sequences may reveal the essential features of these sequences. Thus far, one is not able to recognize a single motif in the fungal promoters known.

Functional analysis of the 5'-upstream sequences of the *A.nidulans pki* gene is presently in progress. The strategy is to fuse the 5'-upstream sequence with the *E.coli lacZ* gene and the *trpC* termination region (van Gorcom *et al.*, 1985). This fusion was recently constructed and was strongly expressed in *A.nidulans*. Defined deletions are now being made to determine which sequences are involved in the regulation of the level of transcription.

The gene structures of the four *A.nidulans* glycolytic genes mentioned above, contain introns in contrast to the corresponding genes from *Saccharomyces cerevisiae*. The intron/exon structure of these fungal genes represents an intermediate state in gene evolution. The corresponding

genes in prokaryotes and unicellular eukaryotes, like yeast do not contain introns, whereas in *Aspergillus* these same genes are found to contain introns. In *A.nidulans* two introns are found in *pgk*, five in *tpi*, seven in both *gpd* and *pki*. However, the genes encoding these enzymes in higher eukaryotes contain ten, six, nine and nine introns respectively. Thus the number of introns found in fungi is less than in the corresponding genes from higher eukaryotes. In addition, to fewer introns, the intron length is also generally found to be smaller in fungi. The introns found in *Aspergillus* vary in length from 50 bp up to about 120 bp, whereas intron lengths in the glycolytic genes of higher eukaryotes are sometimes several thousand bp long. This is easily illustrated by comparing the lengths of the structural genes encoding for pyruvate kinase in yeast, *A.nidulans*, rat and chicken (Fig. 1). The proteins encoded by those four genes vary in length from 500 amino acids (yeast) to 530 amino acids (chicken), whereas the length of the structural genes varies much more. The structural gene in yeast is 1500 bp (Burke *et al.*, 1983), in *A.nidulans* 1968 bp, in rat 8360 bp (L type gene)(Cognet *et al.*, 1987) and in chicken approximately 13,600 bp (M type gene)(Lonberg and Gilbert, 1985). Similar comparisons can be made for all genes mentioned above, with essentially the same result. This suggests that in *Aspergillus* the genome size was reduced by a decrease in intron length, and to some extent by a decrease in intron number.

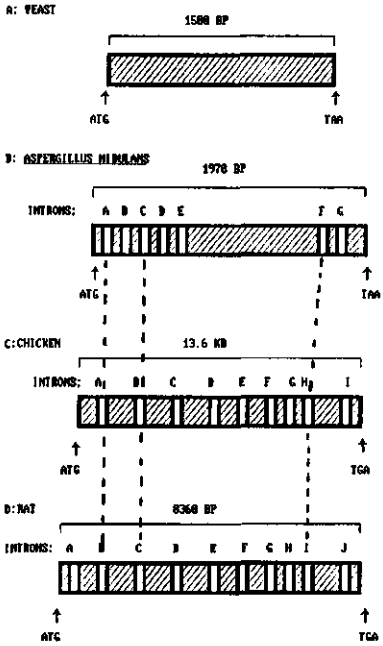

Fig. 1 Schematic representation of the intron position in genes encoding pyruvate kinase.

A: yeast (Burke et al., 1983)

B: *A.nidulans* (Chapter 4)

C: chicken M-type (Lonberg and Gilbert, 1985)

D: rat L-type (Cognet *et al.*, 1987). The figure indicates only the position of the introns, not the intron length. The introns found in an equivalent position in these genes are connected by a dashed line.

Another result of this investigation is the predicted amino acid sequence for the *A.nidulans* and *A.niger* pyruvate kinases, which has been used to build a preliminary structural model for these enzymes. These structural models give insight into the regions and residues involved in the allosteric regulation of the enzyme.

An important question to be answered in the future is whether *in vivo* under specific physiological conditions pyruvate kinase really functions as a major control point in glycolysis. If this turns out to be the case, the pyruvate kinase gene will become an interesting system to test the possibilities which genetic manipulation offers in influencing important pathways in metabolism. Alterations in gene dose are easily realized in these fungi (see Chapters 2 and 5). The impact of this, at the controlling level of the metabolic pathways, certainly needs to be analyzed. Moreover, the structural model presented here, combined with further kinetic analysis, can provide a starting point to alter the enzyme's characteristics by site-directed mutagenesis.

The results presented in this thesis contribute to the extension of basic knowledge in the organization and expression of genes in fungi. In addition, the increased knowledge of the expression of these genes will provide the opportunity to apply this knowledge to industrially important organisms like *A.niger*, and thereby produce improved strains for industrial use.

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Samenvatting

Samenvatting:

Dit proefschrift "Het pyruvaat kinase gen en zijn expressie in *Aspergillus nidulans* en *Aspergillus niger*" beschrijft het onderzoek naar de structuur en expressie van het pyruvaat kinase gen in deze schimmels.

De doelstelling van dit onderzoek was de opheldering van de genstructuur van het pyruvaat kinase gen, vergelijking met de genstructuur van het overeenkomstige gen uit andere organismen, en de studie naar de expressie van het genproduct, pyruvaat kinase.

