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PURIFICATION AND PROPERTIES OF ADENYLOSUCCINATE SYNTHETASE FROM YOSHIDA SARCOMA ASCITES TUMOR CELLS

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Summary

Adenylosuccinate synthetase (IMP:L-aspartate ligase (GDP-forming), EC 6.3.4.4) was purified about 750-fold to a homogeneous state from Yoshida sarcoma ascites tumor cells. A yield of 38% purified enzyme was achieved by a procedure including affinity chromatography on hadacidin-Sepharose 4B.

Ultracentrifugal analyses showed that the molecular weight of the native enzyme was 102 000 with an $s_{20,w}$ value of 4.5 and that the molecular weight in 6 M guanidine-HCl was 47 000. These values indicate that the native enzyme is composed of two subunits.

The isoelectric point was determined to be 5.9 by isoelectric focusing. The optimum pH for activity was 6.8—7.0. The K_m values for IMP, aspartate and GTP were calculated to be 4.1, 9.8 and $0.7 \cdot 10^{-4}$ M, respectively.

The antibiotic, hadacidin was strongly inhibitory, causing competitive inhibition with respect to aspartate with a K_i value of $2.5 \cdot 10^{-6}$ M. Nucleoside mono- and diphosphate also inhibited the enzyme activity, but their inhibitions were not apparently specific.

The purified enzyme showed full activity in the presence of Mg^{2+}, and Mg^{2+} could be partially replaced by Mn^{2+}, Co^{2+}, Ca^{2+} or Cu^{2+}. Divalent metal ions, such as Cd^{2+}, Pb^{2+}, Zn^{2+}, Cu^{2+} and Mn^{2+}, interfered with the activity by antagonizing Mg^{2+}. Hg^{2+} or PCMB inactivated the enzyme, suggesting that an SH-group may be important for activity.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

Introduction

Adenylosuccinate synthetase (IMP:L-aspartate ligase (GDP-forming), EC 6.3.4.4) catalyzes the following reaction IMP + aspartate + GTP = adenylosuccinate + GDP + P_i, and is regarded as a key enzyme in the purine nucleotide biosynthetic pathway. Jackson et al. [1] examined this enzyme activity in various hepatomas and showed that the activity increased in parallel with the degree of neoplastic transformation. Clark and Rudolph [2] compared partially purified enzyme from Novikoff ascites tumor cells with enzyme from normal rat liver, finding significant differences in kinetic properties.

Previously [3,4] we have shown that rat liver contains two types of adenylosuccinate synthetase (Type L and Type M) and suggested that the regulatory properties of the former favor adenine nucleotide biosynthesis, while those of the latter favor ammoniogenesis. To characterize the regulatory and kinetic properties of adenylosuccinate synthetase from neoplastic tissues, we attempted to purify the enzyme from Yoshida sarcoma ascites tumor cells. This paper reports the purification of the enzyme and a comparison of its properties with those of the Type L and Type M isozymes.

Materials and Methods

Materials. AMP, ATP, GTP, creatine phosphate and creatine kinase were purchased from Boehringer. Other nucleotides, fructose 1,6-diphosphate, dithiothreitol, Trizma base, imidazole and Tricine were from Sigma. CNBr-activated Sepharose 4B was from Pharmacia. Cellulose DE-52 was from Whatman. Ultrogel AcA 34 and ampholines were from LKB. Hadacidin was kindly donated by Dr. W.B. Gall, Merck Sharp and Dohme Research Laboratories.

Yoshida sarcoma ascites tumor cells. The cells were kindly supplied by Dr. Shirasaka, Division of Regulation of Macromolecule Functions of this Institute. The tumor cells were grown intraperitoneally in 5-week-old Donryu-strain rats and were harvested after 5—7 days. The ascitic fluid was washed with 0.9% NaCl to remove lysed red cells and then the tumor cells were collected by low-speed centrifugation and stored at —20°C.

