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The Role of the *TRP1* Gene in Yeast Tryptophan Biosynthesis*

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Transcription of the gene for phosphoribosyl-anthranilate isomerase (*TRP1*) from the *TRP1* promoter is initiated only approximately half as frequently as, for example, from the *TRP3* promoter, but *TRP1* mRNA is approximately twice as stable as *TRP3* mRNA. Therefore, the steady state amount of *TRP1* mRNA in yeast cells, grown without amino acid limitation, is similar to the steady-state amount of *TRP3* mRNA. The protein concentration of both enzymes in yeast cells is about the same, but the basal specific enzyme activity in permeabilized cells of the *TRP1* gene product *N*-(5′-phosphoribosyl-1)-anthranilate isomerase is about 2–3 times higher than that of any of the other *TRP* enzymes. According to the kinetic parameters of the purified isomerase protein, the enzyme is more active than, for example, the purified *TRP3* enzyme indoleglycerol-phosphate synthase. It is suggested that the *TRP1* gene of *Saccharomyces cerevisiae* might be the result of a rearrangement event, separating the *N*-(5′-phosphoribosyl-1)-anthranilate isomerase domain from the indoleglycerol-phosphate synthase domain and putting the catalytically more active isomerase domain behind a weak and nonregulated constitutive promoter.

The *de novo* biosynthesis of tryptophan in all prokaryotic and eukaryotic organisms studied so far proceeds through an invariable series of reactions. A set of seven enzymatic activities is necessary to perform the five biosynthetic steps from chorismic acid to tryptophan (Crawford, 1975). In bacteria, the genes that encode the tryptophan biosynthetic enzymes are combined in clusters of one, two, or three transcriptional units on the chromosome (Crawford, 1975). In contrast, in all eukaryotic microorganisms studied so far, the *TRP* genes are scattered over the genome. On the other hand, the encoded enzymes appear to be more highly organized in eukaryotic than in prokaryotic organisms, resulting in multifunctional proteins. Different patterns of fusion have produced multifunctional enzymes with different combinations of functional domains (Hütter *et al.*, 1986).

In most ascomycetes, four genes encode the seven functional domains of the tryptophan pathway. One of these genes codes for a trifunctional polypeptide, NH_2-glutamine amidotransferase-InGP synthase[1]-PRA isomerase-COOH. Several genes with this arrangement have been cloned and characterized further from different ascomycetes such as *Neurospora crassa* (Schechtman and Yanofsky, 1983), *Aspergillus nidulans* (Mullaney *et al.*, 1985), *Aspergillus niger* (Kos *et al.*, 1985), *Cochliobolus heterostrophus* (Turgeon *et al.*, 1986), and *Penicillium chrysogenum* (Sánchez *et al.*, 1986). On the contrary, the yeast *Saccharomyces cerevisiae* carries five genes for tryptophan biosynthesis instead of four (Braus *et al.*, 1985). No gene was found coding for the trifunctional polypeptide: the two functions glutamine amidotransferase and InGP synthase are encoded by the *TRP3* gene (Aebi *et al.*, 1984), whereas PRA isomerase is encoded by the separate *TRP1* gene (Tschumper and Carbon, 1980).

The *TRP1* gene is of special interest because a yeast *ARS* (autonomously replicating) sequence is located adjacent to the 3′-end of the *TRP1* gene (Beggs, 1978). Therefore, the *TRP1* gene is used in many yeast vectors as a selectable marker.

The yeast tryptophan pathway is part of the complex general control regulatory network which couples the derepression of at least 30 structural genes (involved in multiple amino acid biosynthetic pathways) to starvation for any one of a number of different amino acids (Schürch *et al.*, 1974; Hinnebusch, 1986). Four of the five *TRP* genes of *S. cerevisiae* can be derepressed under the general control. The *TRP1* gene is the only exception; its expression is not regulated by amino acid availability (Miozzari *et al.*, 1978a). It has been demonstrated that binding of the *GCN4* activator protein to the promoter regions of the genes regulated by general control causes an increased initiation of transcription (Hill *et al.*, 1986; Hope and Struhl, 1985; Arndt and Fink, 1986). The *TRP1* promoter does not bind the *GCN4* activator protein (Hope and Struhl, 1985) and represents an example of a constitutive, weakly expressed promoter of a structural gene (Kim *et al.*, 1986).

The *TRP1* gene product PRA isomerase catalyzes a practically irreversible Amadori rearrangement, the third step in tryptophan biosynthesis. The basal enzyme activity of the PRA isomerase in permeabilized cells was observed to be higher than that of all the other *TRP* enzymes (Niederberger *et al.*, 1984).

The aim of this work is the analysis of the role of the *TRP1* gene and its gene product PRA isomerase in the tryptophan biosynthesis of *S. cerevisiae*. The expression of the *TRP1* gene

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[1] The abbreviations used are: InGP synthase, indoleglycerol-phosphate synthase; PRA isomerase, *N*-(5′-phosphoribosyl-1)-anthranilate isomerase; CDRP, 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate; kb, kilobase; bp, base pair; PRPP, 5-phosphoribosyl 1-pyrophosphate; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; GAP, glyceraldehyde 3-phosphate; ONPG, *o*-nitrophenyl β-D-galactoside; SDS, sodium dodecyl sulfate; FPLC, fast protein liquid chromatography.

was compared with that of the normally regulated *TRP3* gene. The rate of transcription initiation (promoter strength) of both promoters, the half-life of the mRNAs, and the total amount of transcripts were determined and compared. Furthermore, the *TRP1* gene product PRA isomerase was purified, characterized, and compared to the purified *TRP3* gene product InGP synthase (Prantl *et al.*, 1985).

EXPERIMENTAL PROCEDURES[2]

RESULTS

TRP1 and TRP3 Promoter Expression—As a first approach to compare the transcription initiation frequencies of the *TRP1* and *TRP3* promoters, *lacZ* fusions expressed by both *TRP* promoters were constructed. β-Galactosidase is a convenient enzyme for constructing translational fusions because removal of the first 27 amino acid codons does not affect β-galactosidase activity (Bassford *et al.*, 1978; Guarente, 1983). As basic vectors, the pNM480/1/2 plasmids described by Minton (1984) were used. So that the *TRP1* and *TRP3* fusion proteins would be similar, *TRP* gene fragments carrying the complete promoter, the start codon plus a similar number of amino-terminal codons, were cloned upstream of the promoterless *lacZ* gene. The resulting *TRP1-lacZ* and *TRP3-lacZ* fusion proteins contained 29 and 30 amino-terminal amino acids of PRA isomerase and glutamine amidotransferase, respectively. Both *TRP-lacZ* fusions were finally cloned into the yeast low copy number *CEN4* vector YCp50 (Johnston and Davis, 1984) in order to prevent significant fluctuations in the copy number. The final plasmids were named pME587 and pME588 (Fig. 1).

