Biochimica et Biophysica Acta, 309 (1973) 1–10

BBA 66874

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

III. KINETIC STUDIES OF ADENYLOSUCCINATE SYNTHETASE

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(Received November 6th, 1972)

SUMMARY

1. High levels of adenylosuccinate synthetase (IMP: L-aspartate ligase (GDP), EC 6.3.4.4) have been found in the yeast *Schizosaccharomyces pombe* and the enzyme was partially purified from the *ade*-8 mutant, lacking succino-AMP lyase (EC 4.3.2.2).

2. The enzyme is strongly inhibited by AMP and GMP. Whereas GMP seems to act by a direct competition for the GTP binding site, AMP appears as an allosteric effector, showing at non-saturating substrate concentrations a homotropic effect as well as a heterotropic effect upon the GTP and aspartate binding.

3. The initial rate experiments indicate a fully random mechanism of substrate binding.

INTRODUCTION

Adenylosuccinate synthetase (IMP: L-aspartate ligase (GDP), EC 6.3.4.4) is the first enzyme on the AMP-specific branch of the purine *de novo* pathway. The enzyme has been studied in a number of organisms[1–3], its sensitivity to various nucleotides has been shown, but in no case have allosteric properties been observed.

In the kinetic study of the yeast *Schizosaccharomyces pombe* enzyme, described in this report, two types of conditions have been used: those, in which a strictly Michaelian behaviour is observed, allowing an estimation of the mechanism of action, and those in which the allosteric nature of the enzyme can be demonstrated.

MATERIALS AND METHODS

Materials

An adenine auxotrophic strain *ade*-8h$^-$, lacking succino-AMP lyase (EC 4.3.2.2) and derived from the wild type of *S. pombe* 972h$^-$, by treatment with nitrosomethylurethane, has been used. Except when otherwise indicated, the cells were

grown aerobically at 30 °C in complete yeast extract medium and harvested at the end of the log phase.

Chemicals

All purine derivatives as well as the pyruvate kinase were purchased from Boehringer.

Enzyme preparation

All subsequent operations were performed at 3 °C. 10 g of cells are suspended in 15 ml of 1 mM potassium phosphate buffer (pH 8) and disrupted by shaking with 20 g of glass beads in a refrigerated Braun shaker. The resulting homogenate is centrifuged for 45 min at 71 000 × *g*. To 10 ml of the supernatant (crude extract), 2.6 ml of a 5% streptomycine sulfate solution are added with stirring. The precipitate of nucleic acids is discarded and the supernatant is further purified about 3-fold by precipitation between 45 and 65% $(NH_4)_2SO_4$ saturation. The precipitated material is redissolved in 1 ml of distilled water. This partially purified enzyme preparation has a specific activity of about 30–50 nmoles/mg per min and can be stored for a week at −20 °C without any loss of activity. However, by repeated thawing or by conservation at 3 °C for several days, loss of sensitivity towards inhibitors was observed.

Standard enzyme assay conditions

Adenylosuccinate synthetase is assayed by the method of Lieberman[4], modified as follows: the reaction mixture (0.5 ml) contains 140 mM glycine buffer (pH 8), 6 mM $MgCl_2$, 4 mM IMP, 0.3 mM GTP (sodium salt), 40 mM sodium aspartate, 0.6 mM phosphoenolpyruvate, 15 units of pyruvate kinase and 5 μl (about 800 μg of protein) of the partially purified enzyme. After incubation at 37 °C for 20 min, the reaction is stopped by adding 4 ml of 2.5% $HClO_4$. The absorbance at 280 nm of the centrifuged supernatant is measured in the Acta III Beckman spectrophotometer and the increase in absorbance is determined by comparison with a control mixture in which aspartate was omitted.

No changes in absorbance at 280 nm were observed when the enzyme was incubated in a reaction mixture to which one of the three substrates was added after $HClO_4$. The linearity of the reaction rate as a function of time and of protein content was verified.

RESULTS

Binding of substrates

In the absence of any effector, the plots of reaction rate *versus* one substrate concentration at saturating or non-saturating concentrations of the two other substrates show Michaelis–Menten kinetics. The apparent K_m values, determined under standard conditions, from the double-reciprocal plots, are: IMP, 0.2 mM; GTP, 0.02 mM; L-aspartic acid, 1.5 mM (Figs 3 and 6).

