The Substrate Specificity of Purine Phosphoribosyltransferases in *Schizosaccharomyces pombe*

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1. The activities of the purine phosphoribosyltransferases (EC 2.4.2.7 and 2.4.2.8) in purine-analogue-resistant mutants of *Schizosaccharomyces pombe* were checked. An 8-azathioxanthine-resistant mutant lacked hypoxanthine phosphoribosyltransferase, xanthine phosphoribosyltransferase and guanine phosphoribosyltransferase activities (EC 2.4.2.8) and appeared to carry a single mutation. Two 2,6-diaminopurine-resistant mutants retained these activities but lacked adenine phosphoribosyltransferase activity (EC 2.4.2.7). This evidence, together with data on purification and heat-inactivation patterns of phosphoribosyltransferase activities towards the various purines, strongly suggests that there are two phosphoribosyltransferase enzymes for purine bases in *Schiz. pombe*, one active with adenine, the other with hypoxanthine, xanthine and guanine. 2. Neither growth-medium supplements of purines nor mutations on genes involved in the pathway for new biosynthesis of purine have any influence on the amount of hypoxanthine-xanthine-guanine phosphoribosyltransferase produced by this organism.

Biochemical evidence suggests the existence of a single-protein hypoxanthine-guanine phosphoribosyltransferase in brewer's yeast (Miller & Bieber, 1968) and in human erythrocytes (Henderson, Brox, Kelley, Rosenbloom & Seegmiller, 1968). Partial separation of IMP synthesis from GMP synthesis has been reported in Ehrlich ascites-tumour cells (Atkinson & Murray, 1965). Results for xanthine phosphoribosyltransferase vary from one organism to another. The hypoxanthine-guanine phosphoribosyltransferase of human erythrocytes can also use xanthine as substrate (Kelley, Rosenbloom, Henderson & Seegmiller, 1967; Krenitsky, Papaionnou & Elion, 1969). A partially purified extract of brewer's yeast that transformed hypoxanthine and guanine into their respective nucleotides showed no activity in the presence of xanthine (Miller & Bieber, 1968). A xanthine phosphoribosyltransferase from *Salmonella typhimurium* was separated from the three other purine phosphoribosyltransferase activities by column chromatography (Kalle & Gots, 1961a). Various examples of resistance to purine analogues accompanied by an alteration or a lack of the corresponding phosphoribosyltransferase activity have been described: mutants of *Salm. typhimurium* resistant to 2,6-diaminopurine and to 8-azaguanine showed altered properties of the adenine phosphoribosyltransferase and the guanine

phosphoribosyltransferase respectively (Kalle & Gots, 1963; Adye & Gots, 1966), and some of the purine-analogue-resistant mutants of *Salm. typhimurium* and of *Streptococcus faecalis* lack one or more of the four natural purine phosphoribosyltransferase activities (Kalle & Gots, 1961b; Brockman, Debavadi, Stutts & Hutchison, 1961).

Preliminary results on the substrate specificity of the purine phosphoribosyltransferases of *Schizosaccharomyces pombe* came from a study of purine-analogue-resistant mutants of this yeast (De Groodt, Heslot, Pourquoié, Poirier & Nagy, 1969). The present paper reports the results of the experiments performed on enzyme extracts from the wild-type strain and from several mutant strains. The new findings are in agreement with the existence of two distinct purine phosphoribosyltransferases in this organism, one transforming adenine into AMP and one utilizing hypoxanthine, xanthine and guanine as substrates.

MATERIALS AND METHODS

Strains. Optimum conditions for the assay of the purine phosphoribosyltransferase activities were established with extracts of *Schiz. pombe* (Leupold, 1955), wild-type strain.

After mutagenesis of the wild-type strain by use of 0.01% nitrosomethylurethane, mutants resistant to 8-azaguanine (*aza* 1, *aza* 2 and *aza* 3), to 2,6-diaminopurine

(*dap* 1, *dap* 2 and *dap* 3) or to 8-aza-2-thioxanthine (*pur* 1) were selected for their rapid growth on the minimal medium (see below) supplemented with one of these analogues at 200 mg/l. The mutant *aza* 1, which has an altered phosphoribosyl pyrophosphate amidotransferase, is described in detail by Heslot, Nagy & Whitehead (1966a). The mutants *ade* 1, *ade* 6 and *ade* 2 (Leupold, 1957) are adenine auxotrophs blocked in the pathway for new biosynthesis of purine as indicated in Scheme 1. The biochemical evidence for the enzymic block occurring in these mutants is given by Heslot, Nagy & Whitehead (1966b) for *ade* 1 and by Leupold (1957) and Fisher (1969) for *ade* 2 and *ade* 6 respectively. The crosses between compatible strains of *Schiz. pombe* and mass analysis of the progeny cells were performed by the techniques described by Leupold (1955).

