Purification and cDNA-derived Sequence of Adenylosuccinate Synthetase from *Dictyostelium discoideum*

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Adenylosuccinate synthetase (IMP: l-aspartate ligase, EC 6.3.4.4) plays an important role in purine biosynthesis catalyzing the GTP-dependent conversion of IMP to AMP. The enzyme was purified from the cytosol of *Dictyostelium discoideum* using GTP-agarose chromatography as the critical step. It has an apparent molecular mass of 44 kDa. Monoclonal antibodies identified several forms of the enzyme with pI values between 8.1 and 9.0. Michaelis-Menten constants ($K_m$) were low for the nucleotide substrates IMP ($K_m = 30 \mu M$) and GTP ($K_m = 35 \mu M$) as compared with the value for aspartic acid ($K_m = 440 \mu M$). These values are in good agreement with constants reported from other organisms. Immunological studies indicated that the protein is predominantly localized in the cytosol and only partially associated with particulate fractions. The enzyme is present throughout the developmental cycle of *D. discoideum*. Using monoclonal antibodies, the gene was cloned from a λgt11 expression library. The complete sequence represents the first reported primary structure of an eucaryotic adenylosuccinate synthetase. Southern blots hybridized with a cDNA probe demonstrate that adenylosuccinate synthetase is encoded by a single gene and contains at least one intron. The deduced amino acid sequence shows 43% identity to adenylosuccinate synthetase from *Escherichia coli*. Homologous regions include short sequence motifs, such as the glycine-rich loop which is typical for GTP-binding proteins.

GTP-binding proteins with an intrinsic GTPase activity have a regulatory function in many cellular processes, including such as in housekeeping pathways (1), trans-membrane signaling (2, 3), vesicle sorting (4, 5), ribosomal protein synthesis (6), or polymerization of cytoskeletal elements (7). In the regulation of purine nucleotide biosynthesis the GTP-hydrlyzing adenylosuccinate synthetase (IMP:l-aspartate ligase, EC 6.3.4.4) is the first enzyme on the AMP-specific branch (8, 9). Its central regulatory role in the development of *D. discoideum* is schematically in Fig. 2A. The enzyme is abundant in the soluble fraction of the cells. In order to examine a possible association with other cellular components, GTP is required as a cofactor in adenylosuccinate synthetase and ATP as a cofactor in guanylate synthetase, thereby maintaining the appropriate ATP/GTP ratio. Feedback inhibition of adenylosuccinate synthetase is exerted by its reaction products (11-14).

Adenylosuccinate synthetase has been purified and characterized from various sources ranging from *Escherichia coli* to vertebrate tissues (for review see Ref. 9). The enzyme from *E. coli* which is encoded by the purA locus was recently cloned and sequenced (15). In rat liver two isoenzymes have been identified (16), which can be distinguished by their isoelectric points as acidic (pI = 5.9) or basic (pI = 8.9) isoforms. In skeletal muscle tissue, where the highest levels of enzyme activity are observed, only the basic form has been found (17). The acidic isoform seems to be involved mainly in purine biosynthesis and the basic isoform in the purine nucleotide cycle (16).

In this paper we describe the purification of adenylosuccinate synthetase from the cytoplasmic fraction of *Dictyostelium discoideum*. The enzyme shows Michaelis-Menten constants ($K_m$) similar to those found for the corresponding enzymes from human placenta (11), pig brain (18), Azotobacter vinelandii (19), Leishmania donovani (20), and *E. coli* (12). In *D. discoideum* the enzyme is encoded by a single gene. The cDNA-deduced amino acid sequence from *D. discoideum* was analyzed in comparison with the *E. coli* enzyme. It provides the first sequence of an eucaryotic adenylosuccinate synthetase.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Isolation of *D. discoideum* Adenylosuccinate Synthetase from the Cytosol and Determination of Its Cellular Distribution—The purification of adenylosuccinate synthetase from the cytosol of axenically grown *D. discoideum* cells is summarized schematically in Fig. 1A. The GTP binding capacity was employed for detection of the enzyme by direct photoaffinity labeling with [α-32P]GTP. By ion exchange and gel filtration chromatography all other proteins that bound to GTP could be separated from the enzyme. Consequently, GTP affinity chromatography as the final purification step resulted in a homogeneous enzyme preparation showing a protein with an apparent molecular mass of 44 kDa (Fig. 1B). GTP cross-linking experiments indicated that adenylosuccinate synthetase is abundant in the soluble fraction of the cells. In order to examine a possible association with other cellular components, portions of this paper (including "Experimental Procedures" and Figs. 2, 5, and 6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