The derived clones were identified by their blue color on 5-bromo-4-chloro-3-indolyl β-D-galactoside indicator plates with *Escherichia coli lac* deletion strain MC1061 (Casadaban *et al.*, 1983) as host. Before transformation in yeast, the nucleotide sequences of the *TRP-lacZ* linkage regions of the final clones pME587 and pME588 were determined as described under "Experimental Procedures." The sequence confirmed that the gene fusions had preserved the reading frames (data not shown).

The final constructs pME587 (*TRP1-lacZ*) and pME588 (*TRP3-lacZ*) or the parental vector YCp50 was transformed into the yeast strains RH1244 (*ura3-251 ura3-328 ura3-378*) and RH1310 (*ura3-251 ura3-328 ura3-378 gcd2-1*). The specific β-galactosidase activity of the plasmid-carrying yeast strains was assayed, and the results are shown in Table I.

The level of β-galactosidase directed by the *TRP1-lacZ* fusion gene was less than half of the level directed by the nonderepressed *TRP3-lacZ* fusion. The constitutive regulatory mutation *gcd2-1* in the yeast strain RH1310 was used as a control. As for the original genes, this mutation caused derepression of the *TRP3-lacZ* gene, but had no effect on the *TRP1-lacZ* fusion.

Comparative Analysis of TRP1 and TRP3 Transcripts—The results obtained from *TRP-lacZ* fusions could reflect effects either on transcription or on translation, or on both. In order to distinguish between these possibilities, the *TRP1* and *TRP3* transcripts were further analyzed.

The half-lives of *TRP1* and *TRP3* mRNAs were determined by quantitative hybridization of yeast RNA labeled *in vivo* against plasmid DNA. Exponentially growing cultures of the

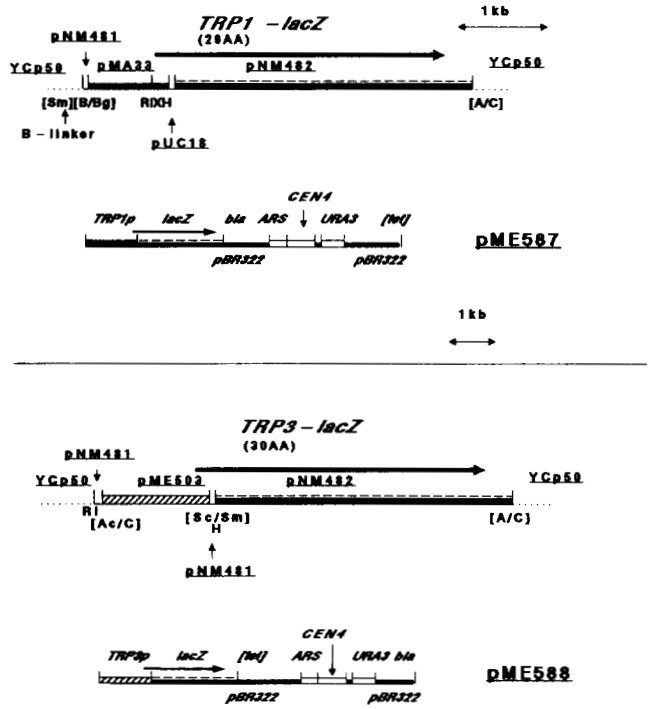

FIG. 1. ***TRP1-lacZ* and *TRP3-lacZ* fusions.** The plasmids pME587 and pME588 are based on the pBR322 derivative *CEN4 URA3* plasmid YCp50. The entire plasmids (*lower line*) and the *TRP-lacZ* fusions in detail (*upper line*) are shown. The different fragments are indicated by the plasmid designations (*underlined*) from which they are derived. *E. coli* sequences are indicated as *black boxes*; yeast sequences are boxed with different patterns for *TRP1*, *TRP3*, and other yeast sequences. The origin of the plasmids is described under "Experimental Procedures." For pME587, the *TRP1* promoter fragment (*Bgl*II (≈−850)-*Xba*I (+85)) from pMA33 was inserted into the multiple cloning site of pUC18. The promoter was recloned as an *Sma*I-*Hin*dIII fragment into pNM481, and a *Bam*HI linker was inserted into the *Sma*I site. After recloning the *TRP1* promoter as a *Bam*HI-*Hin*dIII fragment into pNM482, the *TRP1-lacZ* fusion was inserted as a *Bam*HI-*Asu*II fragment into YCp50. For pME588, the *TRP3* promoter fragment (*Eco*RI (pBR322 sequence, including the ≈900-bp 5′-upstream region of *TRP3*)-*Sca*I (+88)) from pME503 was inserted into the multiple cloning site of pNM481. The *Eco*RI-*Asu*II fragment was cloned into YCp50. The *TRP3* promoter was recloned as a *Cla*I-*Hin*dIII fragment into pNM482. Finally, the *TRP3-lacZ* fusion was cloned as an *Eco*RI-*Asu*II fragment into YCp50. *Ac*, *Acc*I; *A*, *Asu*II; *B*, *Bam*HI; *Bg*, *Bgl*II; *C*, *Cla*I; *H*, *Hin*dIII; *RI*, *Eco*RI; *Sc*, *Sca*I; *Sm*, *Sma*I; *X*, *Xba*I; *AA*, amino acids.

TABLE I

β-Galactosidase activity directed by TRP1-lacZ and TRP3-lacZ fused genes

Strain	β-Galactosidase activity[a]
	milliunits/mg protein
RH1244 (pME587/*TRP1-lacZ*)	0.3
RH1310 (pME587/*TRP1-lacZ*)	0.3
RH1244 (pME588/*TRP3-lacZ*)	0.8
RH1310 (pME588/*TRP3-lacZ*)	1.5
RH1244 (YCp50)	<0.05[b]
RH1310 (YCp50)	<0.05

[a] Values of at least three independent cultivations, each measured twice; the standard deviation did not exceed 20%.

