Thiamin auxotrophy in yeast through altered cofactor dependence of the enzyme acetohydroxyacid synthase

Kerry L. Byrne and Peter A. Meacock

Author for correspondence: Peter A. Meacock. Tel: +44 116 252 2261. Fax: +44 116 252 5101.
e-mail: mea@le.ac.uk

Department of Genetics,
University of Leicester,
University Road, Leicester
LE1 7RH, UK

The *THI1* gene of *Saccharomyces cerevisiae* has been identified and found to be allelic with the previously characterized gene *ILV2* that encodes acetohydroxyacid synthase (AHAS). This enzyme catalyses the first step in the parallel biosyntheses of the branched-chain amino acids isoleucine and valine, using thiamin pyrophosphate (TPP) as a cofactor. The *ilv2-thi1* allele encodes a functional AHAS enzyme with an altered dependence for the cofactor TPP resulting in the thiamin auxotrophic phenotype. Nucleotide sequence analysis and site-directed mutagenesis revealed that the *thi1* mutation is a single base substitution which causes the conserved amino acid substitution D176E in the AHAS protein. This study therefore implicates aspartate 176 as another amino acid residue important either for the efficient binding of TPP by AHAS or for the functional stability of the holoenzyme.

Keywords: acetolactate synthase, thiamin pyrophosphate, *THI1*, *ILV2*, *S. cerevisiae*

INTRODUCTION

Thiamin pyrophosphate (TPP), the biologically active form of vitamin B₁, is an essential metabolite that serves as cofactor for several key enzymes of intermediary metabolism. These include pyruvate decarboxylase (PDC) [EC 4.1.1.1], pyruvate dehydrogenase E1 subunit (PDH-E1) [EC 1.2.4.1; E1], transketolase (TK) [EC 2.2.1.1] and acetohydroxyacid synthase (AHAS) [EC 4.1.3.18]. In recent years AHAS, which catalyses the first step of branched-chain amino acid biosynthesis, has become the subject of detailed research since it is the target of a number of commercial herbicides including the sulfonylureas and imidazolinones (reviewed by Duggleby & Pang, 2000). Alignment of the amino acid sequences of various TPP-dependent enzymes including PDC, PDH-E1, TK, AHAS, pyruvate oxidase (POX) [EC 1.2.3.3] and 2-oxoglutarate dehydrogenase [EC 1.2.4.2] from yeast, *Escherichia coli*, humans and plants has revealed a common conserved sequence, GDGX_(24–27)NN now termed the ‘TPP-binding motif’ (Hawkins *et al.*, 1989). These residues are primarily concerned with binding the pyrophosphate end of the

TPP molecule through interactions with a divalent cation, generally magnesium.

Thiamin is synthesized in micro-organisms and plants from two precursors, hydroxyethyl thiazole phosphate (HET-P) and hydroxymethyl pyrimidine pyrophosphate (HMP-PP), that are condensed together to form thiamin monophosphate (TMP). In the bakers’ yeast *Saccharomyces cerevisiae* TMP is then dephosphorylated to free thiamin, which is subsequently pyrophosphorylated to form TPP, the endproduct of the pathway. Although some of the enzymes and genes involved in thiamin biosynthesis have been characterized in yeast (reviewed by Begley, 1996; Estramareix & David, 1996; Hohmann & Meacock, 1998), much is still unknown. Therefore, in order to learn more about thiamin biosynthesis and metabolism in *S. cerevisiae* we have embarked upon a genetic analysis of strains carrying thiamin auxotrophic mutations. These included strain Y02587, which carries the first thiamin auxotrophic mutation isolated in *S. cerevisiae*, *thi1* (Mortimer & Hawthorne, 1966). Apart from its nutritional requirements and localization of the *thi1* mutation to the right arm of chromosome XIII, no other information was available regarding this strain.

In this paper we describe the isolation of the wild-type *THI1* gene. Surprisingly, we found that *THI1* is allelic with *ILV2*, which encodes AHAS. Here we show that thiamin auxotrophy can arise from an alteration in

Abbreviations: AHAS, acetohydroxyacid synthase; ALS, acetolactate synthase; PDC, pyruvate decarboxylase; POX, pyruvate oxidase; TK, transketolase; TPP, thiamin pyrophosphate.

cofactor dependence of a TPP-utilizing enzyme rather than through mutation of a structural or regulatory gene of the biosynthetic pathway. We also report that an amino acid residue that does not form part of the consensus TPP-binding motif has a role in determining the cofactor dependency of the enzyme AHAS.

METHODS

Microbial strains and plasmids. Strains of *S. cerevisiae* used in this study are listed in Table 1. Since the *thi1* mutant strain Y02587 was already uracil requiring (*ura1*) it was first converted to uracil prototrophy by crossing with a *Ura*⁺ derivative of strain W303B, and a *thi1* *URA1* derivative was isolated from the meiotic progeny. This strain, Y02587U⁺, was then crossed against a *ura3* strain, PMY3, and KBY6 was isolated from the meiotic products of this cross. Strain W303Δ*ilv2* was made by transforming W303A with a linear *ilv2Δ::URA3* DNA fragment from plasmid pKB22 (see below) and selecting uracil prototrophs. Strain KBY8 was created by transforming W303Δ*ilv2* with the linear fragment of insert DNA from plasmid pKB19h (Fig. 1), which carried the allele of the *ILV2* gene (*ilv2ΔXbaI-XbaI*) with a deletion of the 1.352 kb *XbaI-XbaI* section. Cells were plated on medium containing 5-fluoro-orotic acid to select for *ura3* derivatives. Transformants were screened for uracil auxotrophy to identify those that had the *ilv2Δ::URA3* allele replaced by the *ilv2ΔXbaI-XbaI*. One of these was designated KBY8.

Escherichia coli strains employed as hosts for constructing and propagating plasmids were NM522 (Gough & Murray, 1983)

or the *dam* strain JM110 (Yanisch-Perron *et al.*, 1985). Luria broth was used for the culture of *E. coli* and supplemented with ampicillin for the selection of the transformants (Sambrook *et al.*, 1989).