Enzyme assay. The activity of adenylosuccinate synthetase was measured by the method of Ogawa et al. [5] with some modification. The standard reaction mixture contained 10 μmol imidazole-HCl (pH 6.8), 0.25 μmol IMP, 0.12 μmol GTP, 2 μmol aspartate, 2 μmol $MgCl_2$, 0.6 μmol creatine phosphate, 10 μg creatine kinase and enzyme solution in 250 μl. After incubation for an appropriate time (10—40 min) at 37°C, the reaction was stopped by adding 2.5 ml 5% $HClO_4$ and denatured proteins were removed by centrifugation. A control mixture without aspartate was run simultaneously. Increase in absorbance at 280 nm was determined in a Hitachi model 200-20 spectrophotometer. The difference between the molar absorbancies at 280 nm of adenylosuccinate and IMP was 11 700 · cm^{-1} [6]. 1 unit of enzyme activity was defined as the amount catalyzing the formation of 1 μmol adenylosuccinate/min. Protein concentration was determined by the method of Lowry et al. [7] with bovine serum albumin as a standard.

Preparation of hadacidin-Sepharose 4B. All subsequent operations were per-

formed at 0—4°C. 3 g CNBr-activated Sepharose 4B were rinsed with 600 ml of 1 mM HCl and washed with water. Aminohexyl-Sepharose 4B was prepared by the method of Cuatrecasas [8], as follows: the beads were allowed to swell to about 8 ml and then mixed with 8 ml of a solution of 16 mmol of 1,6-hexanediamine in cold distilled water adjusted to pH 10 with 4 M HCl. The mixture was stirred gently overnight at 4°C, and then the beads of aminohexyl-Sepharose 4B were washed with a large volume of water to remove free 1,6-hexanediamine. Next hadacidin was coupled to this aminohexyl-Sepharose 4B by a modification of the method developed by Hughes et al. [9] for coupling oxidized GMP to 3,3′-iminobispropylamine agarose. A solution of 78 mg hadacidin (*N*-formyl hydroxyaminoacetate) in 5 ml water was adjusted to pH 8.2 with 1 M NaOH and then mixed with 5 ml aminohexyl-Sepharose 4B, equilibrated with 0.2 M Tricine-NaOH (pH 8.2) containing 1 M NaCl. The mixture was stirred gently for 1 h. Then 44 mg $NaBH_4$ in 4 ml of 0.2 M Tricine-NaOH (pH 8.2) containing 1 M NaCl were added and 1 h later a further 60 mg of $NaBH_4$ (powder) were added. After one more hour the reaction was stopped by filtration and washing with water. The hadacidin-Sepharose 4B thus formed was packed into a column and washed successively with the following solutions; (1) 10 vols. of 10 mM potassium phosphate buffer (pH 7), (2) 2 vols. of 1 mg/ml bovine serum albumin, (3) 10 vols. of 1.5 M KCl and (4) 10 vols. of 10 mM potassium phosphate buffer (pH 7) containing 0.1 mM dithiothreitol.

Isoelectric focusing. Isoelectric focusing was carried out by the method of Matsuo et al. [10]. The sample was applied to a column (120 ml) of a linear gradient formed with solutions of 0 and 50% (w/v) sucrose solutions containing 0.75 and 2.25 ml of 40% ampholine, respectively, and subjected to electrophoresis at 700 V for 42 h.

Ultracentrifugal studies. Ultracentrifugation was carried out as described by Chervenka [11]. For sedimentation equilibrium analysis, a standard sector cell with a 12-mm centerpiece of filled Epon and a split-beam photoelectric scanner were used. Sedimentation velocity was measured in the conventional way with a single sector cell and schlieren optics. The molecular weight was determined assuming that the partial specific volume ($\bar{v}$) of the purified protein was 0.74 (in aqueous solution).