De pyruvaat kinase genen van *A.nidulans* en *A.niger* zijn geisoleerd met behulp van heterologe hybridizatie, waarbij het sterk overeenkomstige pyruvaat kinase gen uit gist gebruikt is om beide genen op te sporen. De identiteit en functionaliteit van het pyruvaat kinase gen uit *A.nidulans* is onderzocht door een transformatie systeem te ontwikkelen, gebaseerd op een pyruvaat kinase mutant. Deze mutanten kunnen niet groeien op media met een glycolytische koolstofbron, zoals glucose of sucrose. Als gevolg hiervan kunnen transformanten, die een functioneel gen in hun genoom geïntegreerd hebben, op deze media geselecteerd kunnen worden.

Het *A.nidulans* pyruvaat kinase gen is gelocaliseerd op een 2,9 kb *Eco*RI/*Bam*HI fragment. Transformatie van *A.nidulans* WG231, met een zodanige mutatie in het pyruvaat kinase gen, dat geen pyruvaat kinase eiwit meer gevormd wordt, leidt tot volledig herstel van de pyruvaat kinase activiteit. Er wordt een rechtstreeks verband gevonden tussen het aantal kopiëen van dit fragment dat in het genoom geïntegreerd is, en de gemeten pyruvaat kinase activiteit. Hieruit wordt geconcludeerd dat het genoemde fragment het gehele gen bevat, inclusief de gebieden op het DNA die betrokken zijn bij de expressie. Hoewel het gen gereguleerd wordt afhankelijk van aangeboden koolstofbron, zijn er aanwijzingen gevonden dat er regulerende DNA sequenties ontbreken in dit 2,9 kb *Eco*RI/*Bam*HI fragment. Uit de gevonden waarden van de pyruvaat kinase activiteit na groei op een gluconeogenetische koolstofbron als acetaat, welke in de

transformanten verhoogd is ten op zichte van die in de wild type stam, wordt geconcludeerd dat de gen activiteit gereguleerd wordt door repressie van de expressie.

Na transformatie wordt het transformerende DNA in ongeveer 90% van de gevallen geïntegreerd aangetroffen op de plaats van het pyruvaat kinase gen binnen het genoom. Slechts bij uitzondering vindt integratie op een andere, willekeurige plaats in het genoom plaats. Dit komt overeen met de resultaten, zoals die door andere onderzoekers gevonden worden, namelijk dat hoofdzakelijk homologe integratie plaats vindt daar waar rechtstreeks op de betreffende genetische eigenschap geselecteerd wordt. De hier omschreven resultaten en conclusies, worden beschreven in hoofdstuk 2.

Het pyruvaat kinase eiwit uit *A.nidulans* is geïsoleerd uit de wild type stam, en uit een transformant met een verhoogde expressie, ten gevolge van een hoger aantal copiën van het pyruvaat kinase gen. Dit eiwit is gedeeltelijk gekarakteriseerd, en beschreven in hoofdstuk 3. Karakteristieken van het eiwit, zoals het molekuulgewicht en de aminozuursamenstelling, zijn vergeleken met waarden welke zijn afgeleid zijn uit de basenvolgorde in het coderende gebied van het gen. Voor deze karakteristieken is een goede overeenkomst gevonden (hoofdstuk 4). Het gen codeert voor een eiwit bestaande uit 526 aminozuren, dat sterke homologie vertoont met pyruvaatkinase uit gist en zoogdieren.

De coderende sequentie van het pyruvaat kinase gen, wordt onderbroken door zeven intronen. Deze worden niet gelijkmatig verdeeld binnen de coderende sequentie gevonden, maar voornamelijk aan het begin van het gen, het 5' uiteinde. De positie van deze intronen, is vergeleken met de positie van de intronen in de pyruvaat kinase genen afkomstig uit onder andere kip, en rat. Uit deze vergelijking blijkt dat drie van de zeven intronen in het *A.nidulans* gen, in dezelfde, of in een vrijwel gelijke positie gevonden wordt. Het aantal en de lengte van de intronen in het pyruvaat kinase gen van *A.nidulans* reflecteert de intermediaire positie

welke *Aspergilli* in de evolutie innemen, tussen lagere eukaryoten zoals gist enerzijds en de hogere eukaryoten, zoals kat, kip en rat anderzijds.

Het pyruvaat kinase gen van *A.niger* is op soortgelijke wijze geisoleerd als dat van *A.nidulans* (hoofdstuk 5). De functionaliteit van dit gen is aangetoond door middel van co-transformatie, waarbij het *pyrA* gen uit *A.niger* gebruikt is als selectie marker. In de gevonden transformanten werden verhoogde expressie niveau's gevonden, tot het dertigvoudige van het wild type niveau. In alle gevallen blijkt het pyruvaat kinase gen in dit geval op een willekeurige plaats in het genoom geintegreerd te zijn, mogelijk als gevolg van de gekozen experimentele condities.

