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REGULATION OF PURINE METABOLISM IN *SCHIZOSACCHAROMYCES POMBE*

IV. VARIATIONS IN THE STABILITY AND KINETIC PARAMETERS OF AMIDOPHOSPHORIBOSYLTRANSFERASE DEPENDING ON GROWTH PHASE AND GROWTH CONDITIONS

MARIA NAGY, UWE REICHERT* and ANNE-MARIE RIBET

Laboratoire de Génétique, Institut National Agronomique, 16, rue Claude Bernard, Paris 5° (France)

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SUMMARY

1. Markedly different stabilities of amidophosphoribosyltransferase (5-phosphoribosylamine:pyrophosphate phosphoribosyltransferase (glutamate-amidating), EC 2.4.2.14) are found in *Schizosaccharomyces pombe* cells depending on the growth rate and on the growth phase of the culture: relatively stable extracts are prepared from wild-type cells harvested at the end of the log phase, whereas very unstable extracts are obtained from cells of a slowly growing mutant devoid of adenylosuccinate synthetase (IMP:L-aspartate ligase (GDP-forming), EC 6.3.4.4) harvested at the end of the growth phase.

2. The inactivation rate at 3 °C of the unstable enzyme preparation is considerably lowered by phenylmethylsulfonyl fluoride, an inhibitor of proteases. This last has, however, no effect on the rate of thermal inactivation at 48 °C. Otherwise, addition of the unstable extract to the stable one does not modify the rate of inactivation of the last: these results indicate that the stable and unstable extracts differ in their susceptibility to proteolysis and to heat inactivation.

3. The kinetics of thermal inactivation and the Sephadex G-200 filtration of the unstable preparation indicate a molecular heterogeneity of amidophosphoribosyltransferase. The apparent molecular weights of the two forms separated on a Sephadex column are 360 000 and 180 000.

4. Increased affinity for the substrate 5-phosphoribosyl-1-pyrophosphate and for the endproducts of the de novo pathway AMP and GMP correlated with a decrease in the cooperativity of their binding is observed with the unstable enzyme preparation as compared to the stable one.

Abbreviation: *P*-Rib-*P*-*P*, 5-phosphoribosyl-1-pyrophosphate; PMSF, phenylmethylsulfonyl fluoride.

* Permanent address: Zentralinstitut für Biochemie und Biophysik der Freien Universität, D 1000 Berlin.

INTRODUCTION

In the course of our studies on the regulation of purine biosynthesis in *Schizosaccharomyces pombe*, we have tried to demonstrate a control of the first enzyme of the pathway, amidophosphoribosyltransferase (5-phosphoribosylamine:pyrophosphate phosphoribosyltransferase (glutamate-amidating), EC 2.4.2.14), by the repression–derepression mechanism. The material used for this work was an auxotrophic mutant strain *ade* 2 h^- devoid of adenylosuccinate synthetase activity. The choice of this mutant was determined by an hypothesis of Dorfman [1] assuming that adenylosuccinate synthetase (IMP:L-aspartate ligase (GDP-forming), EC 6.3.4.4), the first enzyme on the AMP specific branch, plays the role of apo-repressor for one early enzyme of the de novo pathway.

The specific activities of amidophosphoribosyltransferase measured in the mutant cells as well as in the wild-type cells were found nearly constant in all repressive or derepressive growth conditions, as well as in different phases of the culture. However, striking differences were observed in the stability of the enzymic extracts, depending on the growth conditions and the growth phase in which cells are harvested. The present communication deals with these conditions and correlates the stability of amidophosphoribosyltransferase to variations in the kinetic parameters, reported earlier [2].

MATERIALS AND METHODS

Yeast strains

Wild type 972 h^- and the purine auxotroph *ade* 2 h^- devoid of adenylosuccinate synthetase activity (unpublished results of our laboratory) are used.

