



Cite this: *Phys. Chem. Chem. Phys.*, 2020, 22, 16325

# The multifaceted effects of DMSO and high hydrostatic pressure on the kinetic constants of hydrolysis reactions catalyzed by $\alpha$ -chymotrypsin<sup>†</sup>

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The use of cosolvents and high hydrostatic pressure (HHP) has been described as an efficient means to modulate the stability of enzymes and their catalytic activity. Cosolvents and pressure can lead to increased reaction rates without affecting the stability of the enzyme. Here, we studied the combined effects of one of the most used organic cosolvents, dimethyl sulfoxide (DMSO), and HHP to reveal their combined effect on the kinetic constants of an  $\alpha$ -chymotrypsin-catalyzed peptide hydrolysis reaction. The Michaelis constant and the turnover number of the reaction respond differently to the two variables, and we observed an opposite effect of hydrostatic pressure and the dipolar cosolvent DMSO on the kinetic parameters. The results could be rationalized by determining the volume diagram of the reaction at the different solution conditions. In our case, the use of high hydrostatic pressure in concert with DMSO does not lead to an improvement of the enzymatic activity. However, the advantages of DMSO and HHP to increase the temperature stability of the enzyme and to increase the solubility of more hydrophobic substrates could still be useful.

Received 7th June 2020,  
Accepted 6th July 2020

DOI: 10.1039/d0cp03062g

rsc.li/pccp

## Introduction

Enzymes are specific proteins that act as the cell's own biological catalysts. They catalyze almost all chemical reactions which are necessary to sustain life processes in cells. To this end, not only the great selectivity and catalytic efficiency of the enzymes, but also mild reaction conditions are required.<sup>1</sup> In the fields of biotechnology, pharmaceuticals and the food industry, the interest in such biocatalytic processes has continuously increased in recent years, as they often offer better options than chemically catalyzed pathways.<sup>2–5</sup> One of the major drawbacks of the use of enzymes is their insufficient stability under industrial processing conditions, which demand that an enzyme be also robust with long shelf life and high chemical and thermal stability.<sup>6</sup> Numerous studies, known as protein engineering, are being conducted to develop new, tailor-made enzymes for technical applications.<sup>7</sup> However, the optimum of the process parameters (e.g., temperature, pH) of enzyme-catalyzed reactions is generally within a small range, which can lead to limited stability of the enzymes as well as low reaction rates and yields under industrial process conditions.<sup>8</sup>

By using the pressure variable, the range of process conditions can be extended.<sup>5,7,9–14</sup> Hydrostatic pressures have been shown to be able to stabilize some enzymes at higher temperatures and modulate their conformational dynamics and reaction rates.<sup>5,7,9–16</sup> In fact, nature has explored the effect of pressure on enzymatic reactions, as many organisms thrive at high-pressure conditions in the deep sea, where pressures up to 1 kbar (100 MPa) and beyond are encountered.<sup>17–19</sup> The enzymes operating in the deep sea have adapted themselves to the pressure in mainly two ways. On the one hand, some enzymes can adapt intrinsically to the high pressure, *i.e.* by mutations. On the other hand, enzymes may be stabilized by upregulation of particular cellular natural cosolvents, such as trimethylamine-*N*-oxide.<sup>20,21</sup>

A further possibility to increase the stability of enzymes and to improve the solubility of substrates, suppress water-dependent side reactions and direct the enantioselectivity of the reaction is the use of cosolvents ('solvent engineering'), such as dimethyl sulfoxide (DMSO).<sup>2,7,22</sup> DMSO is known as the most powerful organic solvent currently available due to the wide variety of dissolving organic substances such as carbohydrates, polymers, peptides and many inorganic salts and gases, with loading levels of up to 50–60 wt%. Fundamental to the solvating ability is the high dielectric constant and the chemical-make-up, *i.e.* the dipolar character of DMSO. Further, DMSO is miscible with water and does not form azeotropes. Moreover, DMSO has a low environmental toxicity.<sup>22</sup> Depending on the concentration, DMSO is able to

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/d0cp03062g



stabilize proteins, *i.e.* to enhance the denaturation temperature of proteins, *e.g.* of lipase and amylase.<sup>23,24</sup> Thereby, the hydrophilic properties of DMSO as well as the possibility to enhance the characteristic three-dimensional water structure seem to be of importance.<sup>25</sup> But DMSO may also act as a denaturant, *e.g.* for hen egg white lysozyme.<sup>26–28</sup> Such unfolding is possible if the non-polar side chains are exposed to DMSO and the unfolded structure binds more DMSO.<sup>29</sup> It is also known that DMSO is an inhibitor, activator or molecular chaperone for some enzymatic reactions and may change the enantioselectivity of the reaction.<sup>27,30–39</sup> Eremeev *et al.* describe a critical concentration beyond which the conformational changes induced in the protein globule by the solvent are irreversible. For  $\alpha$ -chymotrypsin, this concentration is 4.2 M DMSO.<sup>40</sup>

