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Enzyme Kinetics in Acoustically Levitated Droplets of Supercooled Water: A Novel Approach to Cryoenzymology

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The rate of the alkaline phosphatase-catalyzed hydrolysis of 4-methylumbelliferone phosphate was measured in acoustically levitated droplets of aqueous tris (50 mM) at pH 8.5 at 22 ± 2 °C and in supercooled solution at −6 ± 2 °C. At 22 °C, the rate of product formation was in excellent agreement with the rate observed in bulk solution in a cuvette, indicating that the acoustic levitation process does not alter the enzyme activity. The rate of the reaction decreased 6-fold in supercooled solution at −6 ± 2 °C. The acoustic levitator apparatus is described in detail.

The goal in cryoenzymology is to use low temperatures to facilitate the investigation of enzymes by slowing the rates of reactions or trapping intermediate states such that the systems become experimentally accessible. Since the natural solvent for enzymes is water (or dilute aqueous solutions), the normal freezing point of water at 0 °C imposes a limitation on cryoenzymology. Typically, this limitation is circumvented by adding a carefully selected antifreeze that is believed to preserve the chemical and physical properties of liquid water or at least does not perturb the function of the enzyme.[1]

A more attractive choice of cryosolvent is supercooled *liquid* water. In its supercooled state, water retains its liquid properties, though its viscosity increases significantly.[2−4] Small volumes of water (<10 μL) can remain liquid down to about −42 °C (i.e., the homogeneous nucleation limit)[4] provided the water does not contain any ice-forming impurities and is not in contact with an ice-forming surface (i.e., heterogeneous nucleation). Since it is the solvent that most closely resembles ordinary liquid water, supercooled water is an ideal medium to use to slow reactions involving proteins, as this solvent is the least disruptive to the native structure of the protein. To avoid nucleation, the volume of water must be small and the water must be free of ice-forming impurities. Supercooled water has been prepared in narrow capillaries,[2,3] in water-in-oil emulsions,[2,3] and in levitated droplets.[5]

Indeed, the use of supercooled water has already been exploited to a limited extent in cryoenzymology. Douzu and co-workers prepared aqueous solutions of various enzymes in supercooled water by forming water-in-oil emulsions with sorbitan tristerate (Span 65) surfactant. This technique was used to measure the low-spin to high-spin ratio in camphor-bound Fe^{3+} cytochrome P450, to stabilize the $Fe^{2+}\cdot O_2$ intermediate in cytochrome P450, and to follow horseradish peroxidase−CO recombination following flash photolysis.[6] More recently, Szyperski and co-workers have applied NMR methods for structural determination of proteins in supercooled water down to −16.5 °C in narrow capillaries.[7,8] Both of these methods of preparing supercooled water have limitations. In the preparation of oil-in-water emulsions, there is the potential for either the oil or surfactant to disrupt the enzyme structure, perhaps even denaturing it. In the case of the capillary method, the large volume of solution limits the extent of supercooling.[2,3] Levitated droplets are free of these limitations.

In acoustic levitation, small liquid or solid particles are suspended in the nodal points of a resonant acoustic field (i.e., a standing wave) established between a high-intensity source and a reflector.[9−11] Recently, there has been considerable interest in applying acoustic levitation to problems in areas of analytical chemistry including several papers in this journal.[12−15] (See Santesson and Nilsson[16] for a recent and thorough review of these applications.) Much of the motivation for this research has been

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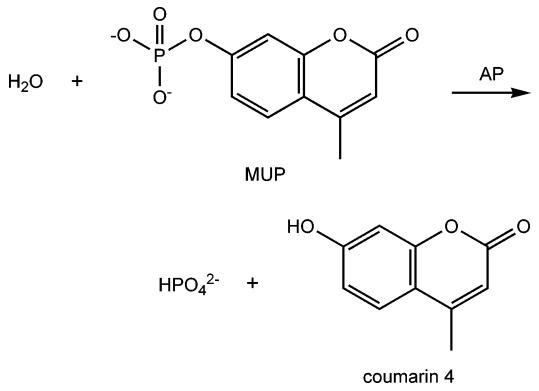
that acoustic levitation provides experimental access to moderately small volumes (~0.1−10 μL) in a containerless (or noncontact) environment. The containerless aspect of the system is driven by the need to eliminate wall effects (adsorption and reaction) when working with small volumes of dilute solutions that are routinely encountered in bioanalytical applications.[16] Here, the noncontact environment of the levitator permits the supercooling of water.

As a model system, we have chosen to work with the enzyme alkaline phosphatase (AP, EC 3.1.3.1) a nonspecific phosphomonoesterase.[17] In our reaction, AP cleaves the phosphate group from 4-methylumbelliferone phosphate (MUP), converting it into the fluorescent product coumarin 4.