[b] −1 frameshift mutations in the *TRP1-lacZ* and *TRP3-lacZ* fusions also resulted in a very low, barely detectable β-galactosidase activity (data not shown).

[2] Portions of this paper (including "Experimental Procedures," Fig. 1S, and Table 1S) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

plasmid-carrying strain RH962 (pME582) with the plasmid-encoded complete *TRP1* and *TRP3* genes were radioactively labeled with [^{3}H]uracil for 15 min, followed by a chase with cold uracil. Total RNA was isolated from samples at the time points indicated (Fig. 2) and hybridized to filter-bound, saturating amounts of *TRP1* and *TRP3* DNAs, and the specifically bound radioactivity was determined. Hybridization to filters carrying pBR322 DNA was used as a control for nonspecific hybridization. The half-life of the mRNAs was calculated from the slope of semilog plots.

The half-life of the *TRP3* mRNA (length = 1.75 kb) was 11 min. For the smaller *TRP1* transcript (length = 0.8–1.0 kb), the half-life was 19 min, indicating a higher stability of the *TRP1* mRNA than that of the larger *TRP3* mRNA.

The steady-state amounts of *TRP1* and *TRP3* mRNAs were determined by quantitative Northern hybridization. In order to obtain radioactive probes of the same specific radioactivity and to avoid uncertainties arising from the different lengths of the *TRP1* and *TRP3* transcripts (Fig. 3), two internal fragments of the structural genes of almost the same size were used as probes: the 661-bp *Hin*fI-*Xba*I fragment of the *TRP3* gene (Zalkin *et al.*, 1984) and the 651-bp *Xba*I-*Pst*I fragment of the *TRP1* gene (Tschumper and Carbon, 1980). Both fragments were isolated from plasmid pME581 simultaneously, labeled by nick translation, and cohybridized against poly(A)$^+$ RNA bound to a nitrocellulose membrane. The specific transcripts were revealed by autoradiography and cut out from the nitrocellulose filters for quantitative determination of radioactivity. Results are shown in Fig. 3. The relative values of the basal mRNA levels of both *TRP1* and *TRP3* genes were within the same range for the chromosomally (strain X2180-1A; *lane 1*) as well as for the plasmid-encoded (strain RH962 (pME582); *lane 3*) genes. The high copy number resulted in higher absolute values for both plasmid-derived transcripts. If one compares the total amount of mRNA of the chromosomal *TRP3* gene in strain X2180-1A, expressed under nonderepressing conditions (*lane 1*), and

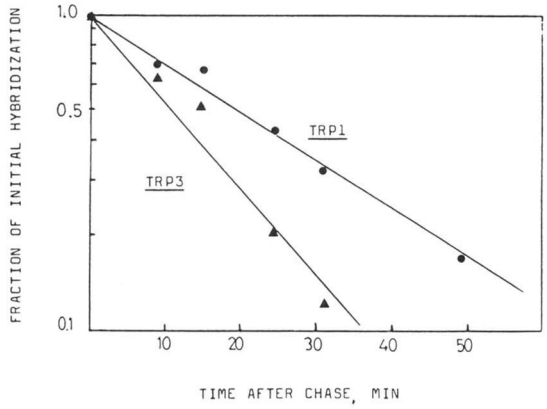

FIG. 2. **Decay rates of yeast *TRP1* and *TRP3* mRNAs.** RNA of strain RH962 (pME582) carrying the plasmid-encoded complete *TRP1* and *TRP3* genes was pulse-labeled for 15 min with 50 μCi of [^{3}H]uracil (53 Ci/mmol)/ml of yeast culture followed by a chase of unlabeled uracil. RNA was extracted from samples taken at intervals thereafter and hybridized to filter-bound pME503 (Aebi *et al.*, 1984; 2.5-kb *TRP3* *Cla*I-*Bam*HI fragment on pBR322), YRp7 (1.45-kb *TRP1* *Eco*RI fragment on pBR322), and pBR322 as a control for nonspecific hybridization. Hybridization is shown as a fraction of the total measured radioactivity isolated at each time point. At least 10^6 cpm were applied to each filter. The 100% values of radioactivity (after substraction of nonspecific pBR322 hybridization) in *TRP3* mRNA were 1300 cpm and in *TRP1* mRNA were 900 cpm (mean of two filters each ±15%). *TRP3* mRNA, $t_{½}$ = 11 min; *TRP1* mRNA, $t_{½}$ = 19 min.

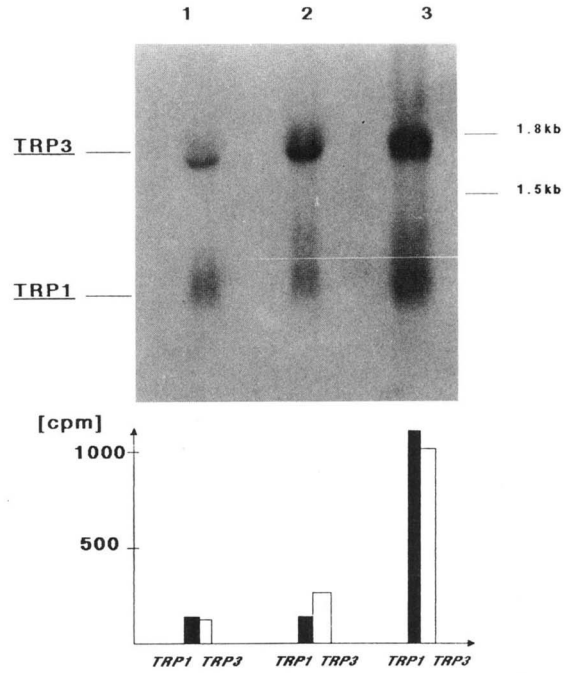

FIG. 3. **Comparative quantitative Northern analysis of transcripts of *TRP1* and *TRP3* genes.** 40 μg of poly(A)$^+$ RNA of *S. cerevisiae* strain X2180-1A (*lane 1*) and RH558-1 (*gcd2-1*) (*lane 2*) and 20 μg of poly(A)$^+$ RNA of the plasmid-carrying strain RH962 (pME582) (*lane 3*) were cohybridized against the radioactively labeled 661-bp *Hin*fI-*Xba*I *TRP3* and 651-bp *Xba*I-*Pst*I *TRP1* fragments of pME581. The radioactivity of the different transcript signals (average of at least three independent experiments) was measured as described under "Experimental Procedures." Transcript size of *TRP1* mRNA was 0.8–1.0 kb and of *TRP3* mRNA was 1.75 kb.

in the constitutively derepressed mutant strain RH558-1 (*gcd2-1*) (*lane 2*), the increase in mRNA correlated well with the derepression factors observed with the *TRP3-lacZ* fusion (Table I) and the gene product InGP synthase (Fig. 4). This served as a control for equivalent poly(A)$^+$ enrichment of both RNA preparations. As expected, no increase in the amount of *TRP1* mRNA could be found under the genetic derepression signal.

