Research paper

The chondroitin sulfate/dermatan sulfate 4-O-endosulfatase from marine bacterium *Vibrio* sp FC509 is a dimeric species: Biophysical characterization of an endosulfatase

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**A B S T R A C T**

Sulfatases catalyze hydrolysis of sulfate groups. They have a key role in regulating the sulfation states that determine the function of several scaffold molecules. Currently, there are no studies of the conformational stability of endosulfatases. In this work, we describe the structural features and conformational stability of a 4-O-endosulfatase (EndoV) from a marine bacterium, which removes specifically the 4-O-sulfate from chondroitin sulfate/dermatan sulfate. For that purpose, we have used several biophysical techniques, namely, fluorescence, circular dichroism (CD), FTIR spectroscopy, analytical ultracentrifugation (AUC), differential scanning calorimetry (DSC), mass spectrometry (MS), dynamic light scattering (DLS) and size exclusion chromatography (SEC). The protein was a dimer with an elongated shape. EndoV acquired a native-like structure in a narrow pH range (7.0–9.0); it is within this range where the protein shows the maximum of enzymatic activity. The dimerization did not involve the presence of disulphide-bridges as suggested by AUC, SEC and DLS experiments in the presence of β-mercaptoethanol (β-ME). EndoV secondary structure is formed by a mixture of α and β-sheet topology, as judged by deconvolution of CD and FTIR spectra. Thermal and chemical denaturations showed irreversibility and the former indicates that protein did not unfold completely during heating.

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1. Introduction

Sulfatases, which cleave sulfate esters in biological systems, have key roles in modulating the sulfation of several molecules involved in many physiological processes. The sulfatase substrates range from small cytosolic steroids, like estrogen sulfate, to complex cell-surface carbohydrates (such as glycosaminoglycans, GAGs). The GAG chains consist of repeating disaccharide units of glucuronic/iduronic acid and hexosamine in a linear chain. Modification of all these molecules is important in signalling pathways, bacterial pathogenesis, hormone regulation and even in lysosomal storage disorders [1,2]. In humans, sulfatases have been involved in genetic disorders and cancer [3,4]. Recently, from a biotechnological point of view, sulfatases have been also used as synthetic tools in the design of new therapeutic compounds or even in the

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remodelling of key sulfonation sites of GAGs [1]. In these industrial applications, understanding protein stability has important implications in shaping enzymatic activity and protein redesign. Furthermore, among hydrolases, sulfatases are unique in requiring a post-translational modification on a particular active site amino acid. This leads to the formation of 3-oxo-alanine (“formylglycine”), the sole natural residue having an aldehyde function [5].

Chondroitin sulfate (CS)/dermatan sulfate (DS) are probably the most abundant GAGs, expressed at the cell surfaces and in extracellular matrices in the animal kingdom. During biosynthesis, D-glucuronic acid can be epimerized at the C-5 position to give L-iduronic acid, and both of them may be sulfated at the C-2 position.

In addition, CS/DS can be sulfated at position 4 on iduronate, and in more less frequently, on glucuronates. Also, N-acetyl-galactosamine moieties are sulfated at positions 4 and 6 [1]. Most of the endosulfatases identified so far are exosulfatases (acting at the sulfated saccharide residues at the terminus of GAGs). Recently, however, a CS/DS 4-O-endosulfatase has been identified in B. thetaiotaomicron [6], and in Vibrio sp FC509 [7]. The biochemical characterization of the latter indicates that the enzyme is only active in a narrow pH-range (from 6.5 to 9.0), with a maximal reaction rate at 30 °C (at pH 8.0). Since, at the best of our knowledge, there are no studies addressing the conformational stability of chondroitin sulfate endosulfatases, we embarked in the description of stability for the endosulfatase of Vibrio sp FC509.

We used several biophysical techniques, namely, fluorescence, CD, FTIR spectroscopies, AUC, DSC, MS, DLS and SEC. Our results suggest that the protein is a dimer with an elongated shape, as shown by SEC, AUC, DLS and MS. Dimerization did not involve the presence of disulphide-bridges as suggested by AUC, SEC and DLS experiments in the presence of β-ME. The protein acquired a native-like structure within a small pH-range (from 7.0 to 9.0), which is similar to that where the protein has the maximum of activity [7]. The structure of the protein is formed by large percentages of α-helix and β-sheet scaffolds, as judged by deconvolution of CD and FTIR spectra. At low pH values, the protein lost a large amount of its secondary and tertiary structures, and it shows solvent-exposure of hydrophobic patches. Thermal and chemical-denaturations at physiologiclal pH were irreversible, and the thermal denaturation was well described by a two-state irreversible model with activation energies in the range of 70 kcal mol⁻¹.

2. Materials and Methods

2.1. Materials

Ultra-pure urea and GdmCl were from ICN Biomedicals Inc. (USA). Concentrations of urea and GdmCl were calculated from the refractive index of the corresponding solution [8]. Trizma acid and base, NaCl and ANS were from Sigma. The high molecular weight marker for gel filtration was from GE Healthcare (Barcelona, Spain). The β-ME was from BioRad; dialysis tubing with a molecular weight cut-off of 3500 Da was from Spectrapore. Standard suppliers were used for all other chemicals. Water was deionized and purified on a Millipore system.

