Purification and Subunit Structure of Argininosuccinate Lyase from *Chlamydomonas reinhardtii*

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Argininosuccinate lyase (EC 4.3.2.1) was purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation, chromatography on DEAE-cellulose and gel filtration on Sephadex G-200. The final enzyme preparation was purified 46-fold compared with the crude extract. Electrophoresis of this preparation revealed three bands, the major one having the enzyme activity. Analysis of the enzyme by gel filtration and by disc electrophoresis (in two different concentrations of acrylamide) gave mol.wts. of 200000 (± 15000) and 190000 (± 20000) respectively. Treatment with sodium dodecyl sulphate and mercaptoethanol dissociated the enzyme into subunits of mol.wt. 39000 (± 2000). The results are indicative of the multimeric structure of the enzyme, which is composed of five (perhaps four or six) identical subunits.

In the green alga *Chlamydomonas reinhardtii*, the argininosuccinate lyase (L-argininosuccinate L-arginine lyase, EC 4.3.2.1) catalysing the splitting of argininosuccinate into arginine and fumarate is the last enzyme involved in the biosynthetic pathway to arginine (Strijkert & Sussenbach, 1969). Several mutants impaired in the activity of this enzyme have been isolated and mapped in a short segment (the *arg-7* locus) of the linkage group 1 (Loppes *et al.*, 1972; Loppes & Matagne, 1972). When some of these mutants were combined in the same diploid cell, the enzyme function was partially restored and the cell was no longer arginine-dependent: such a positive complementation between mutants belonging to the same cistron generally indicates a homomultimeric structure of the enzyme (Catchside & Overton, 1958; Fincham, 1966). Moreover, by studying some properties of the enzyme formed in several heterozygote diploid strains (*arg-7* $\times$ wild-type), we suggested that the argininosuccinate lyase was composed of more than two identical polypeptide chains (Matagne, 1976). A better understanding of the phenomenon of complementation in this system should arise from an analysis of the structure of argininosuccinate lyase in *Chlamydomonas*.

The present paper deals with the purification of the enzyme and with the determination of the number of the constitutive subunits.

Materials and Methods

Growth of the alga

Chlamydomonas reinhardtii strain 137 C was grown in aerated liquid 40 N medium (Loppes *et al.*, 1972) supplemented with 1 g of sodium acetate/litre under

continuous light (5000 lx) and at 23°C. Each culture flask contained 1 or 2 litres of medium; the total volume was 35 litres.

Determination of proteins

Protein was determined by the method of Lowry *et al.* (1951) in purification steps 1 and 2 with crystalline egg-white lysozyme as a standard. In steps 3 and 4, protein was determined by measurement of u.v. absorbance at 280 and 260 nm (Warburg & Christian, 1941).

Enzyme assay

Argininosuccinate lyase was assayed as previously described (Loppes *et al.*, 1972) with argininosuccinate as a substrate and measuring the amount of arginine by the Sakaguchi reaction. The specific activity was expressed in μmol of arginine/h per mg of protein at 37°C.

To detect the enzyme activity in the fractions collected from the chromatography columns, the following rapid micro-method was used: a 50 μl portion of each fraction was incubated with 50 μl of argininosuccinate solution (3 mg/ml of 0.1 M-sodium phosphate buffer, pH 7.5) for 30 min at 37°C; to each fraction was added 50 μl of naphthol plus thymine reagent (Van Pilsum *et al.*, 1965), then 50 μl of NaClO solution (commercial eau de Javel diluted 25-fold). Blanks were made by omitting substrate. Absorbance was read at 515 nm.

Electrophoresis

Polyacrylamide-gel electrophoresis was performed in glass tubes 7 or 10 cm long, internal diam. 6 mm.

