Biochimica et Biophysica Acta, 384 (1975) 168—179

BBA 67440

PURIFICATION OF A DEREPRESSIBLE ARYLSULFATASE FROM *CHLAMYDOMONAS REINHARDTI*

PROPERTIES OF THE ENZYME IN INTACT CELLS AND IN PURIFIED STATE

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(Received October 8th, 1974)

Summary

Arylsulfatase (aryl-sulfate sulfohydrolase, EC 3.1.6.1) has been purified from SO_4^{2-}-starved cells of *Chlamydomonas reinhardti*. The enzyme was isolated from acetone-powder extract by $(NH_4)_2SO_4$ precipitation, Sephadex G-200 filtration and ion-exchange chromatography. Only one fraction of arylsulfatase was found. The final preparation was homogenous by the criteria of sedimentation, diffusion and polyacrylamide gel electrophoresis. The purified enzyme had a molecular weight of about 150 000, estimated by ultracentrifugation and gel filtration, and an isoelectric point of 9.0. The properties of the enzyme as investigated in intact cells and in the purified state were found to be very similar except for the temperature optimum. Imidazole strongly increased the enzyme activity by increasing the V, but reduced the affinity for the substrate. The enzyme activity was competitively inhibited by borate with a greater affinity for borate than for the substrate. The *Chlamydomonas* enzyme is a Type I arylsulfatase since it was inhibited by CN^-, but not by SO_4^{2-} and phosphate.

Introduction

As a part of an investigation of the derepressibility of arylsulfatase during the life cycle of *Chlamydomonas reinhardti* [1], we purified the enzyme and examined the properties.

We present here a method for purification and a comparative investigation of properties of this derepressible enzyme in intact cells and in purified state. Few arylsulfatases from microorganisms have been obtained in a purified state [2], and to our knowledge this is the first report on the properties of purified arylsulfatase from algae.

Materials and Methods

Culture conditions

Asynchronous and synchronous cultures of *C. reinhardti* No. 11-32 (90) from the Algal Collection of the Institute of Plant Physiology, University of Göttingen, Germany, were used throughout this work.

Culturing conditions and synchronization procedure were as described before [3], and the derepressing treatment was as reported in an accompanying paper [1]. Large quantities of SO_4^{2-}-deprived cells (derepressed, or —S cells) for enzyme purification were grown in 1-liter Roux flasks with an inner light path of 45 mm. The cells used as inocculum were taken from a stock synchronous culture (light-dark synchronized) at different hours in the light period and given the derepressing treatment before they were suspended in 5 liters of —S medium [1]. This suspension, containing about $3 \cdot 10^6$ cells per ml, was divided on Roux flasks which were then placed in the same water thermostate as used for the light-dark-synchronized cultures. The cultures of SO_4^{2-}-deprived cells were illuminated and aerated as usual at 35° C. After about 24 h of growth in continuous light the cells were rapidly collected in a small continuously operating centrifuge [4].

Preparation of extract of acetone powder

The harvested —S cells were suspended in 80 ml cold acetone (—20° C) and kept at this temperature for 1 h with occasional shaking. The suspension was thereafter centrifuged and the pellet resuspended in cold acetone followed by centrifugation. The extraction with cold acetone was repeated until most of the chlorophyll was removed from the cells. Thereafter the cells were dried in a gentle stream of air. The dry acetone powder was extracted by suspending it in 80 ml of 0.05 M sodium phosphate buffer, pH 6.5, for 15 min followed by centrifugation. This extraction was repeated until no more sulfatase activity could be removed from the cells. The extracts were pooled and filtered through a glass-fiber filter by suction filtration, and the filtrate was used as the starting material in fractionation experiments.

Arylsulfatase assay

The enzyme activity was measured according to Fromageot [5]. The activity of arylsulfatase in cell-free solutions was measured by incubating a mixture of 1.0 ml *p*-nitrophenylsulfate (4.5 mM in 0.1 M of an appropriate buffer) with 50 or 100 µl of the enzyme solution at 30° C for 30 min. The incubation was stopped with the addition of 4.0 ml 0.25 M NaOH. The intact cells used in this work were taken from a light-dark-synchronized culture at different stages in the light period for derepression of the sulfatase. After about 5 h of derepression (i.e. growth in —S medium) the cells contained in 1.0 or 2.0 ml of the culture suspension ($1.5 \cdot 10^6$ or $3.0 \cdot 10^6$ cells) were collected on a 21-mm diameter membrane filter disc by suction filtration. The cells were washed with 5 ml of distilled water which was drawn through the filter. The filter with the cells was then transferred to a 25-ml Erlenmeyer flask containing 2.0 ml 4.5 mM substrate in 0.1 M of an appropriate buffer of 2° C. The temperature was rapidly elevated to 30° C and the cells incubated with the sub-