2.2. Protein expression and purification

Expression and purification of the protein was carried out as described [7]. For some preparations, we also added 2 mM β-ME in the elution buffer (50 mM Tris, pH 7.8, with 150 mM NaCl) at the last purification step on the semi-preparative Superdex G200 column. This protein stock in 2 mM β-ME was used for spectroscopic, biophysical and hydrodynamic experiments in the presence of the reducing agent.

2.3. Size exclusion chromatography (SEC)

SEC was used to determine the Rs of EndoV at 20 °C under several conditions [9–11]. Protein concentrations ranged from 1 to 10 μM (in protomer units). Samples were loaded in 50 mM, phosphate buffer (pH 7.0), containing 150 mM NaCl to avoid interactions with the column) at 1 ml/min, in the absence and in the presence of 2 mM β-ME, in a calibrated Superdex 200 10/30 HR FPLC column (GE Healthcare) connected to an AKTA basic (GE Healthcare) with monitoring at 280 nm. For the experiments at different pHs, the corresponding buffer (see below) at a concentration of 50 mM was used with 150 mM NaCl. In all chromatograms, the Vs were obtained with UNICORN software (GE Healthcare) in three measurements. The void volume, V0, (6.47 ± 0.06 ml) was determined from blue dextran; the bed one, V1 (19.31 ± 0.03 ml) was from conductivity measurements.

The Vc of EndoV was not protein-concentration-dependent in the range of protein concentrations explored (1–10 μM); the pH- and urea-denaturation experiments were carried out at 10 μM of protein concentration (in protomer units). Urea-denaturations were carried out at pH 7.0 (50 mM phosphate buffer) with 150 mM NaCl.

The protein standards used in column calibration and their corresponding Stokes radii were: thyroglobulin (85 Å), ferritin (61 Å), catalase (52.2 Å), aldolase (48.1 Å) and BSA (35.5 Å) [12]. The Vs of the protein standards were only acquired under native conditions 50 mM phosphate buffer with 150 mM NaCl (pH 7.0). Protein concentrations were 10 μM.
The weight average partition coefficients ($\sigma$) were calculated as:

$$\sigma = \frac{(V_c-V_o)}{V_c}.$$  

The $\sigma$s were transformed by using the inverse error function complement of $\sigma$, erf$^{-1}(\sigma)$, yielding a linear relationship with $R_s$ [9,11]: $R_s = a + b \cdot$ erf$^{-1}(\sigma)$, where $a$ and $b$ are constants.

### 2.4. Analytical ultracentrifugation (AUC)

Experiments were carried out as described [13,14]. Briefly, experiments were performed in a Beckman Coulter Optima XL-I analytical ultracentrifuge (Beckman-Coulter, Palo Alto, CA, USA) equipped with UV–visible absorbance as well as interference optics detection systems, using an An50Ti 8-hole rotor, 12-mm-pathlength charcoal-filled Epoxi double-sector centrepieces. The experiments were carried out at 20 °C in 50 mM Tris (pH 7.9) in the absence and in the presence of 2 mM β-ME. Laser delay was adjusted prior to the runs to obtain high-quality interference fringes. Light at 280 nm was used in the absorbance optics mode. Sedimentation velocity runs were carried out at a rotor speed of 40,000 rpm using 400 μl samples. Sample concentration was 5 μM (in protomer units).

A series of 400 scans, without time intervals between them, were acquired for each sample. A least squares boundary modelling of the sedimentation velocity data was used to calculate sedimentation coefficient distributions with the size-distribution $c(s)$ method [15] implemented in the SEDFIT v13.0b software. The $V_{20,W}$ density ($\rho = 1.0089$ g/ml) and viscosity ($\eta_0 = 1.002$ cP) at 20 °C were estimated with SEDNTERP [16]. The protein partial specific volume, $\bar{V}$, was 0.726 ml/g.

### 2.5. Dynamic light scattering (DLS)

Experiments were performed with a Zetasizer Nano ZS (Malvern Instruments Ltd.) using a thermostatted 12 μl quartz cuvette at 20 °C, as described [13]. Briefly, samples of protein were prepared in 50 mM Hepes buffer (pH 7.0) in the absence and in the presence of 2 mM β-ME. Sample concentration was 15 μM (in protomer units). All the solutions were filtered; immediately before measurements, protein samples were centrifuged for 30 min at 14,000 rpm to remove any aggregates and dust. Data were analyzed using the software developed by Malvern Instruments Ltd. The $R_0$ and $M$ were determined from the Stokes-Einstein equation, assuming a spherical shape for the protein.

### 2.6. Fluorescence

Fluorescence spectra were collected on a Cary Varian spectrofluorimeter (Agilent, USA), interfaced with a Peltier temperature controller, at 25 °C. Sample concentrations were 2 μM (in protomer units) in the pH- and chemical-denaturation experiments. The final concentrations of the buffers were 10 mM. The experiments were prepared the day before and left overnight at 5 °C. A 1 cm-path length quartz cell (Hellma) was used.

#### 2.6.1. Intrinsic fluorescence

The experimental set has been described elsewhere [17], with excitation at 280 and 295 nm. Blank corrections were made in all spectra.

