Thiamine repression and pyruvate decarboxylase autoregulation independently control the expression of the *Saccharomyces cerevisiae PDC5* gene

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Received 12 March 1999

Abstract The *Saccharomyces cerevisiae* gene *PDC5* encodes the minor isoform of pyruvate decarboxylase (Pdc). In this work we show that expression of *PDC5* but not that of *PDC1*, which encodes the major isoform, is repressed by thiamine. Hence, under thiamine limitation both *PDC1* and *PDC5* are expressed. *PDC5* also becomes strongly expressed in a *pdc1*Δ mutant. Two-dimensional gel electrophoresis of whole protein extracts shows that thiamine limitation stimulates the production of *THI* gene products and of Pdc5p. Deletion of *PDC1* only stimulates production of Pdc5p. We conclude that the stimulation of *PDC5* expression in a *pdc1*Δ mutant is not due to a response to thiamine limitation.

Key words: Thiamine; Pyruvate decarboxylase; Autoregulation; Gene expression; Yeast

1. Introduction

Pyruvate decarboxylase (Pdc, EC 4.1.1.1) catalyses the conversion of the glycolytic end product pyruvate to acetaldehyde and CO_2 in alcoholic fermentation [1,2]. The enzyme has been identified in a number of organisms [3] and more than 20 Pdc sequences are deposited in the databases. Pdc consists of four identical subunits and requires thiamine diphosphate (ThDP) and Mg^{2+} as cofactors [3,4]. The three-dimensional structure of yeast Pdc has been determined from crystals produced in the absence and in the presence of the substrate analogue pyruvamide [5,6], which activates the enzyme similar to its substrate pyruvate [7]. The cofactor ThDP is very firmly but non-covalently bound to the enzyme and two subunits within a dimer both contribute to the binding of two molecules of ThDP [3,8]. The enzyme seems to exist in a dimer-tetramer equilibrium with only the tetramer being active [3,8,9].

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Abbreviations: Pdc, pyruvate decarboxylase; *PDC*, genes encoding Pdc or regulators thereof; ThDP, thiamine diphosphate; *THI*, genes encoding enzymes or regulators of ThDP metabolism; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; MOWES, molecular weight search

Haploid yeast strains possess three highly homologous isoforms of Pdc encoded by the genes *PDC1*, *PDC5* and *PDC6* [10–14]. Each isoform independently can form active enzyme and hence expression of only one of the three genes is sufficient to confer Pdc activity [14–16]. The physiological role of Pdc6p is unknown and the enzyme is not involved in sugar catabolism [14,15]. *PDC1* is strongly expressed in rich glucose medium where expression of *PDC5* is hardly or not all detectable [11,13,17–19]. In a *pdc1*Δ mutant strain grown in glucose medium, *PDC5* becomes strongly expressed [13,17,19,20]. Since also the promoter activity of *PDC1* is stimulated in a mutant lacking the coding region of *PDC1*, this phenomenon has been termed Pdc autoregulation [13,19,21]. Recent work has demonstrated that Pdc1p but not its catalytic activity is required to mediate repression of *PDC5* [19]. Thus, a property of Pdc1p independent of catalysis appears to mediate autoregulation.

Pdc is not the only ThDP-dependent enzyme in yeast metabolism. There are a total of five well-characterised yeast enzymes that need ThDP for catalysis and genome sequencing has identified another three, whose functions remain to be characterised [22]. Thus, thiamine is essential for yeast growth. Yeast utilises thiamine from the growth medium, which is taken up by the cell via the transporter Thi10p [23,24] and converted into ThDP by a thiamine pyrophosphokinase encoded by *THI80* [25]. Alternatively, yeast cells can also produce thiamine themselves [22]. Thiamine is synthesised from two precursor molecules, hydroxyethylthiazolephosphate, whose production requires Thi4p [26], and hydroxymethylpyrimidinephosphate, which requires for its synthesis Thi5p or its almost identical isoforms Thi11p, Thi12p and Ydl244p [27]. The two precursors are used by Thi6p [28] to form thiamine phosphate, which is dephosphorylated to thiamine and then converted to ThDP by Thi80p [22]. The *THI* genes are among the most strongly expressed yeast genes but exogenously supplied thiamine represses their expression [22,29]. Since Thi80p is needed for the production of ThDP both from external and from internal thiamine and since only thiamine but not thiamine phosphates are taken up by the cell, *THI80* is an essential gene and is expressed under all growth conditions [22,25].

