Modulation of Enzymatic Activity by Aqueous Two-Phase Systems and Pressure - Rivalry between Kinetic Constants

*Rosario Oliva[#], Sudeshna Banerjee[#], Hasan Cinar and Roland Winter**

Physical Chemistry I - Biophysical Chemistry, Faculty of Chemistry and Chemical Biology, TU Dortmund University, Otto-Hahn Strasse 4a, D-44227 Dortmund, Germany

[#] These authors contributed equally to this work

*e-mail: roland.winter@tu-dortmund.de

Materials

The lyophilized powder of the enzyme α-chymotrypsin (α-CT) from bovine pancreas, the substrate Ala-Ala-Phe-7-amido-4-methylcoumarin (AAF-AMC), the 7-amido-4-methylcoumarin (AMC), polyethylene glycol (PEG) of molecular weight 4.6 kDa and dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich Chemical. Dextran (Dex) 10 kDa was purchased from Carl Roth. The rhodamine B-labeled Dex 10 kDa was purchased from Thermo Fisher Scientific. The chemicals were used without further purification. All the solutions were prepared in the pressure stable buffer composed of 0.1 M Tris-HCl and $CaCl_2$ 10 mM, pH 7.8. Deionized water was used for the buffer and all sample preparations.

Sample Preparation

A stock solution of α-CT was prepared by dissolving the enzyme in the buffer. The concentration was evaluated by recording the absorbance at 280 nm and using a molar extinction coefficient of 51000 M^{-1} cm^{-1}. The stock solutions of the substrate AAF-AMC (30 mM) and of the standard AMC (50 mM) were prepared by dissolving them in DMSO. Their exact concentrations were determined by means of UV/Vis spectroscopy (UV-1800 spectrometer from Shimadzu Corporation). After dilution in buffer, the concentratipon of AAF-AMC and AMC was determined by measuring their absorbance using an extinction coefficient of $\varepsilon(325) = 16000$ M^{-1} cm^{-1} for AAF-AMC and $\varepsilon(370) = 7600$ M^{-1} cm^{-1} for AMC.[1] The ATPS composed of PEG 4.6 kDa and Dex 10 kDa was prepared by mixing apporpriate amounts of PEG and Dex solutions dissolved in buffer. At these conditions, coexisting Dex-rich and PEG-rich phases were obtained at room temperature.[2,3] The final concentration of PEG and Dex were 12.7 wt% and 5.5 wt%, respectively. The dansyl-labled α-CT was prepared by dissolving the enzyme in a buffer composed of 100 mM $NaHCO_3$, 1 mM $CaCl_2$, pH 8.5. A solution of dansyl chloride at the concentration of 10 mg/mL was prepared by dissolving the dye in acetone. Then, the dye solution was added to the protein solution under stirring and stored at 4 °C for 2 h. After the reaction, the excess dye was removed by using a Sephadex G-25 HiTrapp Desalting column from GE Healthcare.

Fluorescence Spectroscopy - Kinetics

The enzyme kinetics was recorded by using a K2 fluorometer from ISS, Inc. (Champaign, IL, USA) at the temperature of 20 °C. The instrument is equipped with a xenon arc lamp as light source. The excitation and emission wavelengths were set at 370 nm and 460 nm, respectively. The width slits for both the excitation and emission monochromators were set to 8 nm. During the hydrolysis of AAF-AMC catalyzed by α-CT, the release of the reporter

group AMC occurred.[1] The formation of the product AMC was monitored continously as a function of time and could be followed by recording its fluorescence intensity at 460 nm without any interference from the substrate AAF-AMC.[4] In all experiments, the concentration of enzyme was 10 nM. The concentration of the substrate was varied from 20 μM to 135 μM. The final volume of the solution was 1500 μL. The desired concentrations of enzyme and substrate were obtained by diluting the stock solutions with either buffer, ATPS or Dex 30 wt% solution, as required. The amount of DMSO was about 0.4 vol% at the highest substrate concentration. The high pressure measurements were performed using the HHP cell system from ISS and quartz cuvettes. The pressure was applied by means of a manual pump and water was used as pressurizing fluid. The pressure was varied between 1 bar and 2000 bar. The enzyme and substrate were mixed, vortexed and then the solution was filled into the sample cell, which was sealed with DuraSeal™ laboratory stretch film and placed into the high-pressure vessel. Since the whole assembling process took nearly 4 min, this time was 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 is necessary. Briefly, four standard solutions of AMC in buffer, ATPS and 30 wt% Dex at concentrations of 4 μM, 2 μM, 1 μM, 0.5 μM were prepared. Recording their intensities, a calibration curve was obtained which was used to convert the fluorescence intensity to the amount of released product.