Chemical-denaturations at pH 7.0 (50 mM, phosphate buffer), either followed by fluorescence or CD, were carried out by dilution of the proper amount of an 8 M urea or 7 M GdmCl stock solution. Both urea- and GdmCl-denaturations were irreversible.

In the pH-denaturations, the pH was measured after completion of the experiments with an ultra-thin Aldrich electrode in a Radiometer (Copenhagen) pH-meter for each sample. The salts and acids used have been described elsewhere [17]. Chemical-denaturations were repeated three times with new samples at any of the concentrations assayed.

#### 2.6.2. Thermal-denaturations

Thermal-denaturations were performed at constant heating rates of 1 °C/min and an average time of 1 s. Thermal scans were collected by excitation at 280 or 295 nm and collected at 315, 330 and 350 nm from 25 to 85 °C. The rest of the experimental set was the same as described above. Thermal denaturations were not reversible at any pH. The apparent $T_m$ was obtained as described [13].

#### 2.6.3. ANS binding

The experimental set for the pH-denaturations has been described [17]. In all cases, blank solutions were subtracted from the corresponding spectra. ANS was used to monitor the pH-denaturation at 2 μM of protein (in protomer units).

### 2.7. Circular dichroism (CD)

Circular dichroism spectra were collected on a Jasco J815 (Japan) spectropolarimeter fitted with a thermostated cell holder and a Peltier unit. The instrument was periodically calibrated with (+)-10-camphorsulphonic acid. Molar ellipticity was calculated as described [10].

#### 2.7.1. Far-UV spectra

Spectra of EndoV at different pHs, GdmCl or urea concentrations were acquired at a scan speed of 50 nm/min with a response time of 4 s and averaged over six scans at 20 °C. The bandwidth was 1 nm. Measurements were performed with protein concentrations of 2 μM (protomer units) in 10 mM buffer, in a 0.1 cm-pathlength cell. Spectra were corrected by subtracting the corresponding baseline. The chemical-denaturations were repeated three times with new samples. Samples were prepared the day before and left overnight at 5 °C to allow for equilibration.

#### 2.7.2. Thermal-denaturation experiments

They were performed at constant heating rates of 1 °C/min and a response time of 8 s. Thermal scans were collected following the changes in ellipticity at 222 nm from 25 to 80 °C in 0.1 cm-path length cells with a protein concentration of 2 μM (in protomer units). No difference was observed between the scans to test for possible instrumental drift. Thermal-denaturations were not reversible at any pH, as shown by: (i) the comparison of spectra before and after the heating; and, (ii) the changes in the voltage of the instrument [18]. The apparent $T_m$ was obtained as described [13].

#### 2.7.3. Near-UV spectra

Spectra of EndoV at pH 7.0 were acquired at a scan speed of 50 nm/min with a response time of 4 s and averaged over six scans at 20 °C. The bandwidth was 1 nm. Measurements were performed with protein concentrations of 29.6 μM (protomer units), in an 0.5 cm-pathlength cell. We did not follow the pH-denaturation of EndoV by near-UV due to the large amounts of protein used.

### 2.8. FTIR

EndoV at 100 μM (protomer units) in phosphate buffer (pH 7.0, 50 mM) was extensively exchanged to deuterated phosphate buffer in Amicon devices. Spectra were acquired at 25 °C in a Variant Resolutions Pro spectrometer (Agilent, USA) using excavated cells with a 50 μm path (Reflex Analytical) and the series software
licensed under OMNIC (Thermo Scientific). Spectra were acquired at 25 °C and processed as described [17,19].

2.9. Analysis of the pH-denaturation curves

The pH-denaturation experiments were analyzed assuming that both protein species, protonated and deprotonated, contributed to the spectral feature:

\[ X = \frac{X_a + X_b 10^{m(pH-pK_a)}}{1 + 10^{m(pH-pK_a)}} \]  

(1)

where \( X \) is the spectral property being observed (ellipticity or fluorescence intensity); \( X_a \) is that for the acidic species; \( X_b \) is the one at high pHs; \( pK_a \) is the apparent midpoint of the titrating group; and \( n \) is the Hill coefficient (which was close to 1 in all the curves reported in this work). The apparent \( pK_a \) reported (from intrinsic or ANS fluorescence, and CD) was obtained from three different measurements in each technique, prepared with new samples.

Fitting by non-linear least-squares analysis to Eq. (1) was carried out by using Kaleidagraph (Abelbeck software) working on a PC computer.

2.10. Mass spectrometry

MALDI-TOF MS experiments were carried out on a Voyager-DE Pro STR spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). This instrument was equipped with a nitrogen UV laser (\( \lambda = 337 \) nm) pulsed at a 20 Hz frequency. The mass spectrometer was operated in the positive linear ion mode with an accelerating potential of +25 kV and a grid percentage equal to 70%. Mass spectrum was recorded with the laser intensity set at 3100 (arbitrary units), extraction delay was set to 400 ns and mass spectra were obtained by accumulation of 100 laser shots and processed using Data Explorer 4.0 software (Applied Biosystems). The instrument was externally calibrated using mono- and multicharged ions of standard solution of BSA (BSA Calibration Standard Kit for AB SCIEX MALDI-TOF Instrument).