Plasmids used or constructed in this study are listed in Table 1. To construct the *ilv2Δ::URA3* deletion allele, DNA of plasmid pKB19c carrying the minimal *thi1*-complementing fragment was digested with the restriction endonuclease *XbaI* and religated to itself, thereby deleting 1.352 kb of the gene and creating the plasmid pKB19h (Fig. 1). A DNA fragment carrying the *URA3* gene was released from pKB22a by *XbaI* digestion and ligated into *XbaI*-cut pKB19h to give plasmid pKB22, carrying the *ilv2::URA3* construct. This plasmid was then digested with *HindIII* and *EcoRI*, to recover the 2.29 kb disruption cassette for transformation into yeast.

Yeast procedures. All standard media and genetic procedures were as described by Sherman (1991). For selective growth, yeast was cultured in SD medium supplemented with the required amino acids and bases. Sporulation was carried out by growth on acetate-based medium, following transfer from rich pre-sporulation medium. To test for thiamin auxotrophy, strains were grown in Wickerham's minimal medium (Wickerham, 1951), with or without thiamin, and supplemented with appropriate amino acids and bases. High-efficiency transformations of yeast were performed by the lithium acetate procedure as described by Geitz *et al.* (1995). For disruption of the *ILV2* locus the 'one-step gene disruption' method was used (Rothstein, 1983). 'Gap repair' was used as described by Orr-Weaver & Szostak (1983) to recover the *ilv2-thi1* allele.

Table 1. *S. cerevisiae* strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Source or reference
Yeasts		
DG622	<i>MATα his3Δ200 ura3-167 trp1Δ1 leu2Δ</i> (pGTy1-H3 HIS3)	J. Strathern, Frederick Cancer Research Center, MD, USA
PMY3	<i>MATα ura3-52</i>	Praekelt <i>et al.</i> (1994)
PMY3 <i>thi4</i>	<i>MATα ura3-52 thi4::URA3</i>	Praekelt <i>et al.</i> (1994)
STX573-3C	<i>MATα ilv2 ura3 leu2 gal1</i>	YGSC
W303A	<i>MATα ade2-1 leu2-3-112 trp1-1 ura3-1 his3-11-115</i>	R. Rothstein, Columbia University, New York, USA
W303B	<i>MATα ade2-1 leu2-3-112 trp1-1 ura3-1 his3-11-115</i>	R. Rothstein
Y02587	<i>MATα thi1 ura1 trp1</i>	YGSC
Y02587U ⁺	<i>MATα thi1 trp1</i>	This study, Y02587 × W303B
W303Δ <i>ilv2</i>	W303A <i>ilv2Δ::URA3</i>	This study, gene disruption
KBY6	<i>MATα thi1 ura3-52 trp1</i>	This study, Y02587U ⁺ × PMY3
KBY8	W303A, <i>ilv2Δ</i> (0.691 kb <i>XbaI-XbaI</i>)	This study, gene replacement
Plasmids		
pE16/8-C4	YRp17- <i>ILV2</i>	Polaina (1984)
pKB19	Ycp50- <i>THI1</i> library clone	This study
pKB19(c-h)	Ycp50-(<i>ilv2Δ</i> derivatives) (Fig. 1)	This study
pKB22a	pUC19- <i>URA3</i> (<i>XbaI</i> fragment)	This study
pKB22	Ycp50-(<i>ilv2Δ::URA3</i>)	This study
pKB24	Ycp50-(<i>ilv2-thi1</i>)	This study
pKB29	pUC19-(<i>ilv2-thi1:sdm</i>)	This study
pKB30	Ycp50-(<i>ilv2-thi1:sdm</i>)	This study

Fig. 1. Localization of the *THI1* gene within library clone pKB19: restriction map of a section of the yeast chromosomal DNA contained in this clone. Restriction enzyme sites are E, *EcoRI*; H, *HindIII*; Sa, *SalI*; Sp, *SpeI*; Xb, *XbaI*; Xh, *XhoI*. ORFs are represented by the arrows. ORF3 is *YMR107w*. Horizontal lines indicate the segments of DNA contained within each of the subclones pKB19c-h. The ability of the various clones to complement the *thi1* strain KBY6 to thiamin prototrophy is shown. Transformants were tested on Wickerham's medium either containing or lacking 2 μ M thiamin; + indicates growth, - indicates no growth.

Table 2. Synthetic oligonucleotides used in this study

Name	Sequence (5'-3')	Target site
1223	CACTATCGACTACGCGATCA	pBR32 <i>Bam</i> HI clockwise
KB9*	CCCGGATCCCTTTGAGCTAAGAGGAG	<i>ILV2</i> ORF (-43 to -27)
KB10*	CCCGGATCCGAGAAAGAAGTCGTGTCC	<i>ILV2</i> ORF (2356 to 2338)
KB11	GCCTGTACGCTTATGACG	<i>ILV2</i> ORF (2051 to 2033)
KB12	GCTTGAACGGCAGAACTC	<i>ILV2</i> ORF (1697 to 1679)
KB13	CAGACCTCTCCTTAACTGG	<i>ILV2</i> ORF (1336 to 1318)
KB14	CTTGGACCATCTGCATGG	<i>ILV2</i> ORF (953 to 935)
KB15	GGTTGCCAACGACACAGG	<i>ILV2</i> ORF (1446 to 1463)
KB16	CAGAAGGCTACGCCAGAG	<i>ILV2</i> ORF (437 to 454)
KB17	CCAGTCGCACACAAGATG	<i>ILV2</i> ORF (830 to 847)
KB18	GCCAATGGTGGCAGGTGG	<i>ILV2</i> ORF (1956 to 1973)
KB19†	TCCAATGGCAGAGGCCTTTGCAGAC	<i>ILV2</i> ORF (516 to 540)
KB20†	GTCTGCAAAGGCCTCTGCCATTGGA	<i>ILV2</i> ORF (540 to 516)
KB21	GTATTTCTCTTATCTGTTG	<i>ILV2</i> ORF (-289 to -269)
KB22	GTCAGCCTCTTGAAAGC	<i>ILV2</i> ORF (615 to 599)

* *Bam*HI restriction enzyme site (underlined) incorporated at the 5' end.

† Mismatched base (underlined) incorporated at centre of the sequence.

