Isolation and Purification to Apparent Homogeneity of 4,5-Dioxovalerate Aminotransferase from *Scenedesmus obliquus* Mutant C-2A′

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In the present paper the purification of a specific 4,5-dioxovalerate transaminase from pigment mutant C-2A′ of the unicellular green alga *Scenedesmus obliquus* to apparent homogeneity is described. The newly isolated enzyme L-glutamate: 4,5-dioxovalerate aminotransferase is not identical with L-alanine: 4,5-dioxovalerate aminotransferase (EC 2.6.1.43) and L-alanine: glyoxylate aminotransferase (EC 2.6.1.44). A procedure for the purification is described and the resulting homogeneous protein is characterized by its K_M-values for oxo-substrates and amino donors, its pyridoxal phosphate requirement, reversability of the catalysis, pH-optimum, isoelectric point and its molecular weight.

Introduction

When Beale and Castelfranco [1] first reported a new pathway for the biosynthesis of 5-aminolevulinate, the first specific precursor in the formation of all tetrapyrroles [2, 3], the intermediates of this pathway were still unknown. Later 4,5-dioxovalerate [4, 5] and glutamate-1-semialdehyde [6, 7] were controversely discussed as possible precursors of 5-aminolevulinate in the C-5-pathway for the formation of chlorophylls in plants.

In a recent paper Breu *et al.* [8] elaborated a pathway, first discussed by Rüdiger and Schoch [9], which includes both intermediates. This model proposes that initially glutamate-1-semialdehyde is formed from glutamate, bound to a special tRNA species, which then is converted to 4,5-dioxovalerate by glutamate-1-semialdehyde aminotransferase [6]. In a subsequent step this intermediate has to be transferred to 5-aminolevulinate by an amino acid: 4,5-dioxovalerate aminotransferase.

Whether this postulated amino acid: 4,5-dioxovalerate aminotransferase is identical with either L-alanine: 4,5-dioxovalerate aminotransferase (EC 2.6.1.43) or L-alanine: glyoxylate aminotransferase (EC 2.6.1.44) [10–13] will be investigated in this paper.

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Materials and Methods

Chemicals

5-Aminolevulinate was purchased from Fluka, Buchs, Switzerland. 2,3-Diaminonaphthalene was obtained from Sigma, Deisenhofen, F.R.G. and recrystallized from hot ethanol. 4,5-Dioxovalerate was prepared as described earlier [5]. All other chemicals were *p.a.* grade.

Cultivation of cells and preparation of cell-free homogenates

For the isolation of 4,5-dioxovalerate aminotransferase the X-ray induced mutant C-2A′ of the unicellular green alga *Scenedesmus obliquus* [14] was employed throughout all experiments. Cells were grown for 3 days at 30 °C in the dark in a heterotrophic medium as described by Bishop and Senger [14] supplemented with 0.5% (w/v) glucose and 0.25% (w/v) yeast extract and microelements following Kratz and Myers [15]. Cells were harvested by centrifugation ($1400 \times g$, 5 min) and broken as described earlier [16]. The homogenate was then centrifuged for 30 min at 2 °C and $27{,}000 \times g$. The supernatant was employed as cell-free soluble enzyme preparation for the further purification procedure.

Analytical procedures and quantitative determinations

Protein determinations followed the method of Lowry *et al.* [17]. The isolation, purification and

quantitative measurement of 5-aminolevulinate as well as of 4,5-dioxovalerate were performed as recently reported by Breu *et al.* [8]. For the determination of pyruvate the method of Richardson and Thompson [18] was used. The relative molecular weight (M_r) of the isolated polypeptide was determined by two different methods. For rough determination gel filtration on Sephadex G 150 (1.5 × 85 cm) was applied. The column was calibrated with catalase, aldolase, ovalbumine, chymotrypsine A and cytochrome *c*. The separation system was equilibrated to pH 6.8 with 10 mM phosphate buffer and run at a flow rate of 20 ml/h. The administered sample volume was 3 ml of crude supernatant, fraction size after separation was 2.8 ml.