Results

Purification. All operations were carried out at 0—4°C. Buffers were adjusted to the required pH values at room temperature and all buffers used contained 0.1 mM dithiothreitol.

Step 1. Preparation of crude extract. About 75 g packed Yoshida sarcoma ascites tumor cells were homogenized in 3 vols. of 50 mM Tris-HCl (pH 7.4) in a Potter-Elvehjem homogenizer and disrupted by ultrasonic vibration at 20 kHz for 30 s in a Branson sonifier, model W-185. This homogenate was centrifuged at 100 000 × *g* for 60 min and the supernatant was used as 'crude extract' (230 ml).

Step 2. $(NH_4)_2SO_4$ fractionation. To the crude extract, powdered $(NH_4)_2SO_4$ was slowly added while stirring to give 40% saturation and the precipitate was removed by centrifugation at 10 000 × *g* for 20 min. $(NH_4)_2SO_4$ was again

added to the supernatant to 65% saturation and the mixture was centrifuged. The resulting pellet was redissolved in a minimal amount of 10 mM potassium phosphate buffer (pH 7) and dialyzed overnight against two changes of 2 l of the same buffer.

Step 3. Cellulose DE-52 column chromatography. The dialysate thus obtained was applied to a Cellulose DE-52 column (2 × 16 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7). The column was washed with the same buffer until unadsorbed protein had passed through. Then adenylosuccinate synthetase was eluted with a linear gradient of 0—0.3 M KCl in 400 ml of 10 mM potassium phosphate buffer (pH 7) and the effluent containing the enzyme activity was collected.

Step 4. Ultrogel AcA 34 column chromatography. The fraction containing adenylosuccinate synthetase activity described above was concentrated by precipitation with 70% saturation of $(NH_4)_2SO_4$. The mixture was centrifuged and the pellet was dissolved in a final volume of 5 ml in 10 mM potassium phosphate buffer (pH 7). This preparation was applied to an Ultrogel AcA 34 column (2.3 × 100 cm), equilibrated with 10 mM potassium phosphate buffer (pH 7), and eluted with the same buffer. The fractions with enzyme activity were combined.

Step 5. Hadacidin-Sepharose 4B column chromatography. The enzyme solution from Step 4 was applied to a hadacidin-Sepharose 4B column (prepared as described in Materials and Methods). The column was washed with 10 vols. of 10 mM potassium phosphate buffer (pH 7) and with 5 vols. of 50 mM aspartate, and then adenylosuccinate synthetase was eluted with 100 mM aspartate. The elution profiles of the enzyme activity and protein concentration are shown in Fig. 1. The fractions containing adenylosuccinate synthetase activity were combined and dialyzed against 10 mM potassium phosphate buffer (pH 7) to remove aspartate.

The purification procedure is summarized in Table I. Adenylosuccinate synthetase was purified about 750-fold and the purified protein appeared homo-

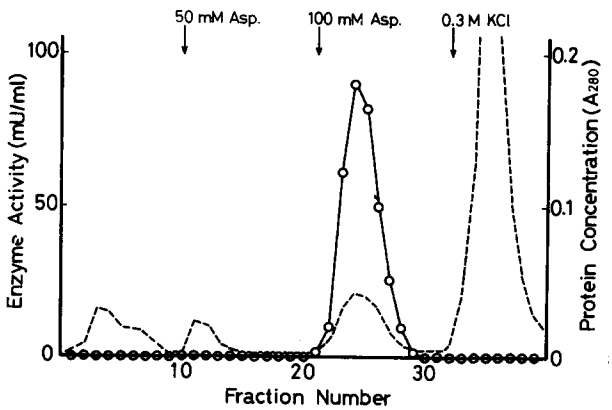


Fig. 1. Hadacidin-Sepharose 4B column chromatography. The enzyme preparation from Step 4 was applied to a hadacidin-Sepharose 4B column (1.2 × 5 cm), as described in the text. The solid line represents the enzyme activity measured under the standard assay conditions and the broken line represents the protein concentration expressed as the absorbance at 280 nm.

