Adenylosuccinate synthase from *Saccharomyces cerevisiae*: homologous overexpression, purification and characterization of the recombinant protein

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Adenylosuccinate synthase (EC 6.3.4.4) catalyses the first committed step in the synthesis of adenosine. We have overexpressed the cloned gene of *Saccharomyces cerevisiae* (ADE12) in *S. cerevisiae*. The recombinant enzyme exhibits similar kinetic behaviour to that of the native enzyme purified from *S. cerevisiae*. This ter-reactant dimeric enzyme shows Michaelis–Menten kinetics only with IMP. L-Aspartate and GTP display a weak negative co-operativity (Hill coefficient 0.8–0.9). This negative co-operativity has not yet been reported for adenylosuccinate synthases from other organisms. Another unusual feature of the enzyme from *S. cerevisiae* is its negligible inhibition by adenine nucleotides and its pronounced inhibition by Cl^- ions.

Key words: ADE12, dimer, negative co-operativity.

INTRODUCTION

Adenylosuccinate synthase [IMP: L-aspartate ligase (GDP-forming), EC 6.3.4.4] is a key enzyme for the synthesis of adenosine *de novo*. It catalyses the reaction:

$$\text{IMP} + \text{L-aspartate} + \text{GTP} \rightarrow \text{adenylosuccinate} + \text{GDP} + \text{phosphate}$$

Adenylosuccinate synthase has been described from various sources ranging from archaebacteria [1] and bacteria [2] to mammals [3,4]. The enzyme from *Escherichia coli* is by far the best characterized enzyme [5–7]. The adenylosuccinate synthase from *E. coli* served Rudolph and Fromm [8] as a model for a ter-reactant enzyme. In their pioneering work they showed by initial rate studies that adenylosuccinate synthase binds its substrate in a sequential and fully random manner.

Isoforms of the enzyme have been described in higher organisms [9]. The liver isoenzyme (L-type) is believed to be involved in adenine synthesis, whereas the muscle type (M-type) contributes to glycolysis and ammoniagenesis through the purine cycle [4].

Adenylosuccinate synthase is a target of herbicides and drugs. The herbicidal action of hydantocidin is explained by the inhibition of adenylosuccinate synthase by the 5′-phosphorylated compound [10]. Likewise the ribonucleotide of the anti-trypanosomal agent allopurinol is selectively aminated by adenylosuccinate synthase from *Leishmania* ssp. [11].

Adenylosuccinate synthases from yeasts are only marginally characterized. Some enzymic characterization has been performed with the partly purified enzyme from *Saccharomyces pombe* [12]. Interestingly, the cadmium tolerance of *S. pombe* is in part explained by adenylosuccinate synthase activity [13].

Our group has identified the adenylosuccinate synthase from *Saccharomyces cerevisiae* as a single-stranded DNA-binding protein, which binds with high specificity to the single-stranded T-rich strand of the core sequence of the autonomously replicating sequence [14,15]. However, until now neither its contribution towards origin recognition nor the amino acid residues involved in DNA binding and sequence recognition have been elucidated. Progress in this field is hampered by the extremely low expression of the enzyme. A 50 litre yeast culture yields only approx. 400 μg of purified enzyme. In addition, purified adenylosuccinate synthase is not stable and loses its activity within weeks.

Here we describe the homologous overexpression of adenylosuccinate synthase and its purification. The recombinant enzyme is enzymically active; it displays negative co-operativity towards GTP and L-aspartate. Only IMP behaves strictly in accordance with Michaelis–Menten kinetics.

EXPERIMENTAL

Material

Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs and Boehringer Mannheim. Radiochemicals were obtained from Hartmann Analytics. Fractogel EMD-SO^{3-}, a cation-exchange resin, was purchased from Merck (Darmstadt). 5-Amino-4-imidazolecarboxamide riboside 5′-monophosphate (AICAribotide) was from Sigma. All reagents were of at least analytical grade.

Plasmid construction

DNA manipulations were performed essentially as described by Sambrook et al. [16] with the *E. coli* strain XL1-Blue as host. The plasmid pEMBLyex2-ASS (Figure 1) was constructed by inserting the coding sequence of the yeast adenylosuccinate synthase into the multiple cloning site of the yeast expression vector pEMBLyex2 [17]. Specifically, pJDBA12 [18], a gift from Dr. Yu.V. Andreichuk (St. Petersburg, Russia), was cut with *Bam*HI and *Sac*I, yielding a 1.3 kb fragment that was ligated into pEMBLyex2 restricted with the same enzymes.

Expression and purification of adenylosuccinate synthase

Cryopreserved competent yeast cells of strain BJ1991 [19] were prepared by the method of Dohmen et al. [20] and transformed with pEMBLyex2-ASS. Ura^+ transformants were detected on minimal agar plates [containing 2% (w/v) glucose, 0.67% yeast

Abbreviation used: AICAribotide, 5-amino-4-imidazolecarboxamide riboside 5′-monophosphate.

