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An inducible arylsulfatase of *Volvox carteri* with properties suitable for a reporter-gene system

Purification, characterization and molecular cloning

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The multicellular green flagellate *Volvox carteri* synthesizes a periplasmic arylsulfatase in response to sulfur deprivation. The inducible enzyme has been purified to homogeneity and characterized. The corresponding gene and cDNA have been cloned. Determination of the sequence of genomic clones and comparisons to the cDNA sequence, revealed sixteen introns and seventeen exons that encode a 649-amino-acid polypeptide chain.

Since the arylsulfatase enzyme is readily assayed using chromogenic substrates, but is not detectable in cells grown in sulfate-containing medium, the gene encoding arylsulfatase may be useful as a reporter gene in *V. carteri*. In addition, the highly regulated promoter of the arylsulfatase gene suggests its suitability as a tool for producing inducible expression vectors for cloned genes.

The green algae of the genus *Volvox* are among the simplest multicellular organisms with only two cell types: somatic and reproductive cells. Therefore, they have been used as a model system for biochemical and physiological investigations (Kirk and Harper, 1986; Schmitt et al., 1992). A number of genes possibly involved in the control of developmental processes have been cloned (Ertl et al., 1992; Sumper et al., 1993; Tschochner et al., 1987; Mages et al., 1988). A large number of developmental mutants have been described (Starr, 1970; Huskey et al., 1979; Callahan and Huskey, 1980). However, a serious drawback for molecular biological studies with this organism was the lack of a transformation system. With the recent establishment of a transformation system (Schiedlmeier et al., 1994), this disadvantage no longer exists. In order to allow the application of efficient molecular genetic approaches, reporter genes as well as appropriate promoters working in this organism are now required.

Studies with the unicellular alga *Chlamydomonas reinhardtii* suggest arylsulfatase as an attractive reporter-gene system, since chimeric genes incorporating the *Chlamydomonas* arylsulfatase-coding sequence and *Chlamydomonas* tubulin promoters can be expressed (Davies et al., 1992). In contrast, attempts to isolate stable transformants, either from the unicellular alga *Chlamydomonas* (Rochaix et al., 1984) or from the multicellular alga *Volvox* (Mages, 1990), failed with heterologous gene constructs. Therefore, a homologous reporter-gene system also appears to be necessary for *Volvox*. The arylsulfatase is an inducible enzyme which is only expressed under conditions of sulfur starvation. A number of chromogenic substrates are available allowing for the easy determination of enzyme activity. An arylsulfatase activity, inducible only under conditions of sulfur starvation, is also detectable in *V. carteri* (unpublished results). The enzyme can easily be assayed in whole cells or in extracts. These features suggest that the arylsulfatase gene may also serve as a valuable tool in transformation experiments in *V. carteri*. The coding sequence of the arylsulfatase could be used as a reporter gene and its promoter would be an attractive regulatory element for fusion with cloned genes, allowing their expression to be induced by sulfate starvation. Attempts to clone the *Volvox* enzyme with the heterologous cDNA probe from *C. reinhardtii* were unsuccessful. Therefore, we obtained amino acid sequence data for the purified enzyme and used this information for cloning both the genomic DNA and the cDNA encoding this enzyme.

MATERIALS AND METHODS

Culture conditions

V. carteri forma *nagariensis* strain HK10 (female) was obtained from the Culture Collection of Algae at the University of Texas (Dr R. C. Starr). Synchronous cultures were grown in *Volvox* medium (Provasoli and Pintner, 1959) at 28 °C in an 8-h dark/16-h light (10000 lx) cycle (Starr and Jaenicke, 1974). In *Volvox* medium lacking sulfate, $MgSO_4$ was replaced by $MgCl_2$.

Assay of arylsulfatase activity

Arylsulfatase activity was measured using either 4-nitrocatechol sulfate or 5-bromo-4-chloro-3-indolyl sulfate. With 4-nitrocatechol sulfate, the assay contained 5 mM substrate, 50 mM Tris/HCl, pH 8.0, and enzyme in a final volume of

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Abbreviations. PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.

Enzymes. Arylsulfatase (EC 3.1.6.1); trypsin (EC 3.4.21.4).

Note. The novel nucleotide sequence data published here have been submitted to the EMBL Data Library and are available under accession number X77214.

100 µl. After incubation at 37 °C for 15 min, the reaction was stopped by adding 1 ml 1 M NaOH and the liberated 4-nitrocatechol was quantified by measuring the absorbance at 515 nm. This assay was performed with extracts and column eluates.

Whole spheroids, incubated in sulfate-free medium for more than 5 h, were cultivated in microtiter wells in 500 µl. 4 µl 30 mM 5-bromo-4-chloro-3-indolyl sulfate was added and cultures were incubated at 28 °C for 2 h. Algae expressing arylsulfatase were identified by their blue color.

For an activity stain in SDS/polyacrylamide gels, arylsulfatase-containing fractions were subjected to standard SDS/PAGE (8% polyacrylamide; Laemmli, 1970, 1977), without heating the probes to 95 °C. After electrophoresis, the gels were soaked in 100 mM Tris/HCl, pH 8.0, for 20 min and 5-bromo-4-chloro-3-indolyl sulfate was added to a final concentration of 0.5 mM. The gels were incubated at 20 °C for the times indicated.