Rudolf and Fromm[1] have determined a sequential, fully random mechanism of action for the *Escherichia coli* succinoadenylate synthetase, using a kinetic approach developed by Fromm[5]. The same mechanism seems to apply to the *S. pombe* enzyme when the following results are considered: (i) The three sets of lines obtained in the

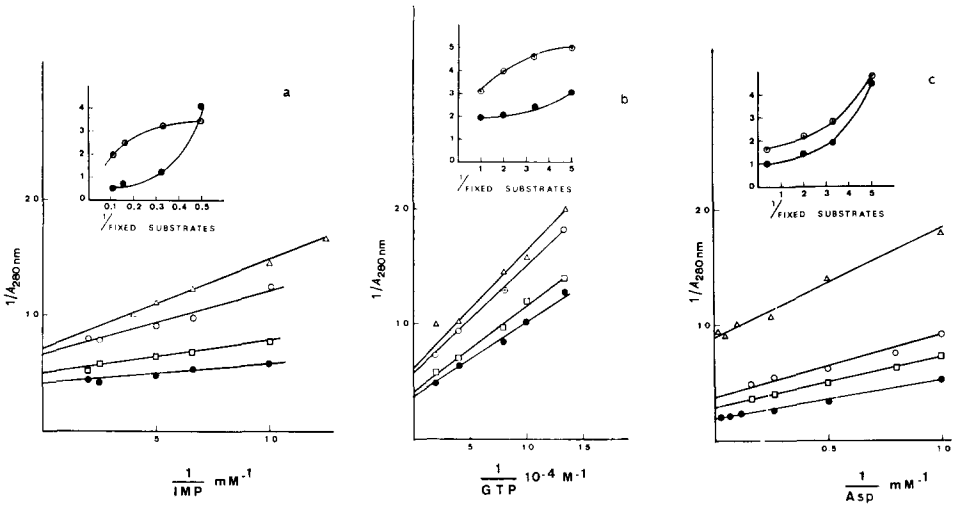


Fig. 1. Initial velocity patterns. Double reciprocal plots. a, Varying IMP concentrations with aspartate and GTP at, respectively, △—△, 2 mM and 20 μM; ○—○, 3 mM and 30 μM; □—□, 6 mM and 60 μM; ●—●, 9 mM and 90 μM. b, Varying GTP concentrations with IMP and aspartate at, respectively: △—△, 0.20 mM and 2.0 mM; ○—○, 0.3 mM and 3 mM; □—□, 0.5 mM; and 5 mM; ●—● 1 mM and 10 mM. c, Varying aspartate concentrations with IMP and GTP at, respectively: △—△, 0.2 mM and 20 μM; ○—○, 0.3 mM and 30 μM; □—□, 0.5 mM and 50 μM; ●—●, 2.5 mM and 250 μM. Inserts: intercept (○—○) and slope (●—●) replots in arbitrary units.

Lineweaver–Burk plots by varying one substrate at four different but fixed concentrations of the two other substrates (the ratio of these two fixed substrates being constant for the four curves) converge at the left of the $1/v$ axis (Fig. 1). This result eliminates the possibility of a ping-pong mechanism, which would give one or more sets of parallel lines.

Between the three types of sequential mechanisms (ordered, partially ordered or fully random), a further distinction can be made: (ii) The inhibition observed for GMP, which is competitive towards GTP (Fig. 6b) appears to be non-competitive relative to IMP and aspartate (Figs. 6c and 6a). This result eliminates the ordered mechanism, in which the inhibitor competitive towards one substrate shows non-

TABLE I

EFFECT OF CULTURE CONDITIONS ON THE SPECIFIC ACTIVITY OF ADENYLOSUCCINATE SYNTHETASE

Enzyme activity was measured under standard conditions.

Strain	*Culture conditions*	*Specific activity (nmoles succinoadenylate/mg per min)*
ade 8 h⁻	Yeast extract medium*	50
ade 8 h⁻	Minimal medium**	37
ade 8 h⁻	Yeast extract* + 50 mg/l adenine	52
ade 8 h⁻	Yeast extract* + 100 mg/l adenine	46
972 h⁻	Yeast extract medium	48

* Standard culture conditions described in Materials and Methods.