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nitrogen base without amino acids (Difco), 20 mg/l L-tryptophan, 20 mg/l adenine, 20 mg/l L-histidine, 20 mg/l L-methionine, 20 mg/l L-lysine and 30 mg/l L-leucine] grown at 30 °C for 3 days and subsequently cultured on minimal agar plates. For large-scale expression of adenylosuccinate synthase, 10–50 ml of minimal medium without uracil and leucine was inoculated with transformed yeast cells and grown for 24–48 h on an orbital shaker at 30 °C. Expression was induced by inoculating the preculture into 10 vol. of complete medium [2% (w/v) galactose/1% (w/v) yeast extract (Roth, Karlsruhe, Germany)/2% (w/v) tryptone (Roth)]. These shaken-flask cultures were grown for up to 72 h at 30 °C and 200 rev./min.

Cells were collected by centrifugation, washed twice with tap water and frozen at −70 °C until use. Frozen cells were resuspended in 3 vol. of lysis buffer [50 mM Tris/HCl/10 mM NH_4Cl/20 mM magnesium acetate/10 mM KCl/0.1 mM PMSF/1 mM 2-mercaptoethanol/0.1 mM EDTA/10% (v/v) glycerol (pH 7.0)] and whole cell extract was prepared by vortex-mixing the cells with prechilled glass beads. Crude extract was obtained by centrifugation at 45000 ***g*** for 60 min. The crude extract was loaded directly at 0.1 ml/min on a 4 cm × 2.6 cm (inner diameter) EMD-SO^{3-} column equilibrated with buffer A [20 mM potassium phosphate/2 mM $MgCl_2$/1 mM 2-mercaptoethanol/0.1 mM EDTA/5% (v/v) glycerol/80 mM KCl (pH 7.0)]. The column was developed with a 100 ml linear gradient of 1 M KCl in buffer A at a flow rate of 1 ml/min. Pure adenylosuccinate synthase was eluted at approx. 200 mM KCl. Pooled fractions were dialysed against 25 mM potassium phosphate/30% glycerol (pH 8.0). The enzyme was stored at −20 °C and remained stable for months.

N-terminal sequencing

Purified adenylosuccinate synthase was spotted on a Polybrene membrane and used directly for the determination of N-terminal amino acid residues with an Applied Biosystems model 476A protein sequencer.

Protein determination

Protein concentrations were determined spectrophotometrically by the method of Ehresmann [21] or colorimetrically by the method of Bradford [22].

Enzyme assays

For the direct assay, the formation of adenylosuccinate (ϵ_{280} 11.7 $mM^{-1} \cdot cm^{-1}$) was monitored for 10–30 min at 280 nm with a Beckman DU 640 spectrophotometer at 25 °C. Adenylosuccinate synthase was preincubated in assay buffer [25 mM potassium phosphate/16 mM magnesium acetate/125 μM GTP/250 μM IMP (pH 8.0)]. The reaction was started by the addition of 2.5 mM L-aspartate. For crude extracts a GTP-regenerating system (1 mM phosphoenolpyruvate/1 unit of pyruvate kinase) was included. The total assay volume was 0.5 ml.

The coupled assay used the regeneration of GDP by pyruvate kinase and phosphoenolpyruvate leading to pyruvate, which was reduced to lactate by lactate dehydrogenase. The concomitant consumption of NADH (ϵ_{340} 6.22 $mM^{-1} \cdot cm^{-1}$) was monitored for 10–30 min with a Beckman DU 640 photometer or an SLT Spectra microtitre plate reader at 340 nm. Adenylosuccinate synthase was preincubated for 5 min in assay buffer supplemented with 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 0.15 unit of pyruvate kinase and 0.2 unit of lactate dehydrogenase. The reaction was initiated by the addition of 2.5 mM L-aspartate. The enzymic reaction was performed at 25 °C. The total assay volume was 0.5 ml (photometer) or 0.15 ml (microtiter plate reader).

Both assays resulted in identical measures of activities. For most determinations the coupled assay was used. The direct assay was performed when ADP had to be included in the reaction mixture. The coupled assay was modified for K_m determination by varying one substrate concentration while keeping the other substrate concentrations constant. For the determination of K_i values the concentrations of a substrate and of an inhibitor were varied. Fitting of the initial rate data was performed with a spreadsheet calculation program or with the DYNAFIT program [23].

Gel filtration

Gel filtration was performed on a Toso Haas TSK G3000SW (60 cm × 0.75 cm, inner diameter) column at a Beckman HPLC system. Adenylosuccinate synthase solution (50 μl; 0.8–80 μM monomer concentration) was loaded at 0.5 ml/min on the column equilibrated with 100 mM sodium sulphate/100 mM sodium phosphate (pH 7.2). Calibration was performed with β-amylase (200 kDa), BSA (67 kDa), ovalbumin (43 kDa), ribonuclease (13.7 kDa) and aprotinin (6.5 kDa). Total volume (V_t) and void volume (V_0) were determined with thyroglobulin (670 kDa) and *p*-aminobenzoic acid respectively. The molecular mass M was calculated by using linear regression between $\log M$ and $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the enzyme. The coefficient of regression, r^2, was at least 0.99.