The hydrolysis of AAF-AMC catalyzed by α-CT follows the Michaelis-Menten kinetics.[5] Thus, catalytic activity of α-CT was evaluated by determining the Michaelis-Menten constant (K_M) and the turnover number (k_{cat}). Briefly, the initial rates (v_0) of the reaction were measured in the regime where the fluorscence intensity changes linearly with time. Then, the data obtained were analyzed using the Lineweaver-Burk equation (Eq. S1):[6]

$$\frac{E_0}{v_0} = \frac{1}{k_{cat}} + \frac{K_M}{k_{cat}} \cdot \frac{1}{[S]}$$

In this equation, E_0/v_0 is plotted against 1/[S], where E_0 is the total enzyme concentration, v_0 is the initial rate, [S] is the total substrate concentration, K_M is the Michaelis-Menten constant and k_{cat} is the turnover number. The K_M and k_{cat} values reported are the average of at least 3 independent measurements.

Pressure Dependent Turbidity

The pressure dependent turbidity measurements were carried out on a PerkinElmer Lambda 25 spectrophotometer with a home-built high-pressure optical cell. As window material, sapphire with a diameter of 20 mm and a thickness of 10 mm was used. Pressure was applied using a high-pressure hand pump and was measured by a pressure sensor (Burster Präzisionsmesstechnik, Gernsbach). As pressurizing medium, water was used.

Pressure Dependent Microscopy

In addition to the turbidity measurements, phase contrast and fluorescence microscopy experiments were carried out using an Eclipse TE2000-U (Nikon Inc.) optical microscope with a Nikon CFI Plan Apo Lambda 10x objective coupled to an TIS DMK 23UX249 camera. For fluorescence microscopy, we used dansyl-labeled α-CT, rhodamine B-labeled dextran and AAF-AMC. For the pressure-dependent microscopy studies, a home-built high-pressure microscopy cell was used. The pressure was generated hydrostatically by a high-pressure hand pump with water as pressure-transmitting fluid. Flat diamond windows were used as optical window material on both sides.

Circular Dichroism Spectroscopy

Far-UV and Near-UV circular dichroism spectra were acquired on a Jasco J-715 (Jasco Corportation, Tokyo, Japan). Far-UV spectra of α-CT (30 μM) were recorded in the range 260-195 nm using a 0.01 cm path length quartz cuvette. The Near UV spectra were taken in the range 320-260 nm. The concentration of α-CT was 15 μM and a 0.1 cm path length quartz cuvette was used. The spectra were recorded for the enzyme dissolved in buffer or in the ATPS. For each sample, a background blank (buffer or ATPS) was subtracted. The spectra recorded are the results of 6 accumulations. The spectra in both the Far- and Near-UV were normalized per mole of residue.[7]

Steady-State Fluorescence Spectroscopy

Fluorescence emission spectra of dansyl-labeled α-CT were acquired by means of a K2 fluorometer from ISS, Inc. (Champaign, IL, USA) at the temperature of 20 °C and using a 1-cm path length quartz cuvette. Briefly, the dansyl-α-CT was dissolved in buffer, ATPS or Dex 30 wt%. Then, the emission spectra were recorded in the range 450-600 nm upon excitation at 325 nm. The position of the emission maximum was evaluated by calculating the spectrum center of mass (CM).[8]

Table S1 Kinetic parameters for the hydrolysis of AAF-AMC catalyzed by α-chymotrypsin at different pressures and at the temperature of 20 °C.