For a more precise determination polyacrylamide gel electrophoresis (PAGE) on slab gels (100 × 150 × 2 mm) was applied using the system of Laemmli [19]. The gel contained 1% (w/v) sodiumdodecylsulfate (SDS) and was 10% (w/v) in polyacrylamide. Separation was performed at room temperature with a constant current of 20 mA. Staining and destaining procedure was carried out following Chua and Benoun [20].

Larger sample volumes which have to be administered to columns are often a critical point in gel filtration. Therefore protein solutions were concentrated by two methods. When larger volumes had to be concentrated PM-30-membranes (Amicon), which retain molecules with $M_r > 30{,}000$, were employed using a N_2-pressure of 2 bar. Otherwise collodion bags could be used, the low molecular weight contaminants and solvent being passed through by suction.

To avoid adsorption of enzyme proteins to these membranes, they were first treated for 30 min with a 1% (w/v) solution of serum albumine and then washed, until no more protein could be recovered in the rinsing buffer.

For the determination of alanine: 4,5-dioxovalerate- and alanine: glyoxylate aminotransferase activity the following assay was applied: in a final volume of 1 ml, 100 µl of 10 mM 4,5-dioxovalerate or glyoxylate and 50 µl of 0.6 M alanine were incubated with 500 µl of enzyme preparation and 350 µl of 0.2 M phosphate buffer, pH 6.8. In some experiments alanine was substituted by glutamate. When K_M-values were determined, concentrations were varied as reported in Results. Incubations were started with 4,5-dioxovalerate or glyoxylate, respectively, continued for 30 min at 37 °C and stopped with 250 µl of 20% (w/v) trichloroacetic acid. The resulting products, 5-aminolevulinate and glycine (pyruvate) were determined as mentioned above [8, 18].

Protein enrichment and separation

In order to separate nucleic acids from the cell-free supernanant 30 µl of a 1% (w/v) protamine-sulfate solution (pH 6.8) were added per mg protein of the crude enzyme preparation. The reagent was added dropwise with stirring. After 15 min the precipitated nucleic acids were separated by centrifugation (3500 × *g*, 10 min).

For a rough pre-separation fractionated ammonium sulfate- and acetone precipitations were carried out under various, mild conditions, but as these methods failed they will not be described here in detail. The attempt to apply the method of Turner and Neuberger [21], who successfully enriched 4,5-dioxovalerate transaminase from *Rhodopseudomonas* by heat denaturing, also failed with *Scenedesmus* and is thus not reported here. Gel filtration was used for desalting, protein separation and determination of molecular weight. For this procedure Sephadex G-25 (Pharmacia) was used in columns of 2.5 × 30 cm or 1.5 × 18 cm size, depending on the sample volume. The gel was equilibrated with 10 mM phosphate buffer, pH 6.8.

Sephadex G-150 (Pharmacia), employed for the latter two aspects of gel filtration, was swollen for 5 h at 95 °C and then a column with a bed volume of 140 ml (1.5 × 85 cm) was casted. It was run at 33 cm water pressure, flow rate being 19 ml/h.

For further purification of 4,5-dioxovalerate transaminase, ion exchange chromatography on DEAE-cellulose and DEAE-Sepharose was employed. DEAE-cellulose (Serva cell, Type 23 SH, Serva) was repeatedly washed with 0.1 M NaOH followed by 1 M HCl and finally equilibrated with 10 mM Na-phosphate buffer to pH 6.8. Protein separations were carried out by batch procedure, increasing stepwise the phosphate amount up to a final concentration of 200 mM or by using a column (3 × 24 cm) proteins being eluted by a linear gradient from 10 to 100 mM phosphate buffer. 250 ml of the supernatant of the protamine sulfate precipitation were administered to the column, the flow rate was 40 ml/h. Fractions of 2.8 ml size were collected. The ion exchanger was regenerated by washing with 3 bed volumes of 0.2 M

NaOH and then re-equilibrated with 10 mM Na-phosphate buffer, pH 6.8.