Evenals het *A.nidulans* gen wordt het *A.niger* gen onderbroken door zeven intronen, die weliswaar verschillen in lengte van die in *A.nidulans*, maar zich op identieke posities in het gen bevinden. Beide *Aspergillus* genen vertonen zeer veel overeenkomst; ongeveer 90% van zowel de basen volgorde als de aminozuur volgorde is identiek, terwijl ook het totaal aantal aminozuren gelijk is, namelijk 526.

In hoofdstuk 6 wordt de ruimtelijke structuur van het pyruvaat kinase gen uit *Aspergillus* beschreven. Deze ruimtelijke structuur is afgeleid uit de structuur van het kattespier pyruvaat kinase. De vergelijking van beide structuren toont verschillen aan m.b.t. de fructose 1,6-bifosfaat bindingsplaats en op het niveau van subeenheid interacties. Met dit laatste is een mogelijke correlatie vastgelegd tussen allosteer en niet allosteer gereguleerde pyruvaat kinase vormen. Op grond van de ruimtelijke structuur wordt verder gepostuleerd dat fosforylering van het pyruvaat kinase uit *Aspergillus* onwaarschijnlijk is.

Curriculum vitae

Curriculum vitae

Leo de Graaff is geboren op 11 januari 1957 in Uitwijk, gemeente Woudrichem, Noord-Brabant. Na het afsluiten van een MAVO opleiding in 1973, werd via een voorbereidend jaar HBO, in 1974 begonnen aan de opleiding Hoger Natuurwetenschappelijk Onderwijs (HNWO), aan het Dr Struycken Instituut te Breda. In het kader van deze opleiding werd een stage gevolgd bij de vakgroep Moleculaire Biologie aan de Katholieke Universiteit Nijmegen, met als thema "In vivo identificatie van M13 eiwitten", onder begeleiding van drs. A.F.M Simons en dr. R.H.N Konings. Hij voltooide deze opleiding in 1978. In datzelfde jaar startte hij met een studie Moleculaire Wetenschappen aan de Landbouwuniversiteit te Wageningen. Het doctoraalexamen, met als hoofdvakken Moleculaire Fysica (Prof. T.J. Schaafsma) en Moleculaire Biologie (Prof. A. van Kammen) en als bijvak Erfelijkheidsleer (Prof. J.H.van der Veen), werd in 1984 afgesloten.

Vanaf 1 april 1984 tot 1 april 1987 was hij werkzaam als promotie assistent bij de vakgroep Erfelijkheidsleer, onder begeleiding van dr.ir J. Visser. Vanaf april 1987 is hij aldaar in dienst als toegevoegd onderzoeker, in het kader van het industrie gerichte deelprogramma Biotechtologie van de Landbouwuniversiteit en het ministerie van Economische zaken (P.C.I.B).

Nawoord

Nawoord

Tal van mensen hebben bijgedragen aan dit onderzoek, en aan de leerperiode die promotieonderzoek inhoudt. Heel veel plezier heb ik gehad, en zeker nog in het begeleiden van stagiaires en doctoraal studenten. De inzet, en onbevangenheid waarmee zij in het onderzoek geparticipeerd hebben, is van wezenlijk belang geweest voor de vorming, en het steeds opnieuw evalueren van het onderzoek. Ik hoop dan ook dat zij met even veel plezier aan deze periode terug denken, als ik doe. Cora van Zeijl, Mieke Lexmond, Swie Lan Oei, Ingrid ten Velde, Raymond Verhaart, Eus Arts, Ab Weijland, Dirk Hondmann, Gern Huijberts, Leonne Plegt, Pascale Rombouts, Cecile Bergmans, Lonneke van der Geest en Stephen Evers. Stephen Evers, heeft door critisch lezen, en corrigeren een grote bijdrage aan het afwerken van de tekst geleverd.

Special thanks to Hilary Muirhead, who was so kind to spend a lot of energy in helping me to model the *Aspergillus* pyruvate kinase.

De mensen op het lab hebben aan dit werk bijgedragen, concreet in werk, Harry Kester en Ronald Busink, en anderen, te veel om op te noemen door hun *esprite de corps*.

Buiten de hier boven genoemde mensen, zijn een tweetal op een meer bijzondere wijze, en ook meer direct bij dit onderzoek betrokken geweest.

Hetty van den Broeck heeft eerst als stagiaire, maar later als analiste, alle experimenten die geleid hebben tot de structuur opheldering van het *A.niger* pyruvaat kinase gen, en de analyse van de *A.niger* transformanten uitgevoerd. Hoewel slechts vervat in een enkel hoofdstuk, is door haar inspanning een wezenlijk deel van dit proefschift tot stand gekomen.

Jaap Visser die door zijn tomeloze inzet de voorwaarden heeft geschapen, waardoor dit proefschift mogelijk was. Zijn inzet en enthousiasme hebben deze periode tot een heel bijzondere gemaakt.

Tenslotte Frouwien Visser, die voor maaltijden heeft gezorgd, en daarnaast ook een heel goed klaverjas maatje bleek.

Iedereen van binnen en buiten het lab die geholpen heeft, op welke manier dan ook, daar voor hartelijk bedankt,