Pressure and Temperature

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Hydrostatic Pressure Increases the Catalytic Activity of Amyloid Fibril Enzymes

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Abstract: *We studied the combined effects of pressure (0.1–200 MPa) and temperature (22, 30, and 38 °C) on the catalytic activity of designed amyloid fibrils using a high-pressure stopped-flow system with rapid UV/Vis absorption detection. Complementary FT-IR spectroscopic data revealed a remarkably high pressure and temperature stability of the fibrillar systems. High pressure enhances the esterase activity as a consequence of a negative activation volume at all temperatures (about −14 $cm^3 mol^{-1}$). The enhancement is sustained in the whole temperature range covered, which allows a further acceleration of the enzymatic activity at high temperatures (activation energy 45–60 $kJ mol^{-1}$). Our data reveal the great potential of using both pressure and temperature modulation to optimize the enzyme efficiency of catalytic amyloid fibrils.*

Amyloid fibrils are linear polypeptide aggregates with a highly periodic cross-β sheet conformation, which abnormally occur inside the body and are associated with diseases such as Alzheimer's and Parkinson's.[1] Increasing evidence shows, however, that amyloid structures may also occur as functional biological units in vivo, and there is considerable interest in the material sciences and bionanotechnology to exploit the unique structural features of these highly stable, nanostructured, and biodegradable assemblies for the generation of novel applications and technical tools, such as protein-based catalysis, conducting nanowires, and devices for water purification.[2]

A key factor limiting the further utility of amyloid materials and their incorporation into technical devices is their so far unclear stability and performance under process-relevant conditions. To establish these characteristics we herein describe the results obtained by exposing two recently described preparations of de novo designed and catalytically active fibril systems to different types of environmental stress. These two systems consist of the peptides Ac-LHLHLRL-$CONH_2$ (AF1) and Ac-IHIHIQI-$CONH_2$ (AF2) that form amyloid fibrils in vitro, which exhibit, upon Zn^{2+} binding, an esterase activity that can be spectroscopically followed by the hydrolysis of the substrate *p*-nitrophenyl acetate (*p*NPA).[3]

Transmission electron microscopy (TEM) showed that the fibril morphology of both samples is markedly different. Both samples contain polymorphic fibrils, as can be inferred from the broad variations of the fibril width (Figure 1), and there are considerable variations in the quaternary structure of the fibrils in these samples. AF1 fibrils are relatively long and twisted ribbons (Figure 1 A), while AF2 fibrils exhibit a more plate-like morphology (Figure 1 B).

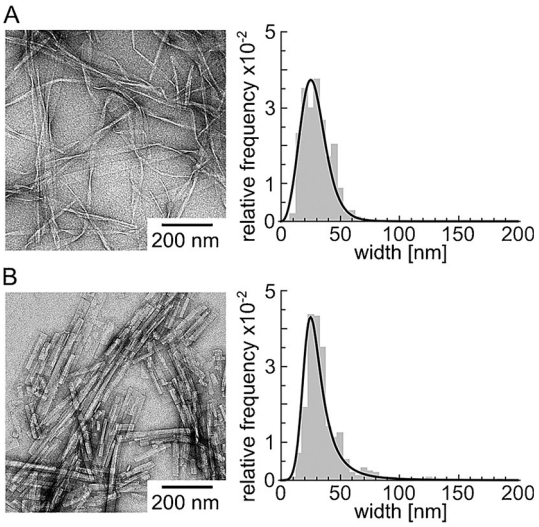

Figure 1. Negative-stain TEM images of AF1 (A) and AF2 peptide fibrils (B) and histograms of the fibril widths ($n = 500$, right side). The histograms were fitted by Burr-type-XII distributions (black lines) with modes 25.2 nm (AF1) and 24.9 nm (AF2).

We then determined the effect of different environmental conditions, such as high hydrostatic pressure (HHP) and temperature, on the structural stability and catalytic activity of the two fibril enzymes. Until now the effects of HHP on enzymatic substrate conversions have been characterized only sporadically.[4] HHP would be expected to accelerate enzymatic reactions, if the activation volume $\Delta V^{\#}$ of the reaction is negative; that is, if the partial molar volume of the transition state is smaller than the sum of the partial molar volumes of the reactants at the same temperature and pressure. By contrast, we would expect HHP to slow down catalysis, if $\Delta V^{\#}$ is positive. Besides the reaction rate, HHP may even alter the stereoselectivity or the substrate specificity

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of an enzyme by favoring the conversion of a substrate whose product possesses a smaller volume than the product of a competing substrate.[4] In addition, as the rate of an enzymatic reaction is often limited by the thermal stability of the corresponding enzyme, it is conceivable that increasing the thermal stability of the enzyme by high pressure combined with an accelerated substrate conversion at increased temperatures could lead to an improved overall reaction rate.