Our objectives in this investigation were (1) to demonstrate that kinetic studies are feasible in levitated droplets, (2) to demonstrate that enzymatic activity is not disrupted in an acoustically levitated droplet, and (3) to measure the rate of an enzyme-catalyzed reaction in supercooled aqueous solution. We describe our acoustic levitator and spectrofluorometer in detail, report results demonstrating that the first two objectives have been achieved in the AP-catalyzed MUP hydrolysis reaction, and present data on the rate of the reaction in supercooled water at −6 °C.

EXPERIMENTAL SECTION

Materials. Tris buffer (50 mM) was prepared from 2 M tris stock (Fisher BP1759) and adjusted to pH 8.50 with ~1 M hydrochloric acid. Alkaline phosphatase from bovine intestinal mucosa was obtained as a lyophilized powder (Sigma P6772). Aliquots of an 80.4 DEA units mL^{-1} stock AP solution in 50 mM tris/0.01% (m/v) bovine serum albumin (BSA, Fisher BP 671-1) were kept at −20 °C until needed. To minimize variability, all assays were prepared from aliquots from a single AP stock solution. There was no evidence of loss of AP activity over a period of two months. Aliquots of a stock 4-methylumbelliferone phosphate (MUP, Acros 41504) solution in anhydrous dimethyl sulfoxide (DMSO) at a concentration of 0.190 M were kept at −20 °C until needed. Standards were prepared in tris buffer from stock solutions of coumarin 4 (Acros 40549) in methanol (HPLC grade, Fisher) and aqueous resorufin sodium salt (Aldrich 230154). All water used was high-purity, deionized grade (18 MΩ, 0.2 μm filtered).

Acoustic Levitator. A diagram of the acoustic levitator apparatus and a photograph of a levitated droplet are shown in

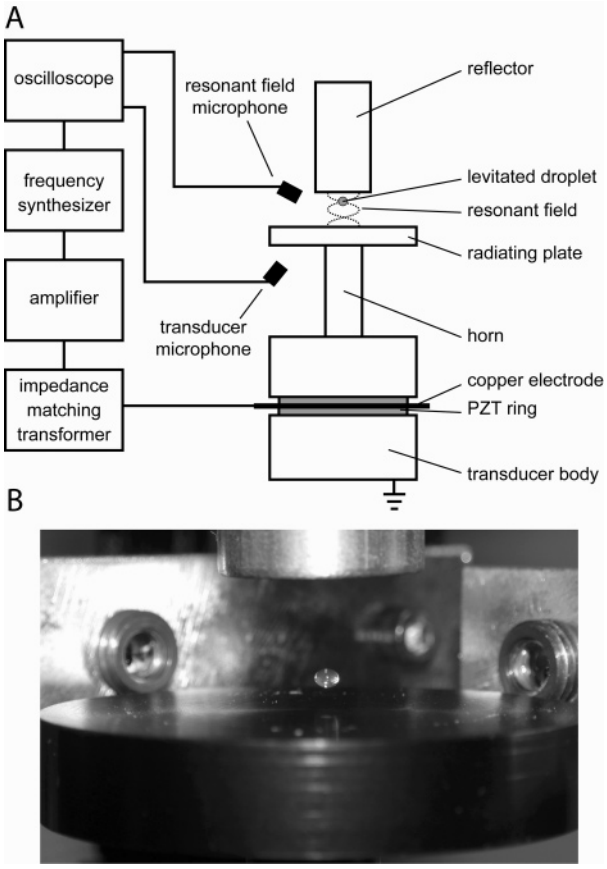


Figure 1. (A) Acoustic levitator apparatus. (B) Photograph of a 10-μL levitated droplet.

Figure 1. A resonant acoustic field (standing wave) was established in the vertical direction (*z* axis) between a high-intensity sound source and a reflector. Small (1−5 mm) droplets could be levitated in the nodal points of the standing wave. A ~29.5-kHz sinusoidal signal was generated by digital function generator (Agilent model 33120A), amplified by a 75-W wide-band amplifier (Krohn-Hite model 7500), and impedance-matched to the ultrasonic transducer with an impedance matching transformer (Krohn-Hite model MT-56R). The ultrasonic transducer[18] consisted of two lead zirconium titanate (PZT) rings compressed in a sandwich configuration between two aluminum cylinders. The acoustic signal was amplified through a stepped horn and emitted by a circular plate. Electrical contact was established through a thin copper electrode sandwiched between the two PZT rings. The two halves of the transducer body were brought into electrical contact and grounded via a bolt that passed through the center of the rings. A polymer sleeve surrounding the central bolt prevented short circuiting between the PZT rings and ground.

