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Exploring Enzymatic Activity in Multiparameter Space: Cosolvents, Macromolecular Crowders and Pressure

Michel W. Jaworek and Roland Winter*[a]

The use of cosolutes and high hydrostatic pressure 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 compromising the stability of the enzyme. Inspired by the multi-component nature of the crowded cellular milieu of biological cells of piezophiles, we studied the combined effects of macromolecular crowding agents, different types of cosolvents and pressure in concert on a hydrolysis reaction catalyzed by α-chymotrypsin. We have seen that crowding agents and cosolvents can have very diverse effects on enzymatic activity. Addition of the deep-sea osmolyte trimethylamine-*N*-oxide displays by far the most positive effect on the catalytic efficiency, k_{eff}, of the reaction, which is even markedly enhanced at high pressures. Addition of the chaotropic agent urea leads to the reverse effect, and PEG and dextran as two representative crowding agents of a different nature show nearly similar values for k_{eff} compared to the pure buffer data. Such information may not only be relevant for understanding life processes in extreme environments, but also for the use of enzymes in industrial processing, which often requires harsh conditions as well.

1. Introduction

Enzymes are the most proficient catalysts for commercial processes, like in the pharmaceutical, food and beverage industries, which offer numerous advantages over traditional chemical processes with respect to sustainability and process efficiency. Hence, it is not surprising that the global market for industrial enzymes grows constantly.[1] Nevertheless, enzyme activity has to be adapted to the desired chemical production process. From an industrial point of view, properties such as the enzyme's long-term stability, processing times, low energy consumption, reduction of production costs taking into account non-toxic and environmentally friendly properties need to be optimized.[1–3] To address these points, present enzyme development mainly deals with protein engineering.[4] But also chemical additives and physico-chemical parameters can be a powerful tool to further improve the yield of the product and cost efficiencies.

Most studies of biochemical processes are examined in dilute solution *in vitro*, although it is well known that up to about 30–40% of the volume in the cytosol *in vivo* is occupied by a variety of macromolecules and solutes.[5] In recent years, it has been shown that macromolecular crowding can markedly impact processes such as protein folding,[6,7] the mobility of components,[8,9] association properties[10,11] and enzymatic activity.[12–14] Hence, to understand also biochemical processes *in vivo*, it seems to be imperative to yield molecular insights into the effect of macromolecular crowders on enzymatic reactions, e.g. by using artificial crowders mimicking cellular environments. Crowding arises mainly from biomacromolecules such as proteins, polysaccharides and nucleic acids, but also from other contributions, such as membraneous organelles, mitochondria and lipid vesicles, the cytoskeleton, and ribosomes. The effect of volume exclusion imposed by crowding has been identified to play a major role.[9] Crowding is generally thought to enhance protein stability because it reduces the conformational entropy of unfolded states but not of the compact native state. Results of the effect of macromolecular crowding on catalytic reactions have been found to be quite diverse, however: enhanced reaction rates,[9,13,15,16] loss of activity,[17,18] and little to no changes[19] have been reported.

As the third thermodynamic parameter next to temperature and chemical activity of the reactants to modulate enzymatic reactivity, high pressure represents another powerful parameter to enhance the stability and activity of enzymes.[20–25] According to Le Châtelier's principle, increased reaction rates are observed when the volume of the transition state of the enzyme-substrate complex, $ES^{\neq}$, is smaller than the sum of the partial molar volume of the reactants (E + S). In some cases, a higher conformational flexibility, which is usually beneficial for increased reaction rates, or a more effective conformational substate of the enzyme can also be induced by pressure application.[21–23]

High hydrostatic pressure is also of biological relevance. A huge fraction of the organisms on Earth is thriving at high hydrostatic pressure (HHP) conditions in the deep sea and subsea floor, where pressures of 1 kbar (100 MPa) and more can be reached.[26–29] Interestingly, deep-sea organisms have also been

[a] *M. W. Jaworek, Prof. R. Winter*
Physical Chemistry I – Biophysical Chemistry
Faculty of Chemistry and Chemical Biology
TU Dortmund University
Otto-Hahn-Strasse 4a, 44227 Dortmund (Germany)
E-mail: roland.winter@tu-dortmund.de