RESULTS

Overexpression and purification of adenylosuccinate synthase

The construct pEMBLyex2-ASS (Figure 1) allowed the expression of adenylosuccinate synthase under the control of galactose in the growth medium. Crude extracts of an induced

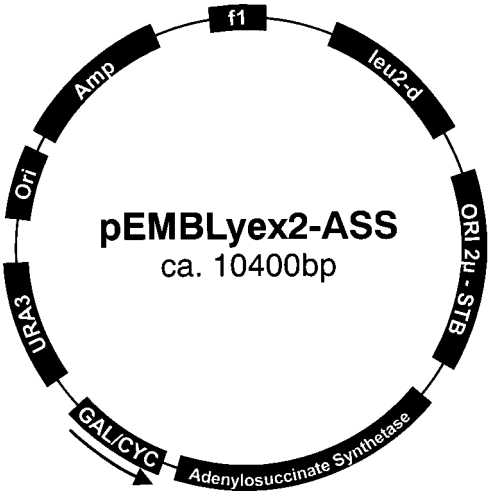

Figure 1 Yeast expression plasmid pEMBLyex2-ASS

The plasmid was constructed as described in Experimental Procedures. The ampicillin resistance gene (*Amp*) and the bacterial plasmid origin *colE1* (ORI) allow the selection and maintenance of the shuttle vector in *E. coli* hosts. Yeast selection markers are *leu2-d* (β-isopropylmalate dehydrogenase gene with a defective promoter) and *URA3* (orotidine-5′-phosphate decarboxylase gene). Replication in yeast cells is ensured in *cir*$^+$ hosts through the ORI 2μ and STB sequences. The expression of adenylosuccinate synthase is controlled by the galactose-inducible *GAL/CYC* hybrid promotor. Polyadenylation and transcriptional termination signals are located between the *Hin*dIII and the STB (*cis*-acting region for stability) sequence.

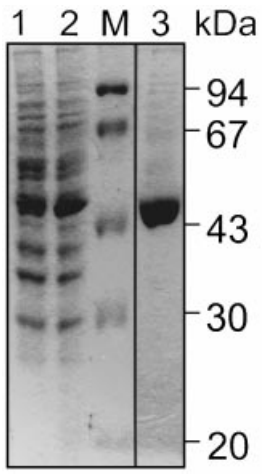

Figure 2 Overexpression of yeast adenylosuccinate synthase

Cells from BJ1991 transformed with pEMBLyex2-ASS were grown under uninduced (lane 1) and induced (lane 2) growth conditions. Crude extracts from these cultures were prepared and separated by SDS/PAGE. Lane 3, purified adenylosuccinate synthase; lane M, molecular mass standards (molecular masses are shown at the right). The gel was stained with Coomassie Blue.

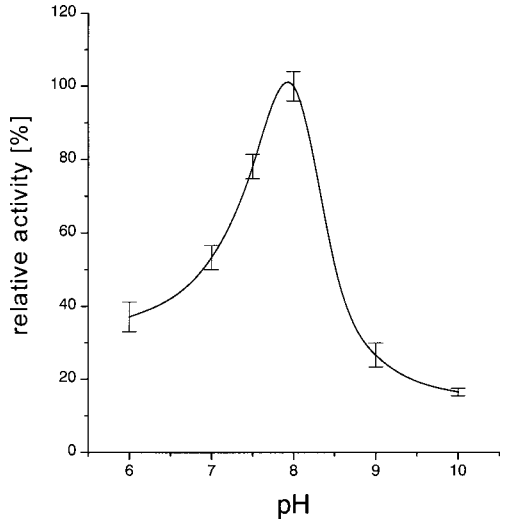

Figure 3 pH–activity profile of adenylosuccinate synthase

Adenylosuccinate synthase (0.3 μM) was added to the assay buffer buffered with 100 mM Tris/acetate in the pH range 6.0–10.0. The enzymic reaction was started by the addition of 2.5 mM L-aspartate. S.D. values for three assays are indicated.

and an uninduced culture were analysed by SDS/PAGE (Figure 2). There was an additional protein of apparent molecular mass approx. 47 kDa, corresponding to the theoretical molecular mass of 48 kDa. The overexpressed adenylosuccinate synthase constituted approx. 20 % of total soluble cell protein as estimated by densitometry of the Coomassie Blue-stained gel.