strate under shaking for 30 min. The incubation was stopped with the addition of 4 ml 0.25 M NaOH, and thereafter the mixture was filtered. *p*-Nitrophenylsulfate was used as substrate throughout this work, but in a few experiments the arylsulfatase activity was also measured by using nitrocatecholsulfate as substrate.

The absorbance of the liberated *p*-nitrophenol (or nitrocatechol) in the reaction mixture after the addition of NaOH was measured at 410 nm (or 480 nm) using an automatic digital spectrophotometer coupled to a sample changer.

The standard deviation of the enzyme assay was found to be ±4%. With incubation times shorter than 50 min there was direct proportionality between enzyme activity and time, and between numbers of cells and amount of enzyme protein per sample.

Ion-exchange chromatography

A satisfactory condition for purification of the enzyme by ion-exchange chromatography was sought using a method of Kirkegaard et al. [6]. The ion-exchange gel DEAE-Sephadex A-50 or CM-Sephadex C-50 was packed in a 10-ml graduated pipette to a column height of 20 cm. Four or five such small columns were used simultaneously. By varying the ionic strength and pH of the solution used both for equilibration of the column and for elution of the proteins, the appropriate condition for separation of arylsulfatase was rapidly found. For preparative fractionation of the enzyme the ion-exchange gel column was 31 cm high and 1.9 cm in diameter.

Absorbance at 280 nm of the eluate from the ion-exchange and gel-filtration columns was continuously monitored with a Uvicord (LKB, Sweden). Absorbances at 260 and 280 nm were also measured on individual fractions with a Beckman Model DB spectrophotometer.

Polyacrylamide gel electrophoresis

The gel systems used were as described by Maurer [7]. Discontinuous alkaline and acid buffer systems with and without 6 M urea and spacer gel were tried. The best results were obtained by using a discontinuous acid buffer system with spacer gel and without urea. This consisted of a 7% separation gel (9 cm) and 3.5% spacer gel (0.6 cm). 0.48 M potassium acetate (with respect to potassium), pH 4.3 and pH 6.7, was used as gel buffer for the separation and spacer gel, respectively. The electrode buffer was 0.035 M β-alanine acetate (with respect to β-alanine), pH 4.5. The gel was polymerized in 10 cm long glass tubes and with 7 mm inner diameter. Electrophoresis was carried out at 4 mA per tube and 4°C for 4.5 h. Bromphenol Blue and Methyl Green were used as front markers for the alkaline and acid buffer systems, respectively. After a run proteins were stained with Coomassie Brilliant Blue. Staining for arylsulfatase activity was done by soaking the gels at room temperature in a freshly prepared solution containing 4 vol. diazoreagent (prepared by diazotation of Basic Fuchsin [8]); 2 vol. 0.25 M NaOH; 5 vol. 20 mM sodium α-naphthylsulfate and 1 vol. 20 mM imidazole solution (in 0.1 M glycine/NaOH buffer, pH 9.8). Incubation time varied from 10 s to 5 min. When the red-band pattern was sufficiently visible, the gels were washed repeatedly with distilled water and stored in distilled water. Scanning of the gels was done with a MPS-50L Shimadzu spectrophotometer.

Isoelectric focusing

Isoelectric focusing was carried out at 4°C according to the method of Vesterberg and Svensson [9] using a 25-ml burette with double-bore stopcock as the electrolysis column (Raa, J., unpublished). Solutions of Ampholine carrier ampholytes covering the pH ranges 3—10 and 7—10 were used. The focusing time was 24 h, and the starting voltage was 350 V and the current about 1 mA. After electrolysis, the content of the column was collected in 1-ml fractions in test tubes for measurement of pH and arylsulfatase activity.

Concentration and desalting of enzyme solutions

After each step during enzyme purification, fractions containing enzyme activity were pooled and thereafter concentrated by ultrafiltration at 4°C in a Dia-Flo Model 52 or Model 402 ultrafiltration cell (Amicon, The Netherlands). Recovery was quantitive with respect to enzyme activity.