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An invited contribution to a Special Collection on Crowded Systems

found to use high concentrations of compatible cosolvents (osmolytes), such as mannosylglycerate and trimethylamine-*N*-oxide (TMAO), to increase the pressure stability of proteins and modulate their activity.[28–33] Obviously, such extremolytes, i.e. compatible cosolutes that are found in extremophiles, may be of particular use. They accumulate to high concentrations in cells in response to diverse environmental stresses, such as heat, cold, osmotic pressure, desiccation, and high hydrostatic pressure without interfering with cellular metabolism and allow their hosts to survive harsh ecological conditions.[28,29,34] Here, we explored the effects of urea and TMAO, which are also widely used in protein folding studies. Urea serves an important role in the metabolism of nitrogen-containing compounds of animals and humans and it is a chaotropic osmolyte commonly appearing next to TMAO.[28,29] High concentrations of urea can significantly reduce the stability of proteins by interacting directly with the protein backbone and side chains. Conversely, TMAO has the ability to protect functional conformations of proteins by stabilizing intrapeptide ionic interactions and by being excluded from the protein backbone.[35] Further, TMAO is able to compensate the destabilizing effect of urea.[30,35,36] As both cosolvents are often present in deep sea organisms, we used both cosolutes in our study. As high TMAO concentrations can lead to a compaction of structures and damping of conformational fluctuations, the addition of low concentrations of urea might be advantageous for enzymatic activity.

In this work, we used the well characterized model enzyme α-chymotrypsin (α-CT) to reveal the combined effects of macromolecular crowding, using polyethylenglycol (PEG) and the polysaccharide dextran, the cosolvents TMAO and urea, and pressure on the activity of the enzyme (Figure 1A). To our knowledge, this is one of the first studies focusing on the combined effect of cosolvents, crowding agents and hydrostatic pressure on an enzymatic reaction. The enzyme α-CT is a serine protease, which hydrolyzes peptide bonds, preferably aromatic amino acids (Trp, Tyr, Phe), and is used to aid in protein digestion. In the active site of α-CT, three amino acids (Ser195, His57, Asp102) form a catalytic triad, which is connected via hydrogen bonds (Figure 1B).

Mozhaev *et al.* reported an improvement of the stability against thermal denaturation and activity of α-CT upon compression. This enzyme is particularly suitable for kinetic studies, since its stability is guaranteed up to 5 kbar[37] and pressure effects up to 4.7 kbar on the enzymatic activity have been shown to be reversible.[38] We have selected the hydrolysis of the substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAAPP*p*NA) by α-CT, forming the products *N*-succinyl-Ala-Ala-Pro-Phe and *p*-nitroanilide (which can be detected by time-lapse absorption spectroscopy), as a model reaction system in the pressure range from ambient pressure up to 2 kbar. The effect of the pure cosolvents on the activity of the enzyme has been reported.[39,40] The focus of this work was mainly on the influence of the crowding agents PEG and dextran on the reaction, and to explore if the combined effects of pressure, cosolvents and crowding agents are additive, synergistic or antagonistic, and if this set of parameters can be used to optimize the activity of the enzyme.

2. Results and Discussion

FTIR measurements were first carried out to check the structural stability of α-CT in presence of 2 M urea and 1 M TMAO. Figure SI 2 shows the normalized amide I' band of α-CT as well as the corresponding changes in secondary structural elements in the different cosolvent solutions. The ratio and position of the subbands in pure buffer are in rather good agreement with FTIR results reported by Meersman *et al.*[41] The amide I' band

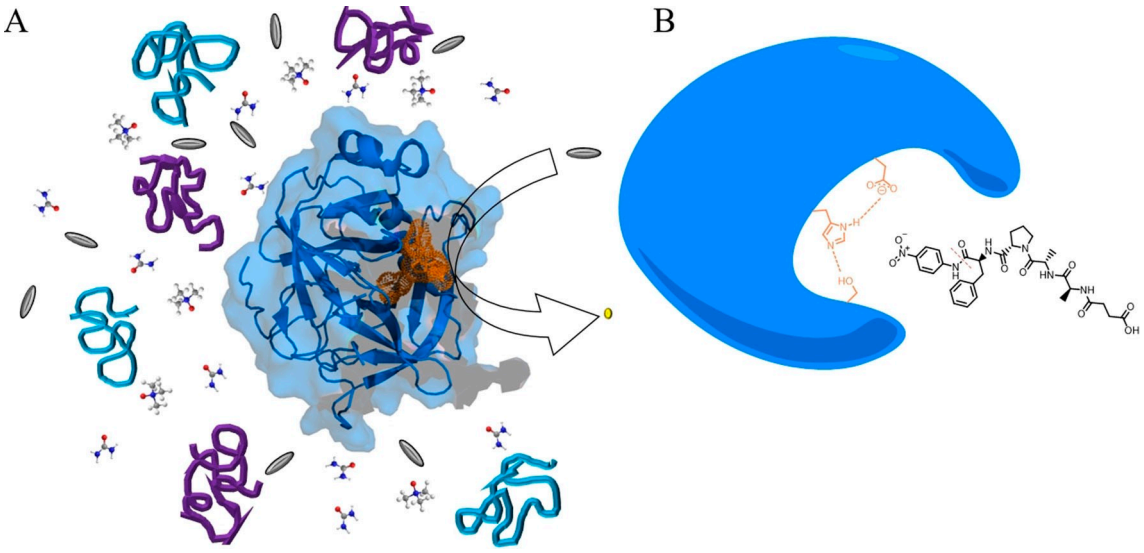

Figure 1. A) Schematic representation of the enzymatic reaction of α-CT (PDB: 1yph; substrate: dark grey; product: yellow) in the presence of crowding agents (dextran: purple, PEG: turquoise) and cosolvents (TMAO and urea). B) Catalytic triad of α-CT in the presence of the substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide.