A more effective purification was expected from DEAE-Sepharose CL-6B (Sigma). Therefore, from pre-swollen gel matrix equilibrated with 10 mM Na-phosphate buffer (pH 6.8), a small column (3 × 6 cm) was prepared. Protein elution was performed with a linear gradient (250 ml) from 0 to 0.3 M NaCl. Samples, which were pre-purified by chromatography on hydroxyapatite (see below) and afterwards concentrated to a volume of 10 ml by filtration on a PM-30 membrane (Amicon B15, Amicon Corp., Lexington, Mass., U.S.A.), were administered to the column, the flow rate being 65 ml/h. Again fractions of 2.8 ml were collected. After washing with two bed volumes of 2 M NaOH and two bed volumes of 1 M Na-acetate solution (pH 3.1) for regeneration the column was re-equilibrated to 10 mM Na-phosphate, pH 6.8.

For pre-purification prior to application to other separation methods, spherical hydroxyapatite (Merck) was used. To remove broken particles, the material was suspended in 10 mM Na-phosphate buffer, pH 6.8. The supernatant containing the broken particles was several times decanted. The column (3 × 8 cm) was then filled with the same buffer and the hydroxyapatite slurry poured into the column to give a bed volume of 42 ml. After equilibration with the above mentioned buffer 50 ml of the supernatant of the protamine sulfate precipitation were applied to the column. Proteins were eluted with a linear gradient (150 ml) from 10 to 100 mM Na-phosphate, pH 6.8. The flow rate was 55 ml/h, fraction size was again 2.8 ml. Regeneration was performed as prescribed by the supplier.

Isoelectric focussing was performed following Maurique and Lasky [22]. Separation was carried out with a flatbed electrophoresis assembly FBE 3000 (Pharmacia) and a power supply ECPS 3000/150 (Pharmacia) which allows to fix the voltage to 1500 V as limiting factor. As carrier a gel bed of Sephadex G-200 SF which was casted in a custom made plastic trough (11 × 22 cm), or a mixture of agarose and Sephadex G-200 SF, which could be fixed to Gel Bond Film (Pharmacia), was applied.

The electrodes were connected to the gel by electrode strips which were soaked, when separating between pH 4 and 6, with a 1% solution of the corresponding ampholyte. When separating between pH 4 and 9, 0.05 M H_2SO_4 and 1 M NaOH were applied as anode and cathode buffer, respectively. The corresponding ampholyte mixtures were obtained from Pharmacia. Separation took about 8 h and was carried out at 10 °C.

For separation 1 ml of sample (= 12 mg protein) which was pre-purified by hydroxyapatite and DEAE-sepharose chromatography were mixed with 20 mg Sephadex G-200 SF and administered to the gel bed as a short band about 3 cm apart of the cathode. When separation was finished, a filter-paper (Whatman # 3) was carefully pressed onto the gel and then stained with Coomassie Blue [23] to localize the protein bands. Protein containing gel parts were scraped off and thoroughly eluted with 10 mM Na-phosphate buffer, pH 6.8. The gel was separated by centrifugation (40,000 × *g*, 20 min) and the supernatant assayed for 4,5-dioxovalerate transaminase activity. Active bands were desalted by gel filtration on Sephadex G-150.

Results and Discussion

Optimization of the enzyme assay

In a previous paper [24] we could demonstrate the existence of 4,5-dioxovalerate transaminase activity in crude enzyme preparations from *Scenedesmus obliquus*. The assay followed the method of Turner and Neuberger [21] containing additionally 1 mM pyridoxal phosphate. To stabilize the enzyme in assays with higher plant material glycerol, dithiothreitol, EDTA and Mg^{2+} were always added to the test mixture [25, 26]. The influence of these compounds on the *Scenedesmus* enzyme was assayed. The substitution of phosphate by Tris was also tested, since phosphate sometimes disturbes anion-exchange chromatography later employed in the separation procedure. Results are shown in Table I. It should be noted that the addition of pyridoxal phosphate at concentrations above 1 mM lowers its activity remarkably. This can only mean that the prosthetic group is tightly bound to the enzyme and is not lost during cell disruption and centrifugation and a surplus of pyridoxal phosphate might block binding of the substrate. This interpretation is in good agreement with the analysis of the enzymes from *Chlorella* [27], *Euglena* [10], *Rhodopseudomonas* [21] and *Clostridium* [11]. Tris also deactivates the enzyme, even at low concentrations, and can therefore not serve as buffer system. EDTA (3 to 15 mM) shows only little effect, glycerol does not effect the enzyme

Table I. Influence of different components of the buffer system on the activity of 4,5-dioxovalerate transaminase in crude cell-free enzyme preparations from *Scenedesmus*. The standard assay (1 mM 4,5-dioxovalerate, 30 mM alanine, 1 mM pyridoxal phosphate) is set as 100%.