To prepare the gels and electrode buffer solutions,

several buffers were used depending on the experiments: (a) 0.1M-sodium phosphate buffer, pH7.2, for electrode buffer as well as for gels [prepared as described by Weber & Osborn (1969), but without SDS* in the system and with a final concentration of 7% (w/v) for acrylamide]; (b) 0.438M-Tris/HCl, pH8.9, for the gel and 0.043M-Tris/glycine, pH8.3, for electrode buffer, with the gel system no. 1 of Maurer (1971), and at two different concentrations (5 or 8% w/v) of acrylamide.

SDS/polyacrylamide-gel electrophoresis was performed in 10cm glass tubes by the method of Laemmli (1970), with a 10% (w/v) acrylamide concentration for the gels. The samples were prepared as follows: 50 μ l of enzyme or reference protein (0.5–1.0mg/ml) was added to 5 μ l of 0.5M- Na_2CO_3 , 10 μ l of 2% (w/v) SDS and 5 μ l of 2-mercaptoethanol. The samples were boiled for 3min. After the addition of some crystals of sucrose and Bromophenol Blue dye, samples (30–50 μ l) were placed over the gels with a micro-syringe. The mobility of the proteins was measured with reference to the mobility of Bromophenol Blue. After electrophoresis, the gels were stained for proteins in a solution of Coomassie Brilliant Blue G-250 (250mg of dye in 100ml of methanol/acetic acid/water, 5:1:4, by vol.) at 4°C for 90min. Destaining was performed in methanol/acetic acid/water (1:1:8, by vol.).

For the determination of the mol.wt. of subunits, the following reference proteins after SDS treatment were used: bovine serum albumin (67000), catalase (60000), ovalbumin (43000), chymotrypsinogen A (27500), myoglobin (17200) and cytochrome *c* (11700).

Determination of molecular weight of argininosuccinate lyase

The mol.wt. of the purified enzyme was determined by gel filtration on Sephadex G-200 (column 1.5cm $\times$ 90cm) equilibrated in 0.01M-phosphate buffer, pH7.5. The flow rate was 7.4ml/h. The following reference proteins (5mg) were used: catalase (240000), aldolase (158000), bovine serum albumin (67000) and ovalbumin (43000).

The molecular weight of the argininosuccinate lyase was also determined by polyacrylamide-gel electrophoresis. Several reference proteins and the purified enzyme were separated by disc gel electrophoresis, by using the buffers of the gel system no. 1 of Maurer (1971) and two concentrations (5 and 8%) of the acrylamide. For each protein, the relative mobility was measured as migration distance divided by that of the Bromophenol Blue. The quotient relative mobility in 8% gel/relative mobility in 5% gel, also called retardation quotient, is completely

independent of charge and depends only on molecular size; by plotting the retardation quotient against the log of the molecular weight for different proteins, one obtains a linear relationship (see Thorun & Maurer, 1971).

Proteins and chemicals

The reference proteins for molecular-weight determination were from Boehringer Mannheim G.m.b.H. (Mannheim, Germany). The chemicals for gel electrophoresis were from Fluka (Buchs, Switzerland). Argininosuccinate (barium salt; 90% pure) was from Sigma (St. Louis, MO, U.S.A.).

Results

Purification

After growth for 5 days, 42g of cells was recovered by centrifugation of 35 litres of culture. The cells were suspended in 150ml of 0.02M-potassium phosphate buffer, pH7.5. They were disrupted by ultrasound (MSE ultrasonic disintegrator) treatment for 2min at 0°C. The suspension obtained was centrifuged at 29000g for 15min; the supernatant, adjusted to 160ml, constituted the crude extract (step 1).

This extract was mixed with 33.3g of $(\text{NH}_4)_2\text{SO}_4$ at room temperature (22°C). After stirring for 30min, the mixture was centrifuged at 29000g for 15min and the pellet discarded. The supernatant (158.5ml) was mixed with 25.75g of $(\text{NH}_4)_2\text{SO}_4$ and stirred for 1h. The mixture was then centrifuged at 29000g for 15min and the precipitate was dissolved in 0.02M-phosphate buffer, pH7.5. After overnight dialysis, 29ml of solution was obtained (step 2).