and percentage of secondary structures in the presence of the cosolvents differ only slightly compared to the pure buffer, indicating that no unfolding/denaturation occurs also in 2 M urea, which would show up in a subband appearing at ~1645 cm^{-1}. Minor changes of the spectral shape could be due to small errors in background correction at these high cosolute concentrations. Of note, Ca^{2+} ions as present in this enzyme reaction have a strong stabilizing effect on α-CT.[42,43] Minor conformational changes, as may be present in the case of urea, which is known to interact directly with the protein backbone and its side chains, cannot be excluded, however.

In the next step, the catalytic activity of the enzyme was monitored through the release of the product *p*-nitroanilide, which is measured through the increase in optical absorbance at 410 nm employing Lambert-Beer's law. For recording fast reactions under high pressure, special equipment is necessary. The high-pressure stopped-flow system used has a deadtime of ~10 ms and operates up to 2 kbar (for details, see the Experimental Section). Figure 2 (A–K) show the time dependence of the absorption data, $A_{410\ nm}$ (t), which can be described by Michaelis-Menten plots for all solution conditions. The initial rate of the reaction, v_0, increases with increasing substrate concentration and reaches in most cases a plateau value at high substrate concentrations, where the enzyme is saturated with substrate. Further, it can be seen that high pressure accelerates the enzymatic activity, as shown by higher reaction rates for almost all solution conditions, indicating an enhancement of the enzymatic activity through a negative activation volume. An exception is only observed for the solutions containing PEG and dextran in the presence of all cosolvents, where differences in the reaction rate from 1 bar to 2 kbar are less pronounced. By comparing the Michaelis-Menten curves of the pure buffer solution with the ones in presence of urea, a significant reduction in slope (p-value < 0.05) is observed, whereas 10 wt.% PEG shows an additional positive effect on the rate of the enzyme reaction. Regarding the Michaelis-Menten plot upon addition of 10 wt.% dextran, no distinct changes in the rate of the enzyme reaction can be observed compared to the pure buffer scenario. Clearly, the two crowder types have a different impact on the reaction rate in the absence and presence of the osmolytes.

In order to draw more detailed conclusions on the enzymatic activity in the presence of the various additives, it is necessary to analyze the progression curves via Michaelis-Menten fits (Eq. 2). Table SI 1 summarizes all kinetic parameters obtained as a function of pressure and Table SI 2 the deduced volumetric data using Eq. 3. Figure SI 1 summarizes the kinetic parameters for all pressures measured. For a better overview, Figure 3 highlights the kinetic parameters (3 A–C) relating to 10 wt.% dextran at 1 bar and 2000 bar and the activation volume (3D), while Figure 4 highlights the kinetic parameters relating to 10 wt.% PEG at 1 bar and 2000 bar and the activation volume in the same order. Table SI 3 compares the pressure dependences of the kinetic parameters. Based on two-sample t-test hypothesis testing, values below 0.05 indicate significance of the results.

With increasing pressure, K_M increases for all solution conditions, i.e. the affinity of the substrate to the enzyme decreases upon compression. The decrease of the affinity is less pronounced in pure buffer and in the presence of 1 M TMAO, and the values are in a similar range as reported literature data.[39,44,45] Urea exhibits a drastic effect on K_M compared to pure buffer, indicating a marked decrease in the substrate binding ability, which has also been observed in this and other enzyme reactions and can be attributed to its ability to bind to the protein and inhibit its active site.[28,29,39] 10 wt.% dextran (Figure 3A) has little to no visible effect on K_M, whereas 10 wt.% PEG, especially 10 wt.% PEG + 2 M urea (Figure 4A), also shows a negative effect on K_M.

Remarkably, the turnover number increases about 1.5–2 fold in all solutions upon compression (Figs. 3B, 4B), with largest values observed for solutions with 10 wt.% PEG and 2 M urea. Addition of 10 wt.% dextran has no significant effect on k_{cat} with respect to the pure buffer solution (p-value > 0.05). As shown in Figs. 3 C, 4 C, the catalytic efficiency, k_{eff}, is largest for the 1 M TMAO, also at high pressures; k_{eff} is similar for pure buffer, pure 10 wt.% PEG and pure 10 wt% dextran, but decreases for the other solvent mixtures, also upon compression. Addition of the deep-sea osmolyte TMAO displays by far the most positive effect on the catalytic efficiency, while addition of the chaotropic agent urea leads to the reverse effect, and PEG and dextran as two representative crowding agents of different nature show nearly similar values for k_{eff} compared to the pure buffer data. A synergistic, but negative effect of a crowding agent and cosolvent could only be observed for the Michaelis constant in the presence of 10 wt.% PEG and 2 M urea, whereas dextran does not show such additional effect. The kinetic parameters catalytic efficiency and turnover number are only significantly affected by one of the cosolutes, PEG or TMAO (p-value < 0.05).