The unusual autoregulation of *PDC* gene expression and the search for the role of Pdc1p in this effect have prompted us to investigate the effects of externally supplied thiamine on

PII: S0014-5793(99)00449-4

the expression of *PDC1* and *PDC5*. Here we show that expression of *PDC5*, but not that of *PDC1*, is repressed by thiamine. However, the deletion of *PDC1* appears not to stimulate *PDC5* expression via a response to thiamine limitation.

2. Materials and methods

2.1. Yeast strains

Two sets of yeast strains were used. The first set of strains is derived from the M5 [17] sibling YSH 6.36.-3B (*MATα leu2 ura3 trp1 SUC GAL*). This strain was transformed with *PDC1-lacZ* and *PDC5-lacZ* promoter reporter gene constructs, which integrate into the *URA3* locus [18], yielding strains YSH 360 (*PDC1-lacZ*) and YSH 361 (*PDC5-lacZ*). Deletions of *PDC1* have been described previously [17,19] and the strains YSH 901 (*MATα pdc1Δ::LEU2 leu2 ura3 trp1 SUC GAL*) and YSH 381 (same genotype plus integrated copy of the *PDC5-lacZ* construct) were used. Some experiments were done with strain YPH499 (*MATa ura3 leu2 lys2 his3 trp1 ade2 SUC GAL mal* [30]) and its sibling T48-3A (*MATa thi80-1 ura3 his3 leu2 trp1 gal*), which carries a leaky and hence not lethal mutation in the gene encoding thiamine pyrophosphokinase [25,31]. Both strains were also transformed with the integrative *PDC1-lacZ* and *PDC5-lacZ* constructs.

2.2. Growth conditions

Yeast cells were grown in Wickerham's synthetic medium with or without 2 mM thiamine [32]. Cells were pregrown in this medium containing 2% ethanol as carbon source and shifted to fresh medium with 8% glucose to stimulate *PDC* gene expression. Samples were taken at the time points indicated to monitor the specific activity of Pdc or β-galactosidase. For analysis of gene expression under steady-state growth, cells were grown in Wickerham's medium supplemented with 2% glucose for 20 generations with periodic re-inoculation into fresh medium.

2.3. Enzyme activity determination

Whole cell protein extracts were prepared with glass beads. Specific Pdc activity was determined according to Schmitt and Zimmermann [10], specific β-galactosidase activity was monitored as described by Rose et al. [33] and protein was measured with the microbiuret method [34]. For all induction experiments, mean values (standard deviation less than 10%) of two independent experiments are given; for steady-state growth mean values of eight measurements plus standard deviation are shown.

2.4. Two-dimensional gel electrophoresis and spot quantification

Cells were pregrown in Wickerham's medium with 2% glucose lacking methionine and with and without 2 mM thiamine overnight, inoculated into 10 ml fresh medium at a cell density of 10^6 cells/ml and grown until 5×10^6 cells/ml. Then cellular proteins were labelled by the addition of 150 μCi of ^{35}S-labelled methionine (15 μCi/μl, >1000 Ci/mmol, Amersham) for 30 min. Protein synthesis was stopped by the addition of 300 μl cycloheximide (1.4 mg/ml). Two-dimensional gel electrophoresis was run as previously described [35]. Extracts equivalent to 2×10^6 cpm were applied by directly dissolving the sample in the rehydration buffer prior to reswelling of the IPG strip [36]. Quantification was done by computer-assisted densitometry [35]. Mean values and standard deviations from two independent gels are shown.

2.5. Mass spectrometry and spot identification

Spots corresponding to proteins whose production rate increased significantly under thiamine limitation were eluted from a preparative gel, trypsin-digested and subjected to mass spectrometry (J. Norbeck, T. Larsson, K.-A. Karlsson and A. Blomberg, unpublished results). Peptide masses were used for MS-Fit searches at http://falcon.ludwig.ucl.ac.uk/ in the non-redundant *Saccharomyces cerevisiae* database. A peptide mass tolerance of ±300 ppm was used to search monoisotopic masses, allowing methionine oxidation and one missing trypsin cleavage site in the calculations.