Compound	Concentration [mM]	mU/mg Protein	% of standard assay
Pyridoxal phosphate	0	0.33	136
	1	0.24	100
	2	0.18	74
	4	0.14	58
Tris	2.5	0.18	73
	5	0.16	66
	10	0.13	54
	15	0.11	45

at all. Dithiothreitol (1 to 10 mM), however, fully deactivates the protein, probably by conformational change. Mercaptoethanol has the same effect, implicating that SH-reagents have to be strictly avoided in the enzyme assay with *Scenedesmus*.

Purification to apparent homogeneity

For a first purification step various common methods have been tested. Ammonium sulfate precipitated the DOVA-transaminase containing protein fraction between 40 and 80% saturation. The enrichment was about 3-fold, but the yield compared to total initial activity was only 12–16%, which is much too low for a first purification step. With acetone the enzyme precipitated between a concentration of 20 and 40%. The enrichment was 2.5-fold, however, only 27% of the activity of the crude preparation could be recovered, which again seemed to be insufficient for further purification. Following the method of Turner and Neuberger [21] who could enrich and purify 4,5-dioxovalerate transaminase from *Rhodopseudomonas spheroides* by heat denaturation, we found no enrichment of the enzyme, but a decrease of activity of about 75%, when the crude protein solution was heated for 10 min to 59 °C. The heating in presence of 1 mM 4,5-dioxovalerate and 200 μM pyridoxal phosphate decreased total enzyme activity again to about 25%, but a specific activity of about 60% could be retained, suggesting that a weak stabilizing effect on part of the enzyme took place.

More successful was chromatography on DEAE-cellulose. In order to prevent the binding of the phosphate groups of nucleic acids to the DEAE-residues of the cellulose, nucleic acids were first precipitated by protamine sulfate. After this procedure the loss in total activity was only 5%, the enrichment factor was 1.3.

Subsequently the remaining protein supernatant was administered to the DEAE-cellulose column, which was equilibrated with 10 mM phosphate (pH 6.8). All proteins could be bound to the column. Elution was performed with a phosphate gradient from 10 to 100 mM, 4,5-dioxovalerate transaminase eluting between 50 and 80 mM phosphate concentration. Enrichment was 6.9-fold, the yield of total activity was 25% of the starting activity.

Because of the relatively high loss in activity on DEAE-cellulose DEAE-sepharose CL-6B was next chosen as gel matrix. When a crude protein solution, pre-purified by protamine sulfate precipitation and on hydroxyapatite (see below), was applied to the column, which was equilibrated to 10 mM phosphate (pH 6.8), all protein was bound. After washing with 1 bed volume of the 10 mM phosphate buffer a NaCl-gradient from 0 to 0.3 M was passed through the column. 4,5-Dioxovalerate transaminase eluted as a single symmetric peak between 0.1 and 0.18 M NaCl. The loss in activity was only about 10% and the enrichment about 7-fold. The elution diagram is shown in Fig. 1.