Cation-exchange chromatography yielded pure adenylosuccinate synthase (Figure 2 and Table 1). The N-terminus of the purified recombinant protein was determined as being Val-Asn-Val-Val, which is identical with the N-terminus of native adenylosuccinate synthase [15]. Both proteins lack the initiator methionine residue.

Enzyme characterization

Preliminary experiments suggested that the adenylosuccinate synthase activity is very dependent on reaction conditions. For example, adenylosuccinate synthase was 4-fold more active in 33 mM potassium phosphate buffer, pH 8.0, than in 33 mM Hepes, pH 8.0. This observation led us to make a careful study of the reaction conditions. We found that the activity was optimal in 25 mM potassium phosphate/16 mM magnesium acetate (pH 8.0). The optimal Mg^{2+} concentration of 16 mM was surprisingly high, but at 8 mM magnesium acetate the enzymic activity was 17 % lower. The greater activity at 16 mM magnesium acetate was not due to the increased concentration of the counter-ion acetate because the addition of sodium acetate did not affect enzymic activity.

Adenylosuccinate synthase exhibited a pronounced pH-dependence with a pH optimum of approx. pH 8.0 (Figure 3). The enzyme activity was independent of the redox potential of the assay solution, which we varied by adding the GSH-to-GSSG ratio between 20:1 and 1:5 (results not shown).

Adenylosuccinate synthase was revealed to be extremely sensitive towards Cl^- concentration (Figure 4), with an apparent K_i of approx. 8 mM. This pronounced inhibition was not observed with other salts that were added to the assay solution (Figure 4).

After having established the optimal reaction conditions we determined K_m for the substrate and also the Hill coefficient, h (Table 2). Only IMP showed strict Michaelis–Menten binding. L-Aspartate and GTP had a weakly negative co-operative binding as can be seen from the curvature in the Eadie–Hofstee plots (Figure 5). The negative co-operativity of L-aspartate and GTP was confirmed by several independent measurements of various fractions and purifications. Negative co-operativity was also found after an additional heparin–Sepharose purification step

Table 1 Purification of adenylosuccinate synthase

A 125 ml yeast culture was purified with a yield of 61 % and a 4.5-fold enrichment.

	Protein		Adenylosuccinate synthase activity	
Fraction	(mg)	(mg/ml)	(μmol/min)	(μmol/min per mg)
Crude extract	214	19.5	12.6	0.06
Purified adenylosuccinate synthase	28.8	1.4	7.7	0.27

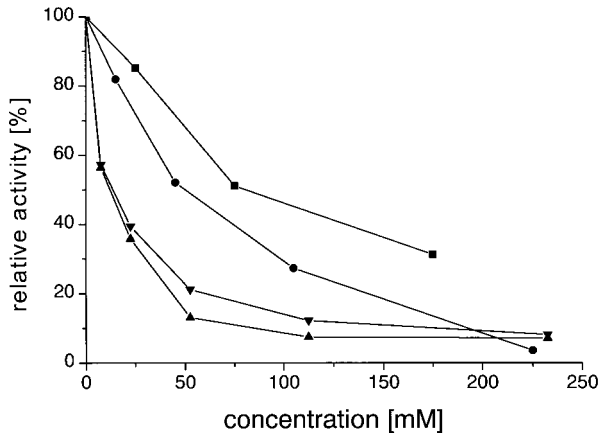

Figure 4 Salt-dependence of adenylosuccinate synthase activity

Adenylosuccinate synthase (0.4 μM) was added to the assay buffer supplemented with four different salts (■, K_3PO_4; ●, Na_2SO_4; ▲, KCl; ▼, NaCl). The enzymic reaction was started by the addition of 2.5 mM L-aspartate. All measurements were made in duplicate.

(results not shown). To exclude the possibility of heterogeneity in the enzyme preparation, chromatofocusing on a Mono P column was attempted. Although the calculated isoelectric point of adenylosuccinate synthase is 8.3 (GCG program), adenylosuccinate synthase was not retarded on the chromatofocusing column (pH 9–6). In addition, the recovery of protein was rather low and the activity was lost. The addition of 0.1% (v/v) Nonidet P40 (Fluka) to the buffers did not improve the recovery of enzyme. Similarly, isoelectric focusing resulted in smeared bands (results not shown).

Inhibition by AMP, ADP, ATP, GMP, adenylosuccinate and AICAribotide 5′-monophosphate

Various adenine nucleotides inhibited yeast adenylosuccinate synthase only marginally (Table 3). The apparent K_i values for these compounds were in the millimolar range and the type of inhibition was not investigated further. ATP was not used by the enzyme as the phosphate donor instead of GTP, and the inhibition by ATP was weak.

Adenylosuccinate was found to be a weak inhibitor. In contrast with a report from Ryzhova et al. [24], we found no inhibition by AICAribotide, an intermediate of the purine synthesis pathway. The lack of inhibition was observed with both the direct assay and the coupled assay.