According to the data presented, the similar total amounts of *TRP1* and *TRP3* transcripts present in yeast cells can be explained in the following way: the lower initiation of transcription of *TRP1* than that of *TRP3* is compensated by the higher stability of the corresponding RNA, resulting in the same total amount of mRNA for the two genes under nonderepressed conditions.

Characteristics of the TRP1 Gene Product PRA Isomerase—The data on *TRP1* promoter and mRNA do not explain the higher specific enzyme activity observed for the *TRP1* gene product PRA isomerase in frozen and thawed, detergent-treated cells as compared to other *TRP* gene products (*e.g.* InGP synthase) (Fig. 4). Assuming equal numbers of PRA isomerase and InGP synthase molecules present in the cells, there are two possible explanations. 1) The high specific PRA isomerase activity in the enzyme assay is a special feature of this particular *in vitro* assay and does not correlate to the corresponding situation *in vivo*. 2) The PRA isomerase molecule is a more active enzyme with a higher specific activity than that of the other *TRP* enzymes.

Enzyme Assays for PRA Isomerase Activity in Permeabilized Cells—Four of the *TRP* enzymes showed a similar basal specific activity at 30 °C (ranging from 1 to 2 milliunits/mg of protein) and could be derepressed by amino acid limitation to approximately 3 milliunits/mg of protein. The only excep-

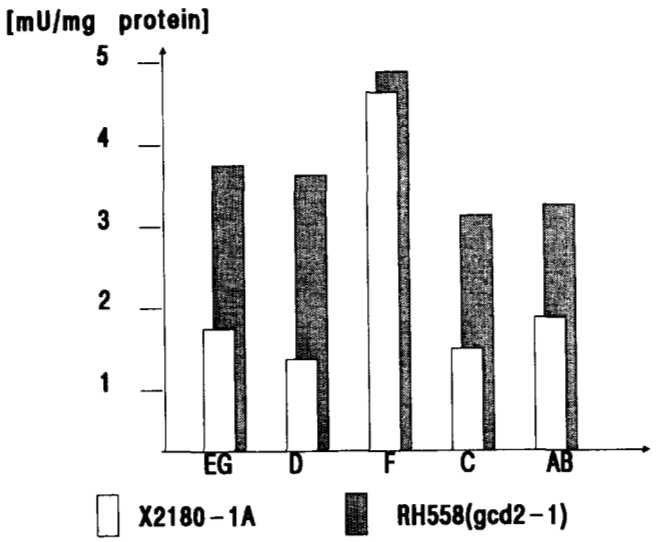

FIG. 4. **Specific enzyme activity of *TRP* enzymes in permeabilized yeast cells.** Cells of *S. cerevisiae* stains X2180-1A and RH558-1 (*gcd2-1*) were permeabilized with Triton X-100, and the specific enzyme activity of all chromosomally encoded *TRP* enzymes was determined. Anthranilate synthase (*EG*) and anthranilate phosphoribosyltransferase (*D*) activities were determined by measuring the change in concentration of anthranilate; PRA isomerase (*F*), InGP synthase (*C*), and tryptophan synthase (*AB*) activities were determined by measuring the change in concentration of InGP as described under "Experimental Procedures." The given values are means of two independent cultivations, and each one was measured twice (the standard deviation did not exceed 20%).

TABLE II

Specific enzyme activity of chromosomally encoded PRA isomerase measured by two different assays in permeabilized cells

S. cerevisiae strain	Specific PRA isomerase activity[a]
	milliunits/mg protein
Measurement of product increase[b]	
X2180-1A	3.0 (4.5)
RH558-1 (*gcd2-1*)	3.1 (4.6)
Measurement of educt decrease[c]	
X2180-1A	3.1
RH558-1 (*gcd2-1*)	3.1

[a] Values are averages of at least two independent cultivations, each measured twice (S.D. ≤ 20%).

[b] The product CDRP was transformed quantitatively to InGP; InGP was measured optically as described under "Experimental Procedures." This stop assay was carried out at 25 °C. The values in parentheses correspond to the values shown in Fig. 4 (30 °C).

[c] The decrease in enzymatically synthesized PRA was measured fluorometrically as described under "Experimental Procedures." This kinetic assay was carried out in a constant temperature cuvette (25 °C) and directly measured.

tion was the *TRP1* gene product PRA isomerase with a nonderepressible, high basal enzyme activity of 4.5 milliunits/mg of protein at 30 °C (Fig. 4; 3.0 milliunits/mg of protein at 25 °C; Table II). In the standard enzyme assay, the highly unstable substrate PRA was prepared nonenzymatically immediately before use from anthranilate and ribose 5-phosphate (Creighton, 1968). The enzyme assay takes advantage of the fact that two relevant reactions are practically irreversible: 1) PRA to CDRP conversion catalyzed by the PRA isomerase itself; and 2) the ring closure to the indole nucleus: CDRP to InGP, CO_2, and H_2O conversion catalyzed by the *TRP3* gene product InGP synthase. By adding enough InGP synthase, the PRA isomerase activity is rate-limiting and can be determined by measuring the increase in the product InGP.

An alternative assay of PRA isomerase activity is the measurement of the decrease of the educt PRA. In the assay developed by Hommel and Kirschner,[3] anthranilate and an excess of 5-phosphoribosyl 1-pyrophosphate were completely converted to PRA by the purified *TRP4* gene product anthranilate phosphoribosyltransferase. The spontaneous decomposition of PRA to anthranilate was balanced by recycling the anthranilate to PRA with anthranilate phosphoribosyltransferase and excess 5-phosphoribosyl 1-pyrophosphate. After zero adjustment of the fluorescence and addition of PRA isomerase, the decrease in PRA concentration was followed fluorometrically. This second PRA isomerase test resulted in values of about 3 milliunits/mg of protein at 25 °C, which are comparable to the data of the standard enzyme assay (Table II).