3. Results

3.1. Expression of PDC5 is repressed by thiamine

We have monitored the expression of *PDC1* and of *PDC5* in wild type cells making use of previously described promoter-*lacZ* fusions [18] and have used specific β-galactosidase activity as a measure for the promoter activity of *PDC1* and *PDC5*. Expression of *PDC1* and *PDC5* is known to be stimulated by the addition of glucose to ethanol-grown cells [11,13]. Time course experiments after the addition of glucose to ethanol-grown yeast cells were conducted in order to more precisely monitor alterations in promoter activity.

In the presence of thiamine, expression of *PDC5* was always much lower than that of *PDC1* (Fig. 1A). However, in medium lacking thiamine, glucose very strongly stimulated the *PDC5* promoter and 2 h after glucose addition the rate of β-galactosidase activity production from the *PDC5-lacZ* construct was similar to that from the *PDC1-lacZ* construct. After 5 h the β-galactosidase activity levelled off (not shown). The promoter activity of *PDC1* was slightly diminished under thiamine limitation (Fig. 1A). Similar results were obtained during steady-state growth in 2% glucose medium of a wild type strain in the YPH499 background (Table 1). In the presence of thiamine the promoter activity of *PDC5* was barely detectable but it increased strongly in the absence of thiamine. The promoter activity of *PDC1* was slightly lower in thiamine-free medium as compared to thiamine-supplemented medium (Table 1).

To distinguish between the lack of external thiamine and that of internal ThDP as a signal for derepression of *PDC5*

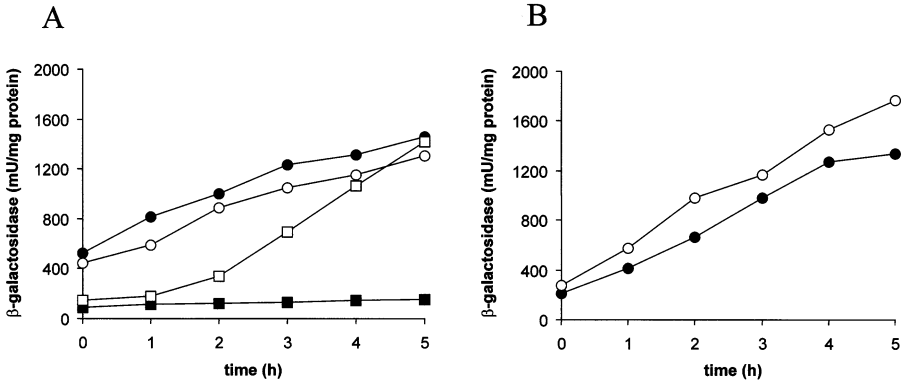

Fig. 1. A: Glucose induction of β-galactosidase activity from *PDC1-lacZ* (●, ○) and *PDC5-lacZ* (■, □) in a wild type strain in the presence (filled symbols) or absence of thiamine (open symbols). B: Glucose induction of β-galactosidase activity from *PDC5-lacZ* in a *pdc1Δ* strain in the presence (●) or the absence (○) of thiamine. The average of two independent experiments is shown.