** Cells cultured under standard conditions were suspended for 8 h at 30 °C in a minimal salt medium.

linear or uncompetitive inhibition with respect to the other substrates. (iii) The replots of the slopes and intercepts of the initial rate experiments in Fig. 1 are all parabolic and do not intersect the origin (see the inserts in Fig. 1). According to Rudolf and Fromm[1] only a fully random sequential mechanism is consistent with this result.

Regulation of adenylosuccinate synthetase activity

A high specific activity has been measured in the mutant *ade*-8 lacking the succinoadenylate lyase. The same level of activity has been found in the wild-type 972h⁻ cells, but in this latter extract the presence of adenylosuccinate lyase makes the limit of the linearity measurement conditions very narrow. As shown in Table I, the activity appears to be constant in all culture conditions: neither did additions of high amounts of adenine to the yeast extract medium repress the enzyme level, nor did an 8-h incubation of the cells in a minimal medium, without any adenine supplementation, allow a further derepression.

Effect of AMP. Measured under the standard conditions described in Materials and Methods, the effect of increasing concentrations of AMP on the initial velocity rate is seen in Fig. 2a. The curve obtained is hyperbolic and a 50% inhibition is reached at 0.35 mM AMP. As seen in Fig. 3 the inhibition is of the non-competitive type towards all three substrates. For aspartate and IMP, the lines intersect on the

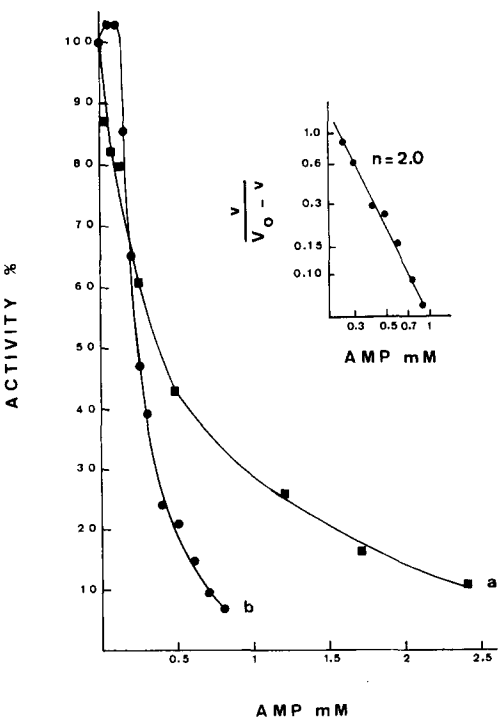


Fig. 2. Effect of increasing AMP concentration on adenylosuccinate synthetase activity. Curve a, under standard conditions and Curve b, at the following substrates concentrations: IMP, 0.45 mM; GTP, 50 μM; aspartate, 2.5 mM. Inserts: the Hill plot of the data in Fig. 2b.