MALDI-TOF MS analysis was achieved by mixing 1 µl of sinapinic acid (SA) matrix at 20 mg/ml in acetonitrile/water (50/50; v:v), 0.1% TFA, with 1 µl of the protein solution (0.6 mg/ml).

2.11. Differential scanning calorimetry (DSC)

DSC experiments were carried out in a capillary VP-DSC differential scanning calorimeter (Malvern Instruments) with a cell volume of 135 µl. Scans were performed in a 4–100 °C temperature range at scan rates of 1–4 °C/min, using 2–10 µM protein (in monomer units). Experiments were performed in Tris 50 mM (pH 8.0) and NaCl 150 mM (oxidizing conditions) or in Tris 50 mM (pH 7.5), NaCl 200 mM and 2 mM β-ME (reducing conditions).

2.12. Analysis of DSC experiments

Thermal denaturations were analyzed using a two-state irreversible denaturation model with first-order kinetics [20] depicted by Scheme 1. where the native protein (N) is irreversibly denatured to a final state (F) that cannot fold back, and this kinetic conversion is characterized by a temperature dependent first-order rate constant (k).

The experimental DSC traces (apparent molar heat capacities versus Temperature) were fitted using Eq. (2), which takes into account the experimental chemical baseline [21]:

\[
C_{p}^{\text{app}} = C_{p}^\text{pre} + \left( C_{p}^\text{post} - C_{p}^\text{pre} \right) (1 - X_N) - \Delta H \left( \frac{dX_N}{dT} \right)
\]

(2)

in which, \( X_N \) is the native state mole fraction, \( \Delta H \) is the denaturation enthalpy, \( C_{p}^\text{pre} \) and \( C_{p}^\text{post} \) are the pre- and post-transition baselines (considered as linear functions of temperature). The first two terms in the right-hand side of Eq. (2) define the chemical baseline, while the last term describes the unfolding transition (excess molar heat capacity, \( C_{p}^\text{exc} \) versus Temperature). The fraction of native protein and its dependence with temperature are given by Eqs. (3) and (4) [21,22]:

\[
X_N = \exp \left[ - \exp \left( \frac{E_a \Delta T}{RT_m} \right) \right]
\]

(3)

and

\[
\frac{dX_N}{dT} = \frac{E_a}{RT_m^2} \exp \left( \frac{E_a \Delta T}{RT_m} \right) \exp \left[ - \exp \left( \frac{E_a \Delta T}{RT_m} \right) \right]
\]

(4)

where \( \Delta T = T-T_m \), \( T_m \) is the denaturation temperature (the maximum in the plot of the excess heat capacity versus \( T \)) and \( E_a \) is the activation energy for the process given in Scheme 1.

Alternatively, when dealing with systems displaying non-first-order kinetics (such as those involving changes in the oligomerization state between the native and transition state for the rate-determining step), there have been developed expressions for \( X_N \) and \( \langle dX_N/dT \rangle \) (Eqs. (5) and (6)). These expressions include a fitting parameter, \( \mu \), that takes into account changes in oligomerization state (note that \( 1/\mu \) is the reaction order) [22,23]:

\[
X_N = \left[ 1 + \frac{1 - \mu}{\mu} \exp \left( \frac{E_a \Delta T}{RT_m} \right) \right]^{\frac{1}{\mu}}
\]

(5)

\[
\frac{dX_N}{dT} = \frac{E_a}{RT_m^2} \exp \left( \frac{E_a \Delta T}{RT_m} \right) \left[ 1 + \frac{1 - \mu}{\mu} \exp \left( \frac{E_a \Delta T}{RT_m} \right) \right]^{\frac{1}{\mu}}
\]

(6)

Note that in the case of a dimeric native protein, \( \mu = 1 \) in first-order kinetics (the transition state is dimeric), while \( \mu = 2 \) when the transition state is monomeric.

Under first-order conditions, the rate constant, \( k \), is obtained by [20]:

\[
k = \frac{vC_{p}^\text{exc}}{\Delta H - \langle H \rangle} \]

(7)

where \( v \) is the scan rate, \( C_{p}^\text{exc} \) is the excess heat capacity at a given temperature and \( \langle H \rangle \) is the heat evolved at that given temperature. The temperature dependence of \( k \) following the Arrhenius equation \( (k = A \exp(-E_a/(RT))) \) yields another way to estimate \( E_a \).

The dependence of the heat evolved with temperature (\( \langle H \rangle \)) can be expressed by Eq. (8), providing an additional method to estimate \( E_a \) [20]:

\[
\frac{E_a}{R} \left( \frac{1}{T_{m}} \right)^{1/2} = \ln \left( \frac{\ln(\Delta H)}{\Delta H - \langle H \rangle} \right)
\]

(8)

Scheme 1. Two-state irreversible denaturation model.
And last, the scan rate dependence of $T_m$ values must follow Eq. (9) and then, it provides another method to determine $E_s$ [20]:

$$\ln \left( \frac{v}{T_m} \right) = C - \frac{E_a}{RT_m}$$

where $C$ is a constant.

3. Results

3.1. EndoV is a dimeric species at physiological pH

To map the hydrodynamic properties of EndoV we used the following complementary hydrodynamic techniques: DLS, AUC, SEC at different protein concentrations, with and without β-ME (2 mM). In addition, protein self-association was addressed by mass spectrometry (MS).