Before subjecting the enzyme solutions to electrophoresis they were concentrated, desalted and transferred to the spacer gel buffer by using a Minicon, Model B 15 ultrafiltration unit (Amicon, The Netherlands).

Ultracentrifugation

Ultracentrifugation experiments were carried out in a Beckman Model E analytical ultracentrifuge. The purified enzyme protein was dissolved (4 mg/ml) in 0.1 M sodium phosphate buffer, pH 6.5, and centrifuged at 20°C. Sedimentation was carried out at 59 780 rev/min. Pictures of schlieren peaks were taken at a bare angle of 50° at intervals of 10, 18, 26 and 34 min after the speed had been attained. The diffusion coefficient was determined at 6166 rev./min. Pictures of schlieren peaks were taken at a bare angle of 45° at intervals of 7, 15, 23, 31 and 39 min after reaching speed. During this time the aera of the peak remained constant. $D_{20,w}$ was calculated with relating the aera:height of peak ratio to time [10].

Chemicals

All chemicals used were of analytical reagent quality. *p*-Nitrophenylsulfate, nitrocatecholsulfate and α-naphthylsulfate were from Sigma Chemical Company, U.S.A. Acrylamide was from Eastman Organic Chemicals, U.S.A. *N,N'*-Methylene-bis-acrylamide (Bis) and *N,N,N',N'*-tetramethylenediamine (Temed) were from K. & K. Laboratories Inc. U.S.A., Basic Fuchsin (triaminotriphenylmethanchlorid), Coomassie Brilliant Blue, Bromphenol Blue and Methyl Green were from Searle Scientific Service, England. Ampholine was from LKB, Sweden. Sephadex G-200, DEAE-Sephadex A-50 and CM-Sephadex C-50 were from Pharmacia, Sweden.

Results

Purification of arylsulfatase

The yield of enzyme and the degree of purification achieved in each step are illustrated in Table I. All purification steps were carried out at 4°C.

Step 1. Derepressed cells were harvested, treated with acetone and the acetone powder (10 g) was extracted with 0.05 M sodium phosphate buffer, pH 6.5, as described in Materials and Methods.

TABLE I

PURIFICATION OF ARYLSULFATASE FROM *CHLAMYDOMONAS REINHARDTI*

The data refer to the extract of 10 g acetone powder. The purification steps in the first column refer to the procedure described under Results. Total protein is expressed as the product of volume (ml) and absorbance at 280 nm. Enzyme units are given as μmol *p*-nitrophenol formed/min at 30°C and pH 9.8 (0.1 M glycine/1 M NaOH buffer). Specific activity is enzyme units divided by $A^{1\,cm}_{280\,nm}$ units of the protein solution.

Enzyme solution from step No.	Volume (ml)	$A_{280\,nm}$ (1 cm cell)	$A_{260\,nm}$ (1 cm cell)	Total protein	Total enzyme units	Specific enzyme activity	Yield (%)
1	665	2.625	4.55	1745	108173	62	100
2	340	1.05	1.39	357	88400	248	82
3	50	0.85	1.0	42.5	70000	1647	65
4	40	0.235	0.102	9.4	29689	3158	27
5	10	0.42	0.185	4.2	22222	5291	21

Step 2. $(NH_4)_2\ SO_4$ precipitation. 259 g solid $(NH_4)_2\,SO_4$ were added slowly to 665 ml of the acetone-powder extract with constant stirring to a final 60% saturation. The extract was stirred until the salt dissolved. After about 20 min at 4°C and occasional stirring, the precipitate was collected on glass-fiber filter discs by suction filtration. The precipitate contained about 20% of the total protein and more than 80% of the total arylsulfatase activity of the acetone powder extract. The glass-fiber filters with the precipitated proteins on were then suspended in 250 ml 0.05 M sodium phosphate buffer, pH 6.5. After 16 h the suspended filters were collected on glass-fiber filter disc by suction filtration, and the filter washed with 90 ml of the sodium phosphate buffer which was drawn through the filter. The filtrate (340 ml) was concentrated to 30 ml by ultrafiltration.