As expected, α-CT exhibits a negative activation volume, $\Delta V^{\neq}$, for all solution conditions (Figs. 3D, 4D), i.e. the volume of the transition state, $ES^{\neq}$, is smaller than the volume of the ES complex, which might be due to filling of some void volume, hydration changes and minor conformational changes inside the enzyme's active pocket in the course of the reaction.[44,46,47] The order of magnitude of $\Delta V^{\neq}$, $-4 \ldots -8$ mL mol^{-1}, is very small, about a factor of 10 smaller than the volume change upon unfolding of a protein,[48] and corresponds to less than one water molecule (18 mL mol^{-1}), only. This nicely demonstrates the power of the method in determining accurate volume changes. Such small $\Delta V^{\neq}$-values of α-CT-catalyzed hydrolysis reactions are in good agreement with literature results for this and other substrates in pure buffer solution.[38–40] The similar $\Delta V^{\neq}$-values for the various solution conditions imply minor, if at all, differences in the transition state complex, i.e. the structure of the reaction center itself.

The lower affinity, i.e. higher K_M value, upon addition of 10 wt.% PEG for this enzymatic reaction may be caused by increased diffusion resistance within the sample[49] or, more likely, interactions of the largely hydrophobic PEG with the hydrophobic substrate and hydrophobic domains of the protein,[9,47,50] leading to activity coefficients strongly deviating

