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REGULATION OF THE BIOSYNTHESIS OF PURINE NUCLEOTIDES IN *SCHIZOSACCHAROMYCES POMBE*

I. PROPERTIES OF THE PHOSPHORIBOSYLPYROPHOSPHATE: GLUTAMINE AMIDOTRANSFERASE OF THE WILD STRAIN AND OF A MUTANT DESENSITIZED TOWARDS FEEDBACK MODIFIERS

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SUMMARY

The kinetic properties of phosphoribosylpyrophosphate:glutamine amidotransferase (EC 2.4.2.14) from the wild strain of *Schizosaccharomyces pombe* and from a mutant (aza-1) resistant to 8-azaguanine are compared.

In the wild type enzyme, the two substrates, phosphoribosylpyrophosphate and glutamine show co-operative saturation kinetics. Inosinic and guanilic acids are powerful inhibitors of the enzyme. The homotropic effect as well as the heterotropic effect of IMP are shown.

In the mutant enzyme, the homotropic effect of glutamine is not observed, the sensitivity towards IMP and GMP is reduced 10 times and the binding of these nucleotides follows first order kinetics.

INTRODUCTION

The first enzyme of the purine biosynthetic pathway, phosphoribosylpyrophosphate:glutamine amidotransferase (EC 2.4.2.14) has been studied in avian, bacterial and mammalian organisms[1–5,20].

We have previously described[6] a mutant of *Schizosaccharomyces pombe*, selected for its resistance to 8-azaguanine and presumably involved in the regulation of the biosynthesis of purines *de novo*. This mutant (aza-1) grows normally on minimal medium and excretes hypoxanthine and inosine. The aza-1 mutation is closely linked to the structural gene ad-4 of the first enzyme of the purine biosynthetic pathway, phosphoribosylpyrophosphate:glutamine amidotransferase.

This paper reports a kinetic study of the phosphoribosylpyrophosphate:gluta-

Abbreviations: AICAR, 5-amino-4-imidazole-carboxamide ribotide; FAICAR, 5-formamido-4-imidazole-carboxamide ribotide.

mine amidotransferase of *Schizosaccharomyces pombe*. We have compared the enzymes of the wild strain and of the mutant strain aza-1.

METHODS

Growth conditions

The wild strain 972 h^- and the strain aza-1 were grown at 30° in a complete yeast extract medium[7]. The cells were harvested during the logarithmic phase of growth after 17 h of culture.

Preparation of crude extract and purification

All subsequent operations were performed at 3°. Cells (17 g wet weight) obtained from a 2-l culture were suspended in 26 ml of buffer composed of 0.1 M Tris–HCl (pH 9.0) containing 20% (w/v) glycerol, 0.1% thioglycerol and 0.01 M EDTA. The cells were broken in a Braun shaker cell homogenizer, and the suspension was centrifuged for 60 min at 120 000 × *g*. The supernatant, containing about 20 mg of protein/ml, was purified by $(NH_4)_2SO_4$ precipitation. The enzyme activity was recovered in the fraction of proteins precipitating between 20 and 45% saturation; dissolved in 6 ml of buffer containing 0.01 M Tris–HCl (pH 9.0), 20% glycerol and 0.1% thioglycerol and dialysed overnight against the same buffer. This treatment raised the specific activity about twice.

The enzyme at this stage retained its activity and its sensitivity towards inhibitors for about a week, when stored at 0°. However, the sensitivity towards inhibitors was lost in 2–3 days at −20°. In the absence of glycerol the activity was lost overnight.

Assay

The assay we performed with our enzyme preparation comprised the two-step procedure of HARTMAN *et al.*[8] based on the following reactions.

1st step

Phosphoribosylpyrophosphate + glutamine → 5′-phosphoribosylamine + PP_i
5′-Phosphoribosylamine + glycine + ATP ⇌ 5′-phosphoribosylglycine amide + ADP + P_i

2nd step

5′-Phosphoribosylglycine amide + IMP + H_2O → 5-amino-4-imidazole-carboxamide ribotide (AICAR) + 5-formamido-4-imidazole-carboxamide ribotide (FAICAR)

AICAR was measured by the colorimetric method of BRATTON AND MARSHALL[9]. The exact assay conditions were derived from those described by NIERLICH AND MAGASANIK[3].