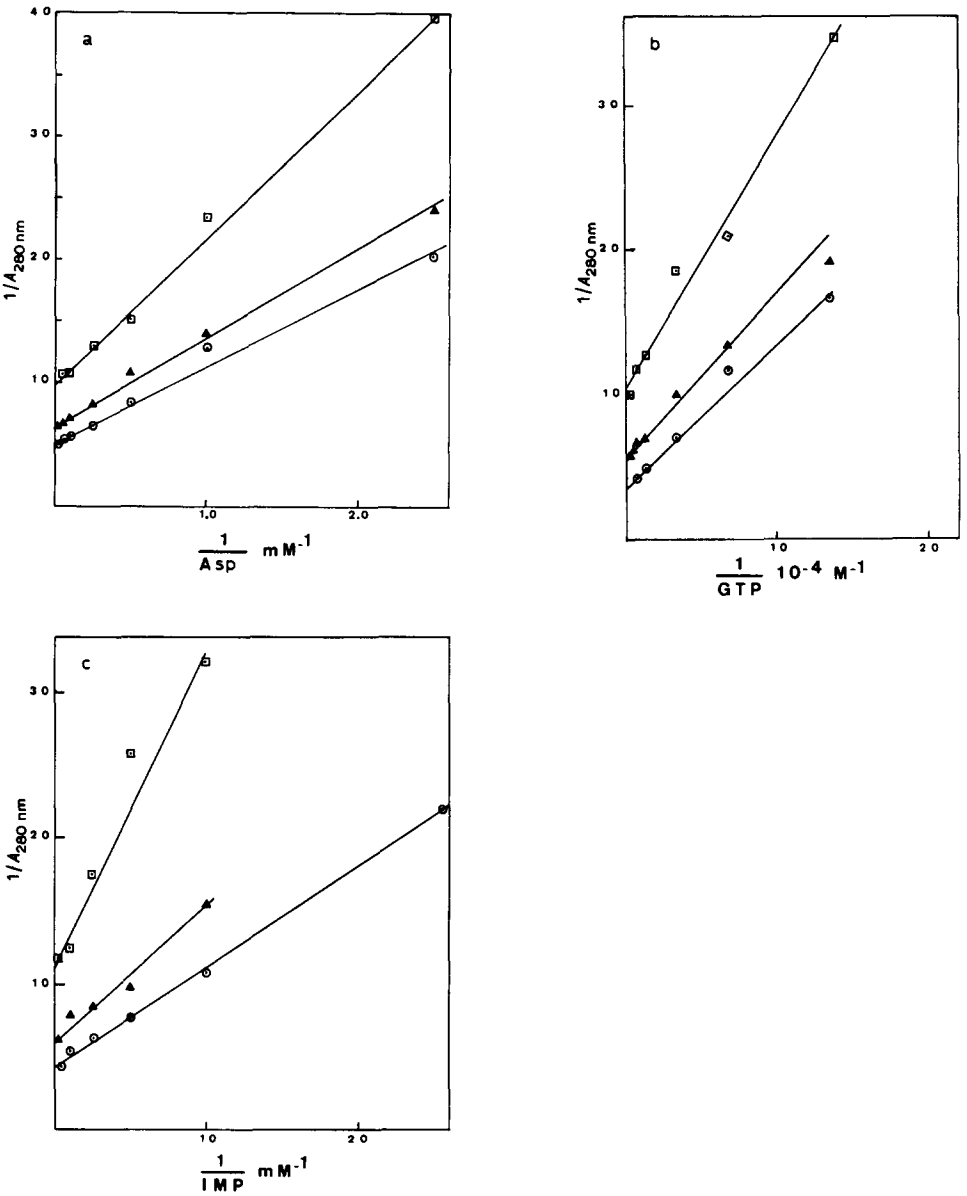
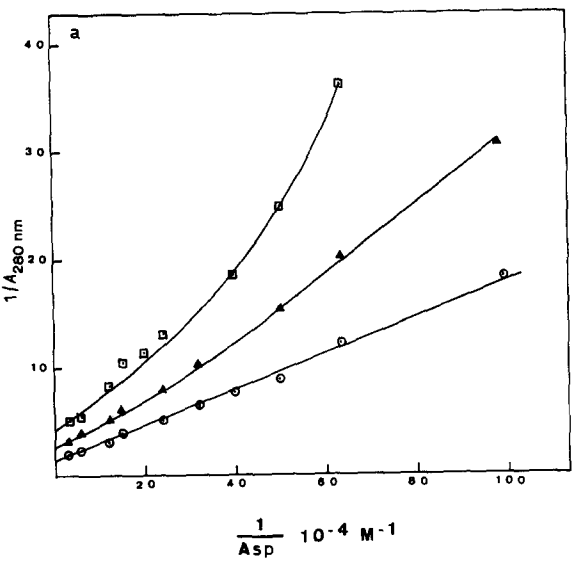


Fig. 3. Effects of AMP on the substrates binding to the enzyme at saturating concentrations of the fixed substrates. a, Effects on the aspartate binding; b, effects on the GTP binding; c, effects on the IMP binding. Standard assay conditions were used, except that AMP was present at the following concentrations: ○—○, 0 mM; △—△, 0.24 mM; □—□, 0.48 mM.