Medium. The culture medium was the synthetic minimal medium described by Leupold (1955). Minimal medium supplemented with 5% (w/v) of agar was used for genetic analysis.

For the growth of the adenine auxotrophs (see above) adenine or hypoxanthine was added to the medium at the concentrations indicated in each experiment.

Growth conditions. The cells were grown aerobically at 30°C for 17 h in the medium suitably supplemented as described above. They were harvested by centrifugation at the end of the exponential phase. The yield was 6 g wet wt. of cells/l of culture.

Purification procedure. The method described is a modification of that described by Kornberg, Lieberman & Simms (1955) for the purification of adenine phosphoribosyltransferase from brewer's yeast. All subsequent manipulations were performed at 4°C. The pellet of cells (100 g wet wt.) was suspended in 0.5 vol. (w/v) of 0.2 M-tris-HCl buffer, pH 8. The cells were disrupted by shaking with glass beads in a refrigerated Braun Shaker and the resulting homogenate was centrifuged for 1 h at 70000g. The supernatant (fraction 1) was kept and nucleic acids were precipitated with 5% (v/v) of 1 M-MnCl₂. The new supernatant was further fractionated by precipitation between 0% and 30% saturation with solid (NH₄)₂SO₄ (176 g/l) without adjustment of pH during addition of (NH₄)₂SO₄. The precipitate was dissolved in 10 ml of 25 mM-glycylglycine-NaOH buffer, pH 6.8 (fraction 2). This fraction was adjusted to pH 6.8 with 0.1 M-NaOH. The enzymes were allowed to become adsorbed for 5 min on alumina-C_γ gel (Sigma Chemical Co., St Louis, Mo., U.S.A.); 1 g of gel/100 mg of protein in fraction 2 was used. The supernatant was discarded and the gel was washed with 1 vol. of 0.1 M-sodium acetate buffer, pH 5.95. The supernatants obtained from three consecutive desorptions of the enzymes from the gel with 4 ml portions of 20 mM-sodium phosphate buffer, pH 7.4, were pooled (fraction 3). A portion (3 g) of calcium phosphate gel (Keilin & Hartree, 1938) was added and the enzymes were allowed to adsorb for 5 min. The supernatant was discarded. The supernatants obtained from three consecutive desorptions with 4 ml portions of 50 mM-sodium phosphate buffer, pH 7.4, were pooled (fraction 4).

Protein concentrations of the different fractions were determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with dry lysozyme as standard protein.

Enzyme assay. The incubation mixture contained (final concentrations in a final volume of 0.6 ml): 0.17 M-tris-

Scheme 1. Biosynthesis of purine nucleotides in *Schiz. pombe*.

HCl buffer, pH 8; 4.6 mM-MgCl₂; 0.2 mM-phosphoribosyl pyrophosphate (ribose 1-phosphate 5-pyrophosphate); 0.1 mM-[8-¹⁴C]adenine, 0.1 mM-[8-¹⁴C]guanine, 0.1 mM-[8-¹⁴C]hypoxanthine or 0.1 mM-[8-¹⁴C]xanthine (5 mCi/mmol; Commissariat à l'Energie Atomique, Saclay, France). The reaction was started by addition of the enzyme. The mixture was incubated for 10 min at 37°C, and the reaction was then stopped by boiling for 1 min. The precipitate was discarded and 0.01 ml of the supernatant was chromatographed on Whatman no. 1 paper with a system retaining the purine nucleotides at *R_F* 0 [descending chromatography for 17 h with butan-1-ol-formic acid-water (15:3:2, by vol.)]. The appropriate area was cut out and the radioactivity of the purine nucleotide formed was counted in a liquid-scintillation spectrometer (Packard Tri-Carb 314 AF). The scintillation liquid contained 5 g of 2,5-diphenyloxazole and 30 mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/l of toluene. The counting efficiency of ¹⁴C was 50%.