Purification of arylsulfatase

Volvox spheroids (HK10) from three 20-l cultures were collected on a nylon screen. After extensive washing with *Volvox* medium lacking sulfate, the algae were incubated in sulfate-free medium under standard conditions for a further three days. At the end of this period, the spheroids were collected. Solid NaCl was added to a final concentration of 400 mM, and the culture was stirred for 15 h at 4 °C. The suspension was filtered through 100-, 40- and 10-µm mesh nylon cloths to remove the *Volvox* spheroids. Ammonium sulfate was added to 40% saturation, and the solution was stirred at 4 °C for 20 min. After centrifugation for 20 min at 8000 *g*, the supernatant was brought to 70% saturation with ammonium sulfate, incubated at 4 °C for 2 h, and centrifuged for 1 h at 11000 *g*. Precipitated protein containing arylsulfatase activity was suspended in 50 mM Tris/HCl, pH 8.0. After extensive dialysis against the same buffer, the solution was passed through a C_{18} (octadecylsilane) cartridge (Millipore) and applied to a column of blue Sepharose CL-6B (Pharmacia) equilibrated with 50 mM Tris/HCl, pH 8.0. The column was washed with two volumes of the same buffer and was developed by applying a linear gradient of NaCl (in 50 mM Tris/HCl, pH 8.0) from 0–400 mM. The arylsulfatase eluted at 130–200 mM NaCl. The protein was collected by precipitation with ammonium sulfate (70% saturation). The pellet was dissolved in a minimal volume of 50 mM Tris/HCl, pH 8.0, 100 mM NaCl and applied to a Superose 12 HR 10/30 FPLC column (Pharmacia) equilibrated in the same buffer. The fractions containing enzyme activity were concentrated and desalted in a microconcentrator (Centricon 30, Amicon). The concentrated material was applied to a 8% SDS/polyacrylamide gel and arylsulfatase was eluted from the gel by diffusion. Typically, the yield was 50–100 µg homogenous arylsulfatase. For peptide analysis, the arylsulfatase was digested without elution from the SDS/polyacrylamide gel.

Proteolytic digestion and separation of peptides

30 µg arylsulfatase was analysed by SDS/PAGE (8% polyacrylamide) and stained with Coomassie blue. The gel slice containing arylsulfatase was crushed and totally destained by incubations in 30% methanol, followed by 7% acetic acid containing 50% methanol and finally by 7% acetic acid (each for 2 h). The gel was soaked in 90% ethanol for 2 h and dried by lyophilization. 4 µg trypsin in 800 µl 0.2 M NH_4HCO_3 was added and the gel material was incubated at 37 °C overnight. The resulting peptide mixture was eluted from the gel by diffusion in 0.2 M NH_4HCO_3 containing 50% acetonitrile. The eluate was passed through a 0.22-µm pore-size filter (Millipore), adjusted to 0.1% trifluoroacetic acid and dried by lyophilization. The peptides were dissolved in 6 M guanidine/HCl/0.1% CF_3CO_2H and fractionated by reverse-phase HPLC (Smart system, Pharmacia) on a µRPC C_2/C_{18}, 3-µm particle-size column (Pharmacia). The peptides were eluted by a 30-min linear gradient of 5–40% acetonitrile in 0.1% CF_3CO_2H with a flow rate of 200 µl/min.

The peptides were sequenced by Edman degradation using an automated gas-phase peptide sequencer (Applied Biosystems, Inc.).

Generation of a cDNA probe by PCR

RNA was extracted as described by Kirk and Kirk (1985) from *Volvox* spheroids incubated in sulfate-free medium for 15 h. A cDNA library covalently linked to magnetic beads was constructed according to the instructions of the manufacturer of the magnetic beads (Deutsche Dynal). Polymerase-chain-reaction (PCR) amplification was performed in 100 µl containing the following reagents: 50 mM Tris/HCl, pH 8.5, 50 mM NaCl, 2.5 mM $MgCl_2$, 2 mM dithiothreitol, 2 µl suspension of the cDNA library covalently linked to magnetic beads, 400 pmol degenerate sense primer TTYGAYTAYAAYAA, 400 pmol degenerate antisense primer ATYTGNGCRTARAA, 100 µM dNTP and 2.5 U *Taq* polymerase. 40 cycles of PCR amplification (Perkin-Elmer cycler model 480) were performed (denaturation at 94 °C for 45 s, annealing at 45 °C for 30 s, and extension at 72 °C for 20 s). The resulting 146-bp DNA fragment was ligated into the *Sma*I site of pUC18 and was sequenced.

Cloning of the arylsulfatase gene

The *V. carteri* genomic library in λEMBL 3 (Frischauf et al., 1983), described by Ertl et al. (1989), was used to clone the arylsulfatase gene. The screening and cloning procedures followed standard techniques (Sambrook et al., 1989). DNA sequencing was performed by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using T7 DNA polymerase (Pharmacia). Synthetic oligonucleotides were used throughout to sequence the arylsulfatase gene.