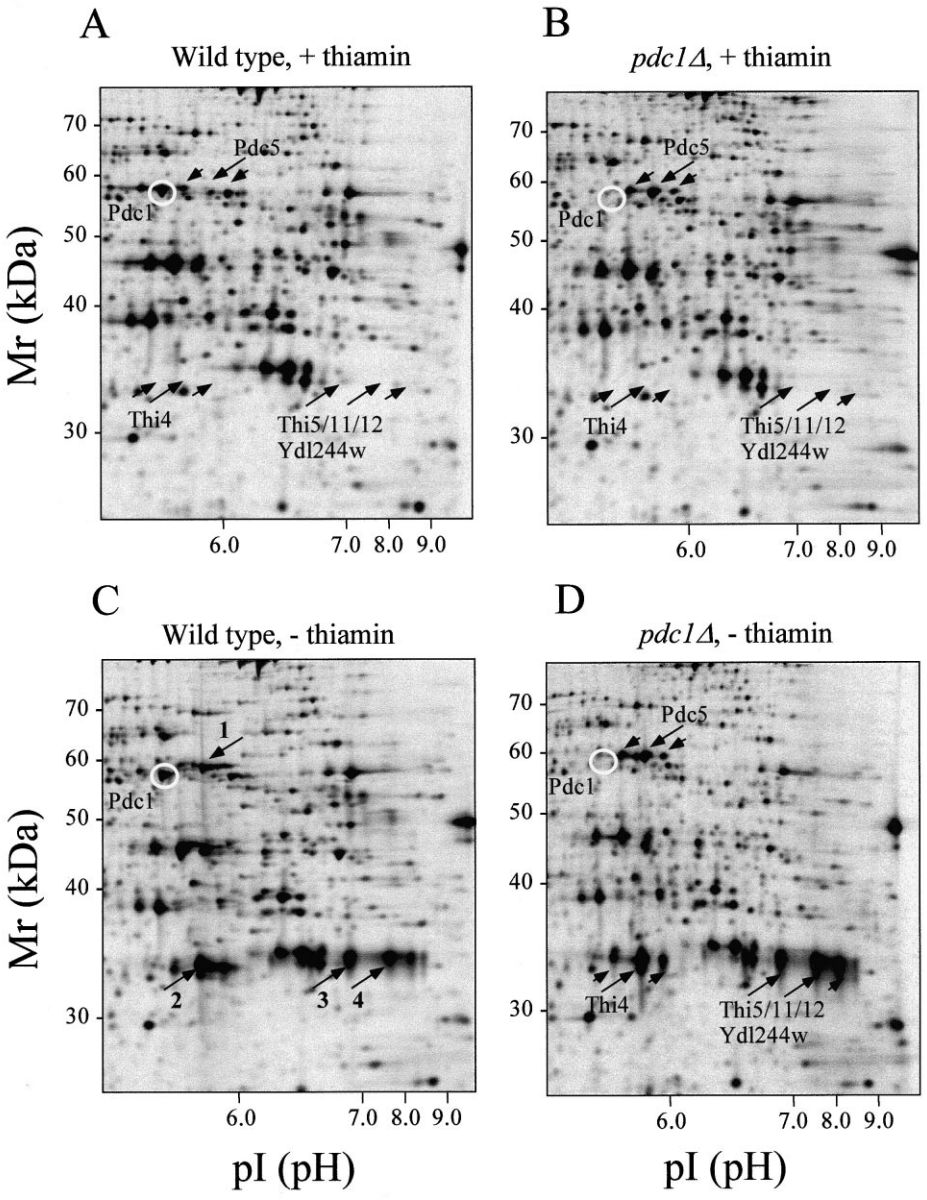

Fig. 2. Two-dimensional gel electrophoresis of total protein extracts of wild type (A and C) and *pdc1*Δ (B and D) cells grown in the presence (A and B) and absence (C and D) of thiamine. Panel C highlights spots 1–4, which were eluted from a preparative gel to determine their identify by mass spectrometry, as detailed in Table 2.

we monitored the promoter activity of *PDC5* in a *thi80-1* mutant. This strain has strongly diminished activity of thiamine pyrophosphokinase and reduced intracellular levels of ThDP and has been reported to derepress *THI* genes even in the presence of thiamine [22,25,31]. After a lag phase of about 3–4 h after glucose addition the promoter activity of *PDC5* increased in the *thi80-1* mutant, but not in the wild type (not shown). During steady-state growth of the *thi80* mutant in the presence of thiamine in 2% glucose medium the promoter activity of *PDC5* reached approximately the same values as in the wild type grown in the absence of thiamine (Table 1). Thus, it seems that a low internal ThDP level triggers expression of *PDC5*, as was observed for the *THI* genes [25].

3.2. Thiamine deficiency also stimulates PDC promoter activity in a pdc1Δ mutant

We have shown previously that the deletion of the *PDC1* gene strongly stimulates the *PDC5* promoter activity. Hence, we next asked if and how thiamine limitation would affect *PDC5* promoter activity in a *pdc1*Δ strain. Therefore the same glucose induction regime in the absence and presence of thiamine was applied to *pdc1*Δ cells harbouring a *PDC5-lacZ* construct (Fig. 1B). Although the difference in promoter activity between the presence and absence of thiamine was much less dramatic than in the wild type, we consistently found that omission of thiamine from *pdc1*Δ cells stimulated the *PDC5* promoter activity even further by 10–30%.