Whereas the effects of DMSO or pressure on enzymatic reactions have been studied in the past, the combined effect of both parameters in concert on enzymatic reactions is essentially unknown. In this work, we studied the effect of high hydrostatic pressure (HHP) and DMSO concentration on the activity of the enzyme  $\alpha$ -chymotrypsin ( $\alpha$ -CT) using the fluorescent model peptide alanine-alanine-phenylalanine-7-amido-4-methylcoumarin (AAF-AMC) as substrate. The enzyme selectively hydrolyzes the amide bond at the C-terminus of aromatic residues (Scheme 1). Tretyakova *et al.* have shown that DMSO at concentrations up to 20 vol% is able to stabilize  $\alpha$ -chymotrypsin against temperature denaturation.<sup>41</sup>

## Materials and methods

### Materials

The lyophilized powder of the enzyme  $\alpha$ -chymotrypsin ( $\alpha$ -CT) from bovine pancreas, the substrate AAF-AMC (alanine-alanine-phenylalanine-7-amido-4-methylcoumarin), the reaction product AMC (7-amido-4-methylcoumarin) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich and used without further purification. All the solutions were prepared in pressure stable buffer (0.1 M Tris-HCl and  $\text{CaCl}_2$  10 mM, pH 7.8). Deionized water was used for the buffer and all sample preparations.

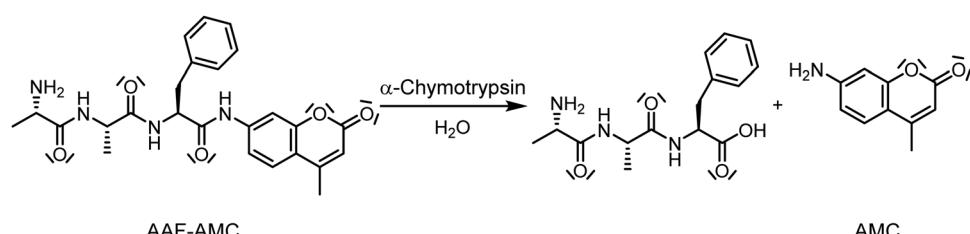
### Sample preparation

A Tris buffer solution with and without DMSO (0, 5, 10 or 20 vol%) was adjusted at a pH value of 7.8 by titration with hydrochloric acid and filtered using a syringe filter (0.45  $\mu\text{m}$ ). A 1 mM stock solution of the product AMC ( $\varepsilon_{370\text{nm}} = 7600 \text{ M}^{-1} \text{ cm}^{-1}$ ) was

prepared by adding a small aliquot of AMC previously dissolved in pure DMSO to the respective buffer (0, 5, 10 or 20 vol% DMSO).<sup>43</sup> Starting from this solution, 2 mL solutions of 1  $\mu\text{M}$ , 2  $\mu\text{M}$ , 3  $\mu\text{M}$  and 4  $\mu\text{M}$  AMC were prepared for the calibration. A 7.5  $\mu\text{M}$  stock solution of the enzyme  $\alpha$ -CT ( $\varepsilon_{280\text{nm}} = 51000 \text{ M}^{-1} \text{ cm}^{-1}$ ) was prepared by dilution in the respective buffer (0, 5, 10 or 20 vol%).<sup>44</sup> From this stock solution, 2  $\mu\text{L}$  were added to each sample to be fluorescence-spectroscopically examined. The substrate AAF-AMC ( $\varepsilon_{325\text{nm}} = 16000 \text{ M}^{-1} \text{ cm}^{-1}$ ) was prepared in DMSO at a concentration of 29.05 mM.<sup>43</sup> A certain amount of this stock solution was added to the sample solution depending on the substrate concentration investigated (in the range 20–135  $\mu\text{M}$ ). The reaction mixture was filled up with the appropriate buffer to the final volume of 1500  $\mu\text{L}$ . For the circular dichroism (CD) spectroscopic measurements, the concentration of  $\alpha$ -CT was set to 100  $\mu\text{M}$  for all water/DMSO mixtures investigated. The exact concentrations of the enzyme, the substrate AAF-AMC and the product AMC were determined by UV spectroscopy (UV-1800 spectrometer from Shimadzu Corporation).

### Fluorescence spectroscopy – kinetics

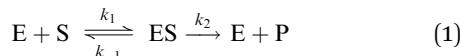
To investigate the catalytic activity of  $\alpha$ -CT, fluorescence measurements were carried out by means of a K2 Multifrequency Phase Fluorometer from ISS Inc. (Champaign, IL, USA) at the temperature of 20 °C. The light source in this instrument was a xenon arc lamp. For the intensity measurements, the slit widths for both the excitation and emission monochromator were set to 8 nm. The excitation wavelength was set at 370 nm while the emission wavelength was set at 460 nm. At 460 nm, the release of the reported group AMC can be followed without any interference from the substrate.<sup>8</sup> For measurements at atmospheric pressure, the prepared samples were filled into a quartz cuvette with a path length of 1.0 cm. To examine the enzyme kinetics of  $\alpha$ -CT under high hydrostatic pressure, the pressure was varied between 1 bar and 2000 bar. To be able to use fluorescence spectroscopy at higher pressures, the HHP cell system from ISS and a special cuvette with a path length of about 0.9 cm were used. After filling, the cuvette was sealed with a polyethylene membrane (Sigma-Aldrich; catalogue no. Z379026) and positioned in the beam path of the high-pressure cell. Afterwards, the pressure was increased by using a manual pump. The fluorescence measurements could only be started after the filled cuvette was correctly positioned in the light path and the high-pressure cell closed. Therefore, the intensities of the