	1 bar		**500 bar**		**1000 bar**		**2000 bar**	
Solvent	K_M **/ μM**	k_{cat} **/ s^{-1}**	K_M **/ μM**	k_{cat} **/ s^{-1}**	K_M **/ μM**	k_{cat} **/ s^{-1}**	K_M **/ μM**	k_{cat} **/ s^{-1}**
Buffer	172±37	0.93±0.14	223±39	0.80±0.12	283±70	1.02±0.23	396±61	1.71±0.23
Dex	87±6	1.00±0.05						
ATPS	201±34	0.33±0.05	233±69	0.32±0.08	209±40	0.22±0.03	241±42	0.30±0.05

Buffer: 0.1 M Tris-HCl and $CaCl_2$ 10 mM, pH 7.8
Dex: Dextran 10 kDa 30 wt%
ATPS: PEG 4.6 kDa 12.7 wt% and Dex 10 kDa 5.5 wt%

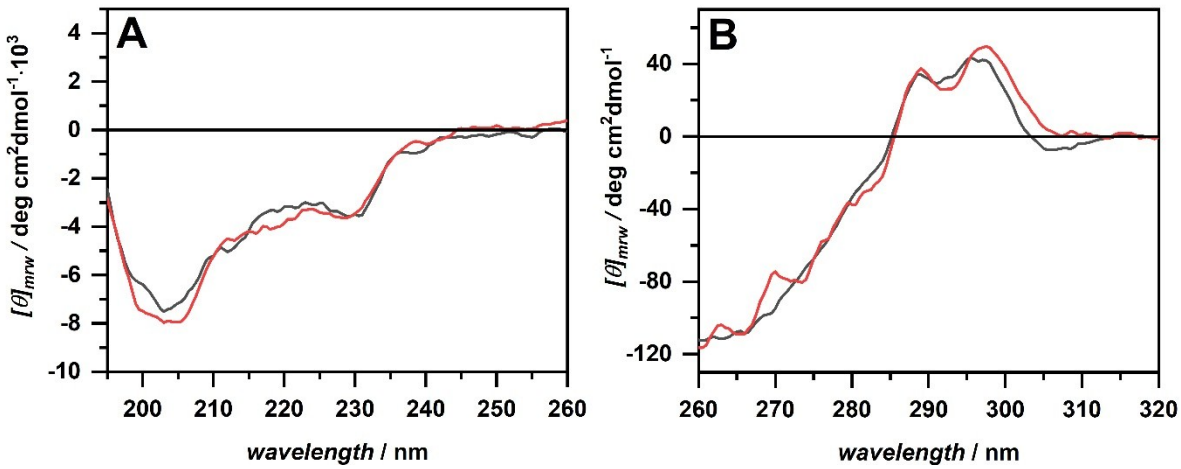

Fig. S1 (A) Far-UV and (B) Near-UV circular dichroism spectra of α-chymotrypsin in buffer (black lines) and in the ATPS (red lines).

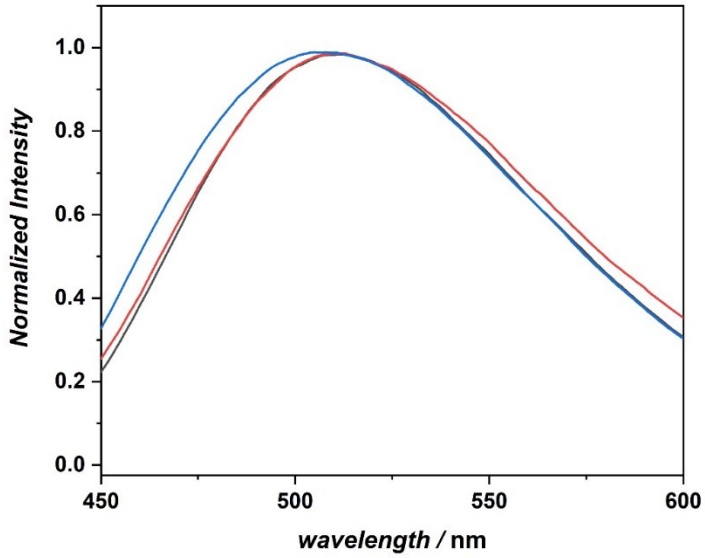

Fig. S2 Normalized fluorescence emission spectra of Dansyl-α-chymotrypsin in buffer (black line), Dex 30 wt% (red line) and in the ATPS (blue line). The experiments were carried out at 20 °C using a 1-cm path length quartz cuvette.