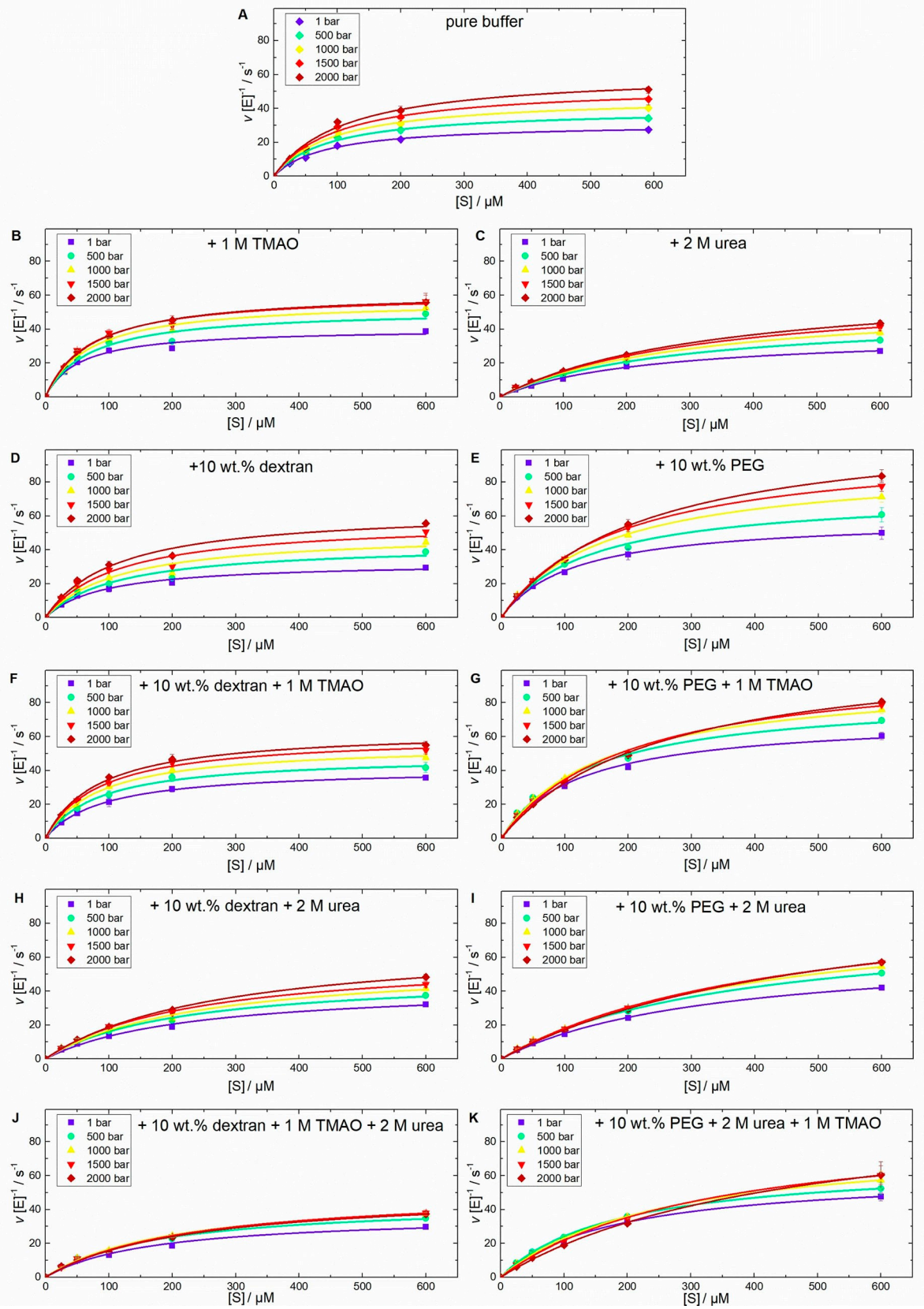

Figure 2. Michaelis–Menten plots for the hydrolysis of SAAPP*p*NA catalyzed by α-CT at different substrate concentrations, [S], and pressures, *p*, at $T = 25\,^{\circ}C$ for all measured solution conditions (pure buffer plus 1 M TMAO, 2 M urea, 10 wt.% dextran, 10 wt.% PEG, and in various combinations).

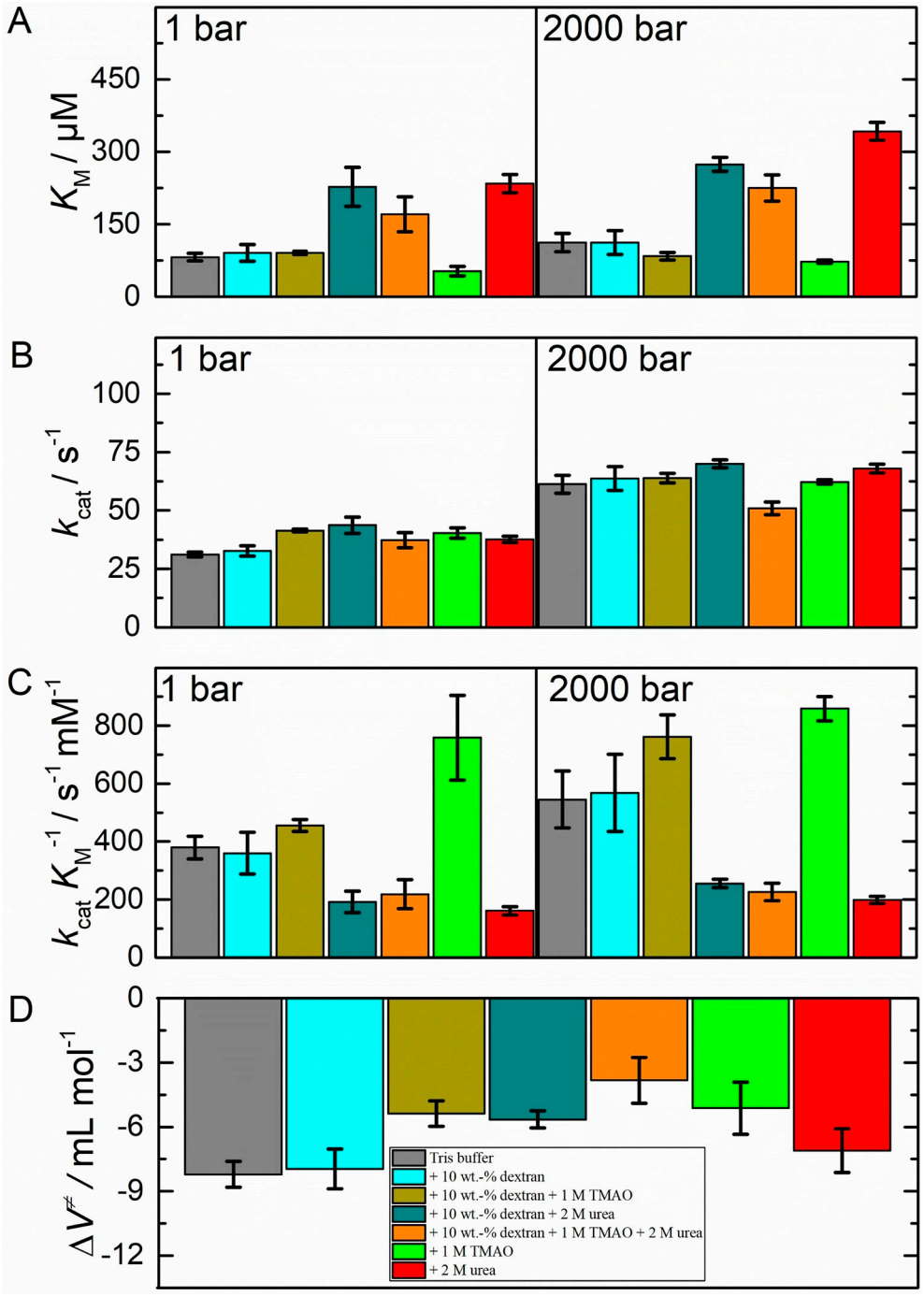

Figure 3. Pressure dependence of the kinetic constants for the hydrolysis of SAAPP*p*NA catalyzed by α-CT in different solutions regarding dextran: A) Michaelis constant, B) turnover number, C) catalytic efficiency and D) activation volume.