**Scheme 1** Representation of the cleavage of the amide bond of the substrate alanine-alanine-phenylalanine-7-amido-4-methylcoumarin (AAF-AMC) by  $\alpha$ -chymotrypsin. After the hydrolysis, the product 7-amido-4-methylcoumarin (AMC) is released.<sup>42</sup>



first 0.5 to 3 min could not be recorded, which has been taken into account in the data analysis. Since it is not possible to correlate directly the intensity of the released AMC to concentration, a calibration of the instrument was necessary by using standard solutions of AMC. The hydrolysis of AAF-AMC catalyzed by  $\alpha$ -CT follows the Michaelis-Menten kinetics, according to the following reaction:



In this scheme, E is the enzyme  $\alpha$ -CT, S is the substrate AAF-AMC, ES is the enzyme-substrate complex and P is the released product AMC.

The initial rate of the enzyme reaction,  $v_0$ , can be determined by following the formation of the product,  $v_0 = d[P]/dt$ , where  $[P]$  is the concentration of the product, which was calculated from the slope of the linear fit of the time dependent fluorescence data at 410 nm.<sup>7,14</sup> Based on the definition of the rate determining step, the rate constant of product formation,  $k_2$ , is also denoted  $k_{\text{cat}}$  or turnover number. To prevent back reaction, the substrate is used in excess so that the enzyme is saturated. The Michaelis constant,  $K_M$ , a measure of the affinity of substrate binding to the enzyme, is given by  $K_M = (k_{-1} + k_2)/k_1$ . By the linear regression analysis of the Lineweaver-Burk plot,  $K_M$  and  $k_{\text{cat}}$  are obtained:<sup>7</sup>

$$\frac{[E]_0}{v_0} = \frac{[E]_0}{v_{\max}} + \frac{[E]_0 \cdot K_M}{v_{\max}} \cdot \frac{1}{[S]} = \frac{1}{k_{\text{cat}}} + \frac{K_M}{k_{\text{cat}}} \cdot \frac{1}{[S]} \quad (2)$$

where  $v_{\max}$  is the maximum rate achievable and  $[S]$  is the initial substrate concentration;  $[E]_0 = [E] + [ES]$  is the total enzyme concentration.<sup>9</sup> The results reported are the average of at least three independent measurements.

### Analysis of the pressure dependence of the enzymatic reaction

Generally, a chemical reaction is defined both by its energy and volume profile. For a one-step reaction, meaning that one transition state is involved only, the reaction volume is given by  $\Delta V = V_p - V_r$ , where  $V_p$  and  $V_r$  are the partial molar volumes of the product and the sum of the partial molar volumes of the reactants under standard-state conditions. At constant temperature,  $\Delta V$  can be obtained from measurement of the pressure dependence of the equilibrium constant of the reaction,  $K$ :<sup>12,21</sup>

$$\left( \frac{\partial K}{\partial p} \right)_T = -\frac{\Delta V}{RT} \quad (3)$$

According to Eyring's transition state theory,<sup>45</sup> the rate constant,  $k$ , of the reaction may be expressed as

$$\begin{aligned} k &= \kappa \frac{k_B T}{h} \exp\left(-\frac{\Delta G^\neq}{RT}\right) = \kappa \frac{k_B T}{h} \exp\left(-\frac{\Delta G_1^\neq + \int_1^p \Delta V^\neq dp}{RT}\right) \\ &= \kappa \frac{k_B T}{h} \exp\left(-\frac{\Delta G_1^\neq}{RT}\right) \exp\left(-\frac{\Delta V^\neq(p-1)}{RT}\right) \end{aligned} \quad (4)$$

where  $\Delta G^\neq = \Delta H^\neq - T\Delta S^\neq$  is the Gibbs energy of activation, and  $\Delta H^\neq$  and  $\Delta S^\neq$  the enthalpy and entropy of activation;  $\kappa$  is

the transmission coefficient across the activated complex ( $\neq$ ), which is assumed here to be temperature and pressure independent.  $\Delta G_1^\neq$  is the corresponding value at atmospheric pressure (1 bar).  $\Delta V^\neq$  is the volume of activation and represents *stricto sensu* the difference between the partial molar volume of the transition state and the initial state. Hence, the magnitude of  $\Delta V^\neq$  informs on the rate when running the reaction at a given pressure. The determination of  $\Delta V^\neq$  proceeds from the relation reported above by plotting  $k$ -values against pressure in a relatively narrow pressure range, usually up to 1–2 kbar (neglecting the 1 in the  $(p-1)$  term):

$$\left( \frac{\partial k}{\partial p} \right)_T = -\frac{\Delta V^\neq}{RT} \quad (5)$$