Fourier-transform (FT) IR spectra of AF1 and AF2 fibrils show no marked secondary structural changes within the analyzed pressure and temperature ranges (0.1–400 MPa and 20–60 °C, Figure S1 in the Supporting Information), rendering the two fibril systems highly suitable for enzymatic studies covering a wide range of temperatures and pressures. Generally, mature fibrillar structures have been shown to reveal a high pressure stability as they are largely devoid of void volume and internal water.[5]

Using a high-pressure stopped-flow (HPSF) system we studied the esterase activity in the pressure range from 0.1 to 200 MPa and at three temperatures (22, 30, and 38 °C). Figure 2 shows Michaelis–Menten plots for the fibril-catalyzed hydrolysis of *p*NPA. At 0.1 MPa, 22 °C and 0.8 M *p*NPA, we find the reaction rates to be $(4.4 \pm 0.7) \times 10^{-3}\ s^{-1}$ for AF1 and $(18.8 \pm 1.7) \times 10^{-3}\ s^{-1}$ for AF2 (Figure 2), which relates to previously published values that were obtained under slightly different experimental conditions.[3] The reaction rate also increased with temperature; for example, raising the temperature from 22 to 38 °C increased the reaction rate of AF1 by about 80 % and of AF2 by approximately 130 %. This effect is seen at all pressure and substrate concentrations used in our study. At 200 MPa, the reaction rate increased about 3.5-fold at all temperatures and substrate concentrations for both AF1 and AF2 compared to the rate at 0.1 MPa. In summary, AF2 fibrils are more active than AF1 fibrils, specifically at high pressures or temperatures, and show a roughly 6-fold higher reaction rate. Figure 3 shows the pressure dependences of the turnover number k_{cat}, of the Michaelis constant K_M, and of the catalytic efficiency k_{cat}/K_M of the catalyzed hydrolysis of *p*NPA. We find that increasing the pressure does not substantially affect K_M, while it potently increases k_{cat} and as a consequence thereof also k_{cat}/K_M. This observation is made consistently with both fibril samples. When we raise the temperature, we find k_{cat} and K_M to be increased, while k_{cat}/K_M is not significantly affected in the case of AF1 and increases only slightly in the case of AF2. Compared to AF1, AF2 displays a three times lower K_M and three times higher k_{cat}, and as a consequence thereof its k_{cat}/K_M value is nine times higher. Interestingly, all these ratios do not change substantially with pressure and temperature.

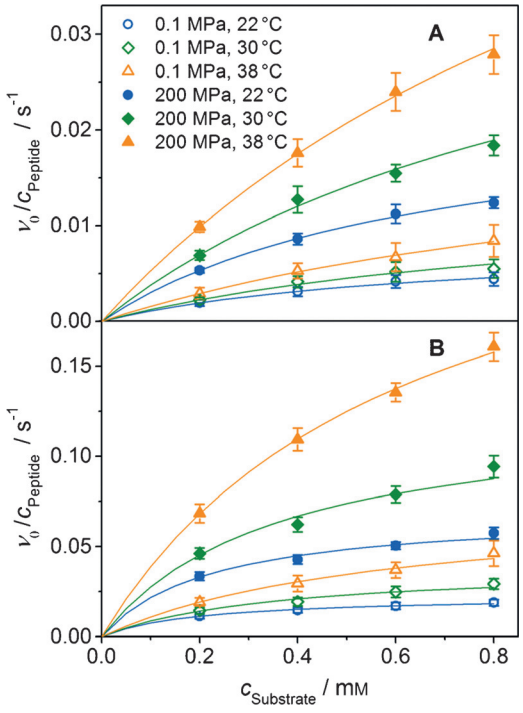

Figure 2. Representative Michaelis–Menten plots for the catalyzed hydrolysis of *p*NPA at different pressures and temperatures: A) AF1, B) AF2. The solid lines are the fits for the respective data derived from linear regression of the Lineweaver–Burk plots.