TABLE I

PURIFICATION OF ADENYLOSUCCINATE SYNTHETASE FROM YOSHIDA SARCOMA ASCITES TUMOR CELLS

	Volume (ml)	Total activity (mU)	Total protein (mg)	Specific activity (mU/mg)	Recovery (%)	Purity (-fold)
Crude extract	230	15 000	4870	3.08	100	1
$(NH_4)_2SO_4$ fractionation	110	13 100	3180	4.12	87	1.34
Cellulose DE-52 column chromatography	95	12 600	243	51.9	84	16.9
Ultrogel AcA 34 column chromatography	52	8 400	47.5	176.8	56	57.4
Hadacidin-Sepharose 4B column chromatography	11	5 700	2.41	2360	38	767

geneous on electrophoresis in 7.5% polyacrylamide gel with and without 0.1% sodium dodecyl sulfate (SDS). On staining the gels with Coomassie brilliant blue, only one protein band was seen.

Molecular weight. Sedimentation velocity analysis: a typical sedimentation pattern of adenylosuccinate synthetase with a single, symmetrical peak is shown in Fig. 2. The $s_{20,w}$ value was calculated to be 4.5. Sedimentation equilibrium analysis: the purified enzyme was subjected to low-speed sedimentation equilibrium analysis, as shown in Fig. 3a. From this, the average molecular weight was determined to be 102 000. A similar experiment was made on the enzyme in 6 M guanidine-HCl. As shown in Fig. 3b, a straight line was obtained and from the slope the molecular weight of the protein was calculated to be 47 000. These data indicate that in the native state adenylosuccinate synthetase exists as a dimer.

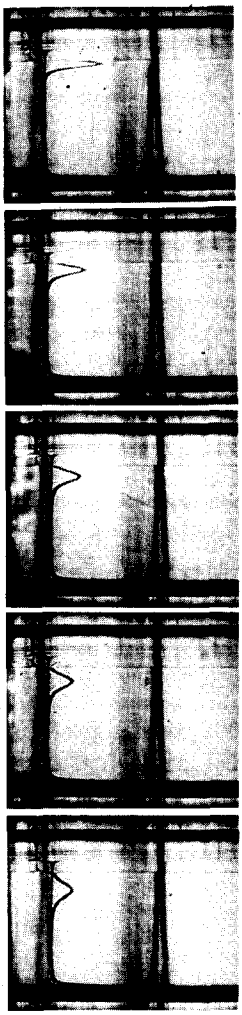
Isoelectric point. The purified enzyme was subjected to isoelectric focusing (pH range, 4—7) as described in Materials and Methods. After electrophoresis, fractions of 30 drops were collected, and the enzyme activity and pH value of each fraction were measured. As shown in Fig. 4, the activity was recovered at pH 5.9 in a single peak.

Stability. Adenylosuccinate synthetase from the tumor cells was most stable at pH 7.0 in phosphate buffer. In crude extract the enzyme was rather stable, and more than 50% of the activity remained after 1 week at 0° C or 6 weeks at —20°C. But the purified enzyme in dilute solution rapidly lost activity when stored at either 0°C or —20°C.

Optimum pH for activity. Adenylosuccinate synthetase activity was determined at various pH values. As shown in Fig. 5, a broad bell-shape pH-activity curve was obtained with a maximum at pH 6.8—7.0.

K_m values for three substrates. K_m values were determined from Lineweaver-Burk plots [12] of the activity with various concentrations of one substrate and fixed concentrations of the other two. From the hyperbolic saturation curves obtained (data not shown), the apparent K_m values for IMP, aspartate and GTP were calculated to be 4.1, 9.8 and $0.7 \cdot 10^{-4}$ M, respectively.