from ideal behavior.[18,51] Surprisingly, k_{cat} increases in the presence of PEG in all solutions, which is quite uncommon for this crowding agent.[10] For this, different activity coefficients of the released products and a change of water activity in the presence of PEG may be likely reasons. Conversely, the more hydrophilic crowding agent dextran shows no significant effect on the kinetic parameters (p-value > 0.05). Literature data on K_M and k_{cat} in the presence of dextran are diversified. The parameters can change in both directions compared to pure buffer, and have been found to depend on the size and concentration of the crowder, but also on the size of the enzyme itself.[18,49,52] In our case, we used a small size (10 kDa) and 10 wt.% of dextran, which has no marked effect on this enzyme reaction. Regarding the catalytic efficiency, urea shows the greatest adverse effect. The increase of K_M can have different reasons. Urea may act as a competitive inhibitor of the

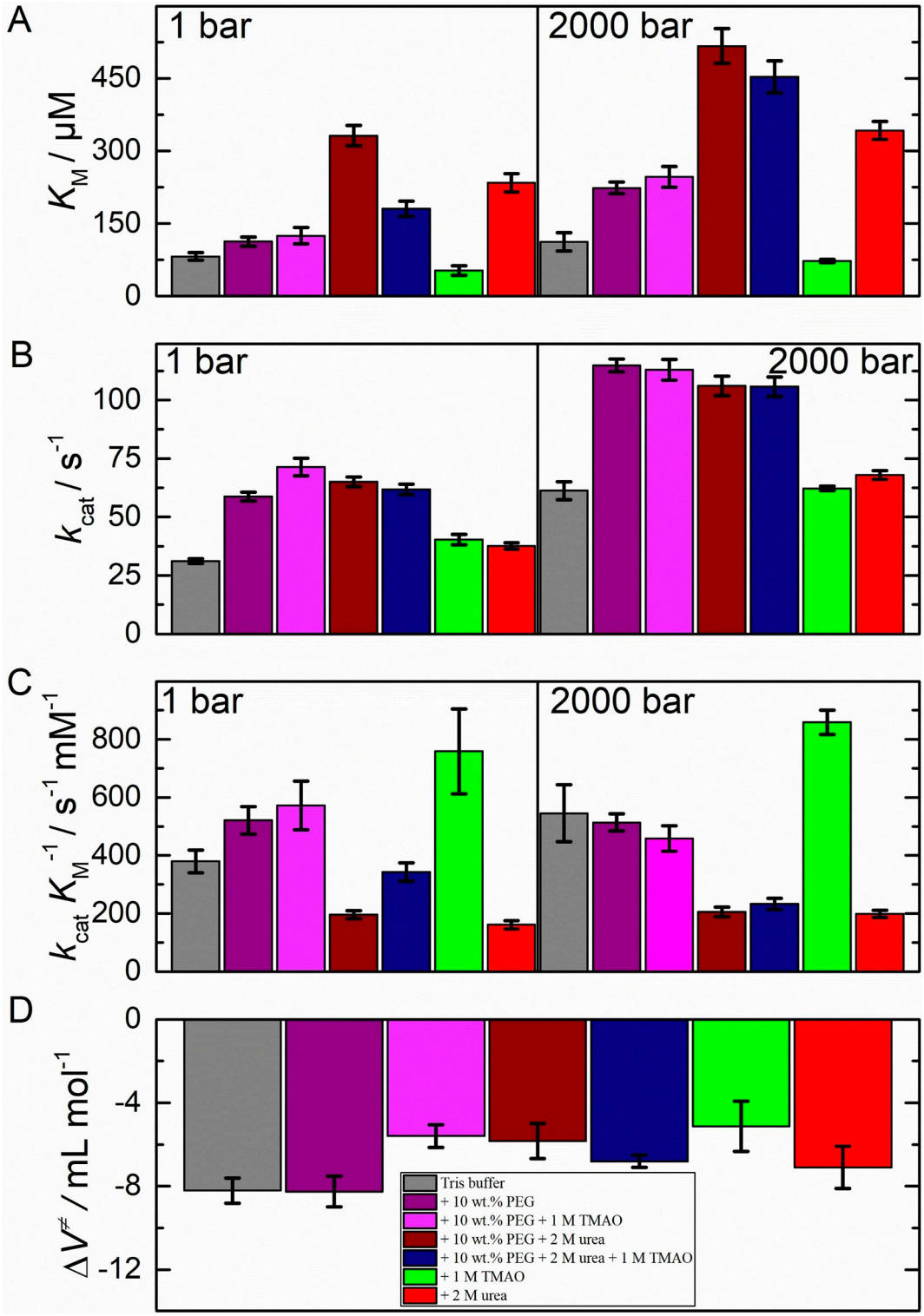

Figure 4. Pressure dependence of the kinetic constants for the hydrolysis of SAAPP*p*NA catalyzed by α-CT in different solutions regarding PEG: A) Michaelis constant, B) turnover number, C) catalytic efficiency and D) activation volume.