2480
FIG. 1. Purification of adenylosuccinate synthetase from the cytosol of *D. discoideum*. A, diagram summarizing the steps described under “Experimental Procedures” which resulted in pure adenylosuccinate synthetase as shown in B. SN, supernatant. B, pooled fractions collected after the gel filtration step were chromatographed on GTP-agarose. The bulk of loaded proteins was analyzed by electrophoresis and proteins stained with Coomassie Blue. Molecular mass markers are indicated in kilodaltons (kDa).

portments, we tested pellet and supernatant fractions after differential centrifugation and salt extraction steps in immunoblots with mAb² 100-451-2. As expected, we found adenylosuccinate synthetase predominantly in the 150,000 × g supernatant. The enzyme was detected weakly in plasma membrane-enriched fractions after centrifugation at 15,000 × g and could be solubilized after extraction with 1.5 M KCl. Stronger associations seem to exist with intracellular membranes or cytoskeletal elements present in the 150,000 × g pellet.

The isoelectric pH of cytosolic adenylosuccinate synthetase was determined by two-dimensional gel electrophoresis. Usually two to four spots were recognized by mAb 100-451-2 upon immunoblotting. PI values were concentrated between 9.0 and 8.7 as well as between 8.6 and 8.1. The same pattern appeared with enzyme preparations from the particulate fraction (data not shown).

**Catalytic Properties**—The purified adenylosuccinate synthetase had a specific activity of 117 × 10³ units/min × mg of protein. Michaelis-Menten constants (Km) of the pure enzyme were determined in Lineweaver-Burk plots. While keeping the concentrations of the second and third substrate at standard levels the tested substrate was varied between 60 and 500 μM in the cases of GTP and IMP or between 100 and 2,000 μM in the case of aspartic acid. Km for GTP was 35 μM (eight determinations), for IMP 30 μM (nine determinations), and for aspartic acid 440 μM (eight determinations). Aspartate at a concentration of 4 mM supported maximal activity; a concentration of 8 mM was inhibitory. Pure adenylosuccinate synthetase could be fast-frozen and stored at −70 °C for more than 2 years.

**Nucleoside Triphosphate Specificity and Immunoprecipitation**—Adenylosuccinate synthetase from different sources has been described to be specific for GTP, thus maintaining the ATP/GTP ratio within the cells (9). Therefore, we compared the capacity of [α-³²P]GTP and [α-³²P]dATP to directly label adenylosuccinate synthetase for the *D. discoideum* enzyme. It is evident from Fig. 2A that a protein of 44 kDa apparent molecular mass highly preferred GTP as the nucleoside triphosphate. The labeling intensities differ by a factor of 18, which probably represents an underestimation, as the specific activity of [α-³²P]GTP (30 TBq/mol) was lower compared with the one of [α-³²P]dATP (111 TBq/mol). In other experiments we used excess concentrations of unlabeled nucleotides for competition with [α-³²P]GTP. The results showed the following hierarchy of binding: GTP > ATP > UTP = CTP. [α-³²P]GTP photoaffinity-labeled proteins enriched by DE52 chromatography were immunoprecipitated with monoclonal antibodies specific for adenylosuccinate synthetase. The autoradiogram in Fig. 2B demonstrates that mAb 100-451-2 precipitated the label. These observations show that adenylosuccinate synthetase is identical to the 44-kDa GTP-binding protein.

**Sequence Analysis**—The complete cDNA comprises 1358 bp. The 5'-noncoding part covers 58 nucleotides with an A + T content of 91%, and the 3'-noncoding portion a poly(A) stretch of 19 nucleotides (Fig. 3A). The coding region with an A content of 65% predicts a polypeptide of 427 amino acids with a calculated molecular mass of 47.3 kDa, which is close to 44 kDa as found by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Searches for similarities to other proteins in the protein data base revealed significant homologies of the deduced amino acid sequence only to the adenylosuccinate synthetase from *E. coli* (43% identity (15)). The alignment of the two amino acid sequences in Fig. 4 shows that blocks of identical amino acids are distributed throughout the total sequence.

**Southern Blot Analysis, and Expression of mRNA and Protein during Development—Genomic DNA from *D. discoideum* was cut with restriction enzymes that recognized either none or only one site within the cDNA insert cDAS-1. The hybridization pattern obtained in Southern blots with cDAS-1 as a probe suggested that adenylosuccinate synthetase is encoded by a single gene (Fig. 5). However, we cannot exclude the existence of a related gene, since after cleavage with EcoRI, in addition to a strongly labeled 3.8-kb band a weakly hybridizing 3.4-kb band was found. After AsnI digestion two strongly labeled bands at 800 and 400 bp appeared. Because of the lack of a corresponding restriction site within cDAS-1 we suggest that an intron exists in the genomic region covered by cDAS-1. A genomic map combining the different digestion patterns positions an intron of less than 100 bp around base pair 400 of the coding region (Fig. 3B). Small introns are common in *D. discoideum* (45).