DNA manipulations. Standard DNA handling procedures were carried out according to protocols described in Sambrook *et al.* (1989). Total yeast chromosomal DNA of high quality, for use in Southern blot analysis, was prepared from spheroplasts (Cryer *et al.*, 1975). DNA samples were sequenced on an ABI model 373A DNA sequencer, following sample preparation using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit. The primer for sequencing DNA cloned into the *Bam*HI site in YCp50 DNA was obtained from New England Biolabs; 1223, pBR322 *Bam*HI site primer (clockwise). Custom-made primers were supplied by PNACL at Leicester University and are listed in Table 2.

Site-directed mutagenesis was performed using oligonucleotide primers with mismatched bases. Thus primers KB19 and KB20 (Table 2) were designed complementary to both strands of DNA to cover the 25 bp region surrounding the *thi1* mutation, T528G, that was to be introduced. These primers, along with KB21 and KB14, which annealed to the flanking *ILV2* DNA either side of the mutation site, were used to amplify the two halves of the *ILV2* gene by PCR. The products of these two reactions were recovered, mixed together, denatured and used as template for PCR using only the two flanking primers KB21 and KB14. The product obtained was a 1.24 kb fragment of *ILV2* sequence with the introduced *thi1* mutation. This was purified and digested with

SpeI, to release a 691 bp fragment, containing the site-directed mutation. This fragment was ligated into *SpeI*-cut pKB18h to make pKB29, which contained the *ilv2-thi1:sdm* gene on vector pUC19. This clone was digested with the restriction endonuclease *StuI*, to confirm that the *thi1:sdm* mutation had been correctly incorporated, and the 691 bp *SpeI* fragment was sequenced using primers KB14, KB16 and KB21. The DNA construct pKB29, which was confirmed to contain no other mutations, was then digested with restriction endonucleases *HindIII* and *Sall* to release the mutant *ilv2-thi1:sdm* gene. This was ligated into *HindIII*-*Sall*-digested YCp50 DNA to create the plasmid pKB30 used for introduction of the site-directed mutant allele into yeast.

Acetohydroxyacid synthase (AHAS) assay. Yeast strains were grown overnight (25 or 50 ml) to a concentration of $1-3 \times 10^7$ cells ml⁻¹, in Wickerham's minimal medium supplemented with tryptophan, histidine, adenine, isoleucine and valine. Cells were harvested at 5000 r.p.m. for 5 min, washed twice in sterile distilled H₂O and resuspended in 1.0 ml 0.1 mM Tris/HCl buffer (pH 7.5), 0.1 M NaCl, 0.1 mM EDTA at 4 °C containing the protease inhibitors chymostatin (1 µg ml⁻¹), PMSF (1 mM) and pepstatin (1 µM). These were permeabilized by adding 100 µl chloroform and vortexing for 30 s, collected by centrifugation and the supernatant was decanted. After resuspension in 1.0 ml of the same buffer the total protein concentration was measured using the Bio-Rad 'Bradford Reagent' method. AHAS was assayed via its acetolactate synthase (ALS) activity. Acetolactate production was measured at 30 °C for 30 min, using a method based on that of Ryan & Kohlhaw (1974). For each assay two tubes were set up, A and B, each containing a mixture of 40 mM sodium pyruvate, 10 mM MgCl₂, 100 mM Tris/HCl (pH 7.5) and varying concentrations of TPP (0–200 µM), to a total volume of 100 µl. The reactions were started by the addition of ~0.5 mg permeabilized cell protein to each tube. They were stopped by the addition of 50 µl 3 M sulphuric acid to tube A and 150 µl 6 M sodium hydroxide to tube B. The yield of acetolactate produced was determined by converting it to acetoin. This involved measuring the total amount of acetoin produced (tube A) and subtracting the amount of background acetoin produced by the cells (tube B). Acetolactate was converted into acetoin by a decarboxylation step that involved heating the sample in an acid environment. Therefore tube A was incubated at 60 °C for 30 min, after which the sample was made basic by the addition of 100 µl 12 M sodium hydroxide. Acetoin was measured according to the method of Westerfeld (1945). Each sample was made up to a total volume of 1 ml with sterile distilled water, then 200 µl 0.5% creatine was added followed by 200 µl 5% α-naphthol (freshly made up in 2.5 M sodium hydroxide) and the solution mixed by inverting the tubes several times. The colour was allowed to develop for 1 h at room temperature, the samples were centrifuged for 2 min and then their absorbance at 540 nm was measured against a blank made up of 1 ml water, 200 µl creatine and 200 µl α-naphthol. The ALS activity was calculated as acetoin produced per mg of permeabilized cell protein per minute.

RESULTS

Molecular cloning of the wild-type *THI1* gene

The first documented thiamin-auxotrophic strain of *S. cerevisiae*, Y02587, isolated in the 1960s, carries a mutation designated *thi1* (Mortimer & Hawthorne, 1966). We obtained Y02587 from the Yeast Genetic Stock Center (YGSC), University of California,

Berkeley, USA, and carried out genetic analysis. When the *thi1* mutant strain was crossed with a Ura3⁺ derivative of the thiamin-prototrophic strain W303B, the heterozygous diploid was found to be prototrophic for thiamin. Therefore the *thi1* mutation is recessive. The diploid strain was induced to undergo sporulation and 12 four-spore meiotic tetrads were dissected. All tetrads showed 2:2 segregation of the Thi⁻ and Thi⁺ phenotypes, confirming that *thi1* is a single-locus mutation.

In order to test whether the *thi1* mutation was allelic with other known mutants which gave thiamin auxotrophy, *thi2* (Kawasaki *et al.*, 1990), *thi3* (Nishimura *et al.*, 1992), *thi4* (Praekelt *et al.*, 1994) and *thi6* (Nosaka *et al.*, 1994), complementation analysis was performed. Appropriate crosses were made between the *thi1* mutant and each of the other mutant strains, and the heterozygous diploids were tested for thiamin requirement; all of the diploids were prototrophic for thiamin. Thus *thi1* is not allelic with the thiamin mutations *thi2*, *thi3*, *thi4* or *thi6*.