Effects of nucleotides and related compounds. Adenylosuccinate synthetase preparations from various species were reported to be inhibited by nucleotides



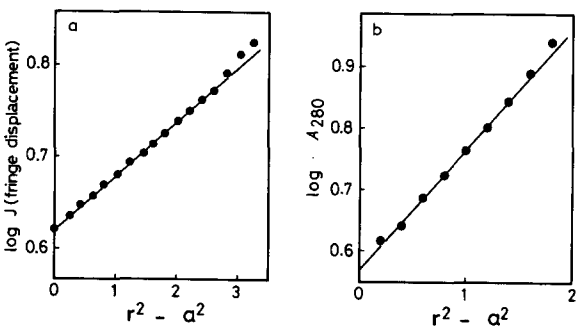


Fig. 2. Sedimentation pattern of adenylosuccinate synthetase. The purified enzyme, which was concentrated with a collodion bag (Sartorius-Membranfilter GmbH) and dialyzed against 10 mM potassium phosphate buffer (pH 7), was run in a Beckman Spinco model E ultracentrifuge equipped with RTIC units at 22.0°C. The concentration of enzyme solution was 0.4%. Photographs were taken 5, 13, 21, 29 and 37 min (left to right) after the rotor speed reached 48 000 rev./min.

Fig. 3. Equilibrium sedimentation analysis of adenylosuccinate synthetase. a, The purified enzyme in 10 mM potassium phosphate buffer (pH 7, 4 mg/ml) was centrifuged at a rotor speed of 5200 rev./min. Data were analyzed with a computor. The common logarithm of the fringe displacement is plotted against the square of the distance from the axis of rotation. b, The purified enzyme (4 mg/ml) was dialyzed for 3 days against 6 M guanidine-HCl and then centrifuged at 17 000 rev./min. The ordinate shows the common logarithm of the absorbance at 280 nm and the abscissa is as in a.

[5,13,14]. As shown in Table II, the tumor enzyme was also inhibited by nucleotides and related compounds. The data indicate that nucleoside mono- and diphosphate were potent inhibitors and that guanine nucleotides were more inhibitory than adenine nucleotides at 2 mM. Pyrimidine nucleotides inhibited the enzyme activity, too, but the data are not shown because the

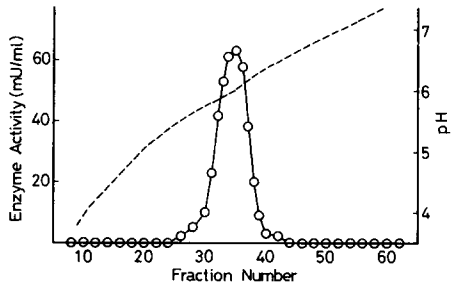


Fig. 4. Isoelectric focusing of adenylosuccinate synthetase. The purified enzyme was applied to an ampholine column and subjected to electrophoresis as described in the text. Ampholine of pH 4—7 was prepared by mixing equal volumes of ampholines of pH 4—6 and 5—7. Enzyme activity is shown as a solid line and the pH gradient as a broken line.

strong absorbances of these compounds at 280 nm interfered with the spectroscopic measurement of the activity.

The inhibitory effects of AMP and GMP were further examined. Enzyme assays were carried out with various concentrations of inhibitors under the standard conditions described in Materials and Methods. From the results the I_{50} value, the concentration for 50% inhibition, of AMP was calculated as 1.7 mM, and that of GMP as 0.37 mM in the presence of saturating concentrations of the three substrates.

Effects of other compounds. Fructose 1,6-diphosphate, an intermediate of glycolysis, was reported to be a potent inhibitor of adenylosuccinate synthetase from rat skeletal muscle and liver [3,15]. Fructose 1,6-diphosphate also inhibited the tumor enzyme and its I_{50} value was estimated to be 0.92 mM under the standard assay conditions.