Step 3. Gel filtration of Sephadex G-200. The concentrated enzyme solution was subjected to gel filtration on a column (1.9 cm × 52 cm) of Sephadex G-200 and eluted with 0.05 M sodium phosphate buffer, pH 6.5, containing 3% NaCl. Two protein peaks were eluted from the column. The first, which was eluted close to the void volume, contained large amounts of material strongly absorbing at 260 nm (presumably nucleic acid). The second peak being somewhat retarded on the column, contained the highest activity of arylsulfatase.

Step 4. DEAE-Sephadex column chromatography. The pooled fractions from Step 3 containing enzyme activity were concentrated and transferred to 0.45 M Tris · HCl buffer, pH 9.3 (at 4°C), by ultrafiltration. The concentrated enzyme solution was applied to the DEAE-Sephadex column, equilibrated with 0.45 M Tris · HCl buffer of pH 9.3 and eluted with the same buffer. Under these conditions three protein peaks were eluted from the column. Arylsulfatase interacted weakly with the gel and appeared in the middle protein peak eluted from the column mainly within the sieving range.

Step 5. CM-Sephadex column chromatography. The pooled fractions from Step 4 containing enzyme activity were concentrated and transferred to 0.1 M sodium phosphate buffer, pH 6.5. The concentrated enzyme solution was then fractionated by ion-exchange chromatography on CM-Sephadex C-50. The en-

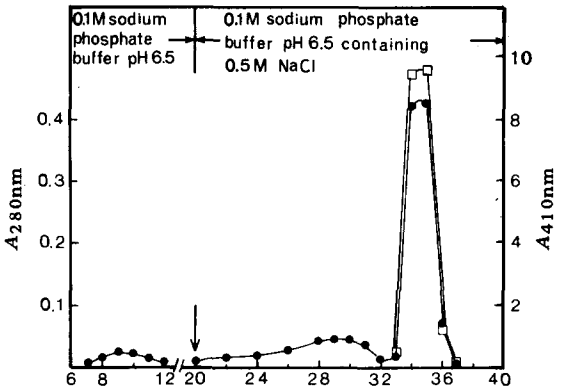

Fig. 1. The elution of the Step 4 preparation from CM-Sephadex (the final step in the purification procedure). The column was equilibrated with 0.1 M sodium phosphate buffer, pH 6.5, and the proteins were eluted in two steps. First with the equilibrating solution and thereafter with the equilibrating solution containing 0.5 M NaCl. □——□, sulfatase activity; ●——●, A_{280nm}.

zyme was attached to the ion-exchange column in 0.1 M sodium phosphate buffer, pH 6.5, and subsequently eluted, as described in the caption to Fig. 1, with 0.5 M NaCl in the same buffer. As can be seen of Fig. 1, the enzyme activity coincided exactly with a peak of ultraviolet absorption (A_{280nm}). The enzyme derived by CM-Sephadex chromatography was checked for homogeneity by polyacrylamide gel electrophoresis and by ultracentrifugation.

Homogeneity of the purified enzyme

The enzyme solution derived from the final step in the purification procedure above, shows a ratio in absorbance at 280 nm and 260 nm of 2.27,

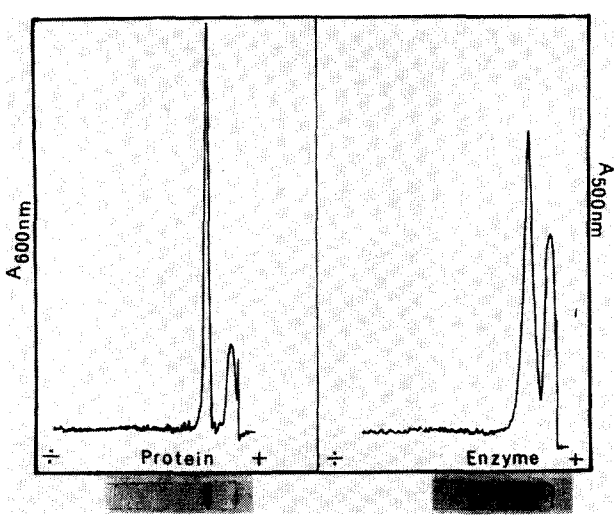

Fig. 2. Electrophoresis of purified arylsulfatase on polyacrylamide gels. Coomassie Brilliant Blue-stained gel (left) and sulfatase activity-stained gel (right). The staining of the gels, the spectrophotometric scanning of the stained electropherograms and the discontinuous acid buffer system used, were as described in Materials and Methods. Electrophoresis was carried out at 4 mA per tube and 4° C for 4.5 h.

suggesting that the enzyme was highly pure with respect to contaminating nucleic acids [11]. Polyacrylamide gel electrophoresis in the acid buffer system (Fig. 2) gave a single protein band corresponding with the single band of enzyme activity. The absorption peak at the anode side of the gels (Fig. 2) is not due to sulfatase activity, but to the opaque spacer gel which absorbed at 500 and 600 nm. In the alkaline buffer system (not shown) the protein band also corresponded with the enzyme activity band.