The first incubation mixture contained, in a total volume of 0.46 ml: 0.1 M Tris–HCl (pH 8.0); 2 mM phosphoribosylpyrophosphate; 20 mM L-glutamine; 20 mM glycine; 1 mM neutralized ATP; 8 mM $MgCl_2$; and 10 μl of a purified preparation of glycine amide ribotide synthetase of pigeon liver containing about 5 mg of protein/ml and the phosphoribosylpyrophosphate:glycine amidotransferase extract of *Schizosaccharomyces pombe*. The reaction mixture was incubated for 15 min at 37°, and the reaction was stopped by the addition of 30 μmoles of EDTA neutralized to pH 7.0 by KOH.

In the second step, the conversion of glycine amide ribotide into AICAR was achieved by a second incubation for 1 h at 37° after the addition of 5 μmoles of IMP and 300 μl of the transformylation enzyme. This latter was prepared immediately before use as follows: 8 mg of the pigeon liver acetone powder (Sigma) were suspended in 0.3 ml of 0.1 M Tris–HCl (pH 7.5) and clarified by centrifugation.

The linearity of the reaction rate in terms of time and of protein content was verified for each enzyme preparation.

RESULTS

Kinetic properties of the wild type enzyme

Binding of substrates

Effect of glutamine on the phosphoribosylpyrophosphate binding. The curves obtained by plotting the variations of initial velocity *versus* various concentrations of phosphoriboxylpyrophosphate at four different fixed concentrations of the second substrate, glutamine, show that the increase of the glutamine concentration essentially affects the v_{max} of the saturation curves, while the phosphoribosylpyrophosphate cencentrations at half maximal velocity ($S_{0.5}$) are only slightly modified. At saturating levels of glutamine $S_{0.5}$ = 0.35 mM (Fig. 1A). The data of Fig. 1A were plotted in terms of the HILL[1]- equation: $\log v/(v_{max}-v) = \log K + n \log$ [phosphoribosylpyrophosphate], where v is the initial velocity, v_{max} is the maximal velocity, k is the apparent overall dissociation constant, n is the interaction coefficient. The slopes of the straight lines thus obtained give values of the interaction coefficient n = 1.7, 2.2, 2.5 and 2.5 at glutamine concentrations respectively of 4, 6, 12 and 20 mM. These results

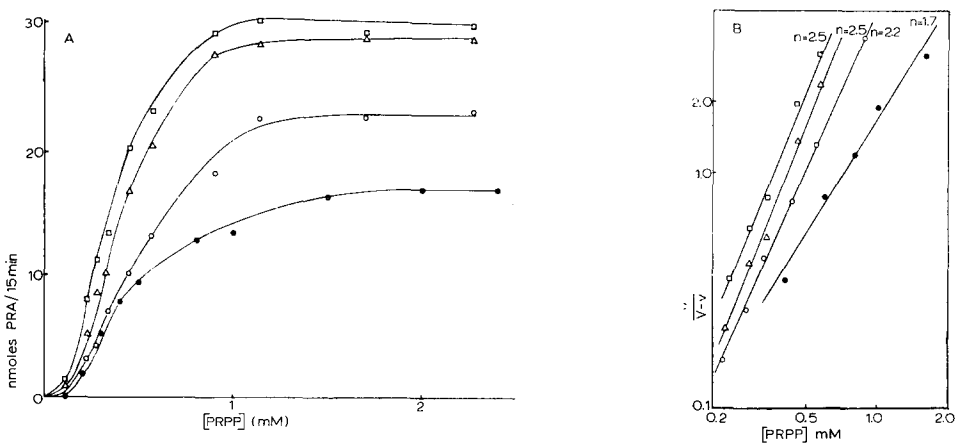

Fig. 1. A. Effect of increasing phosphoribosylpyrophosphate (PRPP) concentration on the initial reaction rates of the wild type phosphoribosylpyrophosphate:glutamine amidotransferase at different glutamine levels. □—□, 20 mM; △—△, 12 mM; ○—○, 6 mM; ●—●, 4 mM. The assay conditions were as described in METHODS. 0.135 mg of enzyme were added. PRA = 5′-phosphoribosylamine. B. Influence of glutamine on the co-operativity of the phosphoribosylpyrophosphate (PPRP) binding to the wild type enzyme, as shown by the empirical HILL[10] plots. □—□, 20 mM glutamine; △—△, 12 mM glutamine; ○—○, 6 mM glutamine; ●—●, 4 mM glutamine. The data were taken from A.

indicate a homotropic interaction between phosphoribosylpyrophosphate molecules and a heterotropic effect of glutamine on the phosphoribosylpyrophosphate binding (Fig. 1B).