PCR amplification of arylsulfatase cDNA fragments

The cDNA library covalently linked to magnetic beads was used to generate cDNA fragments of arylsulfatase by PCR using specific oligonucleotide primers. The rapid amplification of cDNA ends (RACE)-PCR technique was performed as described by Frohman et al. (1988). The PCR fragments were ligated into the *Sma*I site of pUC18. Synthetic oligonucleotides were used to sequence the arylsulfatase cDNA fragments in both directions.

PCR with RNA from twenty *Volvox* spheroids

Twenty *Volvox* spheroids, after their release from the mother spheroid, were selected under the stereo microscope and transferred into 20 µl sterile 50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 5 mM EGTA and 2% SDS (buffer A). After

10 min at 30°C, *Volvox* spheroids were removed under the stereo microscope and RNA was precipitated with 60 µl ethanol. The washed precipitate (70% ethanol), was dissolved in 10 µl reverse-transcriptase buffer, containing 50 mM Tris/HCl (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, 10 U RNAguard (Pharmacia), 1 mM each dNTP and 50 pmol antisense primer (5′-TTATCGGACACGTCAGC, amino acid positions 260–265). Reverse transcription was performed with 100 U Moloney murine leukemia virus reverse transcriptase (Pharmacia) for 60 min at 40°C; 90 µl 50 mM Tris/HCl, pH 8.5, 50 mM NaCl, 2.5 mM $MgCl_2$, 2 mM dithiothreitol (buffer B) containing 50 pmol sense primer (5′-GCTTCTATTCCACTGACG, amino acid positions 177–183) was added, and 38 cycles of PCR amplification (94°C, 45 s; 52°C, 30 s; 72°C, 45 s) were performed.

Assays for substrate specificities

0.01–0.1 µmol different substances were incubated with 0.1 U arylsulfatase for 15 h at 37°C. Subsequently, product formation was analysed by the following steps. Tyrosine and tyrosine sulfate were analysed by TLC (Kieselgel 60, Merck) in 1-butanol/acetic acid/water (4:1:1, by vol.) and were visualized by the ninhydrin reaction. Galactose and galactose sulfate (D-galactose 6-sulfate) were separated by TLC (Kieselgel 60, Merck) in acetonitrile/water (17:3, by vol.) and were visualized by the orcinol/sulfuric acid stain (Vaskovsky et al., 1970). Dextran and dextran sulfate were separated by anion-exchange chromatography. QAE A-25 Sephadex (Pharmacia), equilibrated in 20 mM Tris/HCl, pH 8.0, quantitatively binds dextran sulfate, whereas dextran appears in the flow-through fraction and was assayed with phenol/sulfuric acid (Dubois et al., 1956). Inorganic sulfate was determined by the rhodizonate assay (Terho and Hartiala, 1971). Adenosine 5′-phosphosulfate and AMP were separated by TLC [poly(ethyleneimine)-cellulose with fluorescence indicator dye, Schleicher & Schüll] in 1.75 M LiCl/2 M formic acid and visualized under ultraviolet light at 254 nm. Only tyrosine sulfate was a substrate. Under the conditions used, a quantitative conversion to tyrosine was achieved.

Pulse labelling and preparation of a crude cell lysate

Pulse labelling of *V. carteri* spheroids with [^{35}S]sulfate was performed as described by Wenzl and Sumper (1986). After extensive washing with *Volvox* medium lacking sulfate, the labeled spheroids were disintegrated by ultrasonic treatment (30 s total).

RESULTS

Purification of arylsulfatase

Independent of the developmental stage, synthesis of an arylsulfatase is induced in *V. carteri* in response to sulfur deprivation (unpublished results). This property was used for the isolation of the enzyme. *Volvox* spheroids were cultivated in sulfate-free medium for three days and were harvested. Extraction of the spheroids with 400 mM NaCl removed most of the arylsulfatase activity from the extracellular matrix. The enzyme solution was fractionated and precipitated with ammonium sulfate (40–70% saturation), desalted by dialysis and passed through a reverse-phase C_{18} cartridge (Fig. 1) that did not adsorb the enzyme. The flow-through was applied to a column of blue Sepharose. After extensive washing with 50 mM Tris/HCl, pH 8.0, the column was developed by applying a linear NaCl gradient of 0–400 mM. Arylsulfatase activity was eluted from the column at 130–200 mM NaCl (Fig. 1). The concentrated eluate was further fractionated by gel filtration over a Superose 12 column (Fig. 1). A final purification to homogeneity was achieved by preparative SDS/PAGE (Fig. 1). The yield of enzyme and the degree of purification achieved in each step are shown in Table 1. Remarkably, arylsulfatase activity was not inhibited in the presence of even 1% SDS or 1% *N*-lauroylsarcosine. Therefore, we were able to localize arylsulfatase that had been electrophoresed in standard SDS/PAGE (without prior heat treatment) by staining for enzymic activity (Fig. 1). Native arylsulfatase exhibits an apparent molecular mass of approximately 95 kDa. Heat treatment causes the loss of enzyme activity (data not shown) and, as expected, a higher mobility in SDS/PAGE (apparent molecular mass approximately 70 kDa).

