The Yeast Pyruvate Carboxylase 2 Gene (PYC2) and Structure-function Studies on Yeast Pyc Isozymes

by


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Summary

Pyruvate carboxylase (Pyc) (EC 6.4.1.1) catalyses the ATP dependent carboxylation of pyruvate to produce oxaloacetate, a reaction of importance both for gluconeogenesis and the replenishment of TCA cycle intermediates utilised in other biosynthetic pathways such as isogenesis, porphyrinogenensis and, depending on the species or tissue, also in glutamine and acetyl-choline synthesis. This enzyme belongs to a class of biotin enzymes (class I; Samols et al., 1988) which all catalyse very similar two step reactions involving a biotin carboxylation step, followed by a transcarboxylation step. The Pyc protein itself is a tetramer composed of 4 identical subunits arranged in the shape of a splayed tetrahedron, and each subunit (∼130 kDa) is divided into at least 3 structural domains: an N-terminal ATP binding domain, a central pyruvate binding/transcarboxylation domain, and a C-terminal biotin-carrier domain (Lim et al., 1988). Sequence comparisons have revealed that each of these domains has considerable homology to the corresponding domains of the other class I biotin enzymes, and other functionally related enzymes.

One of the primary research goals of our laboratory over the years has been to characterise the structure-function relationships of pyruvate carboxylase. Prior to my research, F. Lim had sequenced the Pyc gene for Saccharomyces cerevisiae in our laboratory (Lim et al., 1988), and Dr. M. E. Walker, upon disrupting this Pyc gene, found evidence suggesting that there was a second isozyme for pyruvate carboxylase in yeast (Walker et al., 1991).

Hence the primary research objectives of my project were to:

a) clone and characterise the PYC2 gene from Saccharomyces cerevisiae
b) disrupt the PYC2 gene in a pycl null so as to construct a strain with no Pyc activity suitable as a host for the expression of mutant Pyc molecules
c) use site-directed mutagenesis to investigate the role of various amino acid residues or motifs which appear to be important on the basis of resultant sequence comparisons, and/or previous biochemical studies.
The major findings reported in my thesis are presented below.

1.) The existence of two *PYC* genes in yeast was confirmed by the presence of two hybridising bands observed on Southern blots of yeast chromosomes separated by pulse field gel electrophoresis, and probed with *PYC* specific probes from the sequenced yeast gene (*PYCl*). The *PYCl* gene was localised to chromosome VII, and *PYC2* was localised to chromosome II. These genes were each further localised on the physical map of *Saccharomyces cerevisiae* by hybridisation to a set of prime λ-clone grid filters obtained from Professor M. V. Olison (University of Washington School of Medicine, Seattle).

2.) A 3.54 kb *PYC2* clone containing 1.87 kb of the 5' non-coding region, and 2866 bp of the 5' coding region of *PYC2* was isolated from a yeast genomic library. The 3' end of this gene was obtained from a separate genomic library, and both these clones were completely sequenced in both directions. The *PYC2* gene was found to be very similar to *PYCl*, having an identity of 85.4% within the open reading frame (ORF) which encodes for an 1186 amino acid protein (Pyc2) with a 92.6% identity to Pyc1.

Extensive sequence comparisons between the domains of Pyc2 and other related proteins have highlighted a number of motifs which may prove to be important in the function of this enzyme. These sequences and their "proposed" functions respectively, are as follows: conserved cysteine and lysine residues in the ATP domain, iron pair involved in the tautomerisation of biotin; QVEH motif unique to biotin carboxylases, involved in biotin carboxylation; HXHXH motif in the pyruvate domain, Zn⁴⁺ binding site.

During the course of these investigations the sequence of the yeast *PYC2* gene was reported by Stucka *et al.* (1991). Sequence comparisons between the two *PYC2* sequences revealed that the published sequence differed from my data within the ORF by having 36 sequence differences, which include: 35 transversions resulting in 12 predicted amino acid differences, and one insertion at the C-terminus of the published sequence causing a frame shift changing Q1178 to a P, and extending the ORF by 15 bp (5 amino acids). These differences appear to be due to yeast strain variation and/or sequencing errors in the published sequence.
3.) A recombinant Pyc2 C-terminal peptide containing the entire biotin domain was expressed from the clone l had isolated, and the calculated molecular weight of the resultant purified peptide (determined by mass spectrometry) agreed precisely with the theoretical value for the predicted amino acid sequence. The sequence reported by Sucka et al. (1994) differed in this region by a Q1178,R5 amino acid C-terminal extension, and one conservative amino acid difference (R instead of K). Hence, the corresponding recombinant biotin domain peptides containing these different sequences were also expressed, and the effect of these differences on in vivo biotinylation was determined.

4.) Homologous recombination was used to disrupt the PYC2 gene using either the TRP1 or HIS3 selectable marker genes. Using this technique a double null mutant strain (DM18) in which both PYC genes had been knocked out was constructed to use as a host strain for the expression of mutant Pyc proteins. The absence of Pyc in DM18 was confirmed by Western blotting, enzyme assays and aspartate auxotrophy. In addition, studies with the single null mutant strains indicated that Pyc1 is the more abundant isozyme.

5.) There have been a number of chemical modification studies which have suggested that Pyc enzymes contain an essential cysteine residue in the pyruvate binding/transcarboxylation site. Site-directed mutagenesis was used to change individually each of the 4 cysteines in the pyruvate domain of yeast Pyc (to serine) so as to determine whether any of these residues are essential for Pyc activity. None of these mutations produced any major changes in enzyme activity or aspartate dependence of the host strain containing these constructs, indicating that pyruvate carboxylase does not contain an essential cysteine residue involved in base catalysis of the transcarboxylation reaction.