A single symmetrical peak was obtained during diffusion and sedimentation in the ultracentrifuge suggesting a high degree of purity.

All these observations demonstrated the homogeneity of the purified enzyme.

Properties of arylsulfatase in intact cells and in purified state

Effect of pH and imidazole on the sulfatase activity. The results of experiments on the effect of pH and imidazole on the enzyme activity are presented in Fig. 3. As can be seen, the optimum activity was found at pH 9.8 both for intact cells and the purified enzyme when imidazole was absent from the reaction mixture. Fig. 3 shows that the presence of 10 mM imidazole in the reaction mixture led to increased arylsulfatase activity over the whole pH range tested. Maximum increase (10—15 times) above that found with imidazole present was obtained at acid and neutral pH ranges. With 10 mM imidazole the optimum activity was at pH 9.0 both for intact cells and the purified enzyme.

By measuring the enzyme activity at pH 7.8 (in 0.1 M veronal/HCl buffer) in the presence of different concentration of imidazole in the reaction mixture,

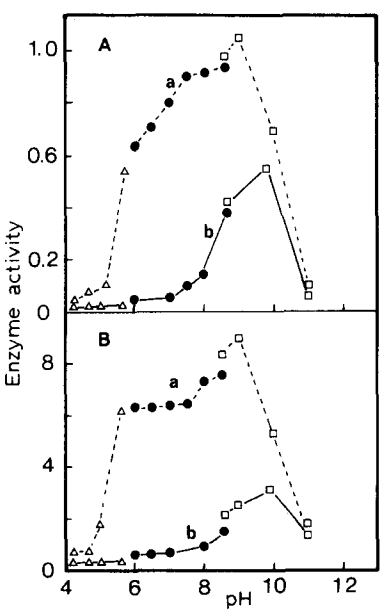

Fig. 3. Arylsulfatase activity as a function of pH. (A) intact cells. (B) purified enzyme. a (- - - - -) with 10 mM imidazole; b (———) without imidazole in the reaction mixture. The enzyme activity was assayed at 30°C over a wide pH range employing the following buffers: pH 4.2—5.5, 0.1 M sodium acetate buffer (△); pH 6.0—8.6, 0.1 M veronal/HCl buffer (●); pH 8.6—11.0, 0.1 M glycine/NaOH buffer (□). The pH was measured at 30°C, and the enzyme activity is expressed as absorbance at 410 nm per 30 min at 30°C.

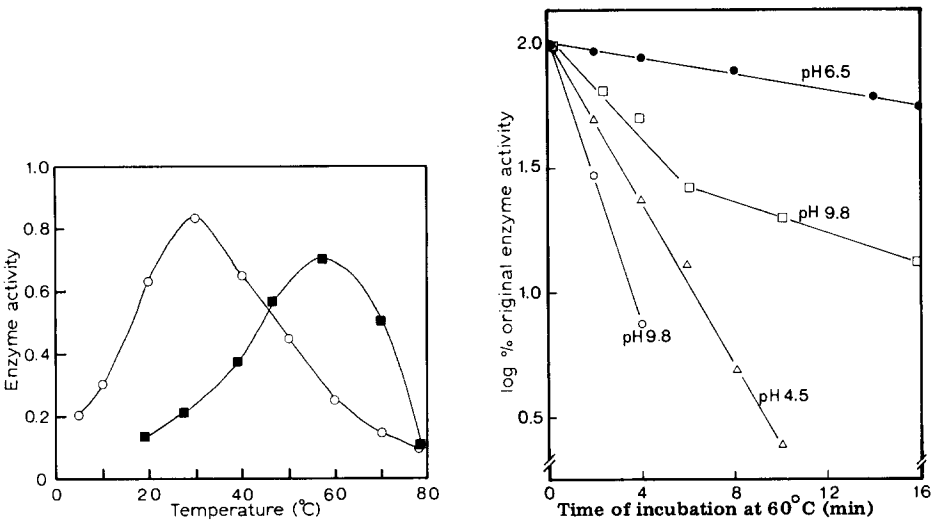

Fig. 4. Effect of temperature on arylsulfatase activity in intact cells (■———■) and in purified state (○———○). The enzyme activity was measured at pH 9.8 (in 0.1 M glycine/NaOH buffer). The incubation time was 10 min.