Growth of cells

Routinely, 10^9 cells from an exponential growing preculture on solid yeast extract medium are used as inoculum for 2 l of fresh yeast extract medium [3] and cultivated on a rotatory shaker. The cells are harvested at the end of the exponential growth (16 h for wild type 972 h^-, and 40 h for mutant *ade* 2 h^-), yielding about 10 g of wet material/l.

Enzyme preparations

To 10 ml of crude extract (made as previously described [2]), 2.6 ml of 5% streptomycin sulfate solution are added with stirring. The precipitate of nucleic acids is discarded and the supernatant is dialysed for 2 h against 1 l of 0.01 M Tris–HCl (pH 9), containing 10 mM thioglycerol and 20% (w/v) of glycerol (Buffer A). The dialysed solution (Stage II, Table I) is brought to 20% saturation of $(NH_4)_2SO_4$ the precipitated material is discarded and the concentration of $(NH_4)_2SO_4$ in the supernatant is raised to 45% saturation. The pellet, isolated by centrifugation, is dissolved in 2.5 ml of Buffer A and dialysed for 2 h against 1 l of the same buffer (Stage III).

When the enzymic extracts are prepared for the Sephadex G-200 filtration, 2 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) is added in the crude extract's buffer and Buffer A.

Sephadex G-200 chromatography

3 ml of the extract at Stage III are applied to a Sephadex G-200 column (2.5 cm × 40 cm) equilibrated and eluted at a rate of 8–9 ml/h with 0.1 M Tris–HCl buffer (pH 8.5), containing 10 mM thioglycerol and 1 mM PMSF. The effluent is concentrated 4-fold on an Amicon-CEC 1 column eluate concentrator fitted with a UM 2 ultrafiltration membrane and 0.7-ml fractions are collected every 20 min.

The enzyme assay and protein determination are as already described [2].

RESULTS

Conditions for obtaining amidophosphoribosyltransferase extracts of different stability

In non-supplemented yeast extract medium, the adenine-requiring auxotrophic strain *ade* 2 h^- shows a growth curve quite different from the wild-type strain 972 h^- (Fig. 1). After a first exponential phase with a growth rate similar to the wild type,

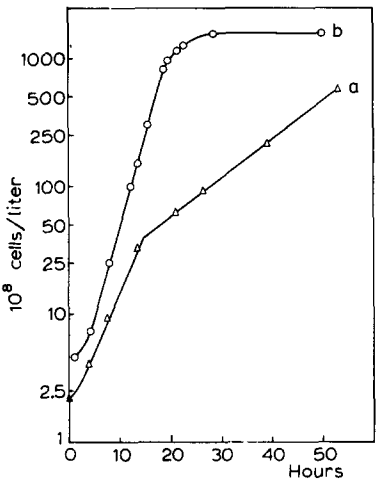

Fig. 1. Growth curves of the wild type 972 h^- (○—○) and mutant *ade* 2 h^- (△—△) strains on complete yeast extract medium [3].

the culture shifts to a second exponential phase of much slower growth, probably due to adenine starvation. Crude extracts from the mutant cells harvested when reaching 20–25% of the stationary cell titer (i.e. 40 h after inoculation) entirely lose their amidophosphoribosyltransferase activity when stored at 3 °C overnight, whereas the extract prepared from wild-type cells cultivated in the same medium and harvested at the same absorbance after 16 h of cultivation remains stable. After purification of the enzyme extracts until Stage III (Table I) the mutant enzyme inactivates completely in 72 h at 3 °C (Fig. 2b). During this time the wild-type enzyme maintains its full activity (Fig. 2a). When equivalent volumes of the stable and unstable crude extracts are mixed and the mixture purified to Stage III, the inactivation of this preparation at 3 °C follows a rate which is near that expected for an independent inactivation rate of each enzyme (Fig. 2c).

Thermal inactivation kinetics performed at 48 °C lead to 50% inactivation of

TABLE I

PURIFICATION STEPS AND RECOVERY OF AMINOPHOSPHORIBOSYLTRANSFERASE

Step I as described in ref. 2, Steps II–IV as described in Materials and Methods.