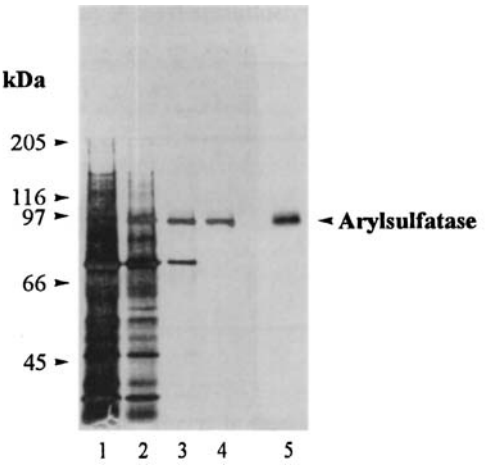

Fig. 1. The purification of arylsulfatase. SDS/PAGE (8% polyacrylamide) was performed without heat treatment of the samples. The preparation after the following steps is shown: C_{18} cartridge (lane 1); blue Sepharose column (lane 2); gel filtration (lane 3); final preparative SDS/PAGE (lane 4). Lanes 1–4 were stained with silver. Lane 5 shows an activity stain of the purified arylsulfatase in the same SDS/polyacrylamide gel (see Materials and Methods section). Each lane of the gel was loaded with the same amount of arylsulfatase activity. The molecular mass of marker proteins are indicated.

Protein chemical studies

Automated Edman degradation of the purified arylsulfatase showed that its N-terminal amino acid sequence was XXRPNFVVIFTDDQ. In order to get additional sequence information, arylsulfatase, localized in a SDS/polyacrylamide gel by the activity stain, was digested by incubating the corresponding gel slice with trypsin. The resulting peptide mixture was eluted from the polyacrylamide gel and chromatographed by reverse-phase C_2/C_{18} HPLC. The components of resolved peaks were directly subjected to amino acid sequence analysis on an automated gas-phase sequencer. The amino acid sequence data obtained are summarized in Table 2. The amino acid sequence of peptide 8 corresponds to the N-terminal amino acid sequence. Peptide 3 includes a post-translational modification at amino acid position 8 which indicates a N-glycosylation site.

Table 1. Purification of arylsulfatase from *V. carteri*. Enzyme units are given as μmol 4-nitrocatechol sulfate hydrolyzed/h at pH 8.0 and at 37°C (15 min assay).

Fraction after	Total protein	Total enzyme activity	Specific enzyme activity	Yield
	mg	U	U/mg	%
NaCl extraction	123	37	0.3	100
C_{18} cartridge	23	28	1.2	76
Blue-Sepharose column	3	14	4.7	38
Gel filtration	0.4	10	25	27
Final SDS/PAGE	0.1	4	40	11

Table 2. Amino acid sequences of tryptic peptides derived from arylsulfatase.

Peptide	Amino acid sequence
1	ILXPEGSVNSWXQSLD
2	TFDYNNPGFSR
3	GQFSHNT–FTDVLGPHGGYAK
4	NLYEADVSDKPAWI
5	XAVAXGKPFYAQISPXAP
6	SLASVDELIDR
7	NGATPNIYPGFYSTDVIADK
8	HQRPNFVVIFTD
9	HWELFSDATLPEGTSHK
10	VVATLQEAGVLDNTYLIY

Generation of a 146-bp cDNA probe by PCR

The amino acid information from the tryptic peptide TFDYNNPGFSR was used to synthesize the sense oligonucleotide primer TTYGAYTAYAAYAA corresponding to amino acids at positions 2–5. The antisense primer ATYTGNGCRTARAA was derived from amino acid positions 9–13 of the peptide XAVAXGKPFYAQISPXAP. Using these primers, a 146-bp fragment could be amplified by PCR from a cDNA library covalently linked to magnetic beads. DNA sequence analysis confirmed that this fragment did indeed encode for a region of the arylsulfatase polypeptide.

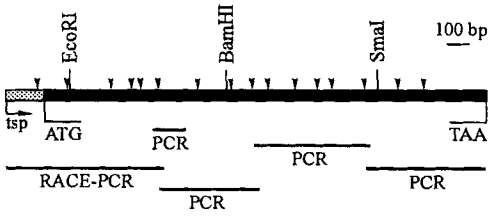

Fig. 3. The PCR strategy used to obtain the complete nucleotide sequence of arylsulfatase cDNA. The positions of introns are given (▼). tsp, transcription start point.