$1/[S]$ axis allowing the determination of K_i values from the intercept and/or slope replots at 0.5 and 0.4 mM, respectively.

When the experiment described in Fig. 2a is repeated at non-saturating (2-fold K_m values) substrate concentrations, the curve obtained is sigmoidal, revealing, under these conditions, the cooperativity of AMP binding (Fig. 2b). The value of the


a
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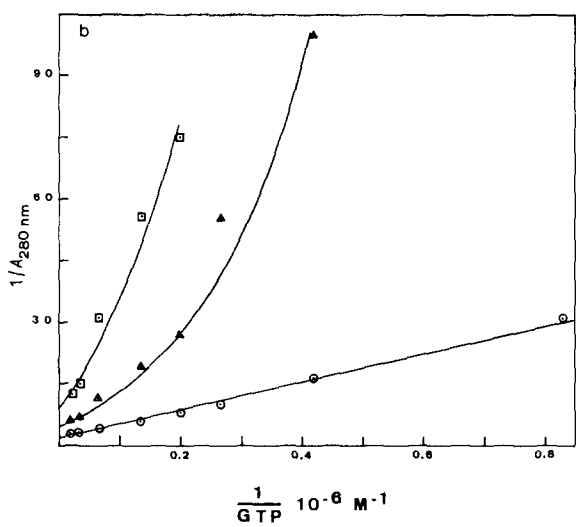
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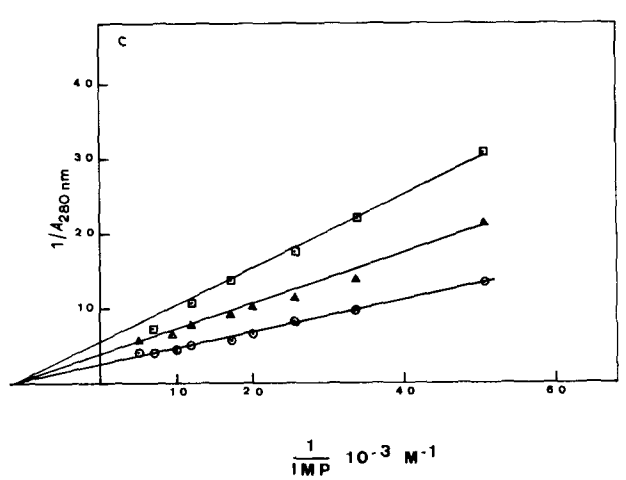
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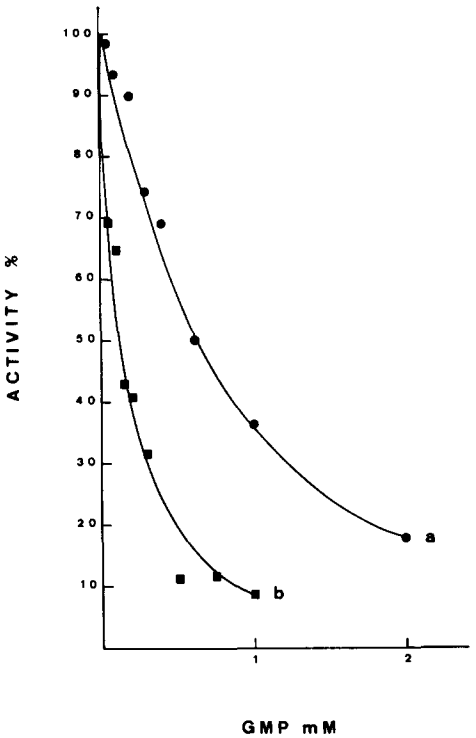

Fig. 5. Effects of increasing GMP concentrations on adenylosuccinate synthetase activity. Curve a, under standard conditions and Curve b, at non-saturating substrates concentrations as indicated in Fig. 2b.

nteraction coefficient, given by the slope of the straight line obtained by plotting log $v/(v_0 - v)$ *versus* log AMP[6] is 2.0, suggesting that at least two AMP binding sites exist per enzyme molecule (see insert in Fig. 2).