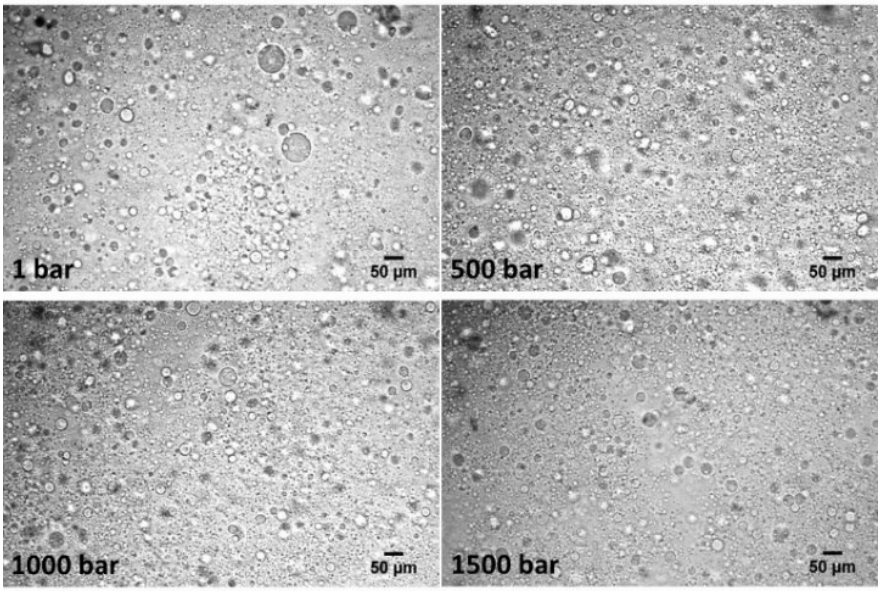

Fig. S3 Phase contrast microscopy pictures of the ATPS at selected pressures. The experiments were performed at 20 1C. The size of the scale bar is 50 mm.

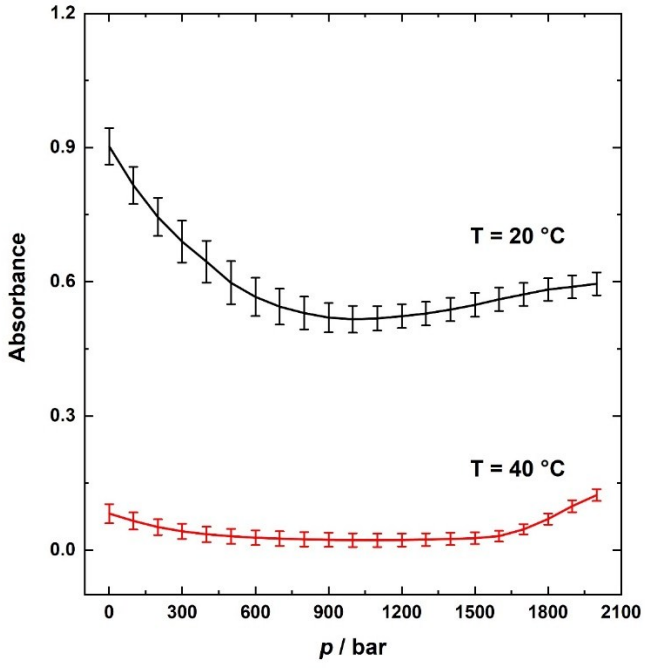

Fig. S4 Changes in apparent absorbance (turbidity) with pressure of the ATPS at 20 °C (black line) and, as a reference, at 40 °C (red line). The employed PEG/Dex mixture is phase separated at 20 °C but not at 40 °C.

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