Over a larger pressure range, the pressure dependence of  $\Delta V^\neq$  must be taken into account, which requires an additional compressibility term,  $(\Delta \beta^\neq p/(RT))$ .<sup>10</sup> In opposition of a simple chemical reaction, the activation volume,  $\Delta V^\neq$ , for an enzymatic reaction is a rather intricate quantity, which may be considered as the sum of several contributions,  $\Delta V^\neq = \sum_i \Delta V_i$ ;  $i$  represents volumetric contributions such as (i) an intrinsic structural term (chemical reaction term) due to changes in intramolecular interactions, invoking bond transformations, steric hindrance and accompanying conformational changes, (ii) a solvation term resulting from the rearrangement of surrounding water (or solvent) molecules and solute–solvent interactions in the active site of the enzyme, *e.g.* due to electrostriction or release of water *via* entropy-driven association of hydrophobic residues, and (iii) pressure-induced changes in protein flexibility and conformation (*e.g.*, *via* selection of a particular conformational substrate).<sup>9–11,46,47</sup>

Henceforth, exploring the temperature parameter together with the pressure variable, it becomes possible to obtain a complete estimate of the thermodynamic quantities relating to the activated complex, namely,  $\Delta H^\neq$  and  $\Delta S^\neq$  and  $\Delta V^\neq$ . Certainly, such kinetic approach is still an approximation for a complex biochemical reaction as the one considered here. Dynamic effects related to pressure-induced changes in transport properties such as diffusion and viscosity might play a role in determining  $k(p)$  as well. A more elaborate treatment would also require knowledge of the particular properties of the solvent, such as by Kramer's theory, which takes into account the viscosity in the preexponential factor, and of preferential adsorption or exclusion of cosolvent molecules from the protein. In dilute aqueous solutions in the low kbar range, the effect of pressure on the viscosity is almost negligible, however.<sup>46,48</sup>

For an enzymatic reaction as given by the Michaelis-Menten formalism (1) and  $k_2 \ll k_{-1}$ ,<sup>10</sup> determination of the pressure dependence of  $K_M$  and  $k_{\text{cat}}$  through

$$\left( \frac{\partial \ln k_{\text{cat}}}{\partial p} \right)_T = -\frac{\Delta V^\neq}{RT}; \quad \left( \frac{\partial \ln K_M^{-1}}{\partial p} \right)_T = -\frac{\Delta V_b}{RT} \quad (6)$$

allows to determine the activation volume,  $\Delta V^\neq$ , and the binding volume,  $\Delta V_b$ . In our case, at high substrate concentrations, the activation volume is defined as the difference between the volume

in the transition state,  $V^\#$ , and the ES complex in the ground state,  $V_{ES}$ :  $\Delta V^\# = V^\# - V_{ES}$ .<sup>7</sup> The volume difference between the ES complex in the ground state,  $V_{ES}$ , and the reactants,  $V_{E+S}$ , represents the binding volume,  $\Delta V_b = V_{ES} - V_{E+S}$ .<sup>8,49</sup>

### Circular dichroism spectroscopy

Circular dichroism (CD) spectra of  $\alpha$ -CT were recorded to obtain information about the tertiary structure of the enzyme in the different media. Near-UV (260 nm to 320 nm) CD spectra were recorded by means of a Jasco J-715 spectropolarimeter (Jasco Corporation, Tokyo, Japan) at the temperature of 20 °C using a quartz cuvette with a path length of 0.1 cm. The concentration of the enzyme was 100  $\mu$ M. For each sample, a background blank (buffer without and with DMSO) was subtracted. The spectra recorded are the results of 5 accumulations. The spectra were normalized per mole of residue. Due to the strong absorbance of DMSO in the far-UV region, it was not possible to evaluate secondary structural changes of the enzyme in this spectral region.<sup>50</sup>

## Results and discussion

### DMSO effect on the hydrolysis of AAF-AMC at ambient conditions

The impact of the organic solvent DMSO on the hydrolysis of the substrate AAF-AMC catalyzed by  $\alpha$ -CT was investigated by means of fluorescence spectroscopy at  $T = 20$  °C. Fig. 1a shows the Lineweaver-Burk plots for the hydrolysis of AAF-AMC in buffer and in the presence of different amounts of DMSO (5, 10 and 20 vol%) at ambient pressure. By fitting the experimental data points to the Lineweaver-Burk equation (eqn (2)), a complete characterization of the kinetic parameters was obtained. The dependence of the kinetic parameters,  $k_{cat}$  and  $K_M$  as well as the catalytic efficiency,  $k_{cat}/K_M$ , on the DMSO concentration at ambient conditions are summarized in Fig. 2a–c.

Fig. 2a shows that increasing DMSO concentration from 0 to 10 vol% leads to a slight increase of the Michaelis constant,  $K_M$ , indicating a decrease of the affinity of the enzyme for the substrate (for all data, see Table S1, ESI†).<sup>8,13,14</sup> At 20 vol% DMSO, a decrease of  $K_M$  to a value comparable to that obtained in neat buffer was detected. Overall, the presence of DMSO has only a small effect on the Michaelis constant, signifying that the formation of the enzyme–substrate complex (ES) is not largely affected even in the presence of high DMSO concentrations. Fig. 2b depicts the change of the turnover number,  $k_{cat}$ , as function of DMSO concentration. Increasing DMSO concentrations lead to a marked decrease of  $k_{cat}$ , which passes from 1.16  $s^{-1}$  in neat buffer to 0.25  $s^{-1}$  at 20 vol% DMSO, *i.e.*, in DMSO, the release of AMC from the ES complex is retarded.