Purification and M_r Determination of the PRA Isomerase—In order to ascertain whether the *TRP1* gene product PRA isomerase catalyzes its reaction more effectively than the other *TRP* enzymes, the protein was purified from strain RH218 (YARp1). This transformant expresses PRA isomerase at about 100-fold higher levels than the wild-type strain (see Miniprint for detailed description of purification of PRA isomerase). A M_r value of approximately 23,000 was calculated from the elution profile of the purified polypeptide from gel filtration of a Superose 12 column. The same value was obtained by analytical ultracentrifugation of pure enzyme[4] in good correlation with the values derived from sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the known nucleotide sequence (Tschumper and Carbon, 1980). These data strongly suggested that the PRA isomerase is a monomer.

Kinetic Studies of the Purified PRA Isomerase—In order to determine V_{max} and K_M, the relevant V_i values were measured by steady-state kinetic experiments. Different limiting concentrations of PRA were enzymatically synthesized (as described above and under "Experimental Procedures"). The initial substrate concentrations ($[S_0]$) in the range of 0.5–5 × K_M were calculated from the spectrophotometrically determined initial anthranilate concentrations (E_{310} = 2.98 mM^{-1} cm^{-1}; E_{240} = 7.17 mM^{-1} cm^{-1}). The decrease of PRA was measured fluorometrically to determine the initial velocities (V_i).

On the basis of these data, a K_M value for PRA of ≈5 μM was determined. With the V_{max} value and the determined protein concentration of the pure enzyme ($[e_0]$), the turnover number (catalytic constant $k_{cat} = V_{max}/[e_0]$) was calculated to be k_{cat} = 60/s. This leads to $k_{cat}/K_M = 1.25 \times 10^7$ μM^{-1} s^{-1}, which is only 50-fold smaller than the diffusion-controlled maximum (Fersht, 1985).

Under "Discussion," these values will be compared with the corresponding values of the *TRP3* gene product InGP synthase.

DISCUSSION

The *TRP1* gene is expressed from a constitutive, unregulated promoter. The *TRP1* promoter is less efficient than the *TRP3* promoter in initiating mRNA synthesis as measured in *lacZ* fusions under the same growth conditions. On the other hand, the *TRP1* transcript (0.8–1.0 kb) is about twice as stable as the *TRP3* transcript (1.75 kb).

A simple explanation for these differences is a reciprocal relationship between mRNA stability and length as proposed by Santiago *et al.* (1986). These authors suggest that mRNA length and at least one additional factor strongly influence

[3] U. Hommel and K. Kirschner, personal communication.

[4] A. Lustig, personal communication.

mRNA stability in yeast. Longer mRNAs presumably present a larger target for an initial random endonucleolytic cut followed by rapid degradation.

The total amount of mRNA at the steady state, as determined by quantitative Northern hybridization, is similar for both genes. Assuming that the messenger stability reflects functional activity, the experimental data can be explained as the compensation between different promoter strengths (*TRP3* promoter approximately twice as strong as *TRP1* promoter) and different mRNA stabilities (*TRP1* transcript twice as stable as *TRP3* transcript) for the two genes. Therefore, a different rate of translation initiation of both genes seems unlikely.

In *S. cerevisiae*, the concentration of the small PRA isomerase protein (M_r = 23,000) is very low. The PRA isomerase was purified from a 100-fold overproducing strain by total 15,000-fold enrichment relative to the chromosomally encoded gene. Therefore, the enzyme comprises not more than 0.007% of total cytoplasmic protein in a wild-type yeast cell. The corresponding fraction of InGP synthase/anthranilate synthase (M_r = 122,000) comes to 0.05% (Prantl *et al.*, 1985). Considering the different molecular weights of both enzymes, PRA isomerase and InGP synthase have comparable numbers of protein molecules per yeast cell. These data are in good agreement with the previous assumption that the translation efficiency for both genes is similar.

During hydroxylapatite chromatography, the PRA isomerase was eluted as a single component. The enzyme of M_r = 23,000 was shown to be a monomer under physiological salt concentrations.[4]

The basal enzyme activity of the *TRP1* gene product PRA isomerase is 2–3 times higher than that of the other *TRP* gene products. The comparatively high enzyme activity was found independently in two assays, in which product formation or substrate decrease was measured. The high basal enzyme level of the PRA isomerase cannot be explained by a higher transcription or translation rate of the *TRP1* gene.

Table III compares the kinetic parameters of the purified yeast PRA isomerase with those of the purified yeast InGP synthase (Prantl *et al.*, 1985). The lower Michaelis-Menten constant (K_M) as a parameter for the affinity for its substrate, the higher catalytic constant (k_{cat}) as a parameter for the turnover of the substrate, as well as the higher value of k_{cat}/K_M characterize PRA isomerase as being more efficient than InGP synthase.

TABLE III

Steady-state kinetics of PRA isomerase and InGP synthase in yeast

Enzyme	k_{cat}	K_M	k_{cat}/K_M
	s^{-1}	μM	$M^{-1}\ s^{-1}$
PRA isomerase	50	4	1.25×10^7
InGP synthase[a]	2	250	8.0×10^3

[a] Values were determined at 30 °C in the complex with the *TRP2* gene product according to Prantl *et al.* (1985).

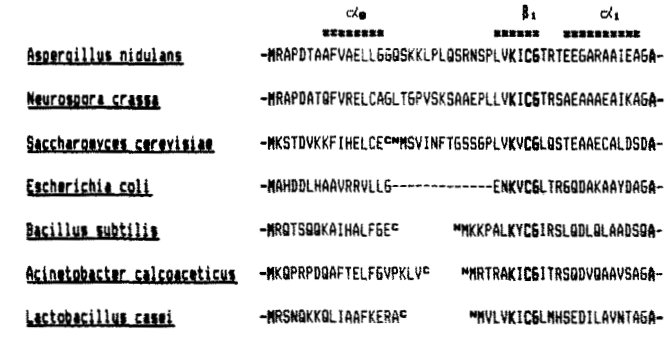

FIG. 5. **Alignment of amino acid sequences in different organisms of corresponding InGP synthase-PRA isomerase connector regions and NH_2 (indicated by superior *N*) and COOH (indicated by superior *C*) termini of separated genes (Priestle *et al.*, 1987).** Identical amino acids are written in ***boldface*** *letters*. Sequences necessary for the formation of the α/β barrel are indicated by the *top line*.