Since aspartate is a substrate, we examined the effects of all other amino acids found in the body, except tryptophan and tyrosine, which interfered with the spectrophotometric assay because of their strong absorbances at 280 nm. None of the amino acids tested at 2 mM had any significant effect on the enzyme activity.

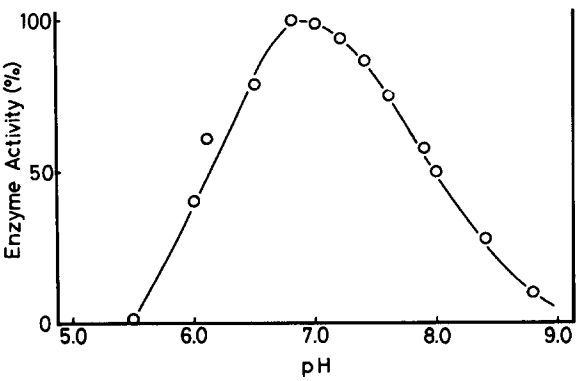


Fig. 5. Effect of pH on the activity of adenylosuccinate synthetase. The enzyme activity was determined at various pH values in imidazole-HCl buffer (below pH 7.0) and Tris-HCl buffer (above pH 7.0), and is expressed as a percentage of the maximum activity.

TABLE II

EFFECTS OF NUCLEOTIDES AND RELATED COMPOUNDS ON ADENYLOSUCCINATE SYNTHETASE

Nucleotides and related compounds were dissolved in imidazole-HCl (pH 6.8) and added at a final concentration of 2 mM to standard reaction mixture without creatine phosphate and creatine kinase.

	% Inhibition		% Inhibition
AMP	49.6	IDP	15.9
ADP	21.1	ITP	0
ATP	0	Adenosine	0
dATP	0	Guanosine	31.6
XMP	42.3	Adenine	0
GMP	80.2	Guanine	3.6
GDP	81.8	Inosine	0
dGTP	38.5	Hypoxanthine	0.9

The antibiotic, hadacidin (*N*-formyl hydroxyaminoacetic acid) was reported to inhibit adenylosuccinate synthetase from *Escherichia coli* [16,17] and Novikoff ascites tumor [2]. Fig. 6 shows double-reciprocal plots obtained by the method of Lineweaver and Burk [12], indicating that hadacidin acts as a competitive inhibitor of the enzyme with respect to aspartate. From this plot the K_i value for hadacidin was calculated to be $2.5 \cdot 10^{-6}$ M. Judging from the ratio of the K_m value for aspartate to the K_i value for hadacidin (about 400), hadacidin is a potent inhibitor.

Effects of metal ions. Adenylosuccinate synthetase required Mg^{2+} for full activity. Mg^{2+} could be partially replaced by Mn^{2+} (37.0%), Co^{2+} (34.8%), Ca^{2+} (14.1%), Ba^{2+} (13.2%) or Cu^{2+} (10.9%) at 2 mM. The effects of various concentrations of metal ions (in the presence of $4 \cdot 10^{-3}$ M Mg^{2+}) are summarized in Table III. At a concentration of $2 \cdot 10^{-3}$ M, Cd^{2+}, Pb^{2+} and Zn^{2+} almost completely inhibited the enzyme activity. Moreover, Cu^{2+}, Ca^{2+} and Mn^{2+} were also strongly inhibitory, but their inhibitions were reversed by lowering their con-

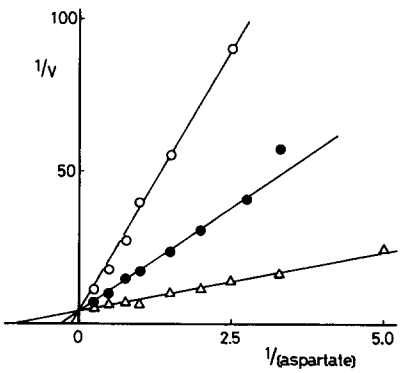


Fig. 6. Kinetic study of the inhibition of adenylosuccinate synthetase by hadacidin. Enzyme was assayed under the standard conditions except that the concentration of aspartate was varied in the presence of a fixed concentration of hadacidin. △———△, without hadacidin; ●———●, 7 μM hadacidin and ○———○, 14 μM hadacidin.