Step	Protein (mg/ml)	Spec. act. (10^{-9} mole/mg protein per min)	Recovery (%)
I Crude extract	25	3.0	100
II Supernatant after streptomycin and dialysis	20	5.0	100
III 20–45% $(NH_4)_2SO_4$ precipitate after dialysis	20	10.0	45
IV Sephadex G-200 filtration	5	20.0	30

the enzyme activity after 34 min for the wild type and after 3 min for the mutant (Figs 3a and 3b).

However, when the yeast extract medium is supplemented with 100 mg/l of adenine, the growth curve of the *ade* 2 h^- strain culture is similar to that of the wild type and the stability of amidophosphoribosyltransferase extracted after 16 h of culture is comparable to that shown in Fig. 2a for the wild-type enzyme.

Thus, the instability of amidophosphoribosyltransferase does not appear to be correlated to the auxotrophy of the *ade* 2 h^- strain but rather to a malnutrition of the cells.

Since the wild type is also exposed to conditions of starvation, at least in the stationary phase, it should also be possible to extract unstable enzyme from strain 972 h^-. We, therefore, checked the stability of amidophosphoribosyltransferase extracts from wild-type cells at different stages of growth. The time for 50% inactivation at 48 °C was taken as a measure of stability. The results (Fig. 4) confirm our

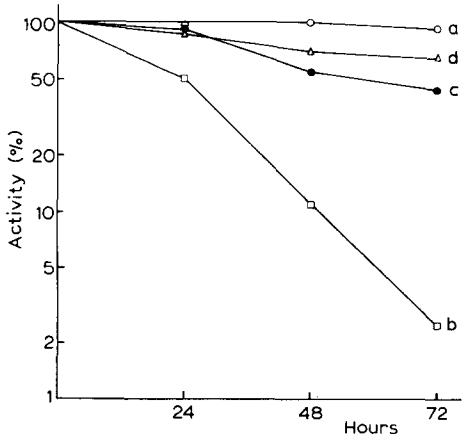

Fig. 2. Conservation of amidophosphoribosyltransferase extracts purified to Stage III at 3 °C. Wild type 972 h^- (○—○); mutant *ade* 2 h^- (□—□); mutant *ade* 2 h^- purified and conserved in the presence of 2 mM PMSF (△—△); 1:1 mixture of crude extracts of wild type 972 h^- and mutant *ade* 2 h^- purified to Stage III (●—●).

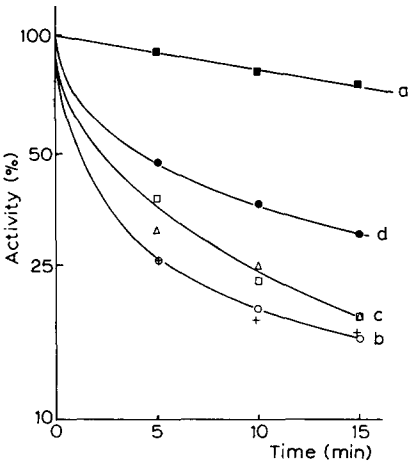

Fig. 3. The effect of the incubation time at 48 °C on the stable and unstable preparations of amidophosphoribosyltransferase and the protecting effect of AMP, adenosine and IMP. Extracts purified to Stage III are used immediately, wild type 972 h⁻ (■—■); mutant *ade* 2 h⁻ (○—○); mutant *ade* 2 h⁻ purified in the presence of PMSF (+—+); mutant *ade* 2 h⁻ containing 5 mM AMP (□—□); mutant *ade* 2 h⁻ containing 5 mM adenosine (△—△); mutant *ade* 2 h⁻ containing 5 mM IMP (●—●); the three purine derivatives tested are added before incubation at 48 °C. 20-μl aliquots are removed for the assay of the residual activity and diluted 23 times in the assay mixture preincubated at 37 °C.