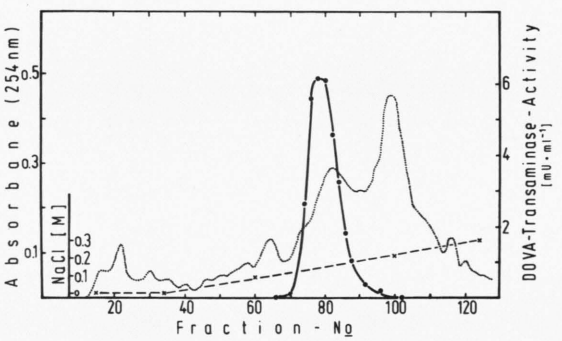

Fig. 1. Elution profile of 4,5-dioxovalerate aminotransferase from a DEAE-sepharose CL-6B column. 10 ml of a protein solution, pre-purified by protamine sulfate precipitation and chromatography on hydroxyapatite were administered to the column, which was equilibrated to 10 mM phosphate, pH 6.8. Proteins were eluted with a gradient from 0 to 0.3 M NaCl, preceeded by one bedvolume of buffer. Column size: 3×6 cm; flow rate 65 ml/h; fraction size: 2.8 ml. x---x NaCl-concentration; ····· absorbance at 254 nm; ●——● 4,5-dioxovalerate transaminase activity.

For pre-purification of protein preparations prior to administration to DEAE-sepharose hydroxyapatite was employed. As it is known that especially double stranded nucleic acids bind to this material, these compounds were precipitated by protamine sulfate prior to applying the soluble protein supernatant to the column. 4,5-Dioxovalerate transaminase did not bind to the separation material, but was eluted with the starting buffer, which was 10 mM in phosphate, pH 6.8. Bound proteins were eluted with a gradient from 10 to 100 mM phosphate. Although the transaminase did not bind to hydroxyapatite, a 2.6-fold purification was obtained. The loss in transaminase activity was only 24%.

For further purification of the enzyme isoelectric focussing was applied. To get a first rough determination of the isoelectric point a gradient from pH 3 to 9 was built up in the gel matrix. Two different carriers were employed: Sephadex G-200 SF and a mixture of agarose and Sephadex G-200 SF. It turned out that the latter carrier yielded much sharper bands.

The first rough determination revealed a pI around pH 5 for 4,5-dioxovalerate transaminase. Twenty protein bands could be well distinguished on the gel, when a filter-paper was pressed carefully on the gel and was afterwards stained with Coomassie Blue [23]. Only one of the bands showed 4,5-dioxovalerate transaminase activity. To get a more precise determination of the pI-value a gradient ranging from pH 4 to 6 was formed in the gel matrix. The pI-value for 4,5-dioxovalerate transaminase turned out to be 4.72.

For desalting and removal of ampholytes gel filtration on Sephadex G-150 was performed. The active band from isoelectric focussing was thoroughly eluted from the gel matrix and then 3 ml of the protein solution were administered to the column, which was again equilibrated with 10 mM phosphate buffer to pH 6.8. 4,5-Dioxovalerate transaminase activity eluted as a sharp band and was later subjected to SDS-PAGE to prove the homogeneity of the enzyme protein. The elution profile of Sephadex G-150 separation is shown in Fig. 2.

By combining the described successful methods for separation and enrichment, 4,5-dioxovalerate aminotransferase could be enriched 104-fold. The yield in activity over all purification steps was 16%. As the main loss in activity occurs during isoelectric focussing, this method still has to be improved.

A compilation of all purification and enrichment data is given in Table II.

Characterization of 4,5-dioxovalerate aminotransferase

Noguchi and Mori [12] could show that 4,5-dioxovalerate transaminase, purified from liver mitochon-