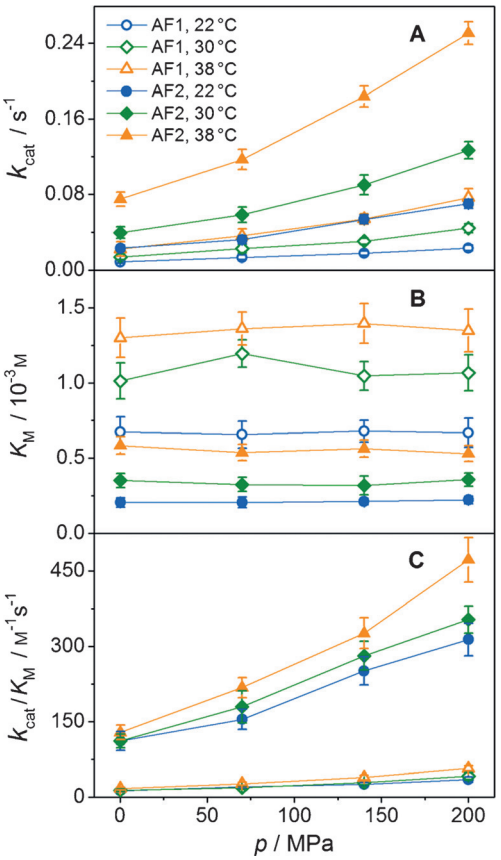

Figure 3. Pressure dependence of the kinetic constants for the catalyzed hydrolysis of *p*NPA at different temperatures: A) turnover number, k_{cat}, B) Michaelis constant, K_M, and C) the enzymatic efficiency, k_{cat}/K_M.

That HHP and temperature are able to increase the enzymatic activity of globular proteins was shown previously for the hydrolysis of the substrates SPNA[4d] and SAAPPNA[6]

by the protease α-chymotrypsin. However, the effect of temperature changes within the range 20–40 °C on the enzymatic activity of α-chymotrypsin was found to be significantly higher than the effect of pressure in the range 0.1–200 MPa. Figure 4 A depicts the temperature dependence of $\Delta V^{\#}$ as obtained from Equation (2) (see the Experimental Section). All $\Delta V^{\#}$ values are negative, indicating that the turnover number of the hydrolysis reaction is enhanced upon compression. For both fibril systems, we find $\Delta V^{\#}$ to be about $-(14 \pm 2)$ cm^3 mol^{-1}, which is in the order of the volume of a single water molecule (18 cm^3 mol^{-1}), and the negative sign indicates a smaller compression of the peptide–substrate complex (ES) relative to the transition state (ES$^{\#}$).

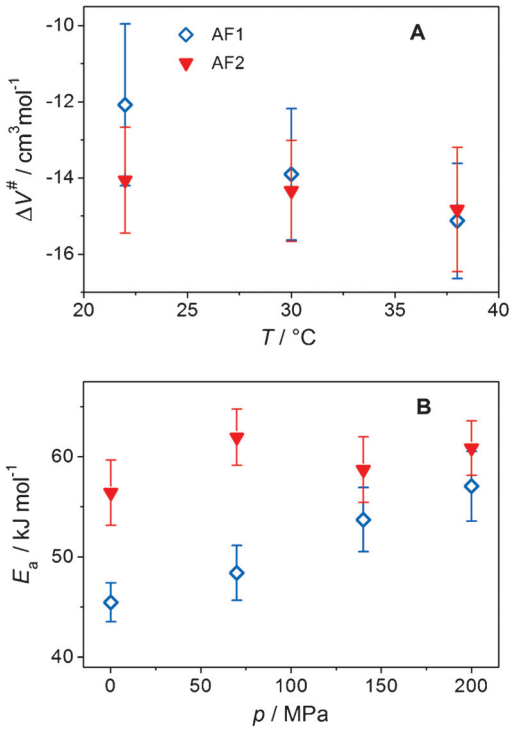

Figure 4. Temperature and pressure dependence of the esterase activity of AF1 and AF2 fibrils. A) Temperature dependence of $\Delta V^{\#}$, B) pressure dependence of E_a.