3.3. Cellular responses to thiamine limitation and deletion of PDC1 monitored by two-dimensional gel electrophoresis

To further investigate the relationship between the mechanisms by which thiamine limitation and deletion of *PDC1* stimulate *PDC5* expression we performed two-dimensional gel electrophoresis (2D-PAGE) of whole cell extracts of wild

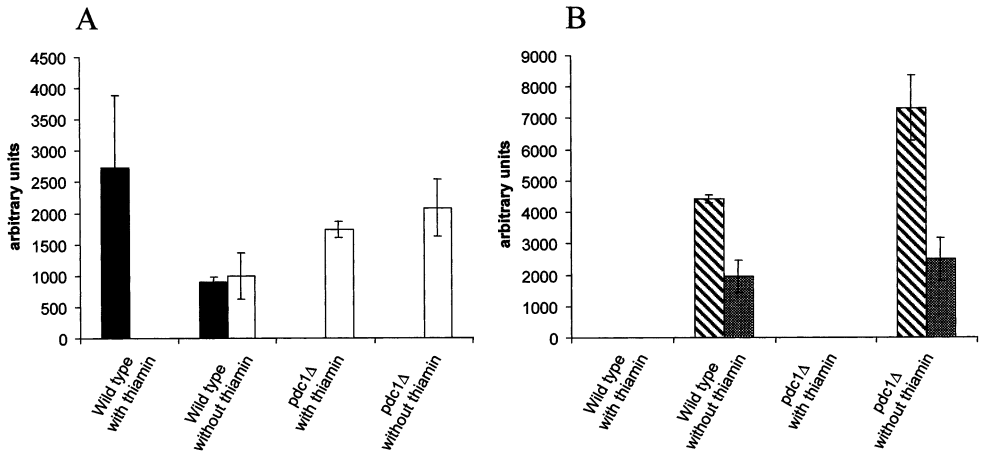

Fig. 3. Computer-assisted densitometric quantification (A) of the Pdc1p (filled bar) and Pdc5p (open bar) spots and (B) of the Thi4p (hatched bar) and a Thi5p (shaded bar) spot. The average and standard deviations of two independent gels are shown.

type and *pdc1*Δ cells grown in the absence and presence of thiamine. Deletion of *PDC1* did not very much alter the pattern of proteins produced, except that the Pdc1p spot [37] disappeared and a new spot appeared in the same area of the gel (Fig. 2A,B; marked as spot 1 in panel C). This new spot was identified by mass spectrometry to correspond to Pdc5p (Table 2).

Thiamine-starved wild type cells showed a significantly different protein expression pattern compared to cells growing in the presence of thiamine. A number of new highly expressed proteins appeared in such cells (Fig. 2C,D). Using mass spectrometry we have identified the prominent spots indicated by numbers in Fig. 2C as Thi4p (spot 2) and as Thi5p/Thi11p/Thi12p/Ydl244p (spots 3 and 4; for identification data see Table 2). *THI4* and *THI5* expression have previously been shown to be derepressed in the absence of thiamine [38]. Thi5p, Thi11p, Thi12p and Ydl244p are more than 99% identical and hence difficult to distinguished with this methodology. However, spot 3 could unambiguously be allocated to Thi12p.

Using computer-assisted densitometry we have quantified the intensity of the spots for Pdc1p, Pdc5p, Thi4p and of one of the Thi5p/Thi11p/Thi12p/Ydl244p spots. The quantification was normalised with respect to the number of methionines in each of the proteins and hence gives a reflection of their molar ratio (Fig. 3). Pdc5p was only detectable either in the *pdc1*Δ strain or in the wild type grown in the absence of thiamine (Fig. 3A). Remarkable, in wild type cells grown in the absence of thiamine, Pdc1p expression was diminished about three-fold and the total Pdc expression (i.e. Pdc1p plus Pdc5p) was not very different from that of wild type cells grown in the presence of thiamine.

Thi4p and Thi5p/Thi11p/Thi12p/Ydl244p were produced only in the absence of thiamine and were undetectable in the presence of thiamine in both wild type and the *pdc1*Δ mutant. However, in the absence of thiamine their production appeared to be somewhat higher in the *pdc1*Δ mutant as compared to the wild type. Remarkably, the Thi proteins studied here are – under thiamine limitation – among the most highly expressed proteins and belong to the same category as the most strongly expressed glycolytic enzymes. Significantly, these proteins, which are required for the biosynthesis of the cofactor ThDP, are about 2-fold more strongly expressed than Pdc1p and Pdc5p. These two proteins are probably the most abundant ThDP-dependent enzymes. The reason for the strong expression of Thi proteins is unclear.