3.1.1. EndoV in the absence of β-ME

In AUC, we could detect two main peaks with sedimentation coefficients in water, $s_{20,w}$, of 4.0 and 6.31 S (Fig. 1(A)), corresponding to $M_s$ of 63.9 and 132 kDa, respectively. These two peaks roughly correspond to the monomer and dimer, respectively (the theoretical $M$ of the monomer is 65.5 kDa). There is a small peak at $s_{20,w} = 10.16$ S, with an estimated $M$ of 271 kDa, which would correspond to the presence of a tetrameric species.

The DLS experiments (in volume) had a single peak with a hydrodynamic radius of $53 \pm 22$ Å, yielding an $M$ of 170 ± 70 kDa (assuming a spherical shape for EndoV) (Fig. 1(B)). That peak had a large poly-disperty: 41%, which indicates the presence of different self-associated species.

In SEC experiments, EndoV eluted at pH 7.0 (50 mM phosphate buffer) as a single peak at 11.10 ± 0.06 ml (the average of three measurements). The $V_c$ did not change in the protein concentration range from 1 to 10 μM. This volume yields a $R_c$ of 46 Å, similar to that of the marker protein aldolase (whose $R_c$ is 48 Å with an $M$ of 158 kDa). Thus, our SEC results further support that EndoV is a dimer at physiological conditions. It is important to note that if we had used the linear relationship between the $M$ and $\sigma$ of a protein [24], then, the apparent $M$ of EndoV should have been 212 kDa. We did not use this relationship, instead of that between the $R_c$ and $\sigma$ (see Materials and Methods section), since in the used column, the linear relationship between the $M$ and $\sigma$ is worse than that with the $R_c$ (regression coefficient of 0.92 versus 0.97).

The MALDI-TOF mass spectrum of EndoV exhibited two main peaks centred at $m/z$ 64,000 and 128,000 (Fig. 1(C)). The former matched very well with the $M$ expected for EndoV based on its cloned sequence. Interestingly, the detection of higher species at $m/z$ 128,000 indicated that EndoV was also present under a dimeric form. The corresponding ion was of high intensity, similar to the intensity of the monomer whatever the concentration of the enzyme used (data not shown). This result evidences the high intensity of the monomer whatever the concentration of the form. The corresponding ion was of high intensity, similar to the 3.1.2. EndoV in the presence of 2 mM β-ME

Since the above experiments were carried out in the absence of reducing agent, we could raise the question whether the observed self-association of EndoV could be due to inter-molecular disulphide bridges. To rule out this possibility, we carried out AUC and SEC experiments in the presence of 2 mM β-ME.

The AUC experiments showed two peaks at $s_{20,w}$ of 3.88 and 6.23 S (data not shown), similar to those observed in the absence of the redox agent. The DLS experiments yielded also a single peak (red line) with similar $R_c$ as that in the absence of β-ME (black line): 47 ± 13 Å (with an $M$ of 126 ± 35 kDa) (Fig. 1(B)), with a polydispersity of 28%. The decrease in the polydispersity (when compared to results in the absence of redox agent) could indicate that the presence of β-ME removes some disulphide-bridged scrambled species. We added β-ME (at 2 and 10 mM concentration) to the sample which had not been purified in its presence (see Materials and Methods section), and we obtained a similar $R_c$ as those in the absence of such agent (Fig. 1(B)). Finally, the $V_c$ of EndoV was not modified by the presence of β-ME in the SEC experiments. Thus, the three techniques suggest that dimerization of EndoV is not disulphide-bridge dependent, and self-association must be due to an intrinsic tendency of the protein.

3.2. EndoV acquired a native-like structure in a narrow pH range

If we wanted to measure the conformational stability of EndoV, we must firstly determine in which pH range the protein acquired a native-like structure. Furthermore, we wanted to find out whether that pH range where the protein acquires a native-like structure matches that where the protein has the maximum enzymatic activity [7]. To that end, we used several spectroscopic and biophysical probes, namely, ANS fluorescence, intrinsic fluorescence, far-UV CD and SEC. All those techniques give complementary information on different structural features of EndoV. We used ANS fluorescence to monitor the burial of solvent-exposed hydrophobic patches [28]. Intrinsic fluorescence was used to monitor changes in the tertiary structure of the protein around its tryptophans and tyrosines. We carried out far-UV CD experiments to monitor the changes in secondary structure. And we used SEC to determine polypeptide chain compactness.

3.2.1. Fluorescence

3.2.1.1. Steady-state fluorescence and thermal denaturations. The fluorescence spectrum of EndoV at pH 7.0 and 20 °C had a maximum at 336 nm (Fig. 2(B), inset), suggesting that: (i) protein fluorescence was dominated by, at least, one of the eleven tryptophans in the primary structure; and, (ii) some of them were fully buried in the structure. The intensity at 330 nm (as at any of the folded spherical protein can be also approximated by [26]: $R_c = (4.75 \pm 1.11)N_0^{0.29}$, where $N$ is the number of residues; for a spherical dimeric EndoV this expression yields $30 \pm 3$ Å. Then, these theoretical results suggest that the shape of dimeric EndoV was not spherical. We further support this conclusion by calculating the frictional coefficient of the protein, $f$. With the values of $s_{20,w}$ for the dimeric species and the $M$, we can calculate the frictional coefficient, $f$ [24,27], and compare with that of a spherical molecule (calculated from $R_c$). For EndoV, the ratio $f/f_0$ was 1.54, when in globular folded proteins is in the range 1–1.20 [24]. This high value could be caused either by a large shell of hydration (which is unlikely [24]) or by an elongated shape.