Several reasons can be invoked to explain the decrease of  $k_{cat}$  with increasing DMSO concentration, such as conformational changes or even denaturation of the enzyme or changes in the hydration shell of the enzyme.<sup>7,51</sup> To proof if conformational changes of the enzyme occur in the presence of DMSO, near-UV circular dichroism (CD) spectra were recorded, which

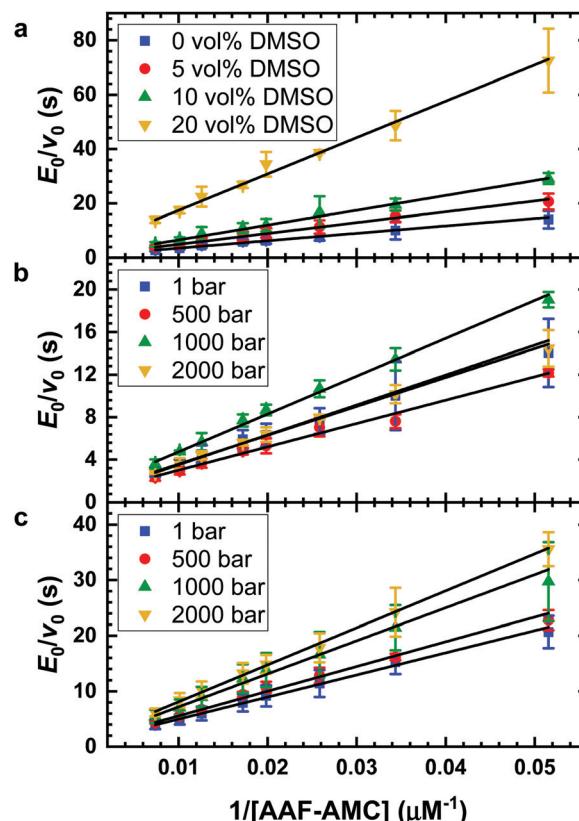
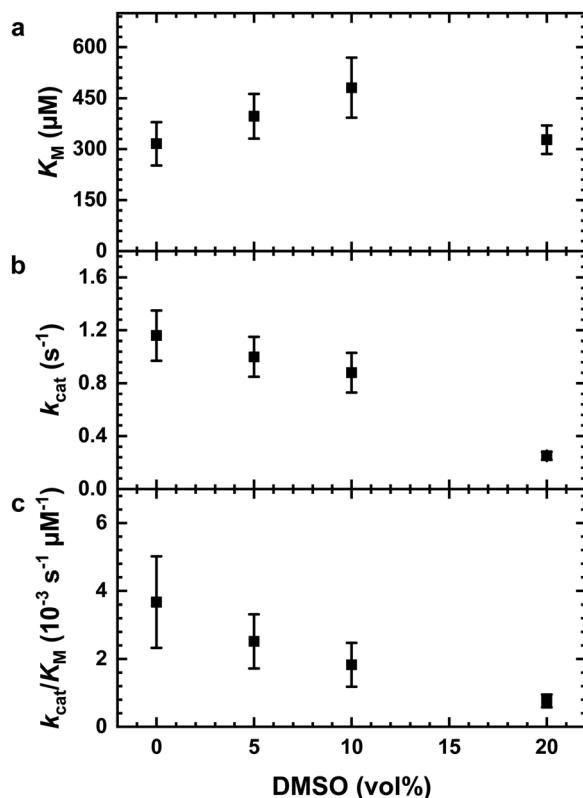


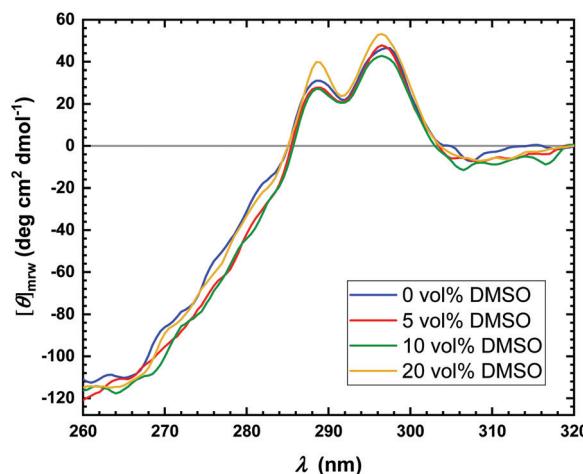
Fig. 1 Lineweaver-Burk plots for the hydrolysis of AAF-AMC catalyzed by the enzyme  $\alpha$ -CT. (a) Plots at ambient pressure as function of DMSO vol%: neat buffer (blue); 5 vol% DMSO (red); 10 vol% DMSO (green); 20 vol% DMSO (orange). (b) Plots in neat buffer at different pressures: 1 bar (blue); 500 bar (red); 1000 bar (green); 2000 bar (orange). (c) Plots in buffer containing 5 vol% DMSO at different pressures: 1 bar (blue); 500 bar (red); 1000 bar (green); 2000 bar (orange).

are reported in Fig. 3. CD spectroscopy is a convenient technique which allows to obtain information on the secondary (far-UV region, 190–260 nm) as well as tertiary (near-UV region, 260–320 nm) structure of proteins.<sup>52</sup> Due to the strong absorbance of DMSO in the far-UV region, it is not possible to explore the effect of DMSO on the secondary structure of the enzyme.<sup>50</sup> Thus, only spectra in the near-UV region were recorded. As evident from Fig. 3, at all concentrations explored, no effect of the DMSO on the tertiary structure of the protein was observed, indicating that the structural integrity of the enzyme is preserved. Therefore, denaturation of the  $\alpha$ -CT even at high DMSO concentrations can be excluded. However, local conformational changes cannot be excluded *a priori*, which might contribute to the decrease of the turnover number.