In *E. coli*, InGP synthase and PRA isomerase are fused in a bifunctional enzyme (without the glutamine amidotransferase domain). The kinetic constants of the purified *E. coli* enzyme agree well with the data found for the yeast enzymes (Kirschner *et al.*, 1980; Kirschner *et al.*, 1987). It has been shown that the two active sites of the bifunctional *E. coli* enzyme are independent and nonoverlapping. Neither channeling of the intermediate CDRP nor cooperative interactions between the two active sites seem to occur (Bisswanger *et al.*, 1979; Cohn *et al.*, 1979; Kirschner *et al.*, 1980). Both functional domains are structured as 8-fold α/β barrels (Priestle *et al.*, 1987) as reported for about a dozen different enzymes (Muirhead, 1983; Lindqvist and Bränden, 1985). The two active sites do not face each other, making any channeling of the substrate between active sites virtually impossible (Priestle *et al.*, 1987). Examination of a multiple sequence alignment for the known PRA isomerase and InGP synthase enzymes from various organisms (including *S. cerevisiae*) is consistent with the notion that all PRA isomerases and InGP synthases have the same topological fold of α/β barrels (Priestle *et al.*, 1987). Thus, in the case of InGP synthase and PRA isomerase, gene fusion seems to affect the respective catalytic efficiencies of either enzyme only to a limited extent.

One question that arises is whether the *TRP1* gene of *S. cerevisiae* resulted from a rearrangement event. Due to the long evolutionary periods involved in the formation of a certain gene arrangement, it is impossible to prove its mode of generation in any specific organism experimentally. For eukaryotic cells, a common hypothesis is that multifunctional proteins encoded by single genes are formed concomitantly with higher development (Hütter *et al.*, 1986). The formation of multifunctional enzymes may confer evolutionary advantages to an organism, such as simultaneous regulation of gene expression, equimolar synthesis of all enzymatic activities, and channeling of intermediates (reduction of intermediate concentration). By contrast, the results obtained for the *TRP1* gene of *S. cerevisiae* support the concept of a late detachment and rearrangement of a promoterless part of a gene (coding for the PRA isomerase domain) from an originally trifunctional general control regulated gene (including the glutamine amidotransferase and InGP synthase functions). The evidences are summarized as follows. 1) The *TRP1* promoter is unusual among the *TRP* promoters; it is the only *TRP* promoter that is not regulated by the general control system. Transcription from the *TRP1* promoter is weaker than from the regulated *TRP3* promoter. This situation is compensated by higher stability of *TRP1* mRNA. The largest *TRP1* transcripts have a 200-bp untranslated leader that presumably impairs translation of the transcript (Kim *et al.*, 1986). 2) The *TRP1* gene product PRA isomerase is a very active enzyme providing the cell with a 2–3 times higher basal enzyme activity than any of the other *TRP* enzymes. 3) Among the lower fungi, separation of the genes of InGP synthase and PRA isomerase is uniquely found only in yeasts (Braus *et al.*, 1985). All other ascomycetes analyzed so far carry a single gene coding for both InGP synthase and PRA isomerase. Comparison of the connector regions and the COOH and NH_2 termini of known fused and separate InGP synthase and PRA isomerase proteins (Fig. 5) reveals a remarkable feature: the

NH_2 terminus of the yeast PRA isomerase contains additional amino acids in comparison to prokaryotic monofunctional PRA isomerases that correspond in length to the natural connector region in the proteins of the ascomycetes *A. nidulans* and *N. crassa*.

If the hypothesis of a rearrangement of the *TRP1* gene is correct, why then are the PRA isomerase and the InGP synthase fused in so many organisms if there is no evident advantage for this arrangement? An answer to this question can only be speculative. Two models were proposed for the evolution of enzymes in multistep pathways. Jensen (1976) proposed that primitive enzymes possessed a broad substrate specificity and were active in several metabolic pathways. Evolution could then have been achieved by gene duplication and subsequent mutations leading to specialization in the substrate specificity of the encoded proteins. This model was supported by Parsot (1986, 1987), who has shown sequence homologies between the tryptophan synthase β chain (last step in tryptophan biosynthesis), threonine synthase, threonine dehydratase, and D-serine dehydratase and has postulated a common ancestor for these enzymes. Horowitz (1945) earlier proposed an alternative hypothesis that biochemical pathways could evolve in a stepwise manner by duplication and evolution of new functions in the reverse direction compared to the direction of synthesis (retrograde evolution). It has been suggested that the eight-stranded α/β barrel is particularly suited for the evolution of new functions since function seems to be determined to a large extent by surface loop modification at the carboxyl termini of the β strands of the basic structure (Lindqvist and Bränden, 1985). Although the α subunit of the tryptophan synthase (second last step in tryptophan biosynthesis), the InGP synthase (third last step), as well as the PRA isomerase (fourth last step) show only a low degree of homology (Priestle *et al.*, 1987), all three enzymes have the topological fold of an 8-fold α/β barrel (Crawford and Kirschner, 1987).

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SUPPLEMENT MATERIAL TO

THE ROLE OF THE TRP1 GENE IN YEAST TRYPTOPHAN BIOSYNTHESIS

by Gerhard Braus, Karolin Luger, Gerhard Paravicini, Tobias Schmidheini, Kasper Kirschner, and Ralf Hütter

Materials. Restriction enzymes and enzymes involved in nucleic acid metabolism were obtained from Boehringer (Mannheim, West Germany), PL-Biochemicals (Uppsala, Sweden), Genofit (Geneva, Switzerland), Biolabs (Schwalbach, West Germany), and Stehlin (Basel, Switzerland). Lysozyme was received from Sigma (St.Louis MI, USA), Helicase from Dr. Gross Chemie (Stettlen, Switzerland). Radiochemicals were all purchased from Amersham Radiochemical Centre (Amersham, UK). CDRP was synthesized according to *Smith and Yanofsky* (1963), PRA according to Creighton (1968). Ethylamino-Sepharose CL-4B was synthesized by the method of Jenissen and Heilmeyer (1975) using Sepharose CL-4B from Pharmacia (Uppsala, Sweden) as matrix. Hydroxyapatite, prepared by the method of Atkinson et al. (1973), was a gift from Ciba-Geigy (Basel, Switzerland). Superose 12 was obtained from Pharmacia. All other chemicals were of the purest grade available from either Fluka (Buchs SG, Switzerland), Merck (Darmstadt, West Germany) or Sigma (St.Louis MI, USA).