assumption. Cells of the lag phase contain an unstable enzyme. During exponential growth the stability increases and attains a maximum at the end of the log phase, thereafter it decreases again. In general, stable enzyme extracts have been obtained every time from actively growing cells, at least in the last third of the exponential

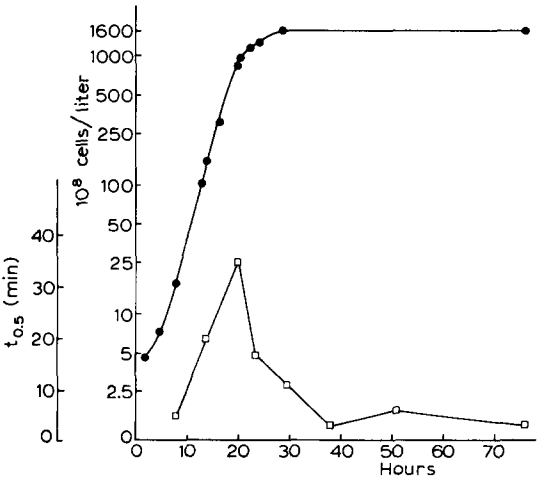

Fig. 4. Variation of aminophosphoribosyltransferase stability during the growth of the 972 h⁻ strain. Enzyme extracts from cells harvested at the times indicated were purified to Stage III and tested for thermostability at 48 °C as described under Fig. 3. t_{50} (min) is the incubation time at 48 °C leading to 50% inactivation of the enzyme (□—□); growth of the culture (●—●).

phase, unstable extracts especially from resting cells or from cells growing under conditions of starvation.

For the experiments described hereafter, the extracts of low stability were prepared from the *ade* 2 h^- strain after 40 h of culture on yeast extract medium and the stable extracts from the wild-type cells after 16 h of growth on the same medium.

Attempts to improve the stability of the unstable extract

A more detailed investigation of the kinetics of inactivation performed at 44 °C reveals that whereas the stable extract of transferase exhibits exponential decay kinetics, the shape of the inactivation curve of the unstable preparation gives evidence of heterogeneity in amidophosphoribosyltransferase molecules (Fig. 5). Attempts to

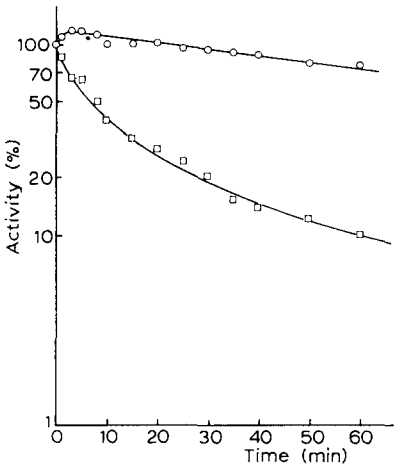

Fig. 5. Kinetics of thermal inactivation at 44 °C of the stable and unstable enzyme preparations purified to Stage III. At the times indicated, 20-μl aliquots are removed for the assay of residual activity. 972 h^- strain (○—○); *ade* 2 h^- strain (□—□).

separate the different forms of enzyme existing in the unstable preparation were for a long time unsuccessful because of its extreme lability. A necessary prerequisite for further study was, therefore, the identification of some stabilizing condition.

(a) Effect of purine derivatives. The effect of various purine derivatives as well as of the substrates of the enzyme on the stabilisation of the unstable preparation was checked. Their presence in all buffers used did not improve the stability of the unstable preparation at 3 °C. Yet a protecting effect of IMP and to a lesser extent of AMP and adenosine against thermal inactivation at 48 °C was observed (Figs 3d and 3c).