To sum up, the three hydrodynamic techniques and MS suggest that, in the absence of a redox agent, EndoV was a dimer with an elongated shape.
other explored wavelengths) showed two transitions as the pH was varied (Fig. 2(A), blank circles). The first one occurred with a $pK_a = 5.1 \pm 0.1$, and the second was probably due to the titration of the phenol group of some of the twenty-seven tyrosine residues; in this transition, no basic baseline was observed, and then we could not obtain its midpoint. We also carried out a pH-titration in the presence of 2 mM β-ME, and, as expected, the results were identical to those in its absence.

Thermal-denaturation experiments at several pHs (2.5, 4.5, 7.4 and 12.1) were carried out by intrinsic fluorescence. At pH 4.5 and 7.4, we observed an irreversible sigmoidal behaviour (Fig. 1A Supplementary Material) from where an apparent thermal midpoint ($T_m$) could be obtained. The apparent $T_m$ ranged from 62.9 ± 0.3 °C (at pH 7.4) to 49.1 ± 0.3 (at pH 4.5). At acidic and basic pHs, no sigmoidal transition was observed. These results suggest that heat probably induced association and formation of aggregated species. Due to the irreversibility at both pHs, we could not further elaborate on the apparent different protein stability.

3.2.1.2. ANS-binding. At low pH, the ANS fluorescence intensity at 480 nm was very large and decreased as the pH was raised (Fig. 2(B), main panel), indicating that EndoV had solvent-exposed hydrophobic regions at low pH. The intensity at 480 nm showed a sigmoidal behaviour, with a $pK_a = 3.75 \pm 0.07$. This value was smaller than that from intrinsic fluorescence, suggesting that the solvent-exposed hydrophobic patches are buried before acquisition of secondary and tertiary structures.

3.2.2. CD

We acquired far and near-UV spectra of the protein at pH 7.0 and 20 °C. The near-UV provides information on the asymmetry (and then on the rigidity) of the aromatic residues (11 Trp, 27 Tyr and 22 Phe). The near-UV of EndoV had two intense negative peaks at 280 and 292 nm (Fig. 2(C), inset), suggesting an asymmetric environment for some aromatic residues. The band at 292 nm was more intense than that at 280 nm, indicating a rigid conformation for some tryptophans [29,30]. The shape and intensity of the spectrum did not change upon addition of 2 mM β-ME (data not shown).

The far-UV CD had an intense minimum at 222 nm (Fig. 2(C)), but it did not show another minimum at 208 nm, suggesting that
the protein is composed of β-sheet and α-helix. Deconvolution of the spectrum by using Dichroweb [31,32] (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) yields percentages of: (i) α-helix going from 60% (in K2D) to 14.7% (in Contin); (ii) β-sheet going from 51.7% (in Contin) to 7% (in K2D); and, (iii) random-coil of 30% in the three programs used. Thus, it seems that EndoV at physiological pH contains larger percentages of ordered structure than random-coil, but we cannot decide, given the large variability among the predictors, which kind of secondary structure (α-helix or β-sheet) has the largest percentage. We also carried out experiments in the presence of 2 mM β-ME, and we did not observe any difference in the shape (and therefore, in the percentages of secondary structure obtained) of the spectrum.

The shape of the far-UV CD spectra changed as the pH was varied. Between pH 6.0 and 8.0, the ellipticity reached a maximum, to decrease at lower and higher pH. At high pH, we could not obtain the pKₐ of the transition due to the absence of a plateau at basic pHs. At acidic pH, the pKₐ was 5.3 ± 0.1, similar, within the error, to that measured by fluorescence (see above).

Thermal-denaturations followed by CD in EndoV (pH 2.5, 4.5, 7.4 and 12.1) showed only sigmoidal, irreversible transitions at pH 4.5 and pH 7.4 (as in fluorescence). The apparent Tₘₛ were 56.4 ± 0.3 °C, and 57.1 ± 0.2 °C (Fig. 2 Supplementary Material), respectively. Since the processes were irreversible, we cannot speculate more on these values and their differences with those measured by fluorescence.

3.2.3. SEC
At pH < 5.0, the Vₑs were larger than the column bed volume (19.31 ml) (Fig. 2(D)). These results suggest that EndoV, even though NaCl was present, was bound to the column at those acidic pHs. From the experiments with ANS (Fig. 2(C)), EndoV had solvent-exposed hydrophobic patches at low pH; we hypothesize that those regions could interact with the column, resulting in larger Vₑs. At 5.0 < pH < 7.0, the Vₑ was decreasing until a constant volume of 11.10 ml was reached. Therefore, the protein did not reach a native-like compactness until physiological pH. The same Vₑ was observed in the presence of 2 mM β-ME.