3.4. Thiamine limitation does not increase specific Pdc activity

The observation that the simultaneous expression of *PDC1* and *PDC5* in thiamine-starved wild type cells did not lead to higher Pdc protein production prompted us to test the specific Pdc activity in such cells. Cells were pregrown in ethanol medium and Pdc production was induced by glucose in the same way as shown in Fig. 1. Indeed, specific Pdc activity was

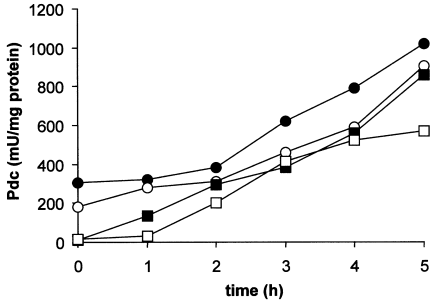

Fig. 4. Glucose induction of specific Pdc activity in wild type (●, ○) and *pdc1*Δ (■, □) cells grown in the presence (filled symbols) or absence of thiamine (open symbols). One out of two independent experiments giving consistent results in two different genetic backgrounds is shown.

Table 1
Relative β-galactosidase activities as a measure for the promoter activity of *PDC1* and *PDC5* under steady-state growth in Wickerham's medium supplemented with 2% glucose and with and without 2 mM thiamine

Strain genotype	Thiamine	Relative β-galactosidase activity (%)
Wild type, *PDC1-lacZ*	+	100 ± 25
Wild type, *PDC1-lacZ*	−	77 ± 7
Wild type, *PDC5-lacZ*	+	1 ± 0
Wild type, *PDC5-lacZ*	−	34 ± 1
thi80, *PDC1-lacZ*	+	97 ± 22
thi80, *PDC5-lacZ*	+	42 ± 4

The strains used are in YPH499 background. The average and standard deviation of eight measurements are shown. The activity of the wild type expressing *PDC1-lacZ* in the presence of thiamine was set at 100%.

Table 2
Identification of the protein spots indicated by numbers in Fig. 2C. Trypsin generated peptide masses was subjected to MS-Fit searches in the non-redundant *S. cerevisiae* database

2D spot[a]	Top ranked protein	Hits in database: masses[b] matched/masses submitted	MOWES score[c]: first on list (second on list)	Experimental[d] MW/p*I*	Theoretical[e] MW/p*I*
1	Pdc5p	5/8	3×10^3 (69)	57.6/5.9	62.0/6.14
2	Thi4p	6/13	2×10^3 (51)	33.9/5.9	35.0/5.8
3[f]	Thi5p	8/14	1×10^5 (68)	34.0/7.3	38.6/8.2
	Thi11p				38.6/8.0
	Thi12p				38.6/8.4
	Ydl244p				38.6/8.2
4[f]	Thi5p	9/15	4×10^5 (176)	34.0/8.1	38.6/8.2
	Thi11p				38.6/8.0
	Thi12p				38.6/8.4
	Ydl244p				38.6/8.2

[a]The numbering of 2D resolved spots is indicated in Fig. 3C.
[b]Number of peptide masses matching the database entry compared to the total number of masses applied to the search.
[c]The MOWES score is a means to compensate for the influence of random score accumulation in large proteins [39]. The higher the score the better the reliability of identification.
[d]Determined from the 2D position according to [35].
[e]Obtained from Yeast Protein Database (YPD; http://www.proteome.com). The order for MW/p*I* values for 2D spots 3 and 4 is the same as for proteins in column two.
[f]For both these spots there were four top candidates. The four proteins are almost identical. However, for spot number 3 (Fig. 2C) a Thi12p unique peptide was identified (amino acids 241–248) with the experimental mass 1067.5 (*m/z*).

not increased but rather slightly diminished in the absence of thiamine (Fig. 4). Identical results were obtained in another genetic background, W303-1A (data not shown).

4. Discussion

We show here that the level of thiamine in the growth medium controls the expression of the *PDC5* gene. This is remarkable since the enzyme encoded by *PDC5*, the minor isoform of Pdc, is not thought to be involved in the production of ThDP but rather uses ThDP as cofactor.

We have now identified three conditions that affect expression of *PDC5*. It seems that the presence of glucose is absolutely required but not sufficient for high level expression of *PDC5* [13,14,18]. In addition, either external thiamine must be absent, as shown here, or the gene for the major Pdc isoform *PDC1* must be deleted [13,14,18]. How these signals in concert control the *PDC5* promoter is under investigation.