Effect of phosphoribosylpyrophosphate on the glutamine binding. When the glutamine concentration was varied, phosphoribosylpyrophosphate levels being kept constant, double reciprocal plots (Lineweaver–Burk) gave straight lines, indicating, in these conditions, the absence of co-operativity between glutamine binding sites. The $S_{0.5}$ values of glutamine variy considerably with phosphoribosylpyrophosphate concentration: an increase of phosphoribosylpyrophosphate concentration from 0.25 to 2 mM decreased the $S_{0.5}$ value from 14.8 to 5 mM (Fig. 2).

Effect of nucleotides

Inhibition by AMP, GMP and IMP. Fig. 3 shows the effects of purine nucleotides on the activity of phosphoribosylpyrophosphate:glutamine amidotransferase obtained from the wild strain. The three nucleotides tested gave complete inhibition when their concentrations were sufficiently high. For AMP this concentration was

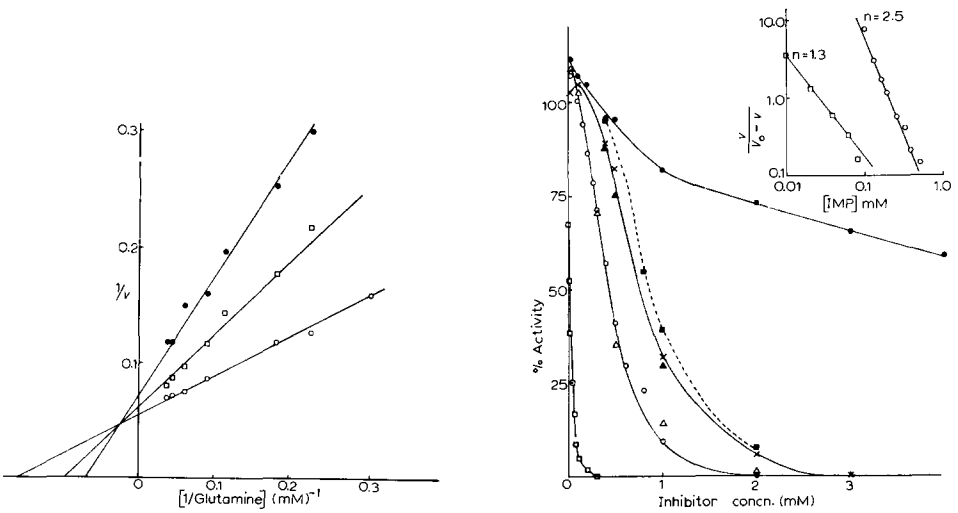

Fig. 2. Effect of increasing glutamine concentration on the initial reaction rates of the wild type phosphoribosylpyrophosphate:glutamine amidotransferase at different phosphoribosylpyrophosphate levels. ○—○, 2 mM; □—□, 0.4 mM; ●—● 0.25 mM. The assay conditions were as described in METHODS. 0.134 mg of enzyme was added. Values for the ordinates are reciprocals of the nmoles of 5′-phosphoribosylamine produced in 15 min.

Fig. 3. Inhibition of the wild type phosphoribosylpyrophosphate:glutamine amidotransferase activity by purine nucleotides. The assay conditions were as described in METHODS, except that the following nucleotides were added as indicated: AMP, ●—●; IMP, ○—○; GMP, △—△; equimolar mixture of AMP + IMP, ×—×; equimolar mixture of AMP + GMP, ▲—▲. The values are plotted in terms of total nucleotides concentrations. The theoretical curve for an additive effect of AMP + IMP or AMP + GMP (■— — —■) was calculated as described by CASKEY *et al.*[1]. The assay conditions were as described in METHODS except the that phosphoribosylpyrophosphate concentration was lowered to 0.3 mM, which is the half maximal velocity value, and IMP was present at concentrations indicated: □—□; 0.364 mg of enzyme was added. In the insert is shown the HILL[10] plot of the data corresponding to the IMP effect on the enzyme activity at saturating (○—○) and half saturating concentrations (□—□) of phosphoribosylpyrophosphate.