Figure 4 B shows the pressure dependence of the activation energy of the reaction, E_a, which was determined according to Equation (4) (see the Experimental Section) from an Arrhenius plot ($\ln k_{cat}$ versus $1/T$). At 0.1 MPa, we found the AF1-catalyzed hydrolysis of *p*NPA to be associated with an E_a value of (45 ± 2) kJ mol^{-1}, which increases slightly upon pressurization to (57 ± 3) kJ mol^{-1} at 200 MPa; this might be due to a minor pressure-induced change in the conformation of the active site. In the case of the more efficient AF2 fibrils, we obtained a value of about (60 ± 3) kJ mol^{-1} that is hardly affected by changes of the pressure, probably owing to a slightly different, more compact structure of the transition state.

These data provide novel evidence for the impact of process-relevant conditions, such as high temperature and pressure, on the structural stability and catalytic activity of amyloid fibril enzymes. Our study does not only show that the catalytic fibrils survive these environments, we even found a dramatically increased performance when applying high pressures. Globular proteins, by contrast, may also respond to pressure, although generally much less than to a temperature rise from 20 to 40 °C.[4d,6] This observation is relevant when considering that amyloid structures may become incorporated into technical flow devices or filtration units applying significant hydrodynamic pressures.

While the molecular basis of these effects remains to be established, it is generally known that proteins are dynamically active molecules[7] and that their thermally fluctuating motions can be crucial to protein folding, ligand binding, and catalytic activity.[8] For example, conformational transitions may allow access for substates with structural and dynamic features that allow the reaction to reach the transition state. Unfortunately, to obtain quantitative information about these conformational substates (CSs) remains a difficult task. In recent years, it has been shown that pressure perturbation can be effectively used to explore intrinsic fluctuations by acting on the volumes of the protein conformers (Figure 5).[9] An increased population of low-lying excited conformers under high pressure implies that these states have a lower partial molar volume, mainly governed by changes in hydration and packing within the protein. In this study, HHP has been shown to populate a CS of the enzyme–substrate complex that is able to promote product conversion by a larger compression of the transition state with respect to the enzyme–substrate complex.

In the long term, identifying CSs relevant for enzymatic conversions by pressure perturbation in concert with atomic-resolution spectroscopies and QM/MM molecular simulations may help to improve our understanding of enzymes and may finally lead to the development of novel enzyme-engineering strategies. Notably, owing to the high temperature and pressure stability of catalytically active amyloid fibrillar structures, significantly higher turnover numbers along with a higher long-term stability of the enzyme manifold may be achieved as demonstrated herein.

To conclude, herein we described the ability of minimal small-peptide arrays in form of amyloid structures, which provide the framework, to support catalytic activities with improved temperature and pressure stability and higher efficiency due to the small size of their active unit with respect to their biological counterparts. Moreover, we showed how their activities may even be further increased through high pressure, such as for the hydrolysis reaction described herein. Finally, our observations may support a potential link between the emergence of protein catalysis during enzyme evolution and the origin of life, which might have started in the deep sea where pressures up to the kbar level are encountered.[10]

Experimental Section

Kinetic assays: All measurements were carried out in Tris-HCl buffer (25 mM Tris, 1 mM $ZnCl_2$, pH 8.0) and at a final peptide concentration of 15 μg mL^{-1}, while the substrate concentration was varied from 200 to 800 μM. The kinetics of product (*p*-nitrophenol, *p*NP) formation