DNA clones that complemented the *thi1* mutation were obtained on the basis of their ability to rescue growth of the mutant on thiamin-deficient medium. A *thi1 ura3* strain (KBY6) was transformed to uracil prototrophy using a YCp50-based *S. cerevisiae* genomic DNA library (Rose *et al.*, 1987). Among the several thousand Ura⁺ transformants recovered, 19 were thiamin prototrophs; in all cases Ura⁻ segregants that had lost the transforming clones displayed thiamin auxotrophy, indicating that the prototrophic phenotype of the transformants was caused by the introduced library plasmid and not by reversion of the original *thi1* mutation. Plasmid DNAs were recovered into *E. coli* from two of the transformants and these were found to complement the *thi1* mutation when retransformed back into the KBY6 strain, confirming that each carried the *THI1* gene.

Restriction enzyme digestions revealed that the yeast genomic DNA fragments in the two plasmids were identical. Subcloning of the insert DNA from one plasmid (pKB19) gave a 5.185 kb *HindIII*-*XhoI* fragment that was able to complement the *thi1* mutation when reintroduced into KBY6 on vector YCp50 (pKB19c, Fig. 1). Nucleotide sequence data were obtained from the ends of the insert DNA and compared with the EMBL nucleotide database. The sequence of the insert DNA was found to match the region of yeast chromosome XIII containing the ORF YMR108w, previously characterized as the *ILV2* gene. Analysis of the sequence of the 5.185 kb DNA fragment revealed three other potential ORFs, all smaller than 420 bp in length (Fig. 1). Further dissection of the DNA fragment carried in pKB19c produced the smaller derivatives pKB19d–h (Fig. 1); phenotypic analysis showed that none of these was able to rescue the thiamin auxotrophy of *thi1*. These subclones included pKB19g, carrying only the internal 2.0 kb *EcoRI* fragment, and the reciprocal derivative, pKB19f, that had this fragment deleted.

Fig. 2. Phenotype of the *thi1* mutant strain: thiamin and isoleucine/valine growth tests of mutant yeast strains. Single colonies were diluted in sterile water and standard aliquots streaked onto Wickerham's minimal medium plus required amino acids and bases, supplemented with nothing (–thi –ilv), or with thiamin (+thi –ilv), or with isoleucine/valine (–thi +ilv), or with both (+thi +ilv). Strains used were as follows: W303 = W303B (Thi⁺ Ilv⁺ control); *thi4* = PMY3*thi4*::*URA3* (Thi[–] control); W303Δ*ilv2* = W303A,*ilv2Δ*::*URA3* (Ilv[–] control); *thi1* = Y02587.

These data suggested that the ORF responsible for *thi1* complementation spanned one or both of these *EcoRI* sites and thus ruled out ORFs 1 and 2 as candidates for the *THI1* gene, as both were contained entirely within the 2.0 kb fragment. Also ORF 3 (*YMR107w*) was eliminated because it was located entirely outside this region. Therefore the only ORF which spanned one or both of the *EcoRI* sites was *YMR108w*, the *ILV2* gene.

Allelism of *thi1* and *ILV2*

Normally mutations at the *ILV2* locus cause auxotrophy for the branched-chain amino acids isoleucine and valine (Falco & Dumas, 1985). However, the *thi1* strain Y02587 did not require isoleucine and valine for growth (Fig. 2). Furthermore, a strain (STX573-3C) previously confirmed to carry a *bona fide ilv2* mutation showed isoleucine and valine auxotrophy but no requirement for thiamin; i.e. the same phenotype as strain W303Δ*ilv2* (Fig. 2). We also found that the *thi1*-complementing clone, pKB19c, conferred isoleucine and valine prototrophy when transformed into strain STX573-3C, indicating that it carried an *ILV2* allele. Likewise a *bona fide ILV2* clone, pE16/8-C4 (Polaina, 1984), was able to restore thiamin prototrophy when transformed into the *thi1* strain KBY6. This gave an intriguing paradox: the *thi1* mutant was not isoleucine and valine requiring and the *ilv2* mutant was not thiamin requiring, yet both mutations were complemented to their respective prototrophies by the same fragment of DNA. One explanation

Table 3. Meiotic segregation of thiamin and isoleucine/valine phenotypes in the cross W303Δ*ilv2* (*Δilv2*::*URA3*) × KBY6 (*thi1*)

Minimal medium supplements	No. of tetrads of segregation pattern (growth:no growth)			
	2:2	3:1	4:0	0:4
+Thi +Ilv	0	0	10	0
–Thi +Ilv	0	0	10	0
+Thi –Ilv	10	0	0	0
–Thi –Ilv	0	0	0	10

could be that *YMR108w* was the wild-type locus for one of the mutations and a suppressor of the other. However the complementing fragments were carried on low-copy-number vectors, which made suppression unlikely. It was therefore important to delete this locus and examine the phenotype of the resultant *ilv2Δ* null mutant. Additionally, examination of the meiotic segregants of diploids made by crossing strains carrying the *ilv2Δ* and *thi1* mutations would help to resolve whether they were allelic.

To this end 'one step' gene replacement (Rothstein, 1983) was carried out at the *ILV2* locus. A linear DNA fragment, comprising the genomic DNA insert of plasmid pKB19c (Fig. 1) with the 1.35 kb *XbaI* fragment from within the *ILV2* gene replaced with a *URA3* gene, was recovered from plasmid pKB22, transformed into the yeast strain W303a, and uracil prototrophs selected. This DNA fragment underwent targeted recombination with the *ILV2* locus because of the homologous sequences flanking the selected *URA3* marker gene. Replacement of the *ILV2* gene by the introduced *ilv2Δ*::*URA3* construct in one of the Ura⁺ transformants, i.e. strain W303Δ*ilv2*, was confirmed by Southern blot analysis (data not shown). This strain was also found to display isoleucine and valine auxotrophy but thiamin prototrophy, i.e. it was phenotypically Ilv[–] Thi⁺ (Fig. 2).

Because of this observation we decided to reinvestigate the phenotype of strains carrying the original *thi1* mutation. We found that the haploid *thi1* strain did not in fact require thiamin for growth when isoleucine and valine were supplied in the medium (Fig. 2). Therefore the true phenotype of the *thi1* mutation is that of thiamin auxotrophy only in the absence of isoleucine and valine.