Microorganisms and plasmids. Yeast strains are derivatives of the Saccharomyces cerevisiae laboratory strains X2180-1A (MATa gal2 SUC2 mal CUP1) and X2180-1B (MATα gal2 SUC2 mal CUP1) obtained from T. Manney (Manhattan KA, USA): RH218 (trp1-218), RH558-1 (gcd2-1); RH962 (trp1-218 trp3B-21); RH974 (trp3-56 leu2-2); RH1244 (ura3-251 ura3-328 ura3-325); RH1310 (ura3-251 ura3-328 ura3-373 gcd2-1). Escherichia coli strain MC1061[Δ(lacIPOZYA) X74 galU galK hsdR Δ(ara leu) strA^R](Casadaban et al., 1983) was used in cloning procedures.

Plasmids YRp7 (Struhl et al., 1979), YARp1 (Zakian and Scott, 1982), pUC18 (Norrander et al., 1983), pME503 (Aebi et al., 1984), YCp50 (Johnston and Davis, 1984), and pNM480/481/482 (Minton, 1984) were described earlier. pMA33 (Dobson et al., 1983) was obtained from S. Kingsman. pME582 is a pUDB207 (Begge, 1978) derivative containing the entire TRP1 and TRP3 genes and was constructed in our laboratory by U. Wirth. For pME581 the 2.5kb TRP3 ClaI-BamHI fragment of pME503 was inserted into plasmid YRp7 (Struhl et al., 1979). Other constructions of plasmids are explained in Results.

Media. YEPD- and MV-medium for yeast were described previously (Miozzari et al., 1978a); E.coli cells were cultivated on Luria-Broth (LB) as complete medium (Miller, 1972). For selective and indicator media 50μg/ml ampicillin, 20ug/ml tetracycline, or 10μg/ml X-gal, respectively, were added to the autoclaved media.

Genetic techniques. Yeast genetic crosses were performed as described by Sherman et al. (1970).

Isolation of nucleic acids. For analytical purposes E.coli plasmid DNA was prepared from 1ml cultures or directly from colonies by the alkaline lysis method of Birnboim and Doly (1979). Preparative isolation of E.coli plasmid DNA was done according to Humphreys et al. (1975).

Total RNA was isolated from 500ml of exponentially growing yeast cells at an OD_{450} of about 2 (≈1.5x10^{8}cells) according to the method of Zitomer and Hall (1976), modified by Furter et al. (1986). For the isolation of in vivo pulse-labelled total RNA a mini-scale method was applied: exponentially growing yeast cells were labelled for 15 min by adding 50μCi/ml of ^{3}H-uracil (53 Ci/mmol, Amersham, UK) and chased by addition of a hundred-fold excess of non-radioactive uracil. At different time points after the chase 4 ml samples of cells were removed, chilled on ice-cold ethanol, sedimented in a bench top centrifuge, and resuspended in extraction buffer (as described above, but containing 1 volume phenol/chloroform and heated to 55°C). Cells were disrupted and total RNA was isolated as described above for the large-scale RNA isolation.

For poly(A)$^+$RNA enrichment the method of Aviv and Leder (1972) was applied.

Transformation of plasmid DNA. Yeast tranformation was carried out by the spheroplast method of Hinnen et al. (1978) with the modifications suggested by Hsiao and Carbon (1979). For E.coli transformations the $CaCl_2$ method of Mandel and Higa (1970) was applied.

Cloning techniques. Restriction of DNA, electrophoresis of nucleic acids, and ligation of DNA were done as described (Aebi et al., 1982). For isolation of DNA fragments, restricted DNA was separated on a low-melting agarose gel (Seaplaque, FMC Corporation, Rockland ME, USA). After staining with ethidium bromide, the desired band was cut out, diluted with TE-buffer and sodium-acetate solution (pH 4.8, final concentration 300mM) to an agarose concentration below 0.3% and melted at 68°C. The solution was then treated twice with water-saturated phenol and the mixture was kept on ice before phase separation to desintegrate the agarose. After chloroform extraction the DNA was precipitated with ethanol.

Labelling of nucleic acids. DNA was labelled by 'nick translation' (Rigby et al., 1977).

Hybridization techniques. For Northern hybridization poly(A)$^+$RNA was separated on a formaldehyde agarose gel, transferred to nitrocellulose (Thomas, 1980) and hybridized according to Wahl et al. (1979). For quantitative determination of radioactivity the relevant bands and appropriate controls were cut out from the nitrocellulose filters, dissolved in Beckman Ready-Solv HP scintillation fluid, and the radioactivity was determined in a Beckman LS-250 liquid scintillation counter.

For quantitative filter hybridization of in vivo pulse-labelled RNA (Zitomer et al., 1979), total RNA, isolated at different times after the non-radioactive chase, was hybridized on nitrocellulose filters carrying equal amounts of plasmid DNA. The filters were produced by using a slot blotter apparatus (Minifold II, Schleicher and Schuell). Hybridization was carried out according to Bach et al. (1979) with some modification in 40% formamide, 2xSSC, 1% bovine serum albumin, 1% polyvinylpyrrolidone K90, 1% Ficoll 400, 1% SDS at 42°C for 42 h. The filters were washed at 40°C in 40% formamide, 2xSSC for one hour, in 2xSSC, 0.2% SDS for an additional hour, and finally in 95% ethanol. After drying the radioactivity per filter was determined in a Beckman scintillation counter LS-250.

Nucleotide sequence analysis. Sequence analysis was done according to Maxam and Gilbert (1980) with the modifications described by Furter et al. (1986).

Enzyme assays. Enzyme activities are specified in International Units [1U= amount of enzyme that leads to the formation of 1μmol product per min]. Specific enzyme activities are given as [mU/mg protein].
β-Galactosidase (EC 3.2.1.23). For measuring β-galactosidase activity in yeast strains carrying plasmids, exponentially growing cells (OD_{450}=1) were harvested and broken by passing them twice through a French Pressure Cell. After incubation with the colorimetric substrate ONPG and stopping the reaction by addition of 1M Na_2CO_3, the broken yeast particulate matter was removed by centrifugation before reading the optical density of the yellow product at 420nm (Miller, 1972).
TRP enzymes. For measuring TRP enzymes in yeast, cells were first made permeable by freezing and thawing in the presence of 0.05% Triton X-100 (Miozzari et al., 1978b).

Anthranilate synthase (EC 4.1.3.27) activity was assayed as described by Fantes et al. (1976).