TABLE III

EFFECTS OF METAL IONS ON ADENYLOSUCCINATE SYNTHETASE

Metals as their chloride or acetate salts were dissolved in imidazole-HCl (pH 6.8) and added to standard reaction mixture, except that the concentration of $MgCl_2$ was 4 mM and creatine phosphate and creatine kinase were omitted.

	Residual activity (%)			
	$2 \cdot 10^{-3}$	$2 \cdot 10^{-4}$	$2 \cdot 10^{-5}$	$2 \cdot 10^{-6}$ (M)
Cd^{2+}	0	17.9	70.0	97.5
Pb^{2+}	0	31.6	70.6	99.8
Zn^{2+}	2.7	73.8	94.8	100
Cu^{2+}	23.5	66.9	97.1	101
Ca^{2+}	31.1	83.5	101	—
Mn^{2+}	46.8	85.7	101	—
Fe^{2+}	80.8	85.2	105	—
Co^{2+}	81.6	105	—	—
Ba^{2+}	95.9	113	—	—
Hg^{2+}	3.5	4.2	10.3	11.7

centrations. These results indicate that these metal ions are antagonistic to Mg^{2+}. In contrast, Hg^{2+} inactivated the enzyme even when added at the low concentration of $2 \cdot 10^{-6}$ M. This inhibition could be reversed by addition of dithiothreitol in large excess. Since Hg^{2+} seemed to react with the catalytically active center of this enzyme, we next examined whether the enzyme was inactivated by modification of its thiol groups with PCMB. In the presence of $2 \cdot 10^{-5}$ M PCMB, adenylosuccinate synthetase from the tumor cells showed hardly any activity. Thus, we conclude that a cysteine residue is present in the active site of the enzyme.

Monovalent cations, such as Li^+, Na^+, K^+ and NH_4^+ did not affect the enzyme activity.

Discussion

Adenylosuccinate synthetase has so far been purified to a homogeneous state from rat and rabbit skeletal muscle [5,13] by conventional methods. This paper reports purification of the enzyme by a procedure involving affinity chromatography on hadacidin-Sepharose 4B. Many nucleotides are reported to be able to bind to adenylosuccinate synthetase as substrates or inhibitors. However, their K_m or K_i values were all more than $1 \cdot 10^{-4}$ M, indicating that their affinities for the enzyme were not necessarily strong. We found that various affinity chromatographies with nucleotides, such as GMP, AMP and IMP as ligands, did not give good purification. On the other hand, as described above, hadacidin was expected to have a high affinity for the enzyme, since its K_i value was estimated to be $2.5 \cdot 10^{-6}$ M, which is two orders of magnitude lower than the values of the nucleotides mentioned above. So we prepared an affinity column with this compound as a ligand. Shigeura [18] compared the inhibitory effects of various hadacidin analogues on adenylosuccinate synthetase activity in *E. coli*. From his data, it is considered that hadacidin in hadacidin-

Sepharose 4B binds to aminohexyl-Sepharose through its formyl group. This column adsorbs many kinds of proteins, but adenylosuccinate synthetase could be specifically eluted with aspartate, while other proteins were subsequently eluted with 0.3 M KCl, as shown in Fig. 1. In this step adenylosuccinate synthetase was purified about 15-fold and obtained as a homogeneous protein.

Ultracentrifugal analysis showed that the molecular weight of adenylosuccinate synthetase from the tumor cells was 102 000 in the native state, and that the enzyme was composed of two subunits with molecular weights of 47 000. These values are similar to those for the enzyme from rat skeletal muscle [5]. However, these two enzymes showed quite different mobilities on SDS-polyacrylamide gel electrophoresis (data not shown); the tumor enzyme moved considerably faster than the muscle enzyme in both 10% and 7.5% polyacrylamide gel. Thus the proteins appear to have different molecular sizes.