Finally, we crossed the strain W303Δ*ilv2* carrying the *ilv2Δ*::*URA3* allele and the *thi1* strain KBY6. The heterozygous diploid exhibited the *thi1* phenotype (thiamin auxotrophy in the absence of isoleucine and valine). Sporulation and analysis of 10 meiotic tetrads was carried out (Table 3). In the presence of isoleucine and valine 4:0 segregation of Thi⁺:Thi[–] phenotypes was observed for all tetrads. However, in the absence of

isoleucine and valine only two spores from each tetrad were viable with thiamin supplementation, and no spores were viable in the absence of thiamin as well as isoleucine and valine. Taken together these observations implied that the *ilv2Δ* and *thi1* mutations showed 2:2 segregation consistent with their being allelic. Moreover the absence of any non-parental ditype or tetratype tetrads confirmed that the *thi1* and *ilv2Δ* mutations were tightly linked.

Characterization of the *thi1* mutation

These paradoxical observations led to the conclusion that the *thi1* mutation defines a unique allele of the *ILV2* gene that has no direct function in the biosynthesis of thiamin. We propose that it be redefined as the *ilv2-thi1* allele. Since AHAS requires TPP as a coenzyme it seemed likely that the *ilv2-thi1* allele encodes a functional AHAS enzyme with an enhanced requirement for thiamin. Such a situation could arise if the AHAS produced had a reduced binding affinity for TPP. By adding exogenous thiamin to the medium the internal concentration of TPP is raised, due to active thiamin uptake and conversion to TPP, which is then stored within the cell (Iwashima *et al.*, 1973; Suzuoki, 1955; Praekelt *et al.*, 1994). Thus, we reasoned that the variant AHAS produced from the *ilv2-thi1* allele only functions effectively in branched-chain amino acid biosynthesis when intracellular TPP levels are high. In contrast, the normal AHAS enzyme, produced from the wild-type *ILV2* allele, has sufficient affinity for TPP that it can function using the normal low endogenous level of cofactor. Supplementation with exogenous isoleucine and valine would completely obviate the need for AHAS function and therefore would allow *ilv2-thi1* mutants to grow without additional thiamin.

To test this hypothesis we decided to investigate the molecular nature of the *thi1* mutation and to characterize the AHAS enzyme produced from the *ilv2-thi1* allele. Recovery of the mutant allele was achieved using the 'gap repair' technique (Orr-Weaver & Szostak, 1983). Cells of the *thi1 ura3* strain KBY6 were transformed to uracil prototrophy with *Xba*I-linearized DNA of the ARS-CEN-based plasmid pKB19h (Fig. 1) which had part of the *ILV2* sequence deleted from the insert DNA. The *in vitro*-generated gap in the transforming DNA was repaired by *in vivo* recombination with the homologous chromosomal sequences. Plasmid DNAs from two different Ura⁺ transformants were recovered into *E. coli* and then transformed back into the *ura3 ilv2Δ* strain KBY8. Transformants carrying each plasmid clone gave the *thi1* phenotype, thiamin auxotrophy in the absence of isoleucine and valine, confirming that we had recovered the *ilv2-thi1* allele.

In order to establish exactly where the *thi1* mutation was located and to determine its effect on the AHAS amino acid sequence, we carried out nucleotide sequencing of the mutant ORF of one plasmid, pKB24, using oligonucleotide primers (Table 2). Only a single nucleotide difference was found within the 2064 bp

Fig. 3. (a) Confirmation of the *thi1* mutation: autoradiograph from Southern blot analysis of genomic DNA (3 µg) digested with restriction endonuclease *Stu*I and hybridized with a probe made from the 2.0 kb *Eco*RI fragment of *ILV2* DNA in plasmid pKB19c. DNAs were isolated from strains W303B (*THI1*) (lane 1), KBY6 (*thi1*) (lane 2), and DG622 (*THI1*) (lane 3). The 11 kb band that appears in lane 2 is the product of incomplete digestion at an adjacent *Stu*I site, and comprises the 4.563 kb fragment with the adjoining 6.331 kb fragment (see b). (b) Restriction map of the *ILV2* region, showing the location of the additional *Stu*I site introduced into the *ILV2* gene by the *thi1* mutation, and sizes of hybridizing fragments. Restriction enzyme sites are E, *Eco*RI; St, *Stu*I.

sequence of the *ilv2-thi1* allele when compared to the published *ILV2* sequence (Falco *et al.*, 1985). The mutation at nucleotide position 528 of the *ILV2* ORF (YMR108w) was a T to G transversion changing codon 176 from GAT to GAG, and thereby replacing the normal aspartate residue with the related glutamate, i.e. D176E. Moreover, the mutation also created a new cleavage site for the restriction endonuclease *Stu*I: 5'-ATGCCT-3' to 5'-AGGCCT-3'. However the *thi1* mutation was located 100 bp upstream from the 5' *Xba*I site used for the 'gap repair' cloning of the *ilv2-thi1* allele. Thus recombination between the transforming gapped plasmid and *ilv2-thi1* sequences must have extended well beyond the ends of the linearized plasmid DNA.

To confirm that strains carrying the original *ilv2-thi1* allele did contain this particular mutation within the *ILV2* ORF we carried out a Southern blot analysis to look for the presence of the additional *Stu*I site. Genomic DNAs were isolated from the *thi1* strain KBY6 and two Thi⁺ *Ilv2*⁺ strains, DG622 and W303B; these were digested with restriction enzyme *Stu*I and subjected to Southern blot analysis using part of the *ILV2* gene as a hybridization probe (Fig. 3). Inspection of the published sequence of chromosome XIII (Bowman *et al.*, 1997)

Fig. 4. Acetolactate synthase assays. ALS activity was measured in permeabilized cells of strain KBY8 containing various alleles of the *ILV2* gene on the vector YCP50. Increasing concentrations of TPP were added to the assay mix. Activity was calculated as acetoin produced [A_{540} units (mg total protein) $^{-1}$ min $^{-1}$]. Duplicate samples were taken, and each measurement is the mean of two independent experiments; error bars indicate standard errors. Symbols indicate the following plasmids: $\square$, pKB19c (*ILV2*); $\bullet$, pKB24 (*ilv2-thi1*); $\circ$, pKB30 (*ilv2-thi1:sdm*); $\triangle$, YCp50 (*ilv2Δ*).

showed that *ILV2* is contained within a 23·279 kb *StuI* fragment. The DNAs of strains W303B and DG622 both yielded a single hybridizing fragment of approximately 23 kb (Fig. 3, lanes 1 and 3). However, DNA of strain KBY6 yielded two smaller hybridizing fragments, of approximately 5 kb and 19 kb (Fig. 3, lane 2). These corresponded to the sizes of fragments (4·563 kb and 18·716 kb) that would be produced if an extra *StuI* restriction site had been created at nucleotide 528 of the *ILV2* ORF. Therefore the mutation generating the *StuI* restriction site was present in strains carrying the original *thi1* allele.