GMP was a potent inhibitor; inhibition was competitive towards IMP (Table 3). In Figure 6 the initial-rate raw data are superimposed on the fitted data for competitive binding. In the inset the linear relationship between K^{IMP}_m (app) and [GMP] is demonstrated.

The inhibition of GTP by GMP seems to be competitive as well. When $1/v$ was plotted against 1/[GTP] (Lineweaver–Burk plot) for various GMP concentrations the curves intersected at the ordinate. With the use of a common maximal velocity for all GMP concentrations a set of parallel lines are obtained in the Hill plot (Figure 7). The inset shows the dependence of the apparent value of K_{Hill} (the intercept of the Hill plot) on GMP concentration.

Quaternary structure of adenylosuccinate synthase

It has been shown previously that the adenylosuccinate synthases from *E. coli* and from *S. cerevisiae* are dimeric [14,25]. The *E. coli* enzyme is active only as a homodimer; its dissociation constant decreases by several orders of magnitude on binding substrate [26]. Analytical gel filtration of overexpressed adenylosuccinate synthase in the absence of substrates yields a molecular mass of 77 kDa, indicating that recombinant adenylosuccinate synthase also dimerizes. Gel filtrations were performed at different precolumn monomer concentrations ranging from 0.8 to 80 μM (Figure 8). The line-shapes of the peaks down to the precolumn concentration of 4 μM were essentially identical, making it very unlikely that a significant dissociation of the dimers occurred during these column runs. At the precolumn concentration of 0.8 μM a broad twin peak was observed. However, at this low concentration the A_{210} was only 5×10^{-3} absorbance units, which was close to the sensitivity limit of the optical system. Taking into account the fact that the proteins were diluted approx. 25-fold during the column run, it is not unlikely that adenylosuccinate synthase dissociates into monomers at concentrations below 0.8 μM. In the absence of substrates the *E. coli* enzyme has a dissociation constant for dimerization of 4.1–11.2 μM as determined by analytical ultracentrifugation [26].

Table 2 Substrate binding constants of adenylosuccinate synthase from yeast and *E. coli*

The apparent K_m, K_{Hill} and h values were determined by the coupled assay. For each determination, eight concentrations were measured in triplicate. The experiments were done at least twice; means ± S.D. are shown. The L-aspartate concentration was varied between 200 μM and 10 mM, that of IMP between 40 and 720 μM and that of GTP between 15 and 750 μM. K_m values were calculated with the DYNAFIT program [23]. K_{Hill} and h are the intercept and slope of the Hill plot of $\log[v/(v_{max} - v)]$ against log[S].

	S. cerevisiae					
Compound	Recombinant K_m (μM)	K_{Hill} (μM)	h	Native‡ K_m (μM)	*S. pombe** K_m (μM)	*E. coli*† K_m (μM)
L-Aspartate	(1650)§	1750 ± 190	0.87 ± 0.03	1000	1500	280/350
IMP	200 ± 18	–	–	300	200	21/60
GTP	(81)§	49 ± 8.7	0.79 ± 0.05	60	20	22/54

* From [12]
† The first value is from [7], the second from [34].
‡ From [14].
§ L-Aspartate and GTP show negative cooperativity; the K_m values are therefore given only for comparative purposes.