Our results were all obtained under conditions of proportionality of formation of reaction product to time and concentration of protein. When crude extracts were assayed for activity they were first dialysed against 1 mM-tris-HCl buffer, pH 8, for 17 h.

Thermal inactivation. The enzyme extract was heated in a water bath maintained at the desired temperature. At different times samples were withdrawn and cooled in an ice bath. Each sample was assayed for phosphoribosyltransferase activity towards several purines.

RESULTS

Purine phosphoribosyltransferases in mutants resistant to purine analogues. The presence of phosphoribosyltransferase activities towards adenine, hypoxanthine, xanthine and guanine was checked in dialysed crude extracts of seven mutants isolated as resistant to 2,6-diaminopurine (*dap*), to 8-azaguanine (*aza*) or to 8-aza-2-thioxanthine (*pur*) (see the Materials and Methods section). Table 1 shows the activities present in each of the mutants studied. Two of the diaminopurine-resistant mutants, *dap* 1 and *dap* 2, lack adenine phosphoribosyltransferase activity, and the azathioxanthine-resistant mutant *pur* 1 lacks hypoxanthine phosphoribosyltrans-

Table 1. *Presence or absence of the purine phosphoribosyltransferase activities in crude dialysed extracts of Schiz. pombe wild-type strain and of mutants resistant to purine analogues*

Enzyme-assay conditions are described in the Materials and Methods section. Presence or absence of activity is shown by + or – respectively.

Strain	Adenine phosphoribosyl- transferase	Hypoxanthine phosphoribosyl- transferase	Xanthine phosphoribosyl- transferase	Guanine phosphoribosyl- transferase
Wild-type	+	+	+	+
Mutant <i>aza</i> 1	+	+	+	+
Mutant <i>aza</i> 2	+	+	+	+
Mutant <i>aza</i> 3	+	+	+	+
Mutant <i>dap</i> 1	–	+	+	+
Mutant <i>dap</i> 2	–	+	+	+
Mutant <i>dap</i> 3	+	+	+	+
Mutant <i>pur</i> 1	+	–	–	–

Table 2. *Mass analysis of the F₁ progeny of the crosses wild-type × mutant *dap* 1 and wild-type × mutant *pur* 1 of Schiz. pombe*

The genotype of the F₁ cells was tested on minimal medium and on minimal medium containing the purine analogue (200 mg/l). Strains, growth media and methods of genetic analysis are described in the Materials and Methods section.

Cross	Genotype of the spores		
	Wild-type	<i>dap</i> 1	<i>pur</i> 1
Wild-type × mutant <i>dap</i> 1	275	276	—
Wild-type × mutant <i>pur</i> 1	331	—	351

ferase, xanthine phosphoribosyltransferase and guanine phosphoribosyltransferase activities. In the other mutants all four activities are present, indicating that the loss of one of the purine (and presumably purine-analogue) phosphoribosyltransferase activities is only one possible alteration responsible for resistance to purine analogues in *Schiz. pombe*.

*Crosses between mutant *dap* 1 or mutant *pur* 1 and wild-type strain.* The results of crosses between the wild-type strain and each of the mutants *dap* 1 and *pur* 1 are given in Table 2. For each of the crosses mass analysis of the F₁ progeny shows an equal segregation between purine-analogue-sensitive and purine-analogue-resistant cells, indicating that each of the mutants differs from the wild-type strain by a single mutation.

*Growth properties of the double mutants *ade* 1 *pur* 1, *ade* 1 *dap* 1, *ade* 2 *pur* 1 and *ade* 2 *dap* 1.* Mutants *dap* 1 and *pur* 1 were also crossed with mutant *ade* 1 and with mutant *ade* 2 (see the Materials and Methods section and Scheme 1). The growth properties of the four corresponding double mutants are shown in Table 3. The capacity

Table 3. *Growth responses of the double mutants *ade* 1 *dap* 1, *ade* 2 *dap* 1, *ade* 1 *pur* 1 and *ade* 2 *pur* 1 of Schiz. pombe on minimal medium and on minimal medium supplemented with adenine or hypoxanthine*

Strains, growth media and methods of genetic analysis are described in the Materials and Methods section. Possibilities for the enzymic transformation of adenine and hypoxanthine in *Schiz. pombe* are illustrated in Scheme 1. +, Presence of colonies after 7 days' incubation at 30°C; –, absence of colonies after 7 days' incubation at 30°C.