(b) Effect of phenylmethylsulfonyl fluoride (PMSF). The recent publications dealing with the inactivation of several enzyme preparations by endoproteases [4–7] prompted us to check the possibility of stabilization of amidophosphoribosyltransferase by the protease inhibitor, PMSF. As seen in Fig. 2d, a considerable improvement in stability is reached when 2 mM PMSF is added to all buffers during the preparation and storage of the unstable enzyme preparation.

However, the susceptibility to heat inactivation of the unstable preparation is

not modified by the presence of PMSF (Fig. 3b). This result (together with the experiment in Fig. 2c, which shows that the unstable extract, rich in proteolytic activity, does not increase the susceptibility to proteolysis of the "stable" enzyme) indicates that the difference between the stable and unstable enzyme extracts does not consist only in the amount of proteolytic material contaminating the two preparations, but rather that increased susceptibility to proteolysis and heat inactivation is a property of amidophosphoribosyltransferase from cells grown in conditions of starvation.

Gel filtration experiments with the stable and unstable amidophosphoribosyltransferase preparations

The addition of PMSF to all buffers used during the partial purification of the unstable amidophosphoribosyltransferase, as well as during the Sephadex G-200 sieving and the immediate concentration of the eluted fractions by means of Amicon thin-channel ultrafiltration enables us to perform the gel filtration experiments with a recovery of about 60% of both the stable and unstable activities. The elution pattern of the stable amidophosphoribosyltransferase shows one main peak (Fig. 6a) eluted

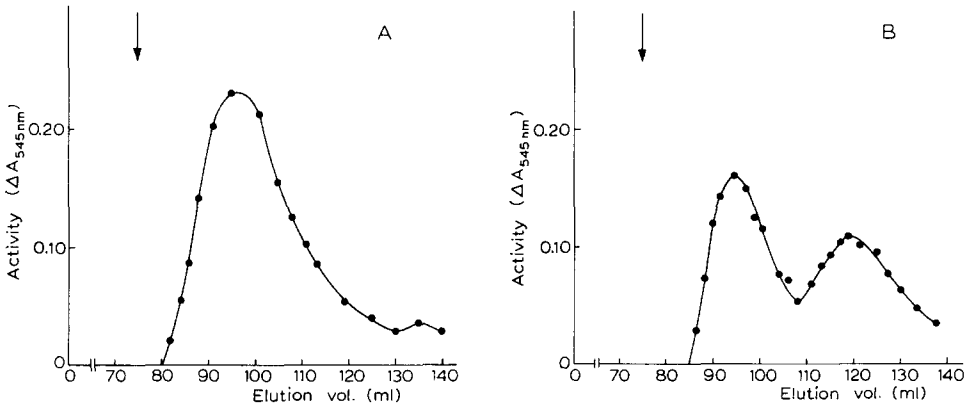

Fig. 6. Elution profile of the stable and unstable preparations of amidophosphoribosyltransferase from the Sephadex G-200 column. The stable (A) or unstable (B) preparation at Stage III are filtered through the column as described in Materials and Methods. Arrow, Blue Dextran elution. The activities expressed for 0.1-ml aliquots are indicated.

at a V/V_0 value of 1.26 $\pm$ 0.05 with an apparent molecular weight of 360 000 (Fig. 7). As seen in Fig. 6b, the elution pattern of the unstable amidophosphoribosyltransferase exhibits a supplementary peak, at a V/V_0 value of 1.35 $\pm$ 0.07 corresponding to an apparent molecular weight of 180 000.

Differences in kinetic properties of the unstable versus stable enzyme preparations

Attempts to perform a comparative kinetic analysis of the two forms separated by Sephadex G-200 gel filtration were unsuccessful because of the strong lability of the enzymes after this treatment. Furthermore, repeated attempts to separate the heterogenous enzyme preparation into more stable fractions by other techniques, such as polyacrylamide gel electrophoresis or isoelectric focussing were, up to the