12 mM (not shown). At low concentrations of AMP, GMP or IMP, between 20 and 100 μM, a stimulation of the enzymic activity of about 10% was found reproducibly. In order to determine the degree of cooperativity between the inhibitor binding sites, the data of Fig. 3 were plotted in terms of the equation of TAKETA AND POGELL[11]:

$$\log v/(v_0 - v) = \log K' - n' \log I$$

where v is the velocity in the precence of inhibitor, v_0 is the velocity in the absence of inhibitor, n' is the apparent number of inhibitor molecules reacting per enzyme molecule, I is the concentration of inhibitor, K' is the apparent overall dissociation constant. The n' value obtained for IMP (see the insert of Fig. 3) and GMP (not shown) was 2.5, indicating a co-operative homotropic effect between the molecules of these nucleotides. However, at half maximal velocity concentration of phosphoribosylpyrophosphate (while glutamine concentration was maintained at saturation), the value of the interaction coefficient n' was 1.3 and the stimulation of the activity was not observed.

The inhibition curves obtained by equimolar mixtures of AMP + IMP, as well as AMP + GMP, are very close to the theoretical curve expected for an additive effect (Fig. 3).

Effect of IMP on the phosphoribosylpyrophosphate binding. The initial velocity patterns for saturation by phosphoribosylpyrophosphate in the presence of different fixed concentrations of IMP show that IMP acts as a competitive inhibitor of the phosphoribosylpyrosphosphate binding (Fig. 4A). A HILL[10] plot of the data in Fig. 4A gives three parallel straight lines having a slope of 2.7 and showing that the presence of IMP lowers the affinity of the enzyme for the substrate, whereas the co-operativity of the phosphoribosylpyrophosphate binding is not modified (Fig. 4B).

Effect of IMP on the glutamine binding. The curve of saturation of the enzyme

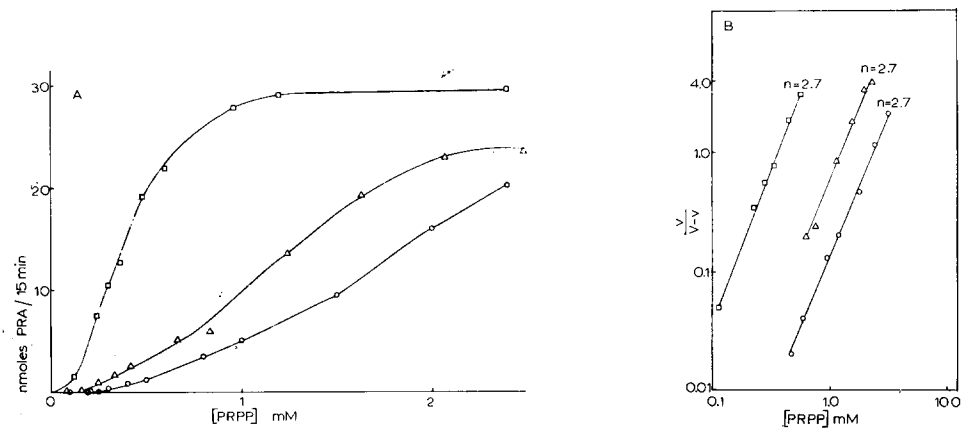

Fig. 4. A. Effect of IMP on the phosphoribosylpyrophosphate (PPRR) binding to the wild type enzyme. The assay conditions were as described in METHODS except that IMP was present at the following concentrations: □—□, 0 mM; △—△, 0.2 mM; ○—○, 0.4 mM. 0.366 mg of enzyme was added. PRA = 5'-phosphoribosylamine. B. Influence of IMP on the co-operativity of phosphoribosylpyrophosphate (PRPP) binding to the wild type enzyme as shown by the empirical HILL[10] plots. □—□, 0 mM IMP; △—△, 0.2 mM IMP ;○—○, 0.4 mM IMP. The data were taken from A.