substrate or may interact with the substrate molecule or favorably with the protein surface. The increase of the other kinetic parameter, k_{cat}, might be explained by favorable changes in the conformational dynamics of the enzyme's active site. A similar behavior has been observed for other enzymatic reactions.[53,54] On the other hand, TMAO reveals the highest k_{eff} compared to all other solutions. Generally, TMAO owns the ability to interact favorably with bulk water, leading to an

increase in hydrogen bonding and structuring of the solvent, and is preferentially excluded from the protein interface, thereby increasing its hydration.[47,55,56]

3. Concluding Remarks

The chemical and pharmaceutical industries depend on measures to protect enzymes against stresses that accompany their production, transport, storage and use. To this end, the full parameter space of thermodynamic variables needs to be explored, including, next to the more common variables temperature, ionic strength and pH, also cosolvents, crowding agents and pressure. In this respect, we might also learn from Nature. The complex cellular milieu is responsible for the so-called quinary structure (and structural dynamics) of proteins in the biological cell, which is the fifth level of their organization. Quinary structure consists of all weaker and more transient interactions between the protein and its surroundings, which are highly modulated by environmental stresses, such as low/high pH, ionic strength, temperature and pressure. Using particular cosolutes, extremophiles – for example piezophiles thriving in the deep sea at high-pressure conditions – have succeeded in stabilizing their enzymes and modulating their activity, which is not only important for understanding life in extreme ecological settings, but might also be useful for optimizing enzyme-based industrial processes.

Generally, crowding agents affect proteins via a combination of entropic (hard-core interactions, exclude volume effect) and enthalpic effects (weak non-specific interactions with enzyme and substrate).[57,58] Enthalpic effects imposed by dextran and PEG are expected to differ because of their different functional groups, while entropic effects might vary to a lesser extent owing to their similar sizes used here (hydrodynamic radii 2.3–2.4 nm), though they have different shapes (dextran-based crowding agents are branched polymers of sucrose and behave more like a random coil compared to the rod-like particles of the linear chain polymer PEG). Owing to the more hydrophobic character of PEG, it is prone to interact weakly with hydrophobic patches of the enzyme surface and substrate. Hence, different from the hydrophilic dextran, a negative effect on the Michaelis constant can be expected, as observed experimentally.

We have seen that crowding agents and cosolvents can have very diverse effects on the enzymatic reaction, which are difficult to predict, in particular when applied together and in concert with pressure modulation. The catalytic efficiency, k_{eff}, of the hydrolysis reaction by α-CT is largest for the TMAO solution, which is further enhanced at high pressures. Further, k_{eff} is similar for pure buffer, pure 10 wt.% PEG and pure 10 wt.% dextran, but decreases for the crowder-cosolvent mixtures, also upon compression. Hence, addition of the deep-sea osmolyte TMAO displays by far the most positive effect on the catalytic efficiency of the reaction, while addition of the chaotropic agent urea leads to the reverse effect, and PEG and dextran as two representative crowding agents of different nature show nearly similar values for k_{eff} compared to the pure buffer data. TMAO is a small cosolute that offers both strongly hydrophilic and hydrophobic solvation regions and has a strong dipole moment (~5 D). Owing to its strong H-bonding capacity, TMAO leads to significant structural changes of the solvent (water) and to a very strong thermodynamically favored exclusion from protein surfaces, i.e., displays a strong solvophobic effect, which increases protein hydration, affects intermolecular distances and may increase intermolecular attractive interactions.[55,56]

To identify the origin of the observed changes of the different kinetic parameters and to quantitatively predict the impact of the various parameters in concert, knowledge of the thermodynamic activity of all reactants of such multi-component solutions as used here could be useful. To this end, the activity coefficients of all reactant species of the enzymatic reaction are required for the various solution conditions, which is a formidable task. Principally, such information can be obtained from experiment and theory via Perturbed-Chain Statistical Associating Fluid Theory (PC-SAFT), as shown for some selected systems, already.[50,51] Such information would also be mandatory to optimize enzymatic reactions in technological applications, such as in the field of biotechnology, pharmaceutical industry and protein engineering. As a future goal, such concepts might then also be applied to even more complex situations, including enzymatic reactions in microcompartments, such as in aqueous two-phase systems, which can serve as *in vitro* model systems for artificial fluid membrane-less organelles, making use of the higher local concentrations of co-localized enzyme and substrate and the concomitant rate increase.[59,60]