Genomic clones encoding arylsulfatase

A genomic library of *V. carteri* constructed in the vector λEMBL 3 was screened using the 146-bp cDNA as a probe. Clones λAr52 and λAr41, hybridizing to the 146-bp cDNA probe, were first isolated. However, neither of these clones contained the 3′ terminal end of the arylsulfatase gene. Therefore, the insert of plasmid pVAr4BS11, derived from λAr41 and containing the region shown in Fig. 2A, was used for an additional screening of the λEMBL 3 library. This produced λAr72, a clone that contains the complete arylsulfatase gene. Partially overlapping fragments from λAr41 and λAr72 allowed the determination of the complete sequence of the *V. carteri* arylsulfatase gene, including the 5′ and 3′ untranslated regions. Exon-intron organization was deduced by comparison of genomic and cDNA sequences. Fig. 2B

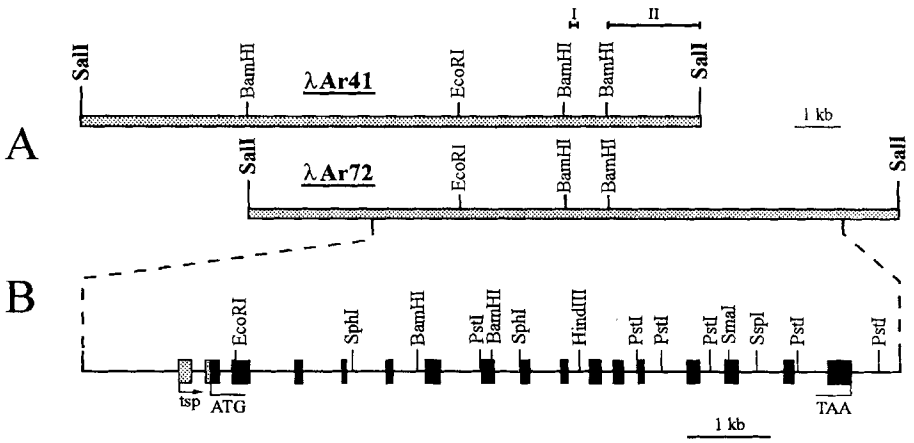

Fig. 2. (A) Physical map of two overlapping arylsulfatase genomic clones λAr41 and λAr72 and (B) derived intron-exon structure of the arylsulfatase gene. In A, the 146-bp PCR probe (I) and the insert of pVAr4BS11 (II) are indicated. Sites linking insert and vector DNA are given in bold-face type. tsp, transcription start point.