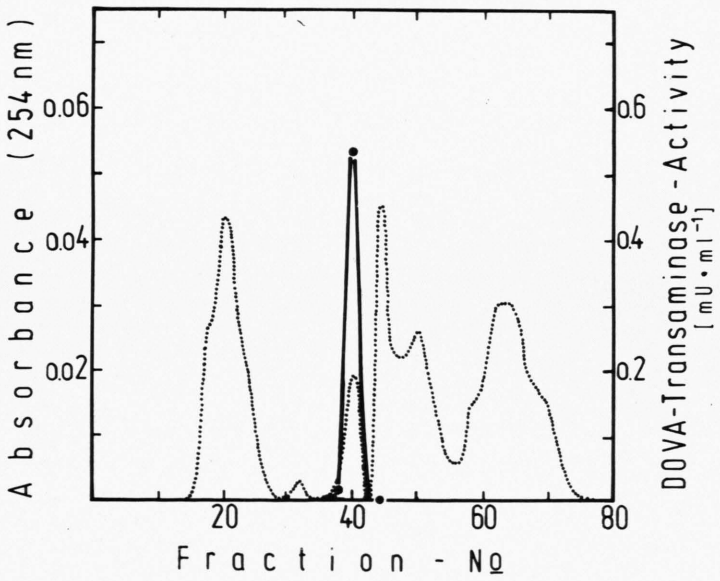

Fig. 2. Elution diagram of 4,5-dioxovalerate transaminase from a Sephadex G-150 column after isoelectric focussing. To the column, equilibrated to 10 mM phosphate buffer, pH 6.8, 3 ml of the eluate from isoelectric focussing were administered. Then the proteins were eluted at a flow rate of 19 ml/h, fraction size being 2.8 ml. Column size was 1.5 × 85 cm. Absorbance at 254 nm: ·····; transaminase activity: •——•.

Table II. Purification of 4,5-dioxovalerate aminotransferase by a combination of different separation methods. Starting material was the supernatant of broken 3-days-old dark-grown cells of mutant C-2A′, prepared as described in Methods.

	Total activity [mU]	Protein [mg]	Specific activity (in U/mg protein)	Purification factor	% Yield of activity
Crude supernatant	600	1935	0.31	1	100
Protamine-sulfate precipitation	582	1617	0.36	1.2	97
Hydroxyapatite	456	556	0.82	2.6	76
DEAE-Sepharose	372	91	4.1	13.2	62
Isoelectric focussing and Sephadex G-150	96	3.0	32.2	104	16

dria by Vartikowski *et al.* [13] is identical with glyoxylate: alanine aminotransferase-isoenzyme 2. This finding raised the question whether 4,5-dioxovalerate transaminase from algae and higher plants is also identical with this glyoxylate transaminating enzyme, especially as several glyoxylate transaminases from plants were already characterized [28].

When elution profiles where tested during the purification procedure for both enzyme activities, it turned out that there were always several protein fractions with glyoxylate transaminase activity, but only one with 4,5-dioxovalerate transaminase activity. A typical elution profile from a hydroxyapatite column is shown in Fig. 3. At each purification step the ratio of glyoxylate- and 4,5-dioxovalerate transaminase activity was determined. The main part of glyoxylate transaminase activities could be separated, as shown in Table III, but a minor final activity remained. This had to be expected, since glyoxylate as a substrate analogue can probably also be transaminated by 4,5-dioxovalerate transaminase. – The three separated glyoxylate transaminase activities, however, were not able to convert 4,5-dioxovalerate to 5-aminolevulinate, clearly indicating that the newly isolated enzyme is a 4,5-dioxovalerate transaminase.

For further characterization of purified 4,5-dioxovalerate transaminase K_M-values for different substrates and amino-donors were determined following the method of Dixon and Webb [29]. Results are compiled in Table IV. The fact that glutamate is about 20-fold more effective as amino-donor for the purified enzyme from *Scenedesmus* clearly demonstrates that it is not identical with L-alanine: 4,5-

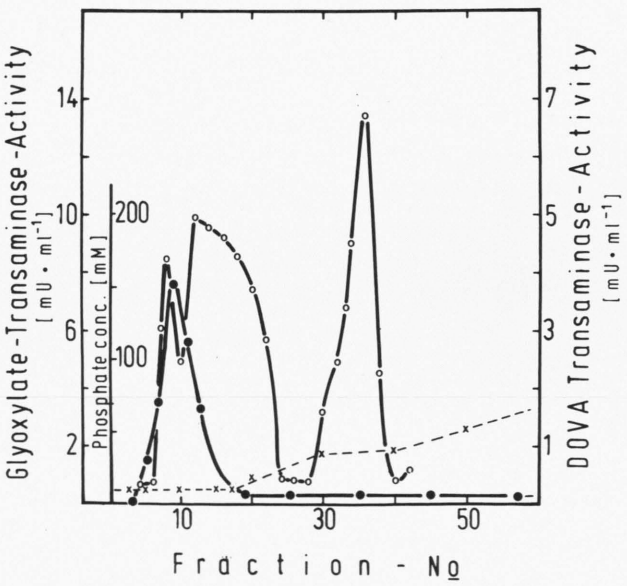

Fig. 3. Elution diagram of 4,5-dioxovalerate transaminase activity from a hydroxyapatite column (3×8 cm) using a crude enzyme supernatant as starting material. Conditions are described in Methods. x---x: phosphate concentration; ●——●: 4,5-dioxovalerate transaminase activity; o----o: glyoxylate transaminase activity.