At non-saturating substrates concentrations the heterotropic cooperative effect of AMP on aspartate and GTP binding can be shown (Fig. 4). In the absence of AMP, when the concentration of one substrate is varied, the concentration of the two fixed substrates being held at the 2-fold K_m value, simple Michaelis–Menten kinetics are observed, and the double-reciprocal plots give straight lines. In the presence of AMP at 0.20 mM and 0.30 mM, the cooperativity of aspartate and of GTP binding appears, while the reciprocal of the IMP saturation curve (Fig. 4c) still gives straight lines.

Effect of GMP. Figs 5a and 5b show the inhibition of the adenylosuccinate syn-

Fig. 4. Effects of AMP on the substrates binding to the enzyme at non-saturating concentrations of the fixed substrates. a, Effects on the aspartate binding at 0.45 mM IMP and 50 μM GTP. AMP concentrations were: ○—○, 0 mM; △—△, 0.2 mM; ⊡—⊡, 0.3 mM. b, Effects on the GTP binding at 0.45 mM IMP and 2.5 mM aspartate. AMP concentrations were: ○—○, 0. mM; △—△, 0.4 mM; ⊡—⊡, 0.8 mM. c, Effects on the IMP binding at 50 μM GTP and 2.5 mM aspartate. AMP concentrations were: ○—○, 0 mM; △—△, 0.2 mM; ⊡—⊡, 0.3 mM.