3.2.4. FTIR
To further confirm the percentages of secondary structure found by CD, we carried out FTIR experiments, and we deconvoluted the Amide I band (Fig. 3 Supplementary Material). We obtained a 45% of α-helix (with the band centred at 1657 cm⁻¹); 37% of β-sheet (1633 cm⁻¹); 7% of turns and high frequency β-sheet (1678 cm⁻¹); and 9% of random-coil (1643 cm⁻¹). Thus, the predictions for the α-helix were larger than those from CD deconvolution, and those for the β-sheet were similar in both techniques. The overestimation of the α-helix component could be due to the fact that its centre is slightly shifted to high frequencies than expected, and thus it may overlap with the component for loops/turns (appearing at 1660 cm⁻¹).

3.3. Thermal denaturations of EndoV by DSC
Denaturation of EndoV by DSC revealed a single denaturation transition, both under oxidizing and reducing conditions (Fig. 3(A)). Due to the irreversibility of the thermal denaturation, and the presence of a slight protein concentration dependence under reducing conditions (Fig. 3(B)), we attempted the fit of the DSC scans to an irreversible two-state denaturation model with non-first-order kinetics (Eqs. (4), (7) and (8)) [20,21]. This model
describes very well the experimental DSC data (Fig. 3(A)). The values of \( m \) were close to unity in the absence and in the presence of \( \beta\)-ME, suggesting that deviations from first-order kinetics were minimal (Table 1). Additionally, the use of different consistency tests (based on different procedures to estimate \( E_a \) values; Fig. 3(C)−(E)), yielded similar values, thus supporting the applicability of the model (Tables 1 and 2).

The average \( \Delta H \) values were 189.6 kcal mol\(^{-1}\) (average \( T_m = 62.7 ^\circ C \); in the absence of the reducing agent) and 143.8 kcal mol\(^{-1}\) (average \( T_m = 62.3 ^\circ C \); in the presence of \( \beta\)-ME); the \( T_m \) values were similar to those from fluorescence measurements (see above). Although we had observed that the dimerization state of the EndoV was not affected by the presence of the reducing agent, thermal unfolding was altered by its presence, probably due to formation of intermolecular disulphide bridges. The theoretical \( \Delta H \) and \( \Delta C_p \) values for a 575-residue-long protein (including the His-tag) at these \( T_m \)s was 444 kcal mol\(^{-1}\) (taking into account that \( \Delta H(60 ^\circ C) = 0.698^N \text{N (kcal \cdot mol}^{-1}\); \( \Delta C_p = 13.86^\text{N (cal \cdot mol}^{-1}\cdot K}^{-1}\); \( 8.36 \text{kcal \cdot mol}^{-1}\cdot K}^{-1}\) [33]. These differences suggest that thermal denaturation did not lead to complete unfolding of the protein.

The \( T_m \) values were similar to those from fluorescence measurements (see above). Although we had observed that the dimerization state of the EndoV was not affected by the presence of the reducing agent, thermal unfolding was altered by its presence, probably due to formation of intermolecular disulphide bridges.

The Arrhenius plots (Fig. 3(C)) allow extrapolation of \( k \) to 37 \(^\circ C\), providing values of \((1.2 \pm 0.5) \times 10^{-5} \) and \((1.0 \pm 0.2) \times 10^{-5} \text{ min}^{-1} \) (obtained from six independent measurements) under reducing and non-reducing conditions, respectively. These values were identical within the error and they correspond to half-lives of 39 ± 16 and 46 ± 9 days for this unfolding reaction.

### 3.4. Chemical denaturations of EndoV

Since thermal-denaturations were irreversible, we carried out urea- and GdmCl-denaturations to measure the conformational stability of EndoV by intrinsic fluorescence and CD. Denaturations were carried out at 25 \(^\circ C\) and pH 7.0 (phosphate buffer), where the protein has acquired a native-like conformation. As the GdmCl or urea concentrations were increased, the ellipticity at 222 nm or the fluorescence intensity were reduced in a sigmoidal manner (Fig. 4).

### Table 1

Energetic parameters for thermal denaturation from DSC measurements.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>( T_m (K) )^{a}</th>
<th>( \Delta H (\text{kcal mol}^{-1}) )^{b}</th>
<th>( E_a (\text{kcal mol}^{-1}) )^{c}</th>
<th>( m )^{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>No ( \beta)-ME</td>
<td>336.70 ± 0.05</td>
<td>180 ± 9</td>
<td>74 ± 7</td>
<td>0.87 ± 0.03</td>
</tr>
<tr>
<td>With ( \beta)-ME</td>
<td>336.3 ± 0.3</td>
<td>144 ± 16</td>
<td>79 ± 6</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

\(^{a}\) Average ± standard deviation (s.d.) from eight independent experiments at different protein concentrations (2−10 \( \mu \text{M} \) in protomer units) and 4 \(^\circ C\)/min.

\(^{b}\) Average ± s.d. from at least ten independent experiments at different protein concentrations and scan rates.

\(^{c}\) Average ± s.d. from four different consistency tests.

Fig. 3. DSC analyses. (A) DSC profiles (excess heat capacity versus \( T \)) under oxidative (circles) and reducing (squares) conditions using 10 \( \mu \text{M} \) of protein (protomer units) and 4 \(^\circ C\)/min scan rate. Lines are fits to a two-state irreversible model. (B) Protein concentration dependence of \( T_m \) values (circles, oxidative conditions; squares, reducing conditions). (C−E) Consistency tests providing values for \( E_a \) from their slopes (symbols are the same as for A and B).
The denaturations were irreversible with both denaturants. The GdmCl-denaturations did not show a long native baseline (Fig. 4(A)); conversely, urea-denaturation curves did not have long enough unfolded baselines, and the transition appeared to be less co-operative (broader) than that followed by GdmCl (Fig. 4(B)), as expected [34]. In both denaturants, we only observed a single transition in the curves.