Table III. Ratio of glyoxylate *versus* 4,5-dioxovalerate transaminase during purification by hydroxyapatite, isoelectric focussing and Sephadex G-150.

Purification step	4,5-Dioxovalerate transaminase activity (DT) [mU/mg protein]	Glyoxylate transaminase (GT) [mU/mg protein]	Ratio GT/DT
Crude enzyme supernatant	0.31 mU/mg	2.63 mU/mg	8.5
Hydroxyapatite	0.82 mU/mg	3.16 mU/mg	3.9
Isoelectric focussing and Sephadex G-150	32.2 mU/mg	28.6 mU/mg	0.9

Table IV. V_{max}-values and K_M-values for amino donors and oxo-substrates for the purified 4,5-dioxovalerate transaminase from *Scenedesmus obliquus*.

Substrate	V_{max} [nmol · min^{-1} · mg protein^{-1}]	K_M-value [mM]
L-Glutamic acid	32.2	0.46
L-Alanine	19.6	10.0
Glyoxylate	17.4	1.05 (with alanine)
4,5-Dioxovalerate	19.6	0.63 (with alanine)
	37.2	0.58 (with glutamic acid)

dioxovalerate aminotransferase (EC 2.6.1.43) [11, 13] and L-alanine: glyoxylate aminotransferase (EC 2.6.1.44) [12]. It is, however, in good agreement with the results of Meisch *et al.* [27], who could demonstrate glutamate to be the natural amino-donor for 4,5-dioxovalerate aminotransferase from *Chlorella fusca*.

A concentration above 1 mM 4,5-dioxovalerate produces a strong substrate inhibition of the transaminase. This is in accordance with Neuberger and Turner [30] and additionally supports the conclusion that 4,5-dioxovalerate is the natural substrate of the purified enzyme from *Scenedesmus*.

As already shown above, the addition of pyridoxal phosphate at concentrations >0.5 mM to the crude enzyme supernatant caused an inhibition of 4,5-dioxovalerate transaminase. Concentrations below 100 μM pyridoxal phosphate revealed a slight activation of the enzyme. The K_M-value for the cofactor was determined to be 8 μM. This value does not take into account that the cofactor is already tightly bound to a portion of the enzyme, as described for other 4,5-dioxovalerate transaminases [10, 21, 27, 28], too.

Also the reversibility of the enzyme catalysis was tested. It could be shown that the purified enzyme could not transfer 5-aminolevulinic acid and pyruvate to 4,5-dioxovalerate and alanine, when concentrations from 0.5 to 10 mM 5-aminolevulinate were applied. This contradicts results of Porra and Klein [31], but is in agreement with all data of other purified 4,5-dioxovalerate transaminating enzymes [10–13, 21, 25–27, 32].

This leads to the conclusion that reversibility (5-aminolevulinate → 4,5-dioxovalerate) in crude preparations is due to unspecific transamination reactions.

The low specificity of transaminases is well known [28] and thus 4,5-dioxovalerate transaminase from *Scenedesmus* was tested for its amino-donor requirement. Results are shown in Table V, activity with glutamate in the presence of 4,5-dioxovalerate being set as 100%. Sulphur containing amino acids were precipitated by Hg^{2+} before determination of 5-aminolevulinate by the Ehrlich-test, because they interfere strongly with the reagent. From the obtained results it becomes obvious that the transaminase from *Scenedesmus* has to be specified as L-glutamate:

4,5-dioxovalerate aminotransferase. Besides 4,5-dioxovalerate also glyoxylate in the presence of alanine was tested. Alanine was chosen as amino-donor, since glyoxylate transaminase is an alanine requiring enzyme (Table V).