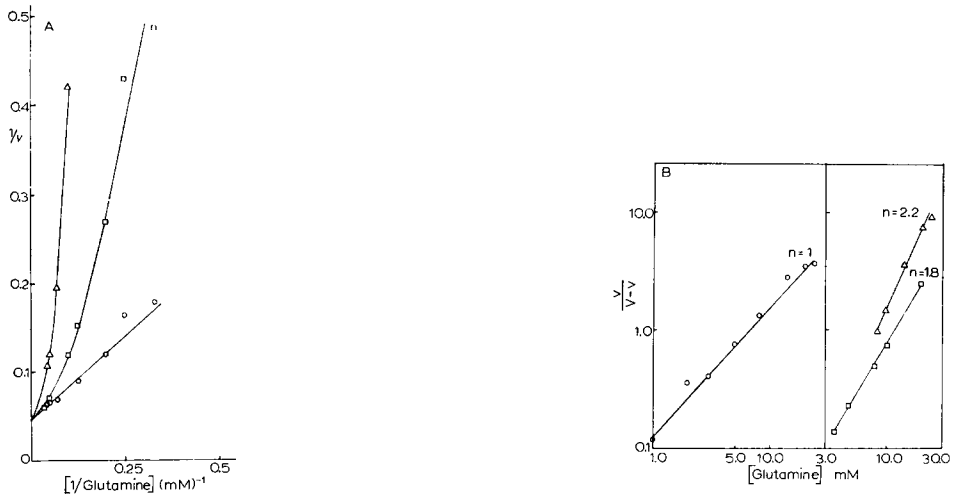

Fig. 5. A. Effect of IMP on the glutamine binding to the wild type enzyme. The assay conditions were as described in METHODS, except that IMP was present at the following concentrations: ○—○, 0 mM; □—□, 0.2 mM; △—△, 0.4 mM. 0.195 mg of enzyme was added. Values for the ordinates are reciprocals of the nmoles of 5′-phosphoribosylamine produced in 15 min. B. Influence of IMP on the co-operativity of the glutamine binding to the wild type enzyme as shown by the empirical HILL[10] plot. ○—○, 0 mM IMP; □—□, 0.2 Mm IMP; △—△, 0.4 mM IMP. The data were taken from A.

by glutamine, which is strictly hyperbolic in the absence of any inhibitor, becomes strongly sigmoid in the presence of a fixed concentration of IMP. The Lineweaver–Burk plot (Fig. 5A) indicates a competitive type of inhibition, and the values of the

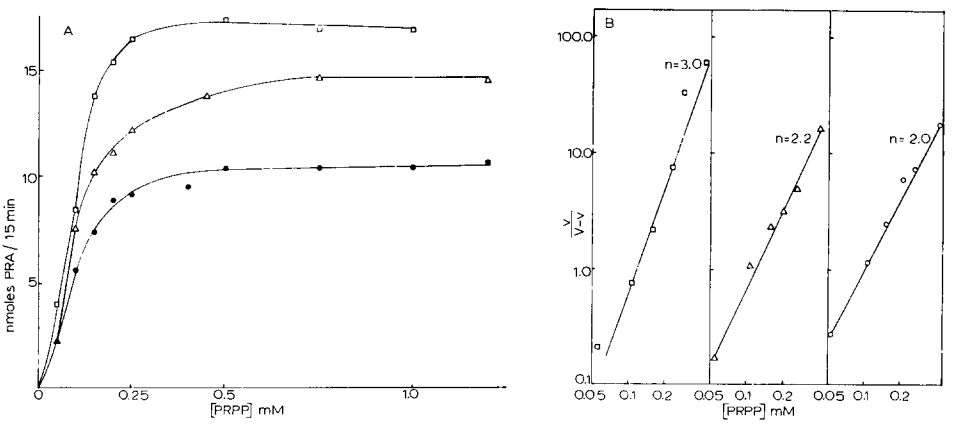

Fig. 6. A. Effect of increasing phosphoribosylpyrophosphate (PRPP) concentration on the initial reaction rates of the mutant enzyme at different glutamine levels. □—□, 20 mM; △—△ 10 mM; ●—●, 4 mM. The assay conditions were as described in METHODS. 0.212 mg of enzyme were added. PRA = 5′-phosphoribosylamine. B. Influence of glutamine on the co-operativity of the 5′-phosphoribosylamine (PRPP) binding to the mutant enzyme as shown by the empirical HILL[10] plot. □—□, 20 mM glutamine; △—△, 10 mM glutamine; ●—●, 4 mM glutamine. The data were taken from A.

interaction coefficient (Fig. 5B) reveal the extent of co-operativity of glutamine binding.