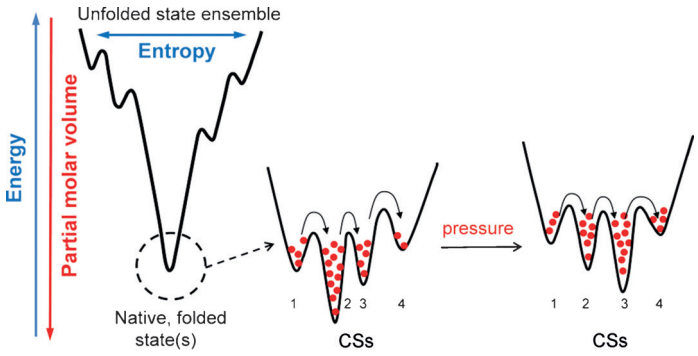

Figure 5. Schematics of the free-energy landscape of proteins. The native ground state contains always cavities (some of which are partially occupied by water molecules, some are empty), therefore has in general a larger partial molar volume than the unfolded state, which is fully hydrated and contains no internal cavities. Hence, pressure destabilizes the ground state compared to partially unfolded and higher-energy states. Mature fibrillar systems, however, are largely devoid of imperfect packing (voids) and are hence rather pressure-stable.[5] Proteins in their native (functioning) folded state are able to sample conformations sharing similar structural and dynamic features (CSs), which correspond to small wells in the energy landscape (red dots illustrate the population level of these CSs). The depths of the CSs as well as their populations may vary with pressure. Intrinsic motions of proteins such as enzymes allow accessing these CSs,[8] which are generally thought to be functionally relevant. Pressure provides a unique window to stabilize low-lying low-volume CSs, for example the state relevant for the transition state of the enzyme–substate complex (here, CS3 in the schematics shown along the reaction path may be such a catalytically productive state en route to the product), and may thereby foster product conversion if $\Delta V^{\#} < 0$.

were followed by optical absorbance at 405 nm based on experimentally determined molar extinction coefficients of *p*NP at different temperatures (Figure S2) and using an HPSF-56 system (Hi-Tech Scientific). The details of the system were described elsewhere.[11] The temperature of the samples was controlled by a thermostat and kept constant at ±0.1 °C around the set temperature during all experiments. The pressure was increased stepwise from 0.1 to 200 MPa. Under the concentration conditions used, the esterase activity of the amyloid fibrils can be described by the Michaelis–Menten kinetics [Eq. (1)], where k_1, k_{-1}, and k_2 are rate constants, E is the enzyme

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

(AF1 or AF2), S the substrate (*p*NPA), ES the enzyme–substrate complex, and P the product.[12] The initial rate of the reaction, v_0, that is, the rate of product formation, was calculated from the slope of the linear fit of the time-dependent absorbance data at 405 nm ($v_0 = d[P]/dt$, where [P] is the concentration of the product). We used a large excess of substrate for saturating the enzyme, which also avoided significant back reaction.

The kinetic parameters $K_M = (k_{-1} + k_2)/k_1$ (Michaelis constant) and $k_{cat} = k_2$ (rate constant of the catalysis or turnover number) were obtained by linear regression analysis of the Lineweaver–Burk plot [Eq. (2)], where [E] is the total concentration of the amyloid fibril, [S]

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

the initial substrate concentration, and v_{max} the maximum achievable rate. The ratio k_{cat}/K_M reflects the enzymatic efficiency.

The pressure effect on the rate of the reaction is obtained from the Eyring Equation (3), where k and k_0 are the rate constants at

$$\left(\frac{\partial \ln k/k_0}{\partial p}\right)_T = -\frac{\Delta V^{\#}}{RT} \quad (3)$$

pressure p and a reference pressure (here 1 bar), respectively, T is the absolute temperature, R the ideal gas constant, and $\Delta V^{\#}$ the activation volume of the reaction. At high substrate concentrations, k is equal to k_{cat} and $\Delta V^{\#}$ the difference of the volume of the transition state and the ground state of the enzyme–substrate complex (ES), that is, $\Delta V^{\#} = V^{\#} - V_{ES}$. If these volumes are compressed to different extents under high pressure, a change of $\Delta V^{\#}$ with pressure will be observed.

The activation energy, E_a, is related to the rate constant of the catalysis through the Arrhenius Equation (4), where A is the

$$k_{cat} = A e^{-\frac{E_a}{RT}} \quad (4)$$

Arrhenius prefactor. From the temperature dependence of k_{cat}, the activation energy of the reaction can be obtained from the slope of the linear regression of the plot of $\ln k_{cat}$ against $1/T$.

TEM: 3 μL of the fibril solution were placed onto glow-discharged, carbon-coated copper grids and incubated for 2 min followed by blotting with filter paper. The sample was washed three times by dipping the grids into 30 μL of water and blotting after each step. Afterwards, the sample was dipped three times into droplets of 2 % (w/v) uranyl acetate, blotted after each step, and dried. Samples were examined under a Jeol-1400 Plus electron microscope operated at 120 kV and using magnifications from 5000 to 25000 times. The micrographs were used to determine for 500 fibrils the width, as measured at the widest position perpendicular to the fibril axis. The widths were measured manually using the program Fiji version 2.0.0 (http://fiji.sc).

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