Two microphones (Radio Shack model 33-3013) were used to monitor the performance of the levitator. The amplitude of the microphone signals was observed on an oscilloscope. The transducer microphone, positioned beneath the radiating plate, was used to monitor the transducer output independent of the reflector position. The resonant field microphone, positioned in the vicinity of an antinode of the resonant field, was used to monitor the quality of the resonant field. The transducer has a sharp resonance

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near 29.5 kHz; however, the resonant frequency varied slightly as a function of temperature, so it was necessary to tune the transducer to resonance just prior to levitation by adjusting the frequency of the input signal until the transducer microphone signal reached a maximum. A 1.9 cm diameter stainless steel cylinder served as the reflector. The reflector was mounted on a tilt/swivel mount (Edmund Industrial Optics K37-922) that was used to ensure that the transducer and reflector faces were parallel. The tilt/swivel mount was attached to a vertical (*z* axis) linear stage (Newport model 423) driven by a linear actuator (Zaber Technologies model T-LA28) controlled by in-house software. Adjustment of the linear actuator changed the vertical gap distance between the transducer and reflector. The linear actuator was mounted onto a pair of orthogonal linear stages (Newport model TSX-1D) aligned in the *x* and *y* directions. These linear stages were used to center the reflector over the transducer. Once the transducer was tuned to resonance, the reflector−transducer vertical distance was adjusted until the signal from the resonant field microphone signal reached a maximum. Initially, the reflector position was adjusted using all five degrees of freedom (i.e., *x*, *y*, *z*, and the tilt and swivel angles) until stable levitation was obtained. Once the reflector was aligned, only the vertical distance required adjustment.

The entire levitator assembly was mounted to a custom-built aluminum scaffold. The scaffold and optics (see below) were mounted onto an aluminum breadboard and enclosed in a light-tight insulated housing. The temperature inside the housing could be varied between −18 and 25 °C by circulating a refrigerated solution of 50% ethylene glycol through three 6-m coils of $^1/_4$-in.-diameter copper tubing placed in three of the corners of the housing. A circulating chiller (Neslab RTE-140) was used to refrigerate the circulating fluid. To slow droplet evaporation in 22 °C experiments, the humidity inside the housing was maintained at 60−70% RH by flowing humidified nitrogen through the housing. The nitrogen was humidified by bubbling the gas through water. Four small fans inside the housing provided mixing to eliminate temperature and humidity gradients. The air flows were not directly incident upon the levitated droplet. The humidity and air temperature inside the chamber were measured with a digital hygrometer (Fisher model 11-661-7B).

Aqueous droplets (10−15 μL, 2.7−3.1-mm diameter) were injected into an acoustic node using a micropipet (Gilson model P20) with a gel loading tip (Rainin GT-060). To reduce adhesion between the droplet and the tip, the tip was rendered hydrophobic by briefly immersing it in a 0.5% (m/v) solution of paraffin in petroleum ether and then allowing it to dry.[19] The pipet tip was positioned just outside of an acoustic node so that as the liquid emerged from the tip it formed a droplet in the node. The droplet was dislodged from the pipet tip by increasing the amplitude of the input signal. Once the droplet was dislodged, the signal amplitude was reduced. A door on the side of the housing and an observation window on the front permitted the injection of droplets into the levitator. To maintain temperature and humidity conditions inside the chamber during droplet injection, the pipet was inserted into the chamber through a pair of polyethylene curtains.

Fluorescence Spectroscopy. The configuration of the optical components is shown in Figure 2. The fluorescence excitation source was a deuterium tungsten/halogen dual light source (Ocean Optics DH2000). The excitation light passed through a variable long-pass filter (Ocean Optics model LVF-H) adjusted to minimize MUP fluorescence (50% cutoff at 321 nm). A pair of fiber-optic collimating lenses (Ocean Optics model 74-UV) served as the excitation and emission optics. The lenses were positioned 3 cm from the droplet and mounted on *xyz* stages (Newport MT-XYZ) so that they could be aligned with the droplet. Fluorescence emission spectra of the droplet were collected using a fiber-optic spectrometer with a charge-coupled device array detector (Ocean Optics USB2000). All optical connections were made with 600-μm-diameter optical fibers (Ocean Optics model P-600-2-UV−VIS); a solarization-resistant fiber was used between the light source and the long-pass filter (Ocean Optics model P600-2SR). Cuvette enzyme assays were performed in a 0.3 cm × 1 cm × 4.5 cm quartz cuvette using the same optical setup except that a cuvette holder (Ocean Optics model CUV-FL-DA) took the place of the levitated droplet. In kinetic assays, an emission spectrum was collected every 8.2 s. A software-controlled shutter in the light source was kept closed between measurements to prevent photodegradation of MUP and coumarin 4.