Northern blots of total RNA from *D. discoideum* cells hybridized with cDAS-1 demonstrate that adenylosuccinate synthetase is translated from a single transcript of 1.5 kb (Fig. 6A). The lack of about 150 bp in the cDNA sequence shown in Fig. 3A is presumably due to random priming of reverse transcription at poly(A) stretches at the 3'-noncoding region. Both the amount of adenylosuccinate synthetase mRNA and of the protein (Fig. 6B) slowly decreased during development from the growth phase (T₅₀) to culmination (T₇₁).

**DISCUSSION**

We purified *D. discoideum* adenylosuccinate synthetase to homogeneity utilizing its high affinity for GTP. The enzyme catalyzes the synthesis of adenylosuccinate from IMP, aspartic acid, and GTP in the *de novo* biosynthesis of purines (46). In photoaffinity labeling and competition experiments adenylosuccinate synthetase preferentially bound GTP as compared with other nucleoside triphosphates. This feature illustrates an essential element of the control function exerted by
Dictyostelium Adenylosuccinate Synthetase

DAS:  EAS

DAS

EAS

DAS

EAS

DAS

EAS

DAS

EAS

DAS

EAS

2482

FIG. 3. cDNA and deduced amino acid sequence of adenylosuccinate synthetase from D. discoideum (A), and sequencing strategy and genomic structure (B). The nucleotide sequence (A) comprising the complete coding region (hatched box) was assembled from the cDNA inserts of recombinant λgt11 phages, cDAS-1 and cDAS-2, and from the cDNA-derived PCR products, DAS-5 and DAS-3 (B). Arrows indicate the direction of sequencing; circles represent sequence-specific primers. DAS-5 was sequenced directly after asymmetric amplification of single strands (ss).

adenylosuccinate synthetase, to coordinate ATP and GTP synthesis by the requirement of GTP as a cofactor in adenylate synthesis (9).

Using monoclonal antibodies for detection of the protein and cDNA probes for the detection of mRNA we observed a steady down-regulation of adenylosuccinate synthetase during the developmental program of D. discoideum. These data are qualitatively consistent with earlier findings of a decrease in specific activity upon starvation of the cells for 2 h (46). However, since the previously reported decrease by 75% is not reflected in an abrupt reduction in the amounts of mRNA and protein during this time period, one has to assume other factors to be involved in this drastic effect. Two-dimensional gel electrophoresis showed that adenylosuccinate synthetase activity from D. discoideum resides in a number of basic isozymes. In vertebrate tissues two isoforms are present; the acidic one represents the general biosynthetic form, whereas the basic form is involved in the purine nucleotide cycle (16).

Typical for the acidic isoform from vertebrate tissues is a low K_M for IMP and a higher K_M for aspartic acid. The same ratio characterizes the D. discoideum enzyme as the enzymes from other lower eukaryotes and from bacteria (9). These data suggest that in more primitive organisms both

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FIG. 4. Comparison of adenylosuccinate synthetase sequences from D. discoideum and E. coli. The numbering on top corresponds to the deduced amino acid sequence of adenylosuccinate synthetase from D. discoideum (DAS), which is aligned to the adenylosuccinate synthetase from E. coli (EAS) according to the FASTP algorithm (54). Identical amino acid residues are boxed.

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functions might be performed by the same isof orm independent of the isoelectric points. The different basic isoforms in D. discoideum might result from posttranslational modifications.

The functional properties of adenylosuccinate synthetase are reflected in its primary structure; 43% of identities in the amino acid sequences of adenylosuccinate synthetases from D. discoideum and E. coli (15) have been found. These identities are distributed over the entire sequences suggesting a high degree of conservation on the level of three-dimensional structure. In both enzymes three motifs are present which are highly conserved among several GTP-binding proteins, such as elongation factor 2, H-ras, or the signal recognition particle receptor (47, 48). The glycine-rich loop GXXXGK typical for nucleotide-binding proteins (37, 49-51) is located between amino acids 11 and 17 of adenylosuccinate synthetase from D. discoideum, motif DXXG between residues 283 and 286, and motif N(T)KXD between residues 329 and 332. GXXXGK and DXXG are necessary for binding of phosphate groups and GTPase activity. N(T)KXD is believed to correspond to the threonine residue instead of asparagine at the first position. This configuration has only been described for the signal recognition particle receptor. This slightly imperfect motif in adenylosuccinate synthetase might allow weak binding of other nucleoside triphosphates.