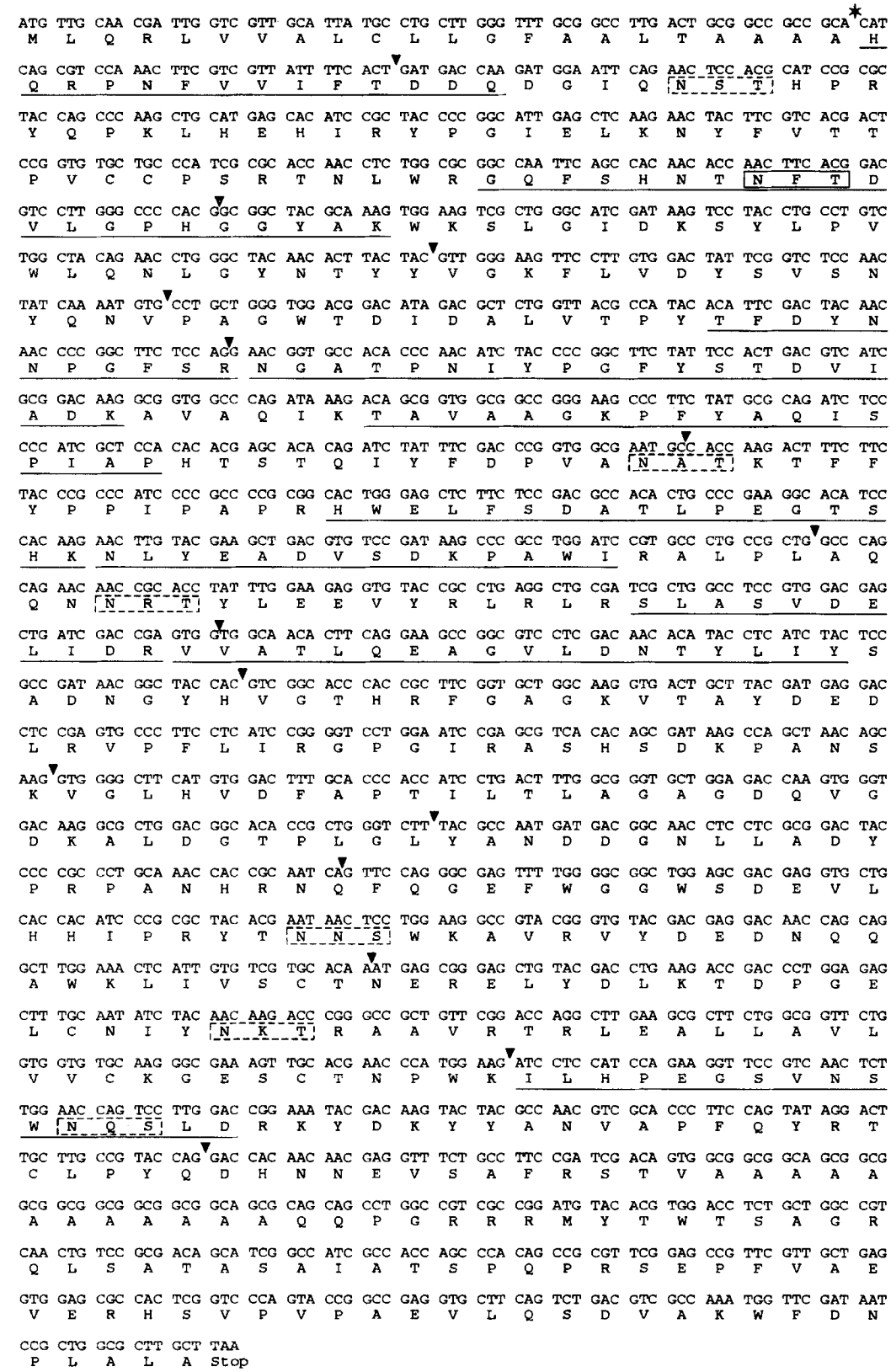

Fig. 4. The nucleotide sequence of arylsulfatase cDNA and deduced amino acid sequence of arylsulfatase. The amino acid sequences confirmed from isolated peptides are underlined. Potential (– –) and confirmed (——) N-glycosylation sites are boxed. The positions of introns are indicated (▼). The cleavage site of the hydrophobic leader sequence is also marked (★).

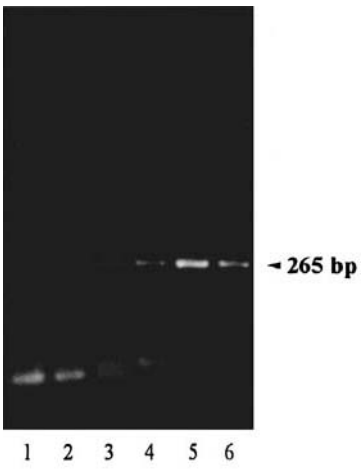

Fig. 5. The transcriptional control of arylsulfatase synthesis. RNA was extracted from twenty *Volvox* spheroids each grown in sulfate sufficient medium (lane 1) and subsequently starved for sulfate for 30 min (lane 2), 1 (lane 3), 3 (lane 4), 6 (lane 5) or 9 h (lane 6). After reverse transcription with an arylsulfatase-specific antisense oligonucleotide, a 265-bp cDNA fragment of arylsulfatase was amplified by PCR. The sense and antisense oligonucleotides were selected from exons 7 and 8, respectively, to prevent amplification of genomic DNA. For details, see the Materials and Methods section.

summarizes the relative locations of the seventeen exons and sixteen introns.

Cloning of arylsulfatase cDNA by PCR

The complete cDNA sequence was determined by PCR using specific oligonucleotide primers, a cDNA library covalently linked to magnetic beads, and the strategy summarized in Fig. 3. The cDNA nucleotide sequence and the deduced amino acid sequence are shown (Fig. 4.); a total of seven potential N-glycosylation sites were found. With the protein chemical data, the glycosylation of at least one of these glycosylation sites has been confirmed (Table 2, peptide 3).

Transcriptional control of arylsulfatase synthesis

PCR was used to determine whether the sulfate-dependent control of arylsulfatase synthesis is exerted at the level of transcription. Using the PCR technique, mRNA from only a few algal spheroids is easily detectable (Ertl et al., 1992). RNA was extracted from twenty *Volvox* spheroids deprived of sulfate for 30 min, 1, 3, 6 and 9 h. Reverse transcription and subsequent PCR amplification of arylsulfatase cDNA yielded the results shown in Fig. 5. mRNA for arylsulfatase is virtually absent in sulfate-sufficient spheroids, but could be detected as early as 1 h after the initiation of sulfate deprivation; arylsulfatase mRNA reached a maximum level after 6 h of sulfate deprivation and subsequently declined. The maximum level of PCR product obtained was comparable to the level obtained with specific actin mRNA primers under the same experimental conditions. Thus, the arylsulfatase promoter should allow a high level of gene expression and the control of arylsulfatase production operates at the level of transcription.

Location of the arylsulfatase

The arylsulfatase gene encodes a N-terminal leader sequence typical of a signal peptide, indicating an extracellular location of the arylsulfatase. However, *V. carteri* spheroids grown in sulfur-deficient medium do not secrete any enzyme with detectable activity into the culture fluid. All the enzyme activity remains associated with the spheroid, but can be extracted with 400 mM NaCl. In a mutant strain called 'dissociator', which is unable to produce a structurally intact extracellular matrix and consequently dissociates into single cells shortly after the end of embryogenesis, large amounts of arylsulfatase activity are secreted into the culture medium. These results suggest deposition of the arylsulfatase within the extracellular matrix of the wild-type strain.