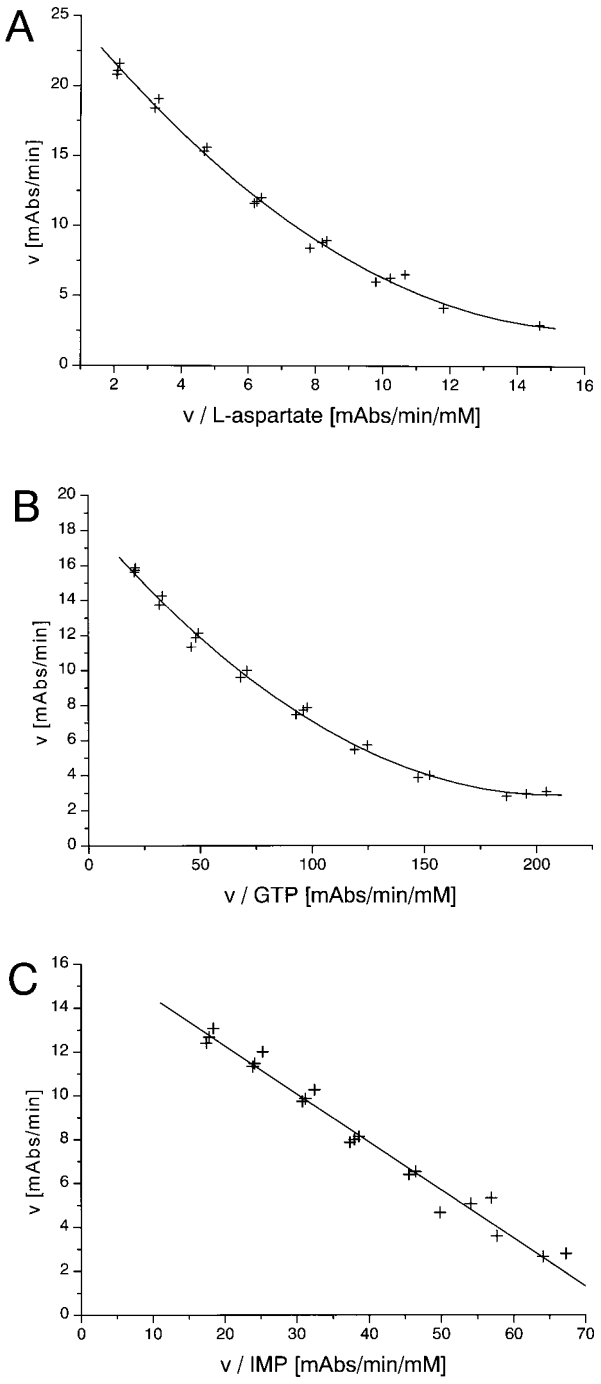

Figure 5 Eadie–Hofstee plots of all three substrates

The measurements were made as indicated in the legend to Table 2; the variable substrates were L-aspartate (**A**), GTP (**B**) and IMP (**C**). A linear relationship was found only for IMP. Abbreviation: mAbs, 10^{-3} absorbance unit.

DISCUSSION

By using the galactose-inducible yeast expression plasmid pEMBLyex2 an efficient overexpression of adenylosuccinate synthase (approx. 20 % of the total soluble protein) was obtained. Adenylosuccinate synthase activity was readily detectable in the crude extract of transformed cells but not in the crude extract of control cultures. The purification of overexpressed adenylosuccinate synthase could be decreased to a single chromatographic step. In contrast, purification of the native enzyme requires four columns [14,15].

Recombinant adenylosuccinate synthase proved to be enzymically fully active (see below). However, in contrast with the native enzyme, the recombinant enzyme did not bind to the T-rich strand of single-stranded autonomously replicating core sequence. The structural basis of the binding activity towards single-stranded DNA is still not known. Although molecular modelling [27] revealed a basic region at the dimer interface that might be involved in binding the phosphate groups of the DNA backbone, there is no hint of how the highly sequence-specific recognition of the autonomously replicating core sequence is achieved by adenylosuccinate synthase. Gallert et al. [14] demonstrated that dephosphorylation of the adenylosuccinate synthase leads to a loss of DNA binding but leaves the enzymic activity unchanged. If adenylosuccinate synthase does indeed have a role in replication initiation at autonomously replicating sequence sites, a cell-cycle-dependent phosphorylation/dephosphorylation is likely. Without further information on the functional significance of the DNA-binding activity and with the still incomplete knowledge of the target sequences of yeast protein kinases [28] it is too early to speculate on which protein kinases could activate the DNA-binding activity of adenylosuccinate synthase. However, it seems that the greatly overexpressed protein is not properly modified post-translationally and is therefore not competent in DNA binding. Unfortunately, adenylosuccinate synthase has 45 serine and threonine residues, making it impossible to compare the phosphorylation patterns of the native and the overexpressed proteins.

To permit a better comparison of the kinetic constant of the recombinant adenylosuccinate synthase with the previously published data on adenylosuccinate synthase from *S. cerevisiae*, *S. pombe* and *E. coli*, we also calculated the Michaelis–Menten constants with GTP and L-aspartate (Table 2), although they show weak negative co-operative binding. The results for the recombinant enzyme are in agreement with the kinetic constants determined for the native enzyme. Owing to the low yield of native adenylosuccinate synthase after purification, a more sensitive assay had to be used for its kinetic characterization. This assay quantifies the L-aspartate-dependent hydrolysis of [γ-^{32}P]GTP. Of course this assay cannot be as precise as a continuous photometric assay and it is highly probable that the weak negative co-operativity that we found for L-aspartate and GTP was overlooked when studying the native enzyme. Nevertheless the Michaelis–Menten constants do not differ by more than a factor of two. In addition the kinetic values are similar to those of the *S. pombe* enzyme. Only the K_m for GTP seems to be considerably lower for the *S. pombe* enzyme. The K_m values of the *E. coli* enzyme are clearly lower for all three substrates. The specific activity of pure adenylosuccinate synthase is 0.27 μmol/min per mg. If we assume a completely active enzyme, a k_{cat} of 0.2 s^{-1} per monomer is obtained. This is approximately one-fifth of that of the *E. coli* enzyme, which is also based on the monomer concentration [26]. A k_{cat} for the *S. pombe* enzyme has not been reported.