Kinetic properties of the mutant strain aza-1

Binding of substrates

Effect of glutamine on the phosphoribosylpyrophosphate binding. The rate concentration curves obtained when phosphoribosylpyrophosphate concentrations were varied at three different fixed levels of glutamine are similar to those of the wild type enzyme (compare Fig. 1A and Fig. 6A): the increase of glutamine concentration increased the v_{max} but did not modify the $S_{0.5}$ value of phosphoribosylpyrophosphate. However, this value (0.1 mM) is 3 times lower compared with that of the wild strain. At glutamine concentrations of 4, 10 and 20 mM, the interaction coefficient n was 2.0, 2.2 and 3.0, respectively (Fig. 6B).

Effect of phosphoribosylpyrophosphate on the glutamine binding. Curves obtained by variations of glutamine concentration at three different fixed concentrations of phosphoribosylpyrophosphate (0.61 mM, which is a saturating concentration, 0.18 and 0.12 mM) give, in the Lineweaver–Burk plots, straight lines that intercept the abcissal axis at the same value ($S_{0.5}$ = 5.5 mM). In the mutant strain, the increase of phosphoribosylpyrophosphate concentration did not modify the [$S_{0.5}$] value of glutamine as it did for the wild type enzyme (compare Figs. 2 and 7).

Effects of nucleotides

Inhibition by AMP, GMP and IMP. Fig. 8 shows the inhibition of the phosphoribosylpyrophosphate:glutamine amidotransferase activity of the aza-1 mutant by AMP, IMP and GMP. Whereas the inhibition curve by AMP did not differ signi-

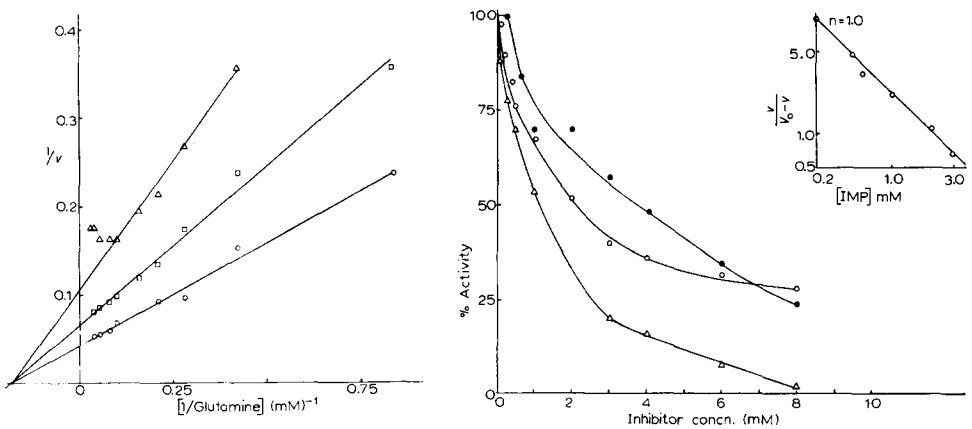

Fig. 7. Effect of increasing glutamine concentration on the initial reaction rate of the mutant phosphoribosylpyrophosphate:glutamine amidotransferase at different phosphoribosylpyrophosphate levels. ○—○, 0.61 mM; □--□, 0.18 mM; △—△, 0.12 mM. The assay conditions were as described in METHODS. 0.180 mg of enzyme was added. Values for the ordinates are reciprocals of the nmoles of 5′-phosphoribosylamine produced in 15 min.

Fig. 8. Inhibition of the mutant enzyme activity by purine nucleotides. The assay conditions were as described in METHODS except that the following nucleotides were added as indicated: AMP, ●—●; IMP, ○—○, GMP, △—△, 0.220 mg of enzyme was added. In the insert is shown the HILL[10] plot of the data corresponding to the inhibition curve by IMP.