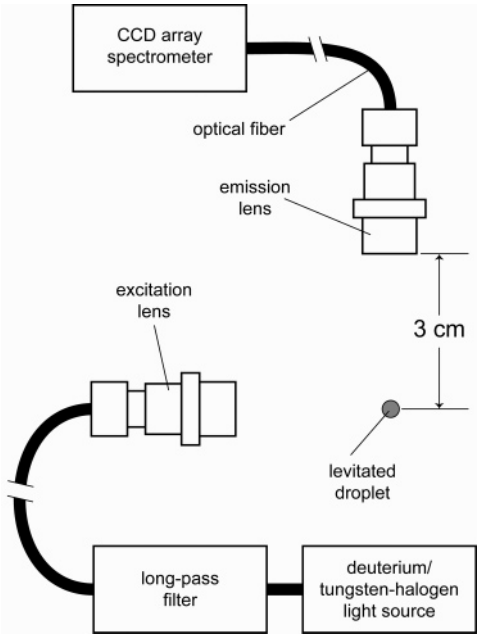

Figure 2. Schematic diagram of the optical components.

Typical fluorescence emission spectra of MUP and coumarin 4 are shown in Figure 3. The emission wavelengths used to monitor MUP, coumarin 4, and resorufin (an internal standard, see Results and Discussion) were 388, 475, and 581 nm, respectively. Although only weakly fluorescent, the MUP substrate was present at a high concentration in the reaction mixture. The shoulder of the MUP fluorescence emission overlapped with the maxima of the coumarin 4 product emission (447 and 475 nm). Since MUP was consumed over the course of the reaction, its fluorescence signal at the coumarin 4 emission wavelength (475 nm) was not constant; thus, it was necessary to subtract it. A two-wavelength mixture analysis method using the emission maxima of both MUP and coumarin 4 was used to subtract the MUP contribution to the fluorescence signal at the coumarin 4 emission wavelength. This is described in the Supporting Information.

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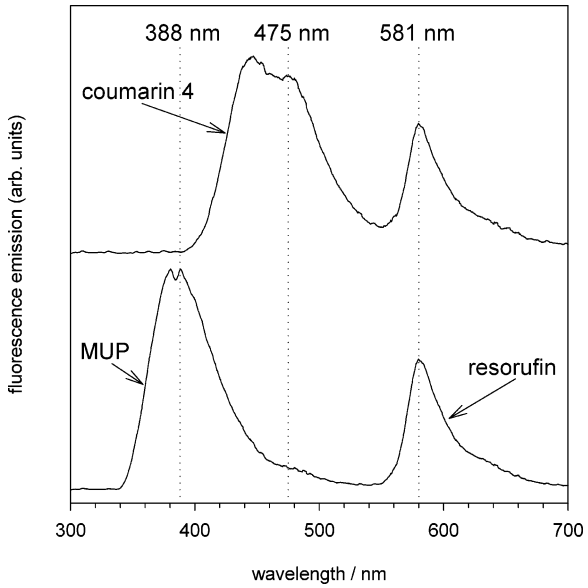

Figure 3. Fluorescence emission spectra of levitated 15-μL droplets of 8 μM coumarin 4 and 200 μM MUP standards. Both droplets also contained resorufin internal standard at a concentration of 200 nM. The vertical lines show the emission wavelengths used for quantitative measurements.

Enzyme Assay. The rate of the AP-catalyzed hydrolysis reaction was measured at pH 8.50 in 50 mM tris buffer. The concentration of AP was 0.017 DEA units mL^{-1} (~33 pM for the homodimer). The MUP substrate concentration was 200 μM (~20K_m, unpublished results). A small amount of dimethyl sulfoxide (0.1 vol %) and BSA (21 μg L^{-1}) was incorporated into the reaction mixture because they were present in the stock solutions of MUP and AP, respectively. Resorufin was added as an internal standard (see Results and Discussion) at a concentration of 200 nM. The reaction was started by substrate initiation. In levitation experiments, substrate initiation was followed immediately by injection of a droplet of the reaction mixture into the levitator. There was no evidence of MUP hydrolysis in the absence of AP, and no emission above background at 475 nm (coumarin 4 wavelength) was observed in the absence of MUP.

Safety Considerations. The acoustic levitator described here has an exposed high-voltage electrode. Touching the electrode could result in serious injury. It is recommended that all exposed metal parts be grounded to reduce the chance of electric shock. The authors have not observed any ill effects from exposure to high-intensity ultrasound.

RESULTS AND DISCUSSION

Calibration. Although droplets could be held in the levitator routinely for 10 min, instabilities in the levitator resulted in both short-term fluctuations in the fluorescence signal (i.e., wiggle) and long-term drift. These effects are evident in the plots of normalized coumarin 4 and resorufin emission versus time shown in Figure 4A. The instabilities cause changes in the position of the droplet. Since the excitation and emission optical paths are fixed in place, these instabilities result in instability in the observed emission intensity. The droplet wiggle is most likely caused by the action of the fans and by the flow of humidified gas in the enclosure. Droplet evaporation is the source of the observed long-term drift.