4. Discussion

4.1. Structure-function relationships in EndoV

EndoV acquired a native-like conformation only between 7 and 9, and did so in a two-step process, with different pH (midpoint) values. First, it buried all the solvent-exposed hydrophobic residues (ANS fluorescence) and second, it acquired concomitantly the secondary (far-UV CD) and tertiary (fluorescence) structures, and a full compactness (SEC experiments). Thus, as the pH is close to 7.0, the protein becomes more compact, and from the variation in the $V_c$ (Fig. 2(D)), it acquired its quaternary structure between pH 5.0 and 7.0. At low pH, the protein has lost approximately one-third of its secondary structure (as judged from the values of ellipticity at 222 nm), and the remnant was not rigid (as concluded from the absence of sigmoidal behaviour in the thermal denaturations at very low pH (pH 2.4)). We could not determine the exact oligomerization state of the protein at very low pH, but the fact that the protein precipitated at concentrations above 2 μM at pH < 4.0, together with: (i) the large amount of solvent-exposed hydrophobic patches (ANS binding experiments); and, (ii) the binding to the column at pH < 5.0, seem to indicate that at low pHs the protein had a tendency to form high-order self-associated species.

The pH interval where the protein acquired a native-like structure is the same where the protein shows its maximum of activity; in fact, the percentage of activity is lower than 50% at pH > 7.0 [7]. Thus, well-rigid secondary and tertiary structures are required for a full enzymatic activity.

We also determined that the $T_m$ was close to 62 °C, and it occurs through an irreversible process with first-order kinetics, thus involving a dimeric denaturation transition state. Interestingly, enzyme activity at pH 8.0 suggests that the maximum activity was happening at 30 °C [7] and that this activity decreased abruptly at lower and high temperatures (for instance, at 40 °C, the activity was only 20% of that observed at 30 °C [7]). Therefore, enzyme inactivation occurs at temperatures lower than those for global denaturation, suggesting lower conformational stability or enhanced flexibility of the active site. In fact, DLS control experiments at 35 °C showed an increase in the poly-dispersity, and in the average $R_b$ of the species in solution with the incubation time at this temperature. Moreover, in FTIR spectra at 35 °C the characteristic bands of β-sheet aggregated species (1616 and 1683 cm⁻¹) [35] were present.

To sum up, our structural studies at different pHs and temperatures indicate that only when EndoV acquired a rigid ordered structure, within a narrow pH range (7.0–9.0), it was fully active.

4.2. Structure of EndoV

Our studies provide the first clues for the structural elucidation of EndoV. We have found that EndoV is a dimer, with monomers with a high percentage of β-sheet and/or α-helix. Although denaturations were irreversible, the apparent two-state nature of chemical and thermal (calorimetric) denaturations might suggest that unfolding of EndoV implies the concomitant dissociation and unfolding of the monomers, although caution must be taken into account since enzymatic inactivation occurs before the main denaturation event by temperature. This result suggests that isolated monomers might not be stable enough [36,37], and then, quaternary structure might be essential for the integrity of native protein monomers. Moreover, we hypothesize that the low stability of the isolated monomers could facilitate rapid switching off signalling, and the low monomer stability could be critical in
modulating the function of the protein.

There are, however, differences in the percentages of the α-helix secondary structure obtained by the FTIR and CD, and there are large variations among the CD deconvolution programs. The differences in the predictions between both techniques could be due to the deconvolution procedures used and in the small changes observed in FTIR in the position of the band corresponding to a pure α-helix. Alternatively, the smaller α-helix percentage in some of the CD programs could be due to the fact that, the spectral ellipticity at 222 nm was used mainly to determine the percentages of helical structure, but at this region the aromatic residues (60 in the sequence of EndoV) also contribute to the spectrum with positive or negative bands, depending of their environments [38–40].

There are several X-ray crystal structures of other sulfatases solved to date [41] and references therein) and all of them show an α-helix. The smaller α-helix percentage in some of the CD programs could be due to the deconvolution procedures used and in the small changes observed in FTIR in the position of the band corresponding to a pure α-helix. Alternatively, the smaller α-helix percentage in some of the CD programs could be due to the fact that, the spectral ellipticity at 222 nm was used mainly to determine the percentages of helical structure, but at this region the aromatic residues (60 in the sequence of EndoV) also contribute to the spectrum with positive or negative bands, depending of their environments [38–40].

Conflict of interest statement

The authors declare they do not have any competing interest.

Author contributions

JLN, AC-A APL, JGH-C, JGT, RD and SV designed the research. JLN, EM-C, JGH-C, LM-G, AC-A and IS acquired the experiments. FL provided biological materials and vectors. JLN, AC-A APL, JGH-C, JGT, RD, FG and SV analyzed the data. JLN, AC-A APL, JGH-C, JGT, RD, FG and SV wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2016.09.015.

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