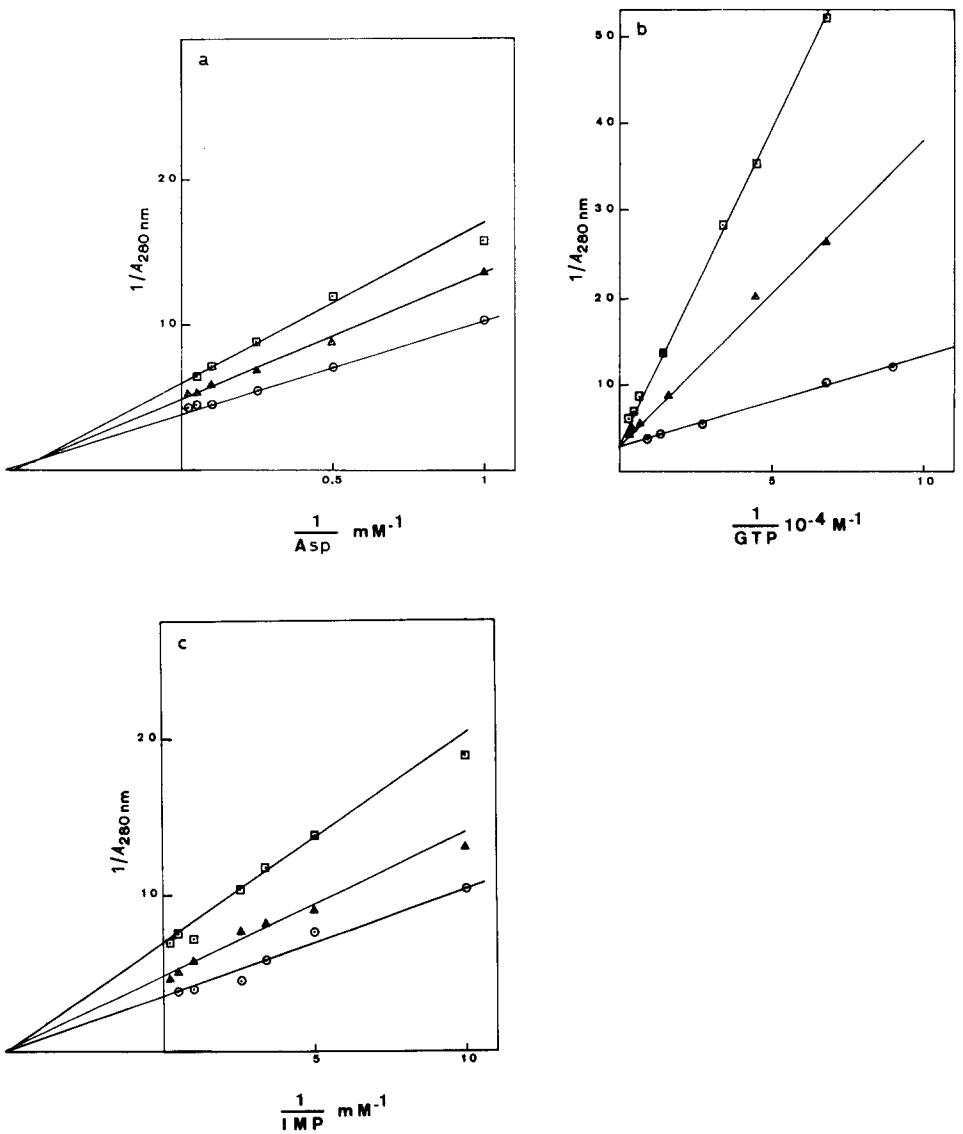

Fig. 6. Effects of GMP on the substrate binding to the enzyme. a, Effects on the aspartate binding; b, effects on the GTP binding; c, effects on the IMP binding. Standard assay conditions were used, except that GMP was present at the following concentrations: ○—○, 0 mM; △—△, 0.3 mM; ⊡—⊡, 0.6 mM.

thetase activity by GMP at saturating and non-saturating substrates concentrations. In both conditions a hyperbolic curve is obtained, the concentrations of GMP giving 50% inhibition being 0.6 and 0.15 mM, respectively.

The inhibition by GMP appears competitive towards GTP (Fig. 6b) non-competitive towards IMP and aspartate (Figs 6c and 6a). K_i values, taken from the secondary plots are 0.1, 0.6 and 1.1 mM, respectively.

DISCUSSION

In *S. pombe*, phosphoribosyl pyrophosphate amidotransferase (EC 2.4.2.14), the first enzyme on the AMP and GMP common pathway, and IMP dehydrogenase (EC 1.2.1.14), the first enzyme on the GMP-specific branch, have already been studied and their allosteric properties and their regulation by feedback effectors have been described[7,8]. This report completes the study of purine biosynthesis regulation in *S. pombe*, demonstrating that the adenylosuccinate synthetase—as the two enzymes above—is subject to feedback regulation by end-products and exhibits allosteric properties.

Only two of the three substrates—GTP and aspartate—show cooperative interactions. The third, IMP, does not. The same result was obtained for the *S. pombe* IMP dehydrogenase kinetics: the IMP saturation curve does not exhibit any homotropic effect, whereas the second substrate, NAD, does.

AMP is an allosteric effector and strongly inhibits adenylosuccinate synthetase of which the activity is reduced to half its value by 0.3 mM AMP. The same extent of inhibition of phosphoribosyl pyrophosphate amidotransferase activity is reached only at a 10-fold higher AMP concentration.

GMP, which is a powerful allosteric inhibitor of both phosphoribosyl pyrophosphate amidotransferase and IMP dehydrogenase, inhibits adenylosuccinate synthetase with an analogous efficiency, but apparently by a direct competitive effect for the GTP binding site.

These findings are in agreement with the AMP and GMP pool values, determined in *S. pombe* wild-type and *ade*-8 mutant cells prepared in the same conditions as for this study: the values of AMP pool are about 10-fold higher as compared to the GMP pool (2 and 0.2 μmoles/g of dry weight, respectively)[9]. In these conditions, in spite of an analogous efficiency of both mononucleotides observed *in vitro*, the regulation of adenylosuccinate synthetase *in vivo* may be ensured principally by AMP.

Concerning the allosteric properties of the enzyme, the interpretation of some of our results by the model of Monod *et al*[10]. is difficult: the enzyme cannot be classified in the V system because of the homotropic effect observed for the binding of two of the substrates. On the other hand, AMP affects essentially the V in both substrates saturation curves, which is incompatible with a K system. However, similar situations have already been described in other systems[8,11]. Another difficulty is the absence of cooperative effect of AMP at high substrates concentrations. Theoretically, an increased cooperativity should be observed with increasing substrates concentrations. This finding could be interpreted by a dissociation of the oligomeric structure into monomers under conditions of higher ionic strength.

An approach to the mechanism of the adenylosuccinate synthetase action has been made which indicates a fully random sequential mechanism. However, a definitive confirmation of this result would require the use of competitive inhibitors towards IMP and aspartate.

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