As final proof that this single base change was solely responsible for conferring the *thi1* phenotype we performed a PCR-based site-directed mutagenesis experiment. Using mismatched oligonucleotide primers (KB19, KB20; Table 2) the T₅₂₈G substitution was introduced into the *ILV2* gene carried on plasmid pUC19 to make pKB29 (Table 1). After confirmation of the mutation by nucleotide sequencing, the mutated gene (*ilv2-thi1:sdm*) was transferred to the vector YCP50 to create pKB30, which was transformed into the KBY8 strain. All of the Ura⁺ transformants exhibited the *thi1* phenotype, confirming that the single base change was indeed solely responsible for the *thi1* phenotype.

AHAS activity in *thi1* mutants

We have postulated that the *thi1* mutant might produce a variant of AHAS that has altered dependence for its cofactor TPP, so that the enzyme can only function in

branched-chain amino acid biosynthesis when intracellular TPP levels are raised by exogenous supply of thiamin. To test this hypothesis we assayed AHAS activity, and its TPP dependency, in yeast strains carrying various alleles of *ILV2*. Acetolactate synthase (ALS) activity, one of the two activities of AHAS, was measured in the KBY8 strain carrying different *ILV2* alleles on low-copy-number plasmids: pKB19c (*ILV2*), pKB24 (*ilv2-thi1*), pKB30 (*ilv2-thi1:sdm*) and YCP50 (*ilv2Δ*).

Permeabilized cells carrying the wild-type *ILV2* allele (pKB19c) were found to have high levels of activity that showed little dependence on TPP concentration over the range 50–200 μM (Fig. 4). Even with no additional TPP high levels of acetolactate were produced, indicating that the cells contained sufficient endogenous TPP for almost full ALS activity. In contrast, permeabilized cells carrying the *ilv2-thi1* allele (pKB24) showed no or little ALS activity in the absence of exogenous TPP; addition of the cofactor stimulated activity but never to the very high levels recorded in the *ILV2* cells. The data obtained from the enzyme produced by the site-directed mutagenesis clone pKB30, *ilv2-thi1:sdm*, were virtually identical to those obtained for AHAS produced from the original *ilv2-thi1* mutant allele. Again, there was no measurable ALS activity when no exogenous TPP was added to the assay mixture, but activity was detected as the TPP concentration was increased.

We did observe a low background level of TPP-dependent acetolactate production in cells containing no copy of the *ILV2* gene, i.e. *ilv2Δ*. This suggests that there may be another enzyme in yeast that can carry out an ALS-type reaction converting pyruvate to α-acetolactate at a very low rate.

DISCUSSION

We have shown that the *thi1* mutation of *S. cerevisiae* defines a novel allele of the *ILV2* gene encoding the first enzyme of branched-chain amino acid biosynthesis, AHAS. The phenotype conferred is that of thiamin auxotrophy in the absence of isoleucine and valine (Fig. 2). We have proposed that the *ilv2-thi1* allele encodes a mutant AHAS enzyme, with altered dependence for its cofactor TPP, that only functions effectively in cells with raised intracellular TPP levels brought about by an exogenous supply of thiamin. Addition of the branched-chain amino acids isoleucine and valine to the culture medium obviates the need for AHAS activity and so also alleviates the thiamin requirement (Fig. 2). Consistent with our hypothesis, enzyme assays showed that, for any given concentration of TPP, yeast cells containing the wild-type *ILV2* allele had a higher ALS activity than cells containing the *ilv2-thi1* allele (Fig. 4). The actual *thi1* mutation is a T to G single base transversion at nucleotide 528 of the *ILV2* ORF, which translates to a conservative amino acid substitution of glutamate for aspartate at residue 176 of the protein, D176E. This change is situated in the amino-terminal one-third of the protein and distant from the conserved TPP-binding

Fig. 5. Alignment of partial amino acid sequences of AHAS and POX proteins. The amino acid sequences of *S. cerevisiae* and *E. coli* AHAS were aligned with that of *L. plantarum* POX, for which the three-dimensional structure has been elucidated. Shown here are two parts of that comparison: (a) the region surrounding the site of the *thi1* mutation in Scilv2p; (b) the loop of AHAS containing residues 518 and 5519, which may interact structurally with D176 in Scilv2p. Sc.ilv2p, *S. cerevisiae* AHAS; Ec.ilvGp, *E. coli* AHAS II; Lp.Poxp, *L. plantarum* POX. Con-1, consensus sequence from Sc.ilv2p, Ec.ilvGp and Lp.Poxp; Con-2, consensus sequence from trans-kingdom comparison of 11 AHASs taken from Chipman *et al.* (1998); in both consensus sequences upper-case denotes the most frequently occurring residue. The symbols #, * and ▼ denote residues E139, D176 and M525 of Scilv2p respectively, and the vertical arrows (↓) indicate potentially interacting residues, R151, T518 and S519 (see text). Structural elements from Lp.Poxp are shown below the sequence: horizontal arrowheads (➤) are β -strands; bold horizontal bars are α -helices. The line-up of Ec.ilvGp and Lp.Poxp is taken from Ibdah *et al.* (1996).

consensus sequence, which is located towards the carboxyl terminus (residues 547–577).