Fig. 5. Thermal inactivation of arylsulfatase. Samples of intact cells and purified enzyme were preincubated at 60°C at different times and in the following buffers: Intact cells (□———□), 0.1 M glycine/NaOH, pH 9.8; purified enzyme (○———○), 0.1 M glycine/NaOH, pH 9.8; purified enzyme (●———●), 0.1 M sodium phosphate buffer, pH 6.5; purified enzyme (△———△), 0.1 M sodium acetate buffer, pH 4.5. The samples were then assayed for residual arylsulfatase activity at pH 9.8 (in 0.1 M glycine/NaOH buffer).

it was found that in intact cells the enzyme activity increased with increasing concentration of imidazole from 0.1 to 10 mM. The purified enzyme showed the largest increase in activity within the concentration range from 1 to 10 mM. Higher imidazole concentrations were not tested since the pH of the incubation mixture was changed when imidazole was more concentrated.

The same stimulating effect of imidazole on the arylsulfatase activity was obtained using nitrocatecholsulfate as substrate.

Effect of temperature on arylsulfatase activity. Fig. 4 shows the different temperature optima for arylsulfatase activity in intact cells and of purified enzyme. For the purified enzyme the optimum was 30°C, but for intact cells it was as high as 60°C. The temperature optima were the same in the presence as in the absence of imidazole in the incubation mixture.

The activation energy at pH 9.8 (in 0.1 M glycine/NaOH buffer) for the purified enzyme was 7.9 kcal/mol (33 054 J/mol) and the activity increased by a factor of 1.6 per 10°C increase in temperature between 0 and 20°C. For intact cells the corresponding values were 8.8 (36 819 J/mol) and 1.6 kcal/mol (between 20 and 50°C), respectively.

Enzyme stability. The purified enzyme was rapidly inactivated at 60°C at alkaline and acid pH ranges. The enzyme was more thermostable at pH 6.5—7.0, and more thermostable at the same pH in intact cells than in purified state. A semilogarithmic plot of the inactivation course (Fig. 5) reveals two phases of inactivation of the enzyme activity measured in intact cells. But the purified enzyme showed typical single-component inactivation kinetics (Fig. 5).

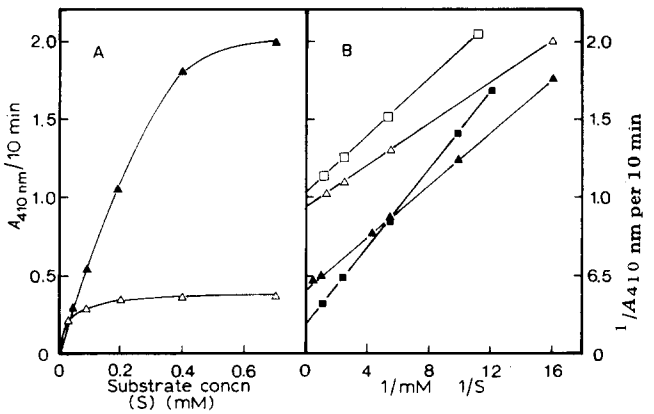

Fig. 6. Arylsulfatase activity as a function of substrate (*p*-nitrophenylsulfate) concentration. (A) Purified enzyme with (▲———▲) and without (△———△) imidazole in the reaction mixture. (B) Lineweaver-Burk plots of arylsulfatase activity. Purified enzyme with (▲———▲) and without (△———△) imidazole. Intact cells with (■———■) and without (□———□) imidazole in the reaction mixture. The enzyme activity in intact cells and in purified state was measured at 38°C in 0.1 M veronal/HCl buffer, pH 7.8.

During a period of 14 days at 3°C and pH 6.5 (in 0.1 M sodium phosphate buffer) 25% of the original enzyme activity was lost. About 80% of the activity of the enzyme in purified state was lost during a storage period of 8 weeks at −20°C. The enzyme was neither stabilized in the presence of mercaptoethanol, nor in the presence of imidazole.