Enzymic properties

Substrate specificity

Naturally occurring organic forms of sulfur constitute a diverse group of compounds, including sulfur-containing amino acids and sulfonates, as well as organic esters of sulfuric acid (Fitzgerald, 1978). Examples of the latter type of sulfoconjugate include the sulfate esters of simple phenols (arylsulfates), of aliphatic alcohols (alkylsulfates and cholin sulfate), of carbohydrates (e.g. chondroitin sulfate), and of amino acids (e.g. tyrosine sulfate). The arylsulfatase of *V. carteri* was assayed using the chromogenic substrates 4-nitrocatechol sulfate and 5-bromo-4-chloro-3-indolyl sulfate. The possibility that the *V. carteri* arylsulfatase may have a broad spectrum of sulfohydrolase activities has been considered. In addition to the chromogenic 4-nitrocatechol sulfate and 5-bromo-4-chloro-3-indolyl sulfate, tyrosine sulfate was also a substrate. In contrast, carbohydrate sulfates such as D-galactose 6-sulfate and dextran sulfate (average molecular

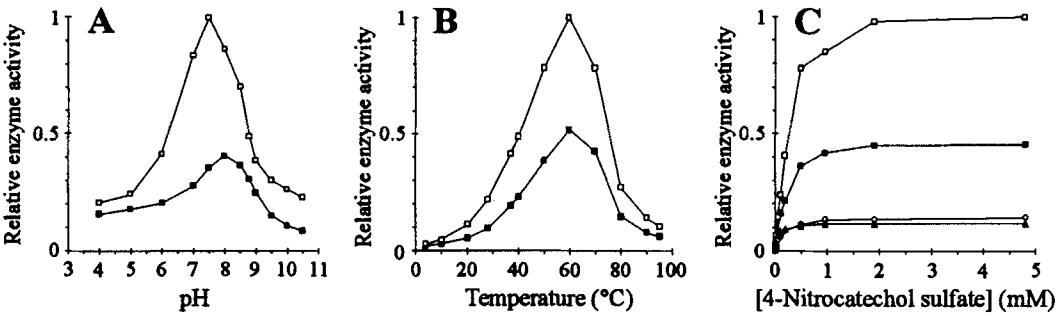

Fig. 6. The enzymic properties of *Volvox* arylsulfatase. (A) Arylsulfatase activity as a function of pH. (B) The effect of temperature on arylsulfatase activity. (C) Arylsulfatase activity as a function of substrate (4-nitrocatechol sulfate) concentration. The final concentrations of imidazole, Na_3BO_3 and KCN were 10, 2 and 10 mM, respectively. Reference (■), imidazole (□); borate (○); KCN (▲).

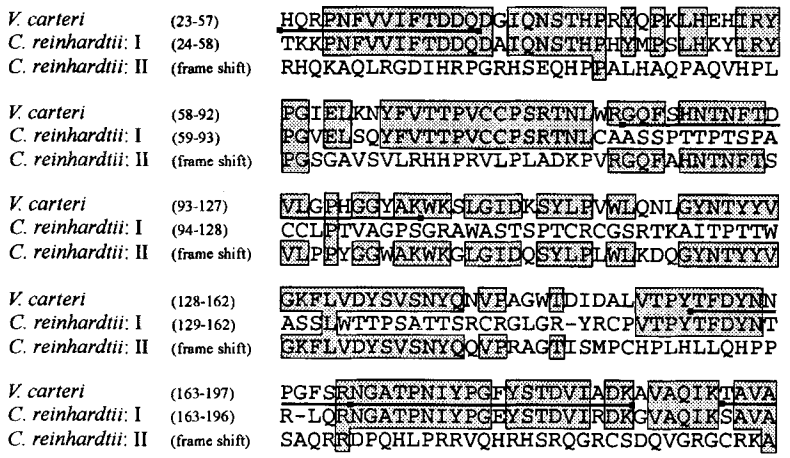

Fig. 7. Alignment and comparision of the *V. carteri* arylsulfatase (amino acid positions 23–197) with the arylsulfatase from *C. reinhardtii* (amino acid positions 24–196). '*Chlamydomonas*: I' indicates the published reading frame. '*Chlamydomonas*: II' is an amino acid sequence deduced from another reading frame of the arylsulfatase cDNA from *Chlamydomonas*. The sequenced peptides are underlined.

mass 5 kDa), alkylsulfates like dodecylsulfate (SDS), and adenosine 5′-phosphosulfate were not accepted as substrates.

A large amount of sulfate is covalently bound to polysaccharides and glycoproteins of the extracellular matrix of *V. carteri* (Wenzl and Sumper, 1987) and might be expected to serve as a reservoir of sulfur in times of deprivation. However, the purified enzyme was unable to release significant amounts of radioactive sulfate from a crude cell lysate previously labeled with $^{35}SO_4^{2-}$. These results indicate that the arylsulfatase from *V. carteri* only utilizes arylsulfates as substrates.

Other properties of the Volvox *arylsulfatase*

The Michaelis-Menten constant (K_m) for the synthetic substrate 4-nitrocatechol sulfate was determined to be 0.27 mM in the presence of 10 mM imidazole and 0.16 mM in the absence of imidazole (Fig. 6C). Imidazole is known to activate the arylsulfatase of *C. reinhardtii* (Lien and Schreiner, 1975). The arylsulfatase from *Volvox* also shows this property; 10 mM imidazole in the reaction mixture led to increased arylsulfatase activity over the whole pH range tested, but shifted the pH optimum from pH 8.0 to pH 7.5 (Fig. 6A).