To carry out the hydrolysis reaction, the presence of water molecules in the enzyme's active site is of key importance.<sup>53</sup> It is possible that DMSO can disrupt hydrogen bonds between water molecules and the enzyme, which in turn may also affect the dynamical properties of the enzyme, leading to a decrease of the observed enzymatic activity.<sup>54</sup> Moreover, it is known that the organic solvent DMSO preferentially solvates tryptophan residues in the active site of the enzyme.<sup>54,55</sup> Thus, a lower



**Fig. 2** Effect of DMSO (in vol%) on the kinetic parameters for the hydrolysis of AAF-AMC catalyzed by the enzyme  $\alpha$ -CT. (a) Michaelis constant,  $K_M$ , (b) turnover number,  $k_{\text{cat}}$ , and (c) catalytic efficiency,  $k_{\text{cat}}/K_M$ . Buffer conditions: 0.1 M Tris, 10 mM  $\text{CaCl}_2$ , pH 7.8;  $T = 20^\circ\text{C}$ ,  $p = 1$  bar.



**Fig. 3** Near-UV CD spectra of  $\alpha$ -CT in neat buffer (blue line) and in the presence of 5 vol% DMSO (red line), 10 vol% DMSO (green line) and 20 vol% DMSO (orange line). The spectra were acquired at  $20^\circ\text{C}$  and at ambient pressure. The spectra were normalized per mole of residue.

turnover number may be explained by a displacement of water molecules from the hydration shell in the active site of the  $\alpha$ -CT by DMSO molecules. To highlight the effect of DMSO on the overall catalytic efficiency of the enzyme, Fig. 2c shows  $k_{\text{cat}}/K_M$  values at different DMSO concentration (data in Table S1, ESI<sup>†</sup>).

The catalytic efficiency decreases with increasing DMSO concentration, reaching a decrease of  $\sim 80\%$  of the catalytic efficiency at 20 vol% DMSO.

#### The combined effects of DMSO and HHP on the hydrolysis of AAF-AMC catalyzed by $\alpha$ -CT

It is known that DMSO can increase the temperature stability of  $\alpha$ -CT. An increase of the denaturation temperature,  $T_m$ , was observed up to 20 vol% DMSO.<sup>56</sup> Thus, a fundamental reason to use DMSO–water mixtures in enzymatic reactions for biotechnological applications is that the reaction can be carried out at higher temperatures to improve the reaction yield. Further, it has been shown that the application of high hydrostatic pressure (HHP) can accelerate enzymatic reactions as well, including hydrolysis reactions catalyzed by  $\alpha$ -CT.<sup>5,7,8,13,14,49</sup> To reveal the combined effect of both parameters, we explored the effect of HHP on the reaction in the presence of 5 vol% DMSO, the DMSO concentration which exhibited the highest catalytic efficiency. For comparison, the effects of HHP on the hydrolysis reaction at neat buffer conditions were also evaluated. The Lineweaver-Burk plots for the reaction carried out at different pressure conditions (1, 500, 1000 and 2000 bar) in neat buffer and in buffer containing 5 vol% of DMSO are reported in Fig. 1b and c, respectively. The kinetic parameters  $k_{\text{cat}}$ ,  $K_M$  and  $k_{\text{cat}}/K_M$  are shown in Fig. 4 and Table S2 (ESI<sup>†</sup>).

Before discussing the effect of HHP on the kinetic parameters, it is important to recall that the structure of the enzyme  $\alpha$ -CT is stable in the whole pressure range covered.<sup>7,57</sup> As reported in Fig. 4a, increasing pressure has a minor, if any, effect on the Michaelis constant in neat buffer condition. The  $K_M$  values at 1 bar, 500 bar and 1000 bar are similar within the experimental errors. Only at 2000 bar,  $K_M$  seems to have slightly increased. In 5 vol% DMSO, the value of the Michaelis constant remains roughly constant throughout the whole pressure range explored. However, the  $K_M$  values are slightly higher compared to the  $K_M$  value obtained in neat buffer solution. Hence, the application of pressure imposes only minor effects on the formation of the enzyme–substrate complex for both solution conditions.

Fig. 4b depicts the effect of HHP on the turnover number,  $k_{\text{cat}}$ , in neat buffer and 5 vol% DMSO. In neat buffer, the application of HHP leads to a slight increase of the turnover number, from  $1.16 \text{ s}^{-1}$  at 1 bar to  $1.43 \text{ s}^{-1}$  at 2000 bar. Conversely,  $k_{\text{cat}}$  decreases in 5 vol% DMSO significantly with increasing pressure, from  $1.00 \text{ s}^{-1}$  at 1 bar to  $0.69 \text{ s}^{-1}$  at 2000 bar. Thus, HHP has an opposite effect on  $k_{\text{cat}}$  in neat buffer and the DMSO solution. These observations reveal a rather complex behavior of the reaction when carried out both under pressure and in the presence of the cosolvent DMSO. As an acceleration of the reaction upon pressurization is favored by small activation volumes and DMSO affects the activities of both enzyme (e.g. by binding to the active site) and reactants, which may also be pressure dependent (see below), simple additivity of both parameters on  $k_{\text{cat}}$  is not expected to be seen.