Negative co-operativity has not previously been observed for any of the characterized adenylosuccinate synthases. Apparent negative co-operativity is also found in heterogeneous enzyme preparations containing isoenzymes with differing substrate affinities. The presence of isoenzymes is very difficult to exclude experimentally. We found negative co-operativity in all fractions tested. In addition, negative co-operativity is not shown for IMP. This would imply that the presumed isoenzymes have different affinities for GTP and L-aspartate but not for IMP. Furthermore,

Table 3 Apparent K_i values of recombinant adenylosuccinate synthase from yeast and *E. coli*

K_i (app) values were determined with all substrates at a concentration of twice the K_m. AMP and ATP concentrations were varied between 1 and 8 mM, and those of ADP and adenylosuccinate between 0.3 and 3 mM. AICAribotide concentration was varied between 0.16 and 5 mM. Because ADP absorbs strongly at 280 nm, the determination of K_i^{ADP} had to be performed in cuvettes with 1 a mm pathlength. K_i (app) values were calculated from a linear regression of the plot of inhibitor concentration against the reciprocal of activity. The concentration of GMP was varied between 30 and 240 μM. K_i values were calculated with the DYNAFIT software.

Compound	K_i (app) (mM) *S. cerevisiae*	K_i (μM) *S. cerevisiae*	*S. pombe**	*E. coli*†
AMP	6		350	95
ADP	$\gg$ 3‡			
ATP	4			
AICAribotide	$\gg$ 5§			
Adenylosuccinate	2.7			
GMP		57 $\pm$ 3 (against IMP)	300	74 (against IMP) 92 (against GTP)

* From [12].
† From [35].
‡ No inhibition up to 3 mM ADP.
§ No inhibition up to 5 mM AICAribotide.

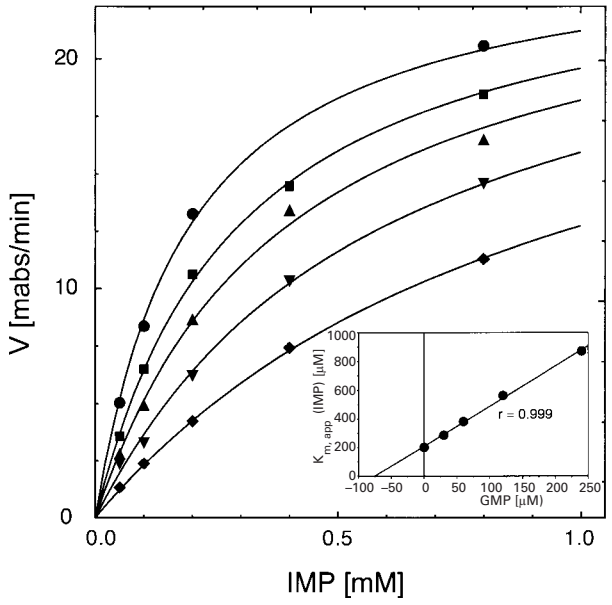

Figure 6 GMP is a competitive inhibitor towards IMP

The inhibition was studied by varying [IMP] in the range 0.05–0.8 mM and [GMP] in the range 0–240 μM (●, 0 μM; ■, 30 μM; ▲, 60 μM; ▼, 120 μM; ◆, 240 μM). Top: the initial rate data are plotted against the IMP concentration. Bottom: linear dependence between $K_{m,app}$ (IMP) and the GMP concentration. Abbreviation: mabs, 10^{-3} absorbance unit.

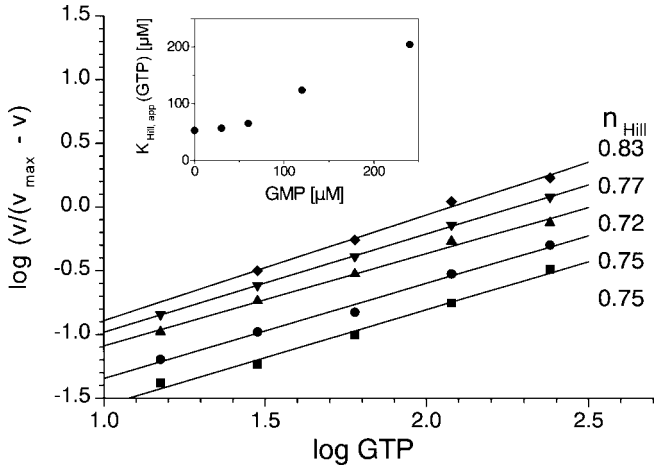

Figure 7 GMP shows negative co-operativity towards GTP

Inhibition was studied by varying GTP in the range of 15 to 480 μM and GMP in the range 0 to 240 μM (◆: 0 μM, ▼: 30 μM, ▲: 60 μM, ●: 120 μM and ■: 240 μM). Shown is a Hill plot. The Hill coefficient of each curve is indicated. In the inset the apparent K_{Hill} is plotted against the GMP concentration.

the N-terminus of the recombinant enzyme was found to be homogeneous. There is only one gene for adenylosuccinate synthase in the *S. cerevisiae* genome. We are therefore sure that the negative co-operativity that we observed is not an experimental artifact. If we take into account the fact that the *E. coli* adenylosuccinate synthase dimers have two shared active sites [25] and that the active site is close to the dimer interface [27], it is not unlikely that binding on one dimer half changes the structure of the other dimer half. In *E. coli* it was even observed that substrate binding promotes dimer formation [26]. However, the *S. cerevisiae* enzyme might be active as both a monomer and a dimer. In that case, depending on the dissociation constants of the monomer and the substrate-bound monomer, negative co-operativity might be observed [29].