Double mutant	Growth response		
	On minimal medium	On minimal medium + adenine (20 mg/l)	On minimal medium + hypoxanthine (20 mg/l)
<i>ade</i> 1 <i>pur</i> 1	–	+	–
<i>ade</i> 1 <i>dap</i> 1	–	+	+
<i>ade</i> 2 <i>pur</i> 1	–	+	–
<i>ade</i> 2 <i>dap</i> 1	–	–	–

of the double mutant *ade* 1 *dap* 1 to grow on minimal medium plus adenine, although the corresponding phosphoribosyltransferase activity is lacking, is explained (see Scheme 1) by the transformation of adenine into hypoxanthine by adenine aminohydrolase (EC 3.5.4.2). The existence of this enzyme in *Schiz. pombe* has been confirmed in our laboratory (unpublished results). The simultaneous loss of the enzymes catalysing the conversion of adenine into AMP and of IMP into AMP accounts for the lethality of the double mutant *ade* 2 *dap* 1 (see Scheme 1).

*Hypoxanthine phosphoribosyltransferase, xanthine phosphoribosyltransferase and guanine phosphoribosyltransferase specific activities in mutants *ade* 6 and *aza* 1.* Table 4 compares the hypoxanthine phosphoribosyltransferase, xanthine phosphoribosyltransferase and guanine phosphoribosyltransferase specific activities in mutants *ade* 6 and *aza* 1 (see the

Table 4. *Hypoxanthine phosphoribosyltransferase, xanthine phosphoribosyltransferase and guanine phosphoribosyltransferase specific activities in dialysed crude extracts of mutants ade 6 and aza 1 relative to those of the wild-type strain of Schiz. pombe*

Mutant properties and enzyme-assay conditions are described in the Materials and Methods section.

Strain	Growth medium	Specific activity relative to that of the wild-type strain grown on minimal medium		
		Hypoxanthine phosphoribosyltransferase	Xanthine phosphoribosyltransferase	Guanine phosphoribosyltransferase
Wild-type	Minimal medium	(1.0)	(1.0)	(1.0)
Mutant <i>aza 1</i>	Minimal medium	1.1	1.0	1.0
Wild-type	Minimal medium + adenine (200 mg/l)	1.1	1.2	1.1
Wild-type	Minimal medium + hypoxanthine (200 mg/l)	1.1	1.0	1.1
Mutant <i>ade 6</i>	Minimal medium + hypoxanthine (200 mg/l)	0.9	0.9	1.0
Wild-type	Minimal medium + xanthine (200 mg/l)	0.9	0.9	0.9
Wild-type	Minimal medium + guanine (200 mg/l)	1.1	1.0	0.9

Materials and Methods section) with that in the wild-type strain grown on minimal medium. It was verified that addition of purines to the growth medium did not induce higher hypoxanthine phosphoribosyltransferase, xanthine phosphoribosyltransferase and guanine phosphoribosyltransferase specific activities. No significant differences were found among the specific activities of the three enzymes in the wild-type strain and in the strains with an altered pathway of new biosynthesis of purine.

Purification of the purine phosphoribosyltransferases. The purification procedure is described in the Materials and Methods section. Table 5 gives the specific activities of the purine phosphoribosyltransferases and the purification factors for each of the steps. The activity towards adenine is completely removed in the last fraction; the purification factors for the other three activities are closely comparable.

Further purification of the hypoxanthine phosphoribosyltransferase, xanthine phosphoribosyltransferase and guanine phosphoribosyltransferase activities was achieved by DEAE-Sephadex column chromatography, but the loss of enzymic activity after elution was considerable and made accurate measurements of enzyme activities impossible.

Thermal inactivation of the purine phosphoribosyltransferases. Fig. 1 shows the thermal-inactivation curves of the purine phosphoribosyltransferases plotted as percentage of initial activity (on a logarithmic scale) versus time of inactivation. These

experiments were performed at two different purification stages of the enzymes (fraction 2 and fraction 4) and at different pH values (6.8 and 7.4) and temperatures (48°C and 46°C). The adenine phosphoribosyltransferase is more heat-sensitive than are the three other activities, which give coincident plots (see Fig. 1).