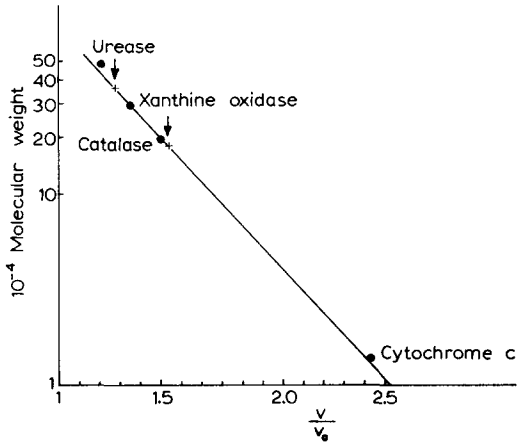

Fig. 7. Determination of the molecular weights of the two forms of amidophosphoribosyltransferase separated on the Sephadex G-200 column. Standard proteins used for the calibration of the column are: (1) urease from soya beans (mol. wt 480 000); (2) milk xanthine oxidase (mol. wt 290 000); (3) beef liver catalase (mol. wt 195 000) and (4) cytochrome *c* (mol. wt 14 000), all from Boehringer, Mannheim. The peaks of the enzymes were determined by assaying their activity. The cytochrome *c* peak was checked by its absorbance at 555 nm. The void volume (V_0), as determined by the elution of Blue Dextran 2000, was at 75 ml. V is the elution volume for each protein. The peaks of amidophosphoribosyltransferase activity are indicated by arrows.

present, unsuccessful. Therefore, a kinetic comparison was performed between the stable and unstable preparations partially purified to Step III (Table I). Important differences are revealed. The kinetics of 5-phosphoribosyl-1-pyrophosphate (*P*-Rib-*P*-*P*) binding to both amidophosphoribosyltransferase preparations is illustrated in Fig. 8. The data are treated in terms of the Hill equation [9]. The values obtained from the slopes of the straight lines are assumed to indicate the degree of cooperativity between the *P*-Rib-*P*-*P* binding sites. The concentration giving rise to a 50% inhi-

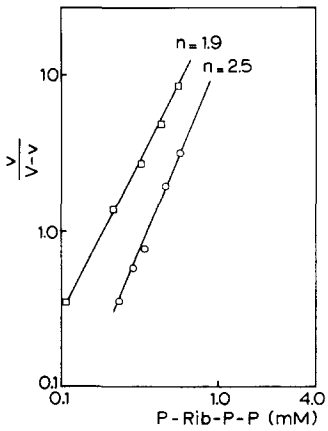

Fig. 8. Effect of increasing *P*-Rib-*P*-*P* concentration on the initial reaction rates of the stable and unstable preparations of amidophosphoribosyltransferase. 0.35 mg of the stable (○—○) and 0.20 mg of the unstable (□—□) extracts, purified to Stage III were used.

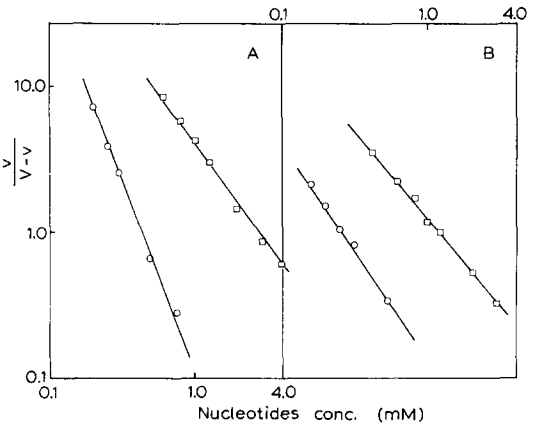

Fig. 9. Effect of increasing GMP and AMP concentrations on the initial reaction rates of the stable (A) and unstable (B) enzyme preparations. The concentrations of GMP (○—○) and AMP (□—□) are as indicated. Enzymic extracts are as in Fig. 8.