The arylsulfatase was thermostable, exhibiting a temperature optimum at 60°C that was unaffected by the presence or absence of imidazole in the incubation mixture (Fig. 6B). The half-life of enzyme activity was 60 min at 60°C and pH 8.0.

The presence of substances like 50 mM Na_2SO_4, 5 mM Na_2SO_3, 5 mM cysteine, 5 mM methionine, 10 mM 2-mercaptoethanol, 10 mM sodium citrate, 10 mM EDTA or 50 mM sodium phosphate (which are known to inhibit many arylsulfatases from other sources) had no effect on the *Volvox* enzyme. However, Na_3BO_3 and KCN inhibited the enzyme (Fig. 6C). 1 mM Na_3BO_3 or 5 mM KCN in the reaction mixture gave 50% inhibition of the arylsulfatase activity.

DISCUSSION

Much of the sulfur in soil is present as sulfate esters or as sulfonates, rather than the inorganic sulfate that predominates in oceans and rivers (Fitzgerald, 1978). Arylsulfatase activity is found in many organisms and plays an important role in the mineralization of sulfate (Speir and Ross, 1978). The properties of the *Volvox* arylsulfatase (like its inducible synthesis and its extracellular location) indicate that mineralization of sulfate, by hydrolyzing sulfate esters in response to sulfur deprivation, seems also to be the function of the *Volvox* enzyme.

Dodgson and Spencer (1957) proposed to subdivide arylsulfatase enzymes into two major groups, the type-I and the type-II arylsulfatases. Type-I arylsulfatases are insensitive to product inhibition (SO_4^{2-}) and inhibition by phosphate, but are inhibited by CN^-, whereas type-II arylsulfatases are inhibited by sulfate or phosphate, but not by CN^-. By these criteria, the inducible arylsulfatase from *V. carteri* clearly represents a type-I arylsulfatase.

Based on amino acid sequence data, we have cloned both the genomic DNA and the cDNA encoding arylsulfatase. The derived amino acid sequence is consistent with known properties of the enzyme. The presence of a hydrophobic leader sequence confirms secretion of this enzyme, and the existence of seven potential N-glycosylation sites indicates glycosylation. The protein chemical data (peptide 3, Table 2) prove the post-translational modification of Asn89.

A comparison of the arylsulfatase sequence with sequences in the Swiss-Prot Protein Sequence Database revealed a number of sequence similarities. As expected, the highest similarity found (55.4% identity over 635 residues) was with the arylsulfatase from *C. reinhardtii* (de Hostos et al., 1989). In addition, significant sequence similarities exist to the human *N*-acetylglucosamine-6-sulfatase (26.2% identity over 279 residues) as well as to human arylsulfatase A (22.3% identity over 220 residues). The region of sequence similarity to both the human enzymes was localized within the C-terminus of the *Volvox* enzyme (residues 260−520). In contrast, the complete amino acid sequence of the *Volvox* enzyme shared a high degree of similarity with the *Chlamydomonas* enzyme. However, there were two significant exceptions. No sequence similarity was found at amino acid positions 81−152 and 252−294. As there is no obvious reason for such localized deviations, the possibility of sequencing errors was considered. The deduced amino acid sequence of the *Volvox* enzyme at amino acid positions 81−152 and 252−294 was confirmed by the sequence data obtained earlier from peptides 3 and 4 (Table 2, Fig. 4). There-

fore, we examined the different reading frames of the published *Chlamydomonas* cDNA (de Hostos et al., 1989) and recognized that a frame shift would create a sequence highly similar to the *Volvox* sequence at amino acid positions 81–152 (Fig. 7). The same is true for the region at amino acid positions 252–294 (data not shown). Thus, the deviations in both these sequences are most probably caused by sequencing errors. After removal of these deviations by adding or deleting single bases at appropriate places in the mentioned regions of the *Chlamydomonas* cDNA, an amino acid sequence can be deduced that shares a much higher similarity (69.2% identity over 633 residues) to the *Volvox* arylsulfatase than the published *Chlamydomonas* arylsulfatase sequence does. At present, however, we cannot rule out the possibility that the discrepancy between the amino acid sequences of the *Volvox* and the *Chlamydomonas* enzyme, which lies in the N-terminus is the result of four compensating mutations that have occurred during algal evolution.

A remarkable property of the arylsulfatase from *Volvox* is its insensitivity towards detergents like dodecyl sulfate. Concentrations as high as 1% SDS do not affect the enzyme activity. This property enables easy detection of the enzyme even in crude cell lysates. Together with a simple enzyme assay using chromogenic substrates, these properties qualify this enzyme as a suitable reporter gene product for the study of differently regulated promoters. The genomic clones now available for this enzyme also include up to 8.5 kb upstream DNA (clone λAr52) and therefore most probably encode the information required to obtain sulfur-regulated expression. The highly regulated promoter of the arylsulfatase from *Volvox* is likely to be a helpful tool for expression experiments of cloned genes. Expression should be inducible simply by suspending the transformed *Volvox* spheroids in sulfate-free growth medium.

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