The isoelectric point of the tumor enzyme was determined to be 5.9, which is identical with the value of the Type L enzyme in rat liver [3], but significantly different from those of the Type M enzyme and the rat skeletal muscle enzyme (pH 8.9) [5]. These results suggest that the tumor enzyme may be the same protein as the Type L isozyme in normal liver. The optimum pH of the tumor enzyme, however, was similar to that of the rat muscle enzyme, but on storage the tumor enzyme was not so stable as the muscle enzyme.

The K_m values of the tumor enzyme for the three substrates were similar to those of the Type L enzyme of liver [3], in that the K_m value for IMP was lower, and the K_m value for aspartate was higher than those of the Type M enzyme or the muscle enzyme. Clark and Rudolph [2] reported that partially purified adenylosuccinate synthetase from Novikoff ascites tumor cells had a lower K_m value for IMP and a higher K_m value for aspartate than those of the normal tissue, and suggested that the lower K_m for IMP might reflect more efficient biosynthesis of adenine nucleotides in the tumor cells. Our data support their suggestion on the regulatory properties of the tumor enzyme.

Various nucleotides, especially nucleoside monophosphates, strongly inhibited the enzyme activity. However, the I_{50} values of AMP and GMP were rather higher than their physiological concentrations. Therefore, it seems unlikely that feedback regulation of the enzyme by nucleotides is important in nucleotide biosynthesis in the tumor cells, as in rat skeletal muscle [5] and human placenta [14].

Hadacidin was strongly inhibitory, causing competitive inhibition with respect to aspartate. Its K_i value (2.5 μM) was as low as the value for the enzyme from Novikoff ascites tumor cells (K_i 1.5 μM). The K_i values of hadacidin for the Type L and Type M enzymes from rat liver were determined to be 2.5 μM and 0.77 μM, respectively, and the ratio of the K_m value for aspartate to the K_i value for hadacidin was calculated to be about 400 in both cases. From these results it can be concluded that hadacidin does not affect the tumor enzyme specifically.

Fischer et al. [19] investigated the effects of metal ions on adenylosuccinate synthetase from rabbit skeletal muscle, liver and heart. They reported that Zn^{2+} inactivated the enzyme (I_{50} value, 5—7 μM) and that Mg^{2+} and dithiothreitol did not affect this inactivation. Ca^{2+} and Mn^{2+} were antagonistic to Mg^{2+}. However, our data (Table III) on the tumor enzyme indicate that the inhibition by

Zn^{2+} was reversed as its concentration was reduced. Consequently, Zn^{2+} seems to act antagonistically to Mg^{2+}, like Ca^{2+} and Mn^{2+}. In addition, we found that Cd^{2+}, Pb^{2+} and Cu^{2+} also interfered with the activity in the same manner as Zn^{2+}. On the other hand, the tumor enzyme was inactivated by Hg^{2+} at a concentration of $2 \cdot 10^{-6}$ M. PCMB also significantly inactivated the enzyme at $1 \cdot 10^{-5}$ M. As previously reported [5], $2 \cdot 10^{-4}$ M PCMB inactivates the rat skeletal muscle enzyme only 30%. From these findings, we conclude that thiol groups are essential for the catalytic activity of the tumor enzyme, unlike that of the skeletal muscle enzyme.

As discussed above, adenylosuccinate synthetase from Yoshida sarcoma ascites tumor cells seems to be different from the skeletal muscle enzyme, but similar to the Type L isozyme of rat liver in its isoelectric point and kinetic properties. These results are consistent with our previous suggestion [3,4] that the Type L enzyme mainly works where the nucleotide biosynthesis is active.

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