Further characterization of the enzyme was achieved by the determination of its molecular weight (M_r). By gel filtration on Sephadex G-150 68,000 ± 5,000 Da were determined as relative molemass. The separating column was calibrated with several proteins of known molecular weight. When the determination of M_r was performed under denaturing conditions by SDS-PAGE, a value of 61,700 Da was found, indicating that 4,5-dioxovalerate aminotransferase from *Scenedesmus* does not consist of subunits, as the relative molemass under non-denaturing and denaturing conditions is very similar. The results of SDS-PAGE is shown in Fig. 4. The value is in good agreement with the enzyme from *Chlorella fusca*, a very similar organism, which was determined to be 60,000 Da [27]. The enzymes of *Scenedesmus* and *Chlorella* do, both, not consist of subunits. Other 4,5-dioxovalerate transaminases [10, 11, 17] showed higher molecular weights, some of them consisting of subunits. SDS-PAGE also revealed that the enzyme was purified to apparent homogeneity, which is shown in Fig. 5.

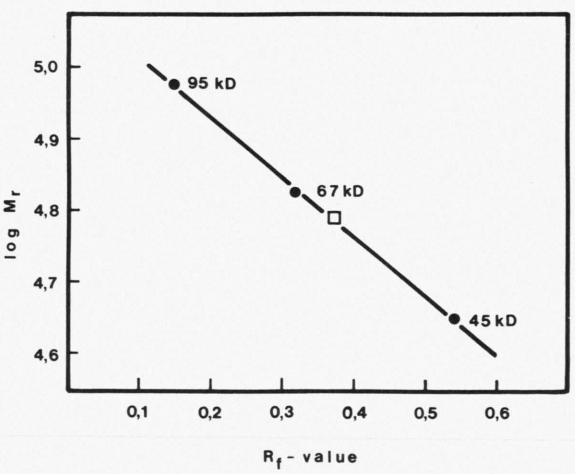

Fig. 4. Determination of the relative molecular weight of 4,5-dioxovalerate aminotransferase by SDS-PAGE. As standards (●) – gelatine (95 kDa), bovine serum albumine (67 kDa), ovalbumine (45 kDa) and chymotrypsin A (25 kDa, not shown here) were employed. 4,5-dioxovalerate transaminase (□) revealed a M_r of 61,700.

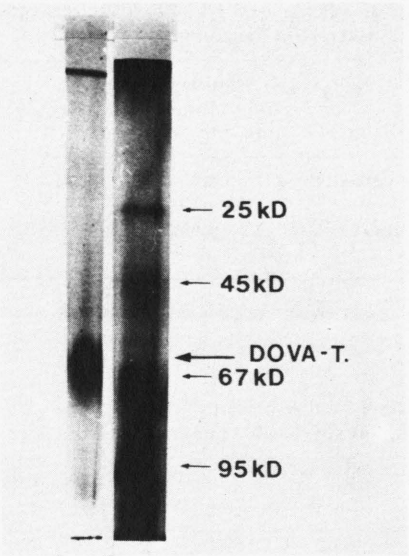

Fig. 5. SDS-PAGE of 4,5-dioxovalerate transaminase demonstrating apparent homogeneity of the enzyme protein. Experimental conditions are described in detail in Methods. The right track represents calibration proteins, as indicated in Fig. 4, the left track shows the purified 4,5-dioxovalerate transaminase.

Finally the pH-optimum of 4,5-dioxovalerate transaminase from *Scenedesmus* was determined. It showed a broad optimum between pH 6.5 and 7.5. This is in good agreement with all so far described 4,5-dioxovalerate transaminating enzymes [10–13, 25–27, 32]. The pH-optimum for the transaminase from *Scenedesmus* ranges among lower pH-values reported for higher plant chloroplasts [33, 34]. As the transaminase from maize showed an optimum of pH 6.8, Harel *et al.* [25] concluded that the enzyme is localized in the cytoplasm and can thus not be responsible for 5-aminolevulinate synthesis. Final evidence for the location of 4,5-dioxovalerate aminotransferase could only be deduced from studies on isolated chloroplasts. Since intact chloroplasts cannot be prepared from *Scenedesmus* we can only assume that 4,5-dioxovalerate transaminase from this organism is a plastid enzyme.

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