This solution was applied to a column (6cm $\times$ 3.2cm) of DEAE-cellulose (Whatman DE 52) which had been equilibrated with 0.02M-potassium phosphate buffer, pH7.5. The column was washed with this buffer until the A_{280} of the eluate was 0.06. The column was then eluted with a linear gradient of NaCl in 0.02M-phosphate buffer, pH7.5 (200ml of phosphate buffer + 200ml of phosphate buffer containing 0.3M-NaCl). Fractions (8.8ml) were collected at a flow rate of 30ml/h with an Isco fractionator connected to a u.v. recorder. Fractions 9–29 (NaCl concentration 0.06–0.19M) containing enzyme activity were pooled (184ml; step 3) then concentrated to 3.8ml by using Sartorius membranes.

This fraction was filtered through a column (90cm $\times$ 1.5cm) of Sephadex G-200 equilibrated with 0.02M-phosphate buffer, pH7.5, containing 0.02% NaN_3 . A constant fraction volume of 2.6ml and a flow rate of 7.4ml/h were maintained during elution with a peristaltic pump. After concentration of the argininosuccinate lyase-containing fractions (21–32) to a volume of 4ml, the chromatography was repeated

* Abbreviation: SDS, sodium dodecyl sulphate.

on the same Sephadex column. The elution of protein and enzyme activity from this column is shown in Fig. 1. Fractions 23–28 were concentrated to 4 ml (step 4) by using Sartorius membranes and stored at 4°C.

The purification procedure, with protein and enzyme-activity determinations, is presented in Table 1.

Homogeneity and molecular weight

Homogeneity of the purified enzyme was checked by polyacrylamide-gel electrophoresis. Gels were used for protein staining or enzyme activity determination. As Tris buffer interferes with the determination of arginine by the Sakaguchi reaction, sodium phosphate buffer (pH 7.2) was used as gel and electrode buffer.

One gel (40 µl of enzyme preparation) was stained for protein and revealed three bands, the slower one being the major band (Fig. 2a). A corresponding gel (100 µl of enzyme) was scanned in the u.v. and showed

a major band in the same position as in Fig. 2(a). This gel was sliced and the slices (3 mm thick) were incubated in 0.1 M-phosphate buffer, pH 7.5, in the presence of argininosuccinate; the enzyme activity was only found at the level of the major band.

The molecular weight of the enzyme was determined by gel filtration on Sephadex G-200 as

Fig. 1. Elution of argininosuccinate lyase from the second Sephadex G-200 column

The argininosuccinate lyase-containing fractions from the first Sephadex G-200 column were concentrated to 4 ml then chromatographed on the same column with 0.02 M-phosphate buffer, pH 7.5 (see the text). ----, A_{280} (protein); —, enzyme activity.

Fig. 2. Electrophoretograms of the purified argininosuccinate lyase (a) and of the purified argininosuccinate lyase after dissociation into subunits by SDS/mercaptoethanol treatment (b)

(a) Enzyme preparation (40 µl, 38 µg of protein) was separated by polyacrylamide-gel electrophoresis in sodium phosphate buffer pH 7.2, as described in the Materials and Methods section. The enzyme activity was only detected in the major band. A 50 µl sample was treated with SDS/mercaptoethanol and submitted to SDS/polyacrylamide-gel electrophoresis as described in the Materials and Methods section. When the major band of gel (a) (containing enzyme activity) was extracted, treated with SDS/mercaptoethanol and submitted to polyacrylamide-gel electrophoresis in the presence of SDS, only the major band (arrow) was detected (see the text).

Table 1. Purification of argininosuccinate lyase from *Chlamydomonas reinhardtii*
For details see the text.