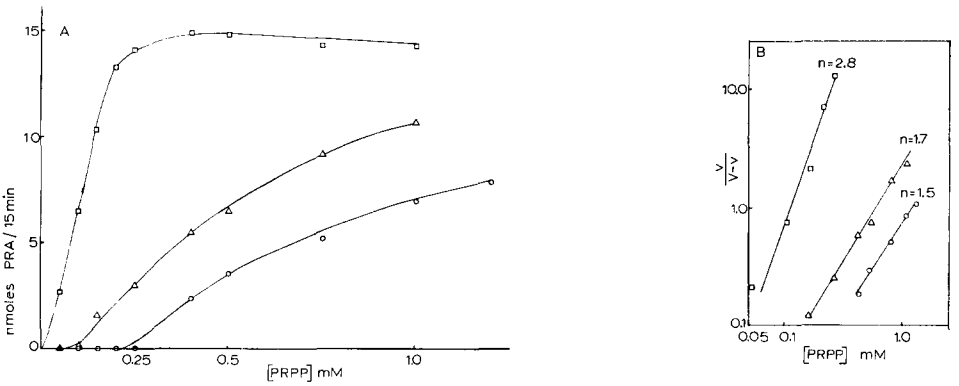

Fig. 9. A. Effect of IMP on the phosphoribosylpyrophosphate (PRPP) binding to the mutant enzyme. The assay conditions were as described in METHODS except that IMP was present at the following concentrations: □—□, 0 mM; △—△, 0.6 mM; ○—○, 1.5 mM. 0.212 mg of enzyme was added. PRA = 5′-phosphoribosylamine. B. Influence of IMP on the co-operativity of the phosphoribosylpyrophosphate (PRPP) binding to the mutant enzyme as shown by the empirical HILL[10] plot. □—□, 0 mM IMP; △—△, 0.6 mM IMP; ○—○, 1.5 mM IMP. The data were taken from A.

ficantly from that of the wild type enzyme, the $[I_{0.5}]$ value for IMP and GMP was about 10 times higher. Furthermore, there was no co-operativity between IMP (and GMP, not shown) binding sites of the mutant enzyme, the n' value of the HILL[10] plot being 1. (See the insert in Fig. 8.)

Compared with the inhibition by IMP of the enzyme from the wild strain, two other differences appear: no total inhibition by IMP could be achieved even at IMP concentrations as high as 8 mM, and no stimulation of activity was found at low concentrations of IMP (compare Figs. 3 and 8).

Effect of IMP on the phosphoribosylpyrophosphate binding. As for the wild type enzyme, the presence of IMP affects the affinity of the enzyme for phosphoribosyl-

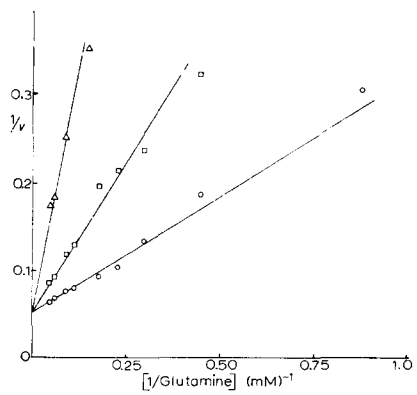

Fig. 10. Effect of IMP on the glutamine binding to the mutant enzyme. The assay conditions were as described in METHODS, except that IMP was present at the following concentrations: ○—○, 0 mM; □—□, 1.2 mM; △—△, 4 mM. 0.236 mg of enzyme was added. Values for the ordinates are reciprocals of the nmoles of 5′-phosphoribosylamine produced in 15 min.

pyrophosphate, without altering the v_{max} value (compare Figs. 4A and 9A). In contrast to the wild type enzyme, the Hill[10] plot of these data shows that the presence of IMP lowers the co-operativity of the phosphoribosylpyrophosphate binding. (Compare Figs. 4B and 9B.)

Effect of IMP on the glutamine binding. The presence of IMP did not induce the co-operativity of the glutamine binding which was observed with the enzyme from the wild strain (compare Figs. 5A and 10). The straight lines obtained in the Lineweaver–Burk plot intercept the ordinate axis at the same value, indicating a competitive type of inhibition.