The structures of three enzymes that use TPP as a cofactor have been resolved by X-ray crystallography. These are PDC from *S. cerevisiae* (Dyda *et al.*, 1993), TK from *S. cerevisiae* (Lindqvist *et al.*, 1992; Nilsson *et al.*, 1993) and POX from *Lactobacillus plantarum* (Muller & Schultz, 1993). AHAS has homology to all of these but is most closely related to POX, sharing 29–30% amino acid identity. Indeed, *E. coli* AHAS II (EcilvGp) has previously been modelled on the crystal structure of LpPoxp (Ibdah *et al.*, 1996); both enzymes use the same cofactors, substrate and initial catalytic step and *E. coli* POX has been found to have a low AHAS activity (Chang & Cronan, 1988). Alignment of the amino acid sequences of AHAS from *S. cerevisiae* and *E. coli* (AHAS II) with that of *L. plantarum* POX (LpPoxp) revealed that the aspartate residue that is altered by the *thi1* mutation is conserved between these three sequences; D176 of yeast AHAS aligns with D84 of AHAS II of *E. coli* and with D96 of *L. plantarum* POX (Fig. 5). Moreover, in a trans-kingdom comparison of AHAS sequences from eubacteria, archaea and eukaryotes (Chipman *et al.*, 1998) this aspartate was conserved in eight out of the eleven polypeptides, with an identical substitution by threonine in the other three.

The POX enzyme is a homotetramer, arranged as a pair of dimers, with each monomer comprising three domains: core (residues 9–191); FAD-binding domain (residues 192–342); and TPP-binding domain (residues 343–593) (Muller & Schultz, 1993). The homology modelling of AHAS has indicated that, as in POX, two TPP molecules are bound by each dimer in pockets between the first domain of one subunit and the third domain of the other subunit, in a head-to-tail fashion (Ibdah *et al.*, 1996). The POX D96, and by analogy D176 in yeast AHAS (Fig. 5), is located in the amino-terminal core domain along with the catalytic glutamate residue, E59 in POX and E139 in yeast AHAS (Fig. 5), that is conserved in all TPP-dependent enzymes. The core domains of the four monomers are closely packed at the centre of the holoenzyme and, by implication, hold the subunits together. Therefore the D176E mutation, which introduces an extra methylene group into the amino acid side chain, may affect dimerization and/or oligomerization of the AHAS subunits, and so affect TPP binding.

To try to gain more information about the structural effects of the *thi1* mutation we used the RasMol program (Sayle & Milner-White, 1995), taking the LpPoxp structure as a template, to model the predicted three-dimensional structures of the Scilv2p in the wild-type

Fig. 6. Structural changes to Scilv2p caused by the *thi1* mutation. Left-hand panel, wild-type Ilv2p monomer; right-hand panel, mutant Ilv2p (D176E) monomer. Using the RasMol v2.6 program the three-dimensional structures of monomers of wild-type and mutant Scilv2p were predicted, based on LpPoxp as a model. The view shows the part of the polypeptide affected by the *thi1* mutation, and the 'core', 'TPP-binding' and 'FAD-binding' domains are located at the top, centre left and bottom right respectively. The polypeptide backbone is shown as a 'cartoon' diagram; ligands TPP and FAD are shown as purple and violet 'stick' models, respectively. Key amino acid residues (see text) are denoted by coloured 'spacefill' structures: D176, red; E176, green; R151, blue; T518, orange; S519, olive; M525, yellow; D550, magenta; N577, cyan.

(D176) and mutant (D176E) forms (Fig. 6). A loop of the TPP-binding domain, containing residues T518 and S519, was found to lie at the interface with the core domain, between the D176 residue and the two ligands TPP and FAD. This serine residue, S519 of Scilv2p, is conserved in all 11 AHAS sequences of the trans-kingdom analysis (Chipman *et al.*, 1998) and in LpPoxp (Fig. 5), and lies close to the conserved methionine residue (M525 in Scilv2p) that has been implicated in determining the conformation of the bound TPP (Duggleby & Pang, 2000); residues of Scilv2p that interact with the phosphate of TPP via a co-ordinated Mg^{2+} anion are D550 and N577 (Duggleby & Pang, 2000). In the wild-type configuration residue D176 can form a hydrogen bond across the domain interface with the residue S519. However, the mutant residue E176 does not form this hydrogen bond but instead forms a salt-bridge with R151, also of the core domain. In wild-type AHAS, R151 forms a hydrogen bond with the backbone oxygen of T518 but this interaction probably does not occur in the mutant because of the interference by E176. Thus the *thi1* mutation, which substitutes a glutamate for an aspartate residue at position 176, is likely to break the hydrogen-bonding network across this domain interface of the monomer, ultimately reducing the binding affinity of AHAS enzyme for the TPP ligand.

To the best of our knowledge our study has shown, for the first time in a micro-organism, that thiamin auxotrophy can arise from an alteration in cofactor dependence of a TPP-utilizing enzyme rather than through mutation of a component of the biosynthetic pathway. This is somewhat analogous to the situation in human

patients suffering from Wernicke–Korsakoff syndrome, where an assembly defect of the TPP-dependent enzyme transketolase, TK, causes a neurological disorder potentiated by thiamin deficiency (Wang *et al.*, 1997).

ACKNOWLEDGEMENTS

This work was supported by BBSRC Research Grants GR/J66362 and 91/G08756 to P.A.M. The *ILV2* plasmid pE16/8-C4 was a kind gift from Professor M. Keilland-Brandt (Copenhagen). We are grateful to Simon Elliston-Elhinn for helpful discussions about the *thi1* mutant phenotype and to Mike Sutcliffe, Department of Chemistry, for guidance on use of the RasMol program and interpretation of the structural data.

REFERENCES

- Begley, T. P. (1996).** The biosynthesis and degradation of thiamin (vitamin-B1). *Nat Prod Rep* **13**, 177–185.
- Bowman, S., Churcher, C., Badcock, K. & 18 other authors (1997).** The nucleotide sequence of *Saccharomyces cerevisiae* chromosome XIII. *Nature* **378** (suppl), 90–93.
- Chang, Y. Y. & Cronan, J. E. (1988).** Common ancestry of *Escherichia coli* pyruvate oxidase and the acetoacetyl-CoA synthetases of the branched-chain amino-acid biosynthetic pathway. *J Bacteriol* **170**, 3937–3945.
- Chipman, D., Barak, Z. & Schloss, J. V. (1998).** Biosynthesis of 2-aceto-2-hydroxy acids: acetolactate synthetases and acetoacetyl-CoA synthetases. *Biochim Biophys Acta* **1385**, 401–419.
- Cryer, D. R., Eccleshall, R. & Marmur, J. (1975).** Isolation of yeast DNA. *Methods Cell Biol* **12**, 39–44.
- Duggleby, R. C. & Pang, S. S. (2000).** Acetoacetyl-CoA synthetase. *J Biochem Mol Biol* **33**, 1–36.