DISCUSSION

The fact that several of the mutants resistant to a purine analogue (*dap 1*, *dap 2* and *pur 1*) lack one or more active purine phosphoribosyltransferases (Table 1) indicates that in the analogue-sensitive strains of *Schiz. pombe* the enzymes transform these drugs into toxic derivatives. Similar findings have been reported for *Salm. typhimurium* (Kalle & Gots, 1961b) and *Strep. faecalis* (Brockman *et al.* 1961).

The characteristics of mutant *pur 1*, which differs from the wild-type strain by a single mutation (Table 2) and has lost the hypoxanthine phosphoribosyltransferase, xanthine phosphoribosyltransferase and guanine phosphoribosyltransferase activities (Table 1), and the growth responses of the double mutants *ade 1 pur 1* and *ade 2 pur 1* (Table 3) suggest that in *Schiz. pombe* a single protein catalyses the transformation of hypoxanthine, xanthine and guanine into their respective nucleotides. The results obtained in the similar experiments with mutant *dap 1* are consistent with the existence of a distinct adenine phosphoribosyltransferase in *Schiz. pombe*. The nearly equal degrees of purification achieved for the hypoxanthine phosphoribosyl-

Table 5. Specific activities and purification factors during the purification of adenine phosphoribosyltransferase, hypoxanthine phosphoribosyltransferase, xanthine phosphoribosyltransferase and guanine phosphoribosyltransferase from *Schiz. pombe*

Purification step	Specific activity (nmol of nucleotide/min per mg of protein)				Purification factor			
	Adenine phosphoribosyltransferase	Hypoxanthine phosphoribosyltransferase	Xanthine phosphoribosyltransferase	Guanine phosphoribosyltransferase	Adenine phosphoribosyltransferase	Hypoxanthine phosphoribosyltransferase	Xanthine phosphoribosyltransferase	Guanine phosphoribosyltransferase
Crude extract (fraction 1)	9.0	3.2	2.2	5.4	2.7	1.8	2.0	2.1
(NH ₄) ₂ SO ₄ precipitate (fraction 2)	24.4	5.8	4.4	11.0	3.3	2.4	2.3	2.4
Alumina-Cy-gel desorption (fraction 3)	80.0	14.0	10.0	26.0	—	2.8	3.0	2.9
Calcium phosphate-gel desorption (fraction 4)	0	39.0	30.0	75.0	—	—	—	—

Fig. 1. Thermal inactivation at 46°C of adenine phosphoribosyltransferase (Δ), hypoxanthine phosphoribosyltransferase ($\circ$), guanine phosphoribosyltransferase ($\bullet$) and xanthine phosphoribosyltransferase ($\times$) activities in partially purified enzyme fraction 4 (pH 7.4) from *Schiz. pombe*. Purification, enzyme-assay conditions and thermal inactivation procedure are described in the Materials and Methods section.

transferase, xanthine phosphoribosyltransferase and guanine phosphoribosyltransferase activities in the four consecutive purification steps (Table 5) and the coincident thermal-inactivation patterns for those same enzymes (Fig. 1) are in agreement with the existence of a single hypoxanthine-xanthine-guanine phosphoribosyltransferase. The different behaviour of the adenine phosphoribosyltransferase activity during these experiments confirms that this enzyme is a distinct protein. These results are similar to those reported for the substrate specificity of the purine phosphoribosyltransferases of human erythrocytes (Kelley *et al.* 1967). If it is confirmed that the hypoxanthine-guanine phosphoribosyltransferase extracted from eucaryotic cells can use xanthine as a substrate, then the xanthine-analogue resistance will become as useful a tool as the guanine-analogue resistance in the cloning analysis of mutant cell lines lacking this enzyme activity (Littlefield, 1963).

Table 4 gives some useful information on the role of the purine phosphoribosyltransferases in the regulation of purine biosynthesis: the addition of one of the purines to the growth medium does not induce higher specific activities of the hypoxanthine-xanthine-guanine phosphoribosyltransferase in the wild-type strain of *Schiz. pombe* and no

difference was noticed in the specific activity of this enzyme between the wild-type strain and two mutants with an altered pathway of new purine biosynthesis. Rosenbloom, Henderson, Kelley & Seegmiller (1968) reported an accelerated new biosynthesis of purine in skin fibroblasts deficient in hypoxanthine-guanine phosphoribosyltransferase activity.

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