Michaelis constants. Fig. 6A shows the dependency of the activity of arylsulfatase in purified state on the substrate concentration in the presence and absence of 10 mM imidazole. The Michaelis constants (K_m) for the purified enzyme determined from the reciprocal plots shown in Fig. 6B, were $2.0 \cdot 10^{-4}$ M and $0.7 \cdot 10^{-4}$ M with and without 10 mM imidazole, respectively. The K_m values for intact cells with and without imidazole were $5.2 \cdot 10^{-4}$ M and $0.86 \cdot 10^{-4}$ M. Fig. 6A and the K_m values show that imidazole increases the V, but decreases the affinity of the enzyme for the substrate.

Inhibitors. The presence of 0.05 M K_2SO_4, 1 mM Na_2SO_3, 10 mM sodium phosphate, 1 mM methionine, 1 mM cysteine, 10 mM sodium citrate and 1 mM EDTA in the reaction mixture had no effect on the enzyme activity. But Na_3BO_3 and KCN (in mM concentration) strongly inhibited the enzyme activity in intact cells and in purified state, in the absence as well as in the presence of imidazole in the reaction mixture.

Lineweaver-Burk plots (not shown) of the enzyme activity as a function of substrate concentration (*p*-nitrophenylsulfate) with and without 2 mM Na_3BO_3 in the reaction mixture, showed that the enzyme is competitively inhibited by BO_3^{3-}. The values of K_i were $5.2 \cdot 10^{-5}$ M and $4.8 \cdot 10^{-5}$ M for intact cells and purified enzyme, respectively. Since the ratio K_i/K_m is less than 1, the enzyme has a greater affinity for BO_3^{3-} than for the substrate (*p*-nitrophenylsulfate).

A concentration of 2 mM KCN in the reaction mixture gave 50% inhibition of the sulfatase activity. This inhibition appeared to be non-competitive.

Isoelectric focusing. The isoelectric point of the enzyme was found from 8

TABLE II

SOME PROPERTIES OF ARYLSULFATASE IN INTACT CELLS AND IN PURIFIED STATE

Properties		Purified state	Intact cells
Temperature optima (°C)		30	60
Acivation energy (kcal/mol)		7.9	8.8
pH optima	without imidazole	9.8	9.8
	with imidazole	9.0	9.0
K_m (M)	without imidazole	$0.70 \cdot 10^{-4}$*	$0.86 \cdot 10^{-4}$*
	with imidazole	$2.0 \cdot 10^{-4}$*	$5.2 \cdot 10^{-4}$*
K_i (M) with sodium borate		$4.8 \cdot 10^{-5}$**	$5.2 \cdot 10^{-5}$**
Isoelectric point		9.0	
$S_{20,w}$ (s)***		$6.74 \cdot 10^{-13}$	
$D_{20,w}$ (cm^2/sec)		$4.06 \cdot 10^{-7}$	
Molecular weight			
Gel filtration		150000	
$S_{20,w}$ and $D_{20,w}$ values		152000	

* With *p*-nitrophenylsulfate as substrate, and in 0.05 M veronal/HCl buffer, pH 7.8.
** Enzyme activity in 0.1 M glycine/NaOH buffer, pH 9.8, in the presence of 2 mM BO_3^{3-}.
*** With the enzyme in 0.1 M sodium phosphate buffer, pH 6.5.

paralell runs of isoelectric focusing to be at pH 9.0 ± 0.4. This high p*I* indicates a large content of basic amino acid in the enzyme protein.

Molecular weight. The molecular weight of the arylsulfatase was estimated by chromatography on a Sephadex G-200 column (1.9 cm × 52 cm), equilibrated in 0.05 M sodium phosphate buffer, pH 6.5, containing 3% NaCl. Samples were applied to the column in the same buffer system. The following marker proteins were used: jack bean urease, 480 000; beef liver catalase, 250 000; bovine serum albumin, 68 000. A linear relationship was obtained for the elution volumes of the standard proteins and log molecular weights. A molecular weight of approximately 150 000 was determined from the elution volume of arylsulfatase.

From the ultracentrifugation a sedimentation coefficient, $S_{20,w}$, of $6.74 \cdot 10^{-13}$ S and a diffusion coefficient, $D_{20,w}$, of $4.06 \cdot 10^{-7}$ cm^2/s were calculated for the enzyme protein. From these $s_{20,w}$ and $D_{20,w}$ values, assuming a value of 0.730 for the partial specific volume, the molecular weight of the protein was calculated [10] to be 152 000.