We have studied the control of expression of the genes *PDC1* and *PDC5* by thiamine in search of the signal that mediates autoregulation of *PDC* gene expression. Previous work has shown that autoregulation, most conveniently studied by the strong stimulation of *PDC5* expression in a *pdc1*Δ strain, does not involve the catalytic activity of Pdc but an alternative function [2,19,22]. Deletion of *PDC1*, which abolishes the probably main ThDP-dependent enzyme, could lead to higher internal ThDP levels. However, although we find expression of *PDC5* to be controlled by thiamine, it is repressed rather than induced by thiamine. This suggests that autoregulation is not a direct consequence of a signal generated by thiamine or ThDP levels.

This conclusion is supported by additional observations. Significantly, it appears that thiamine depletion only affects expression of *PDC5* but not that of *PDC1*. This is in contrast to deletion of the coding region of *PDC1*, which strongly stimulates the promoters of both *PDC5* and *PDC1* [13,17,20,21]. In addition, 2D-PAGE demonstrates that deletion of *PDC1* significantly stimulates only the production of one detectable protein in the cell, Pdc5p. Thiamine depletion, on the other hand, has pronounced effects on a number of proteins in addition to Pdc5p. Taken together these data strongly argue that deletion of *PDC1* causes a signal that specifically stimulates expression of the *PDC* genes, and that signal seems to be independent of catalysis [19] and of the level of the cofactor ThDP. Also based on the analysis of mutations of *PDC1*, which encode catalytically inactive but active regulatory proteins (i.e. which still repress *PDC5* [19]), we presently favour a scenario in which a certain conformation of the enzyme is recognised by the autoregulatory mechanism, which transfers a signal to the nucleus in an unknown fashion.

Our data provide evidence that the signal for the control of *PDC5* expression by thiamine is related to ThDP rather than thiamine itself. A *thi80-1* mutant, which has been shown previously to accumulate lower levels of ThDP than the wild type due to diminished thiamine pyrophosphokinase activity [22,25], stimulates expression of *PDC5* even in the presence of external thiamine. A possible link between the control of the *THI* genes and that of *PDC5* may be Pdc2p, a known regulator of the *PDC* genes, which has recently been isolated as a positive regulator of the *THI* genes as well [18,22].

The simultaneous expression of *PDC1* and *PDC5* under thiamine limitation does not lead to higher total Pdc protein production nor to higher specific Pdc activity in the cell. The cumulative β-galactosidase-specific activity as a measure for the promoter activity of *PDC1* and *PDC5* suggested a higher Pdc protein and hence activity level, especially when considering the glucose induction experiments. The 2D-PAGE data show that under thiamine limitation Pdc1p is produced to lower levels. It is known that Pdc from brewer's yeast requires ThDP for stability, that binding of the cofactor causes major conformational changes to the enzyme, and that it is required for oligomerisation [3]. Hence, under thiamine limitation it is well possible that a portion of Pdc1p is degraded shortly after production due to the lack of ThDP and hence an inability to oligomerise. If this idea is correct it appears from our data that Pdc5p might be less affected by thiamine limitation, possibly because the enzyme has a higher affinity to ThDP.

The apparently diminished level of Pdc1p under thiamine limitation suggests an alternative possibility for how thiamine

limitation could lead to derepression of *PDC5* expression. While we can exclude that autoregulation controls *PDC5* expression via a ThDP limitation signal, the opposite scenario, i.e. stimulation of *PDC5* expression by low thiamine via an autoregulatory signal due to Pdc1p instability, is not impossible. In this scenario, however, one would expect thiamine limitation to also stimulate the promoter activity of *PDC1*, which was not observed in this study. This could, however, be attributed to the fact that Pdc1p is not completely absent and hence that the level of Pdc1p affects promoter activity of *PDC1* and *PDC5* differently in quantitative terms.

Acknowledgements: E.M. gratefully acknowledges a pre-doctoral fellowship from the Swedish Institute. Parts of this work were supported by the Commission from the European Union via Contract BIO4-CT95-0161 to S.H. Work in the group of P.A.M. was supported by Grants UK BBSRC 91/G08756 and 91/P03151. A.B. acknowledges support from the Swedish Research Council for Technical Sciences, TFR.

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