TABLE II

Substrate or inhibitor	K_m or I_{50} (mM)		n or n' (Hill)	
	Stable	Unstable	Stable	Unstable
P-Rib-*P*-*P*	0.35	0.19	2.5	1.9
AMP	2.7	1.2	1.5	1.2
GMP	0.44	0.25	2.6	1.5

bition by AMP and GMP as well as the degree of cooperativity in the binding of these molecules were taken from the plot in Fig. 9 according to Taketa and Pogell [10].

As seen in Table II the affinity of the unstable enzyme preparation for *P*-Rib-

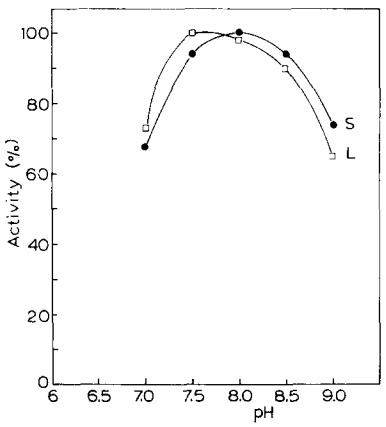

Fig. 10. Effect of pH variations on the initial reaction rates of the stable and unstable amidophosphoribosyltransferase preparations. Tris–HCl buffers were used at the pH values indicated. Stable (●—●) and unstable (□—□) enzyme preparations are as in Fig. 8.

P-P as well as for the endproducts of the pathway AMP and GMP is considerably increased in comparison with the stable enzyme, whereas the cooperativity of its effectors binding is significantly lowered.

Effects of pH on the activity

A difference of 0.5 unit in the pH optimum is repeatedly found between the two enzyme preparations (Fig. 10).

DISCUSSION

The properties of *Schiz. pombe* amidophosphoribosyltransferase depend on the growth rate of the culture and the stage of the growth cycle. This feature, apparently related to the activity of protease, is a new example of a phenomenon recognized in several recent investigations. A similar situation was first observed by Manney [5] with the yeast tryptophan synthase and studied later by T. Katsunuma et al. [6] and A. R. Ferguson et al. [8] who, respectively, established the role of the inactivating enzymes in the instability of tryptophan synthase and the existence, in the yeast cells, of an inhibitor of the tryptophan synthase inactivating enzyme. A proteolytically modified yeast phosphofructokinase was studied by W. Diezel et al. [4]. Our observation of the increased lability of amidophosphoribosyltransferase isolated from the slowly growing mutant in comparison with the exponentially growing wild-type cells and its stabilisation by PMSF can prove useful for the research on this important enzyme very little studied in yeast.

We have shown that the phenomenon observed cannot be interpreted as a simple in vitro occurring artifact due to variable amounts of proteolytic enzymes present in the partially purified extracts: if this would be the case, the addition of PMSF to the unstable extract, which prevents the inactivation by proteases, should simultaneously decrease the susceptibility to heat of the unstable amidophosphoribosyltransferase preparation. Now, the curve of thermal inactivation of the unstable preparation is not modified by the presence of PMSF. Another argument against an in vitro occurring degradation is the experiment in Fig. 2c showing that the "stable" enzyme is not converted to the "unstable one" when mixed with the unstable extract.

The shift of the kinetic constants of the degraded form with respect to the undegraded towards an increased affinity for the substrate *P*-Rib-*P-P* and an increased sensitivity to feedback inhibition suggests that the degradative process of *Schiz. pombe* amidophosphoribosyltransferase could reflect a mechanism of control taking place in the cells under conditions of starvation.

The molecular weights 360 000 and 180 000 of the two forms of amidophosphoribosyltransferase separated from the unstable preparation by the Sephadex G-200 filtration and the decrease of cooperativity in the binding of effectors observed with the same preparation provides an indication of a change in the number of subunits of the enzyme. In human amidophosphoribosyltransferase, two forms with molecular weights of 270 000 and 133 000 have been demonstrated [11]. In pigeon liver, three active forms of the same enzyme with molecular weights of 50 000, 100 000 and 200 000 have been described [12].

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