Finally, Fig. 4c shows the catalytic efficiency in neat buffer and in buffer containing 5 vol% DMSO as function of pressure,

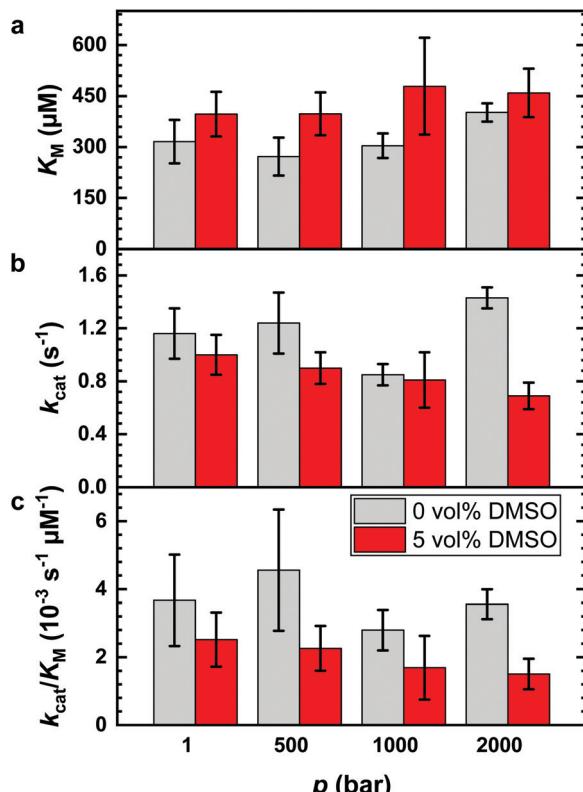


Fig. 4 Pressure dependence of the kinetic parameters for the hydrolysis of the substrate AAF-AMC catalyzed by the enzyme  $\alpha$ -CT at 20 °C in neat buffer (grey bars) and in the presence of 5 vol% DMSO (red bars). (a) Michaelis constant,  $K_M$ , (b) turnover number,  $k_{cat}$ , and (c) catalytic efficiency,  $k_{cat}/K_M$ .

demonstrating the unfavorable effect of HHP in the DMSO solution as well.

To shed more light on the influence of HHP on  $k_{cat}$  and  $K_M$  in both media, a volumetric analysis was carried out. According to eqn (6), it is possible to determine the activation volume,  $\Delta V^\ddagger$ , and the binding volume,  $\Delta V_b$ , from the pressure dependence of  $k_{cat}$  and  $K_M$ , respectively (Fig. S1 and S2, ESI<sup>†</sup>). At our concentrations chosen, the activation volume refers to the volume difference between the transition state ( $ES^\ddagger$ ) and the ground state complex ( $ES$ ). The binding volume refers to the volume difference between the complex  $ES$  in the ground state and the reactants in the ground state ( $E + S$ ). Fig. 5 shows the volume profile for the reaction in the two media.

In the absence of DMSO, the activation volume,  $\Delta V^\ddagger$ , for the reaction is  $-2.5 \pm 0.1$  mL mol<sup>-1</sup>, indicating that the transition state occupies a slightly smaller volume compared to the enzyme–substrate complex in the ground state. The binding volume,  $\Delta V_b$ , amounts to a value of  $5.1 \pm 1.6$  mL mol<sup>-1</sup>, revealing that the reactants have a smaller volume with respect to the  $ES$  complex. In 5 vol% DMSO, the activation volume of the reaction is  $4.5 \pm 0.2$  mL mol<sup>-1</sup> and the binding volume is  $2.0 \pm 0.8$  mL mol<sup>-1</sup>. These values reveal that the transition state  $ES^\ddagger$  occupies a larger volume with respect to the  $ES$  complex in the ground state in the DMSO solution, and that the reactants have a smaller partial molar volume compared to the  $ES$  complex formed.

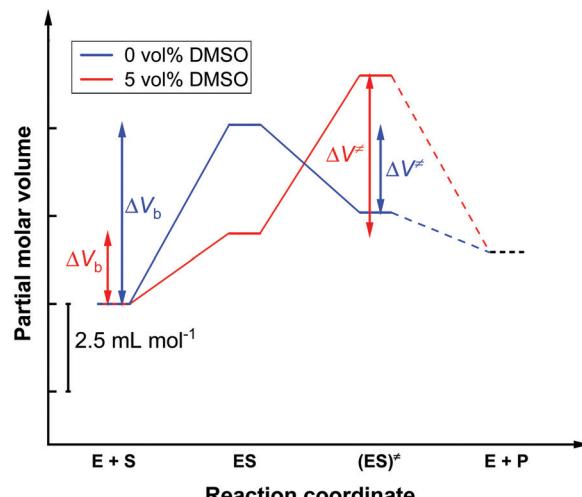


Fig. 5 Schematic representation of the volume profile for the enzymatic reaction in neat buffer (blue line) and in 5 vol% DMSO solution (red line) using as reference the uncomplexed state ( $E + S$ ). The partial molar volume of the product ( $E + P$ ) cannot be given and is therefore shown as a dotted line.