DISCUSSION

The enzyme of *Schizosaccharomyces pombe* exhibits, as do many other regulatory enzymes[12], co-operative rate saturation kinetics. The sigmoid shape of the phosphoribosylpyrophosphate saturation curves was reported previously by HILL AND BENETT[4] for the phosphoribosylpyrophosphate:glutamine amidotransferase from Adenocarcinoma cells, and by NIERLICH AND MAGASANIK[3] for the *Aerobacter aerogenes* enzyme in the presence of inhibitors. However, the co-operativity has not so far been observed for the glutamine binding. Our results with the wild type enzyme reveal both the co-operative homotropic effect of glutamine in the presence of an inhibitor (Fig. 5A) and its heterotropic effect on the phosphoribosylpyrophosphate binding (Figs. 1A and 1B) indicating that glutamine participates, in some manner, in the allosteric transition of the enzyme subunits.

Concerning the order of binding of both substrates, our results are identical with those of HILL AND BENETT[4]. The variation of glutamine concentration does not modify the $[S_{0.5}]$ value of phosphoribosylpyrophosphate, while the increase of phosphoribosylpyrophosphate concentration decreases the $[S_{0.5}]$ value of glutamine. These findings indicate that phosphoribosylpyrophosphate binds to the enzyme before glutamine.

The kinetics of inhibition of the wild type enzyme activity by the nucleotides tested, as well as the effect of IMP on the substrates saturation kinetics, are consistent with the K system as defined by MONOD *et al.*[12] in that: (a) total inhibition is achieved at high inhibitor concentrations; (b) the inhibition is overcome by increasing concentration of substrate; and (c) the inhibitor causes the substrate kinetics to become co-operative, and increasing inhibitor concentrations increase the co-operativity.

All the kinetic results reported here may be accommodated by the models of MONOD *et al.*[12] or KOSHLAND *et al.*[13], if we assume that the equilibrium between the two forms $R \rightleftharpoons T$ in our enzyme preparation, in absence of any effector, is largely in favour of the "tight" inactive form, having the greatest affinity for the negative effector.

Analysis of the kinetic data corresponding to the mutant enzyme compared with those of the enzyme from the wild strain reveals important differences. The bindings of the substrates appear to be completely independent (Figs. 6 and 7): the variation of the concentration of one substrate does not modify the affinity of the enzyme for the other. The affinity of the mutant enzyme for phosphoribosylpyrophosphate is 3 times higher, whereas its affinity for IMP and GMP is 10 times lower, compared with the values obtained for the enzyme from the wild strain. IMP is not

an allosteric effector of the mutant enzyme: neither its homotropic nor its heterotropic effect on the glutamine binding is observed.

Thus, the mutational event conferring the resistance towards 8-azaguanine seems to result in an alteration of at least some of the IMP and GMP binding sites, which prevents the co-operativity of IMP binding and simultaneously modifies the patterns of the substrate binding. However, the remaining co-operativity of the phosphoribosylpyrophosphate binding indicates that the oligomeric structure of the mutant enzyme was preserved.

The modifications observed in the mutant enzyme kinetics, *i.e.* (1) the opposite of the effect of the mutation on the affinities for phosphoribosylpyrophosphate and IMP, (2) the loss of co-operativity of IMP but not of phosphoribosylpyrophosphate binding and (3) the incomplete inhibition of the mutant enzyme's activity by IMP, all support the hypothesis of separate binding sites for the substrate and the feedback modifier on this enzyme.

Concerning the role of the nucleotide monophosphates in the feedback regulation, our results are consistent with the experiments of BURNS[14] with *Saccharomyces cerevisiae in vivo* which suggested that IMP controls the rate of purine synthesis. A regulatory role of IMP is not as apparent in other organisms[3,15,16]. Our previous observation[6] that the mutant aza-1 of *Schizosaccharomyces pombe* excretes hypoxanthine is consistent with our present finding that the mutant enzyme has lost its feedback control by IMP.

The low sensitivity of phosphoribosylpyrophosphate:glutamine amidotransferase to AMP is rather unexpected. Several authors[17–19] have described an alternative enzymic step leading to the 5'-phosphoribosylamine, and using ribose 5-phosphate and NH_4^+ as substrates. It is possible that this step, which seems to exist in *Schizosaccharomyces pombe*, is regulated more specifically by AMP. This hypothesis is presently under investigation.

ACKNOWLEDGMENTS

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