Purification step	Volume (ml)	Total protein (mg)	Specific activity (µmol of arginine/h per mg of protein)	Total activity (µmol of arginine)	Yield (%)
1. Crude extract	160	1020	0.50	501	100
2. (NH ₄) ₂ SO ₄ fractionation	29	180	3.04	547	109
3. DEAE-cellulose	184	40	7.0	280	56
4. Second Sephadex G-200 step	4	3.8	23	87	17

Fig. 3. Determination of the molecular weight of argininosuccinate lyase by polyacrylamide-gel electrophoresis. Plot of the ratio (or retardation quotient) of the relative mobilities in two different gels (8 and 5%) against the log(molecular weight) of standards. —, Value obtained for argininosuccinate lyase. Proteins used were (20 μ g): A, ovalbumin (45 000); B₁₋₄, bovine serum albumin monomer, dimer, trimer and tetramer (monomer 67 000); C, aldolase (158 000); D, fumarase (198 000); E, catalase (240 000).

described in the Materials and Methods section. By this method, one can estimate the mol.wt. of the enzyme as 200 000 ($\pm$ 15 000).

The molecular weight of argininosuccinate lyase was also determined by polyacrylamide-gel electrophoresis. Fig. 3 gives the results from two independent electrophoretic runs; a mol.wt. of 190 000 ($\pm$ 20 000) was obtained by this method.

Subunits of argininosuccinate lyase

The purified enzyme was treated with SDS and 2-mercaptoethanol, then subjected to SDS/polyacrylamide-gel electrophoresis as described in the Materials and Methods section. One major band (representing more than 80% of the total) and several slower faint bands were revealed after protein staining (Fig. 2b).

In one experiment, the enzyme was first separated electrophoretically in phosphate buffer gel. The gel was sliced and the slice containing the argininosuccinate lyase activity was ground and used as a source of enzyme for SDS treatment. After SDS/polyacrylamide-gel electrophoresis, only one faint band, corresponding to the major band of Fig. 2(b) was obtained. The electrophoretic mobility of this band in comparison with reference proteins indicated a mol.wt. of $39\,000 \pm 2000$. The same value was found in SDS/polyacrylamide-gel electrophoresis by the method of Weber & Osborn (1969).

Discussion

From gel-filtration analysis on Sephadex G-200 and from polyacrylamide-gel electrophoresis it was found that argininosuccinate lyase has a mol.wt. of about 200 000. Treatment with SDS and mercaptoethanol gave a major electrophoretic band with a mol.wt. of 39 000. The other bands found in the gel, representing less than 20% of the total protein seem to correspond to contaminants, although the possibility that they are minor constituents of the enzyme cannot be completely excluded.

The present data thus suggest that argininosuccinate lyase of *Chlamydomonas* is a multimeric enzyme composed of probably five (perhaps four or six) identical subunits. The fact that only one gene codes for the enzyme (Loppes & Matagne, 1972) also favours the idea that the subunits are identical. For the ox liver enzyme, Havir *et al.* (1965) found a mol.wt. of 202 000; the minimum size of the enzyme, after 6M-guanidine hydrochloride treatment, was about 50 000 (Englander & Crowe, 1965). There is thus a similarity in size and subunit composition between the two enzymes. However, the K_m value found for the unpurified enzyme of *Chlamydomonas* with argininosuccinate as a substrate (0.43 mM; Matagne, 1976) is lower than the value found for the enzyme from ox liver (1.5 mM; Ratner *et al.*, 1953).

The present results allow a better understanding of the biochemical properties of the enzyme formed by complementation in diploid strains. We previously analysed (Matagne, 1976) the heat sensitivity of argininosuccinate lyase in heterozygotes carrying a wild (+) gene and a mutant (*arg-7-1*, *arg-7-5*, *arg-7-6* or *arg-7-7*) gene: it was found that the enzyme produced by some heterozygotes was much more thermolabile than the wild-type enzyme. In one strain (+/*arg-7-7*), no enzyme form having the thermostability of the wild enzyme was detected. These results can be easily interpreted as follows: if the enzyme is formed of five subunits and if the subunits coded by wild and mutant genes (a and a' subunits respectively) combine at random in the heterozygote, the enzymic form (aaaaa) of the wild-type will be rare enough (3% of the total) to be undetected in heterozygote strains.

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