Anthranilate phosphoribosyl transferase (PRtransferase) (EC 2.4.2.18) activity was measured according to Miozzari et al. (1978a) with the following modification: the disappearance of anthranilate was measured in the presence of an excess of PRAisomerase derived from detergent-treated cells of the 100-fold overproducing strain RH218 [YARp1].

PRAisomerase activity was determined with two different assays.
[1] Assay in permeable cells: A modification of the procedure of Creighton and Yanofsky (1970) was applied. CDRP, the product of the enzyme reaction, was quantitatively converted to InGP by adding an excess of InGPsynthase derived from permeabilized cells of the 50-fold overproducing strain RH974 [pME550]. InGP was determined by the periodate method described by Wegman *and DeMoss* (1965).
[2] Assay of purified enzyme fractions (according to Hommel and Kirschner, personal communication): By adding 1.5mU purified PRtransferase protein to a constant-temperature cuvette (25°C), the anthranilate (10μM) and PRPP (300μM) (in 50mM Tris-HCl pH 7.5, 4mM $MgCl_2$, 2mM dithioerythrol) were converted enzymatically to PRA within 15 sec. The fluorescence (excitation at 310nm, emission at 400nm) dropped during this period to 20% of the original value. When the fluorescence emission was constant for at least 15 sec PRAisomerase (either permeabilized cells or fractions of a crude extract) was added, the further decrease in fluorescence was documented on-line by a recorder.

InGPsynthase (EC 4.1.1.48) activity was determined as described by Fantes et al. (1976).

Tryptophan synthase (EC 4.2.1.20) activity was measured as the A+B reaction as described by Miozzari et al. (1978a).

Protein content of the cells was measured by the method of Herbert et al. (1971). In purified enzyme fractions the protein assay of Bradford (1976) was applied.

Gel electrophoresis of proteins. Polyacrylamide gel electrophoresis in the presence of SDS was performed according to Lämmli (1970). The proteins were stained with silver as described by Ohsawa and Ebata (1983).

Column chromatography of proteins. For the ethylamino-Sepharose and the hydroxyapatite column chromatograpy 30cmx2.5cm columns with a volume of 150ml were used. A superose 12 column (20cmx1cm) on a Pharmacia FPLC was used with a flow rate of 0.1 ml/min. Protein was monitored by measuring OD_{280} in an UV photo cell (Uvicord).

Purification of the PRAisomerase
All steps in the purification procedure were carried out at 4°C. The protease inhibitor phenylmethylsulfonylfluoride as well as dithioerythrol were added to each buffer to a final concentration of 100μM and 400μM respectively.
[1] Preparation of cell-free extract: Yeast cells were cultivated in minimal MV medium supplemented with 0.05% (w/v) casamino acids. Frozen wet cells were washed twice with 100mM potassium phosphate, pH 7.5, containing 5μM diisopropylfluorophosphate, and 500μM K_2EDTA and suspended in the same buffer (0.8ml/g cells). The cells were broken in a MSK-Braun homogenizer by rapid shaking with glass beads under constant cooling with liquid CO_2. Cell debris were removed by centrifugation (18000 x g, 4°C, 40min), yielding supernatant A.
[2] Protamine sulfate precipitation: Protamine sulfate was added to a final concentration of 0.5mg per mg nucleic acid. The precipitate was removed by centrifugation (18000 x g, 4°C, 20min), yielding supernatant B.
[3] Hydrophobic interaction chromatography: The potassium phosphate concentration of supernatant B was increased to 0.5M (pH7.5), K_2EDTA was added to a final concentration of 500μM, and the solution was adjusted to 20% ammonium sulfate saturation (DiJeso, 1968). The precipitate was centrifuged (18000 x g, 4°C, 30min), yielding supernatant C. This solution was loaded on an ethylamino-Sepharose CL-4B column equilibrated previously with buffer containing the same ammonium sulfate concentration. Adsorbed proteins were eluted with a linear gradient of decreasing concentration of ammonium sulfate from equilibration buffer to H_2O. The fractions containing PRAisomerase activity were collected and pooled.
[4] Chromatography on hydroxyapatite: The pooled fractions were dialyzed against 5mM potassium phosphate (pH 7.5), 100mM KCl and loaded on a hydroxyapatite column previously equilibrated with the dialysis buffer. After elution with a linear gradient increasing from 5 to 300mM potassium phosphate, in 100mM KCl, pH 7.5, the PRAisomerase fraction was collected, pooled, frozen in the presence of 10% glycerol by dropping it into fluid nitrogen, and stored at -70°C. At 4°C the enzyme activity was stable for at least one week.

A typical purification protocol is summarized in Tab. 1S. The PRAisomerase was purified 165-fold with a 21% yield. Including the 100-fold overproduction due to the high copy number of the plasmid this corresponds to a more than 15000-fold enrichment with respect to the chromosomally encoded PRAisomerase of strain X2180-1A.

SDS/polyacrylamide gel electrophoresis of the hydroxyapatite fraction yielded a single band on a silver stained gel [Fig. 1S]. For analytical purposes samples in 100mM potassium phosphate, pH7.5 were applied to a gel filtration Superose 12 column.

Table 1S Purification steps of the TRP1 gene product PRAisomerase from the overproducing strain S.cerevisiae RH218 [YARp1]

Step	Protein [mg]	spec.PRAisomerase activity [U/mg]	Purification [-fold]	Recovery [%]
Crude extract	2151	0.62	1	100
Ethylamino-Sepharose chromatography	109	8.61	14	43
Hydroxy-apatite chromatography	4.4	102	165	21

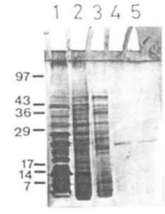

Fig. 1S Analysis by SDS/polyacrylamide gel electrophoresis of successive purification steps of the PRAisomerase

A 12% SDS/polyacrylamide gel was silver stained. Lanes: (1) crude extract (100µg); (2) supernatant fluid from protamine sulfate precipitation (50µg); (3) ethylamino-Sepharose pool (30µg); (4) hydroxyapatite pool (3µg); (5) Superose 12 (FPLC) pool (2µg). M_r markers : phosphorylase b (97.4kd), β_2 trp-synthase (E.coli) (42.9kd), GAPdehydrogenase (35.7kd), α trp-synthase (E.coli) (28.7kd), myoglobin (16.9kd), cytochrome c (13.4kd), bovine pancreas trypsin inhibitor (6.5kd).