Experimental Section

Lyophilized powder of α-chymotrypsin (α-CT) from bovine pancreas, TMAO, urea, dextran (10 kDa), poly(ethylene glycol) (PEG, 4.7 kDa) and *N*-Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAAPP*p*NA) were obtained from Sigma Aldrich and were used without further purification. Pressure-stable Tris-HCl buffer (100 mM tris (hydroxymethyl)amino-methane), containing 10 mM $CaCl_2$ in the absence and in the presence of osmolytes and/or crowder agents was used to prepare the enzyme and substrate solutions for the high-pressure stopped-flow kinetic measurements. The pH was adjusted to 7.8. Both the enzyme and the substrate were dissolved in the respective buffer solutions. The initial concentration of the enzyme for all measurements before loading into the sample cell was 20 nM (10 nM after mixing with the substrate solution). For the substrate, five different concentrations - between 50 μM and 1200 μM (25 μM and 600 μM after mixing with the enzyme solution) – were used. To determine their exact concentrations, UV spectroscopy (UV-1800 of Shimadzu) was performed using molar absorption coefficients of α-CT (51000 M^{-1} cm^{-1} at 280 nm) and SAAPP*p*NA (14000 M^{-1} cm^{-1} at 315 nm), respectively.[39]

Regarding the size of the crowding agents with respect to the size of the 25.5 kDa enzyme α-CT of hydrodynamic radius, R_H, of ~2.45 nm, 10 kDa Dextran has a hydrodynamic radius of about 2.44 nm, and the R_H of 5 kDa PEG is of similar size and amounts to ~2.29 nm.[61–63] Compared to the hydrophilic coil-like polysaccharide dextran, PEG is more hydrophobic in nature and has a rod-like shape.

Enzyme activities at different pressures were carried out using a high-pressure stopped-flow (HPSF) instrument (HPSF-56 of TgK Scientific), which allows recording reactions up to 2 kbar. The catalytic activity of the enzyme was monitored through the release of the product *p*-nitroanilide, which can be measured through the increase in optical absorbance at 410 nm by using the Lambert-Beer law with the molar extinction coefficient $\varepsilon_{410\ nm} = 8800\ M^{-1}\ cm^{-1}$. The HPSF system and the function of the stopped-flow instrument is described elsewhere.[39,40,64] The temperature was kept at 25 °C for all measurements using an external water circuit, and the pressure was increased stepwise in 500 bar-steps up to 2000 bar. Upon achieving the desired pressure, the samples were equilibrated for 5 min. After the rapid mixing process has taken place (dead time ~10 ms), the absorbance was measured at 410 nm up to 100 s. These parameters were chosen to obtain accurate kinetic data in a suitable time window of the high-pressure stopped-flow technique, and to be able to compare our data with previous biophysical studies of this enzyme.[38,65]

Under the reaction conditions chosen, the enzymatic kinetics can be characterized by the Michaelis-Menten scheme [Equation (1)]:[39,40]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \quad (1)$$

(k_1, k_{-1} and k_2 are rate constants, E = enzyme, S = substrate, P = product and ES = enzyme-substrate complex). The initial rate of the enzyme reaction, v_0, was determined by calculating $v_0 = d[P]/dt$, where [P] represents the product concentration, from the slope of the linear fit of the time dependent absorbance data at 410 nm. To determine the turnover number, k_{cat} (equal to k_2) and Michaelis constant, $K_M = (k_{-1} + k_2)/k_1$, the initial velocity is plotted against the substrate concentration, using fits to the Michaelis-Menten equation [Equation (2)]:

$$v = \frac{v_{max} \cdot [S]}{K_M + [S]} = \frac{k_2 \cdot [E] \cdot [S]}{K_M + [S]} \quad (2)$$

([E] = total enzyme concentration, [S] = initial substrate concentration, v_{max} = maximal rate of the reaction). The ratio $k_{eff} = k_{cat}/K_M$ denotes the enzymatic efficiency.

The EYRING-equation describes the pressure effect on the rate of the reaction [Equation (3)]:[20,21]

$$\left(\frac{\partial \ln k_{cat}}{\partial p}\right)_T = -\frac{\Delta V^{\neq}}{RT} \quad (3)$$

(p = pressure, T = temperature, R = ideal gas constant, $\Delta V^{\neq}$ = activation volume). The activation volume, $\Delta V^{\neq}$, which is assumed to be pressure independent in the pressure range covered here, describes the difference of the volume of the transition state ($ES^{\neq}$) and the ground state (ES) of the enzyme-substrate complex (and/or the reactant state (E + S) at low substrate concentrations).[21] Due to different compressibilities of the reactants, the ES complex and the transition state, $\Delta V^{\neq}$-values can be positive or negative, and they can also be dependent on the pressure amplitude.

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Conflict of Interest

The authors declare no conflict of interest.

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