One reason for a negative activation volume can be a higher density of the hydration shell (electrostrictive effect). The density of the hydration shell increases when charged and polar amino acid residues are exposed to the water solvent during the formation of the transition state.<sup>58,59</sup> Furthermore, small conformational changes in the enzyme's active site may be associated with substrate binding. A positive activation volume implicates a retardation of the enzyme reaction, which could be due to a decreased hydration or to packing defects in the active site induced by DMSO binding.<sup>7,13</sup> In the two buffer solutions investigated (0 vol% DMSO and 5 vol% DMSO), a positive binding volume  $\Delta V_b$  was found, this is to say, the volume of the reactants must be smaller than the volume of the  $ES$  complex. This is typically due to the release of hydration water during binding of the substrate to the binding pocket of the enzyme.<sup>60,61</sup> The lower value of the binding volume in 5 vol% DMSO could be explained by a lower degree of hydration of the substrate in the DMSO solution, thereby changing its chemical activity.<sup>7,8</sup>

## Conclusions

In summary, the organic solvent DMSO has a significant influence on the enzymatic activity of  $\alpha$ -CT. The Michaelis constant,  $K_M$ , changes in a non-linear way with the DMSO concentration.  $K_M$  is lowest, *i.e.* the affinity of the enzyme  $\alpha$ -CT to the substrate AAF-AMC is highest at 0 and 20 vol% DMSO.<sup>8,13,14</sup> Cosolvent–substrate interactions may be mainly responsible for the dependence of  $K_M$  on the cosolvent.<sup>7</sup> In addition, weaker hydrophobic interactions between the substrate AAF-AMC and the enzyme could be invoked in the presence of DMSO.<sup>7</sup> Due to the excess of substrate, the catalytic efficiency is mainly controlled by the turnover number,  $k_{cat}$ ,



which decreases with increasing DMSO concentration, as does the catalytic efficiency  $k_{\text{cat}}/K_M$ . This can be explained by a displacement of water molecules in the hydration shell and the active site of the  $\alpha$ -CT by DMSO molecules.<sup>51</sup> In thermodynamic terms, a change in the activity coefficient due to interactions of the enzyme with the substrate, the solvent and the cosolvent may be responsible.<sup>7</sup> A significant change of the tertiary structure of the enzyme upon addition of the DMSO up to 20 vol% could be ruled out.

Regarding the effect of high hydrostatic pressure, no significant change in  $K_M$ , *i.e.* in the affinity of the enzyme to the substrate AAF-AMC, was observed.<sup>8,13,14</sup> Whereas  $k_{\text{cat}}$  increases at high pressure in neat buffer solution, it decreases in 5 vol% DMSO solution, *i.e.* the positive effect of HHP in neat buffer is lost in the presence of DMSO. Accordingly, volumetric analysis of the reaction yields an activation volume,  $\Delta V^\ddagger$ , of  $-2.5 \pm 0.1 \text{ mL mol}^{-1}$  in neat buffer and of  $4.5 \pm 0.2 \text{ mL mol}^{-1}$  in 5 vol% DMSO. For the corresponding binding volume,  $\Delta V_b$ , values of  $5.1 \pm 1.6 \text{ mL mol}^{-1}$  in neat buffer and  $2.0 \pm 0.8 \text{ mL mol}^{-1}$  in 5 vol% DMSO have been obtained. The negative  $\Delta V^\ddagger$  indicates a more compact transition state compared to the ground state of the ES complex, which might be due to an electrostrictive effect and/or a more efficient packing of the activated complex. The positive  $\Delta V^\ddagger$  observed in 5 vol% DMSO indicates a slight volume increase of the  $\text{ES}^\ddagger$  complex, most likely due to binding of some DMSO molecules in the active site of  $\alpha$ -CT.<sup>7,13</sup> The positive binding volumes suggest a release of hydration water when the substrate binds to the active site of the enzyme.<sup>8</sup> Please note, that these volume changes observed here are very small, actually a fraction of the volume of one water molecule ( $18 \text{ mL mol}^{-1}$ ), only. This also demonstrates the strength of the method used here to determine volume changes with high precision.

To conclude, although there is an opposite effect of hydrostatic pressure and the dipolar solvent DMSO on the enzymatic activity of  $\alpha$ -CT, no drastic changes are observed. The use of HHP in concert with DMSO does not lead to an improvement of the enzymatic activity in our case. The advantages of DMSO and HHP to increase the temperature stability of the enzyme and to increase the solubility of more hydrophobic substrates might still be useful, however. To this end, a few %, typically  $<10\%$ , of DMSO are needed, only.<sup>62</sup> Even if many issues remain to be solved, temperature, pressure and non-aqueous cosolvents in combination may be useful to modulate and optimize enzymatic reactions.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

We are grateful for financial support by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy – EXC 2033 – 390677874 – RESOLV.

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