As a key enzyme of adenine biosynthesis that not only represents a committed step in the synthesis of AMP but is also located at the branchpoint of AMP and GMP synthesis, tight regulation of the adenylosuccinate synthase activity is to be expected. However, the inhibition data on the adenylosuccinate synthase enzymes studied so far do not support this notion. There is inhibition by GMP and GDP (Table 3) but neither nucleotide is a product of the adenosine-synthesizing pathway; they are consequently not feed-back inhibitors.

The *S. pombe* and the *E. coli* enzymes are inhibited by AMP. In contrast, the *S. cerevisiae* adenylosuccinate synthase is

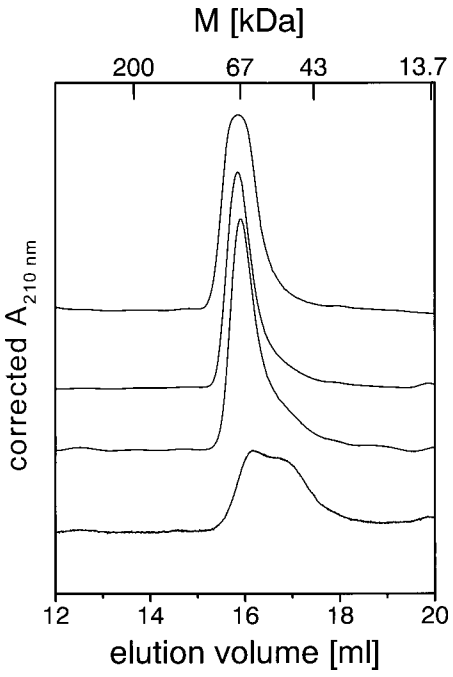

Figure 8 Gel filtration of recombinant adenylosuccinate synthase

The four traces represent gel filtrations at precolumn monomer concentrations of 80, 16, 4 and 0.8 μM (top trace to bottom trace). The absorbances were normalized by protein concentration to compare the different runs. On the top axis the molecular masses of the gel-filtration standard proteins are indicated at the volumes at which they were eluted.

inhibited only in the millimolar range by AMP. Because the concentration of AMP *in vivo* is approx. 0.01 mM [30], this inhibition cannot be of physiological relevance. Only ATP might have a regulatory impact on the enzyme. The K_i of approx. 4 mM is close to the physiological ATP concentration of approx. 2.5–3 mM [31,32].

The type of inhibition of GMP on IMP is clearly competitive. The K_m for IMP is approximately three times the K_i for GMP, which is remarkable. GMP seems to be also competitive towards GTP. Owing to the non-linear substrate binding of GTP a determination of the K_i for GMP is more complex. It is possible that there is also co-operative behaviour of GMP at the GTP-binding sites. As a rough estimate, the apparent K_{Hill} for GTP doubles at approx. 100 μM GMP (Figure 7, inset).

Ryzhova et al. [24] found that their preparation of recombinant adenylosuccinate synthase was inhibited by AICAribotide, an intermediate of the purine metabolism. According to their study AICAribotide is a competitive inhibitor (K_i 0.36 mM) of IMP (K_m 1.7 mM). We have found a value of $K^{IMP}{}_m$ that is one-eighth of this. In addition we did not find any inhibition by AICAribotide. We do not have an explanation for these discrepancies.

Our results suggest that the activity of yeast adenylosuccinate synthase is not tightly regulated by precursors, intermediates or products of the purine-synthesizing pathway. In addition, there are no reports that the activity of adenylosuccinate synthase is regulated by post-translational modifications. Instead, transcription of the adenylosuccinate synthase gene is co-regulated in *S. cerevisiae* with the enzymes for the synthesis of purines *de novo*. The transcription of these genes is efficiently repressed by adenine in the medium; full expression requires the transcription factors BAS1 and BAS2 [33].

We have succeeded in expressing and purifying adenylosuccinate synthase from *S. cerevisiae*. By kinetic studies we have demonstrated that the ter-reactant enzyme displays negative co-operativity towards GTP and L-aspartate. Further studies are required to elucidate the basis of the negative co-operativity and to analyse whether the dimerization of yeast adenylosuccinate is promoted by substrate binding, a property observed for the *E. coli* enzyme. The recombinant enzyme will also be helpful for raising antibodies that will facilitate the purification of larger amounts of native adenylosuccinate synthase.

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