The properties of the arylsulfatase in intact cells and in purified state are summarized in Table II.

Discussion

Primarily on the basis of investigation of the arylsulfatase occurring in the livers of the higher animals it was suggested by Dodgson and Spencer [12] to subdivide arylsulfatase enzymes into two major groups, the Type I and the Type II arylsulfatases. The two types are distinguished by their response to inhibitors and by their substrate specificities. Type I arylsulfatases are insensitive to inhibition by SO_4^{2-} (i.e. to product inhibition), and insensitive to inhibition by phosphate, but are inhibited by CN^-. Type II arylsulfatases are inhibited by SO_4^{2-} and phosphate, but not by CN^-. Furthermore, arylsulfatases I

have a relatively low specificity with affinities both for nitrophenylsulfate and nitrocatecholsulfate. The type II arylsulfatases have relatively high affinity only for nitrocatecholsulfate.

As the derepressible arylsulfatase from *Chlamydomonas* is inhibited by CN^-, but not by SO_4^{2-} or phosphate, and hydrolyzes both nitrophenyl- and nitrocatecholsulfate, it is basically type I arylsulfatase. However, many of the properties of the *Chlamydomonas* enzyme are different from those found with arylsulfatase from other biological systems. SO_3^{2-}, which inhibits all known arylsulfatases [2], had no effect on the *Chlamydomonas* enzyme. Furthermore, no other arylsulfatases have shown a pH optimum as high as 9.8 and an isoelectric point as high as 9.0. As far as we know, the activation by imidazole and inhibition by BO_3^{3-} found with the *Chlamydomonas* enzyme have not been reported for arylsulfatases of other organisms.

The effects of these two compounds are difficult to explain at the present. Imidazole as well as BO_3^{3-} are good chelating agents, and imidazole is known both to activate and to inactivate different enzymes [13—15]. The strong inhibition by CN^- suggests that a heavy metal is involved in the catalytic activity of the enzyme. Thus, it might be possible that BO_3^{3-} and imidazole affect the activity by making complexes with a heavy metal and/or with chemical groups involved in the catalytic activity of the enzyme. There is little information on the nature of the active center(s) of arylsulfatase [2]; however, a detailed investigation of the effect of heavy metals, imidazole and BO_3^{3-} might perhaps give information on the catalytic properties of the *Chlamydomonas* arylsulfatase.

Arylsulfatases have been detected in several microorganisms, especially under conditions of SO_4^{2-} deprivation. Most of these microbial enzymes also appear to be Type I enzymes. The most studied examples are those of *Aspergillus oryzae* [16], *Aspergillus nidulans* [17], *Aerobacter aerogenes* [18,19] and *Pseudomonas aeruginosa* [20]. The two fungi and *P. aeruginosa* contain two or three arylsulfatases while *A. aerogenes* seems to contain only one arylsulfatase. The following evidences point to the conclusion that the activity of arylsulfatase from SO_4^{2-}-starved cells of *Chlamydomonas* is also present in a single protein moiety: (1) The different chromatographic procedures employed in the purification of the enzyme have revealed only one peak with arylsulfatase activity. (2) Isoelectric focusing gave one peak with arylsulfatase activity. (3) Disc electrophoresis in polyacrylamide gels revealed a single band containing arylsulfatase activity. (4) In addition, the heat inactivation kinetics, the electrophoretic patterns with a single protein band coinciding with a single band of enzyme activity, and the sedimentation pattern in the analytical ultracentrifuge, demonstrated the physical homogeneity of the purified enzyme from *Chlamydomonas*.

The properties of the enzyme investigated in intact cells were closely similar to those found with the enzyme in purified state. The ease of assay of the activity in intact cells and the inability to increase the activity by rupturing the cells, or by making acetone powder of them, suggest that arylsulfatase in *Chlamydomonas* is attached to the cell surface. Preliminary results from investigation of the cytochemical location of the enzyme support this indirect evidence.

Acknowledgments

The authors are indebted to Mr K. Bergh, Department of Biochemistry, University of Bergen, for the ultracentrifugation experiments. We wish to thank the Norwegian Council of Science and the Humanities for the technical assistance. The authors thank Dr G. Knutsen for his discussions and suggestions.

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