- Dyda, F., Furey, W., Swaminathan, S., Sax, M., Farrenkopf, B. & Jordan, F. (1993). Catalytic centers in the thiamin diphosphate dependent enzyme pyruvate decarboxylase at 2.4-Angstrom resolution. *Biochemistry* **32**, 6165–6170.
- Estramareix, B. & David, S. (1996). Biosynthesis of thiamine. *New J Chem* **20**, 607–629.
- Falco, S. C. & Dumas, K. S. (1985). Genetic analysis of mutants of *Saccharomyces cerevisiae* resistant to the herbicide sulfometuron methyl. *Genetics* **109**, 21–35.
- Falco, S. C., Dumas, K. S. & Livak, K. J. (1985). Nucleotide sequence of the yeast *ILV2* gene which encodes acetolactate synthase. *Nucleic Acids Res* **13**, 4011–4028.
- Geitz, R. D., Schiestl, R. H., Willems, A. R. & Woods, R. A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast* **11**, 355–360.
- Gough, J. A. & Murray, N. E. (1983). Sequence diversity among related genes for recognition of specific targets in DNA molecules. *J Mol Biol* **166**, 1–19.
- Hawkins, C. F., Borges, A. & Perham, R. N. (1989). A common structural motif in thiamin pyrophosphate-binding enzymes. *FEBS Lett* **255**, 77–82.
- Hohmann, S. & Meacock, P. A. (1998). Thiamin metabolism and thiamin diphosphate-dependent enzymes in the yeast *Saccharomyces cerevisiae*: genetic regulation. *Biochim Biophys Acta* **1385**, 201–219.
- Ibdah, M., Barllan, A., Livnah, O., Schloss, J. V., Barak, Z. & Chipman, D. M. (1996). Homology modeling of the structure of bacterial acetohydroxy acid synthase and examination of the active site by site-directed mutagenesis. *Biochemistry* **35**, 16282–16291.
- Iwashima, A., Nishino, H. & Nose, Y. (1973). Carrier mediated transport of thiamine in baker's yeast. *Biochim Biophys Acta* **330**, 222–234.
- Kawasaki, Y., Nosaka, K., Kaneko, Y., Nishimura, H. & Iwashima, A. (1990). Regulation of thiamine biosynthesis in *Saccharomyces cerevisiae*. *J Bacteriol* **172**, 6145–6147.
- Lindqvist, Y., Schneider, G., Ermler, U. & Sundstrom, M. (1992). Three-dimensional structure of transketolase, a thiamine diphosphate dependent enzyme, at 2.5 Angstrom resolution. *EMBO J* **11**, 2373–2379.
- Mortimer, R. K. & Hawthorne, D. C. (1966). Genetic mapping in *Saccharomyces*. *Genetics* **53**, 165–173.
- Muller, Y. A. & Schultz, G. E. (1993). Structure of the thiamine and flavin-dependent enzyme pyruvate oxidase. *Science* **259**, 965–967.
- Nilsson, U., Lindqvist, Y., Kluger, R. & Schneider, G. (1993). Crystal-structure of transketolase in complex with thiamine thiazolone diphosphate, an analog of the reaction intermediate, at 2.3 Angstrom resolution. *FEBS Lett* **326**, 145–148.
- Nishimura, H., Kawasaki, Y., Kaneko, Y., Nosaka, K. & Iwashima, A. (1992). A positive regulatory gene, *THI3*, is required for thiamine metabolism in *Saccharomyces cerevisiae*. *J Bacteriol* **174**, 4701–4706.
- Nosaka, K., Nishimura, H., Kawasaki, Y., Tsujihara, T. & Iwashima, A. (1994). Isolation and characterization of the *THI6* gene encoding a bifunctional thiamin-phosphate pyrophosphorylase/hydroxyethylthiazole kinase from *Saccharomyces cerevisiae*. *J Biol Chem* **269**, 30510–30516.
- Orr-Weaver, T. & Szostak, J. (1983). Yeast recombination – the association between double-stranded gap repair and crossing-over. *Proc Natl Acad Sci USA* **80**, 4417–4421.
- Polaina, J. (1984). Cloning of the *ILV2*, *ILV3* and *ILV5* genes of *Saccharomyces cerevisiae*. *Carlsberg Res Commun* **49**, 577–584.
- Praekelt, U. M., Byrne, K. L. & Meacock, P. A. (1994). Regulation of *THI4* (*MOL1*), a thiamine biosynthetic gene of *Saccharomyces cerevisiae*. *Yeast* **10**, 481–490.
- Rose, M. D., Novic, P., Thomas, J. H., Botstein, D. & Fink, G. R. (1987). A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere containing shuttle vector. *Gene* **60**, 237–243.
- Rothstein, R. J. (1983). One-step gene disruption in yeast. *Methods Enzymol* **101**, 202–211.
- Ryan, E. D. & Kohlhaw, G. B. (1974). Subcellular localization of isoleucine-valine biosynthetic enzymes in yeast. *J Bacteriol* **120**, 631–637.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sayle, R. A. & Milner-White, E. J. (1995). RASMOL: biomolecular graphics for all. *Trends Biochem Sci* **20**, 374–376.
- Sherman, F. (1991). Getting started with yeast. *Methods Enzymol* **194**, 3–21.
- Suzuoki, J. (1955). Thiamine uptake by yeast cells. *J Biochem* **42**, 27–39.
- Wang, J. J., Martin, P. R. & Singleton, C. K. (1997). A transketolase defect in a Wernicke–Korsakoff syndrome patient. *Alcohol Clin Exp Res* **21**, 576–580.
- Westerfeld, W. W. (1945). A colorimetric determination of blood acetoin. *J Biol Chem* **161**, 495–502.
- Wickerham, L. J. (1951). Taxonomy of yeast. *US Dep Agric Tech Bull* **1029**, 11–56.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains – nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

Received 30 April 2001; accepted 29 May 2001.