Dong and Fromm (52) have recently shown by peptide analysis and site-directed mutagenesis that Lys-140 in the adenylosuccinate synthetase might allow weak binding of other nucleoside triphosphates.

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REFERENCES


**Supplemental Material**

**Dicytostelium Adenylosuccinate Synthetase**

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### EXPERIMENTAL PROCEDURES

Chemical-Labeling Reagents were purchased from Du Pont-New England Nuclear (Wilmington, MA) and Sigma Chemical Co. (St. Louis, MO). Chlorostilbamidine (CSB) and 2,2'-diamino-3,3',4,4'-tetrafluorobenzidine (DTFB) were obtained from Calbiochem-Behring (La Jolla, CA). Nucleoside triphosphates (NTPs) were purchased from New England BioLabs (Beverly, MA). All other reagents were from Sigma.

### Purification of Adenylosuccinate Synthetase from the Soluble Cell Fraction of Dicytostelium discoideum

Cells were harvested and lysed by nitrogen extraction as a powder as described (21). The supernatant from this lysis was then adsorbed to CM-Sepharose FF (Pharmacia, Uppsala, Sweden) as described (25). The enzyme was eluted in a linear NaCl gradient, and fractions containing enzyme were pooled.

### Effects of CSB and DTFB on Adenylosuccinate Synthetase

Inhibition of adenylosuccinate synthetase by CSB and DTFB was examined in cell-free extracts. Adenylosuccinate synthetase activity was measured by following the production of ATP at 340 nm using the coupled assay with ADP and CTP as substrates.

### Inhibition of Adenylosuccinate Synthetase by Dicytostelium discoideum Cytosolic Antisense RNA

The effects of antisense RNA on adenylosuccinate synthetase activity were determined by transfecting Dicytostelium discoideum cells with antisense RNA using the electroporation method described (26). The antisense RNA was coelectroporated with the plasmid pGP-2000 containing the GFP gene to facilitate visualization of transfected cells.

### Conclusions

The results of this study support the hypothesis that adenylosuccinate synthetase is an important regulatory enzyme in Dicytostelium discoideum. The inhibition of adenylosuccinate synthetase by CSB and DTFB suggests that these compounds may be useful in regulating the expression of this enzyme. The inhibitory effects of antisense RNA on adenylosuccinate synthetase activity suggest that this approach may be useful in developing new therapeutic strategies for the treatment of diseases associated with altered adenylosuccinate synthetase activity.

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### References


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### Figures

**Figure 1** Specific binding of GTP. Proteins of fractions enriched in adenylosuccinate synthetase obtained by DEAE-chromatography were incubated with [γ-32P]GTP and gel electrophoresis was performed. The gel was visualized by autoradiography. Lane 1, unlabelled GTP; Lane 2, [γ-32P]GTP.

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**Figure 2** Specific binding of GTP. Proteins of fractions enriched in adenylosuccinate synthetase obtained by DEAE-chromatography were incubated with [γ-32P]GTP and gel electrophoresis was performed. The gel was visualized by autoradiography. Lane 1, unlabelled GTP; Lane 2, [γ-32P]GTP.
Fig. 3 Southern blot analysis. For the restriction enzymes EcoRI (R) and Ascl (A) there is no recognition site. For the enzymes EcoRV (RV), PvuII (P), Ascl (A) and HindIII (Hid) there is one site within the nucleotide sequence of cAD1. Genomic DNA from D. discoideum was digested with one of these enzymes alone or in combination with EcoRI. Fragments were separated in 0.8% agarose gels and blots hybridized with nick-translated cAD1. Markers on the right indicate the position of HindIII-generated fragments of RNA in kb.

Fig. 6 Adenylosuccinate synthetase expression on the mRNA and protein level during the development of D. discoideum. A. Northern blot of total cellular RNA from D. discoideum grown at 4°C for 4 days was hybridized to the adenylosuccinate synthetase transcripts. 16 µg of RNA were loaded per lane. B. Immunoblot of electrophoretically separated proteins from 2 x 10⁸ cell equivalents applied to each lane. MAE 10H-11-2 was used for detection of the adenylosuccinate synthetase protein. Strain AX2 cells were starved on nonnutritive agar plates. 0 h corresponds to growth phase, 6-9 h to aggregation, 9-12 h to the tipped aggregate, 15 h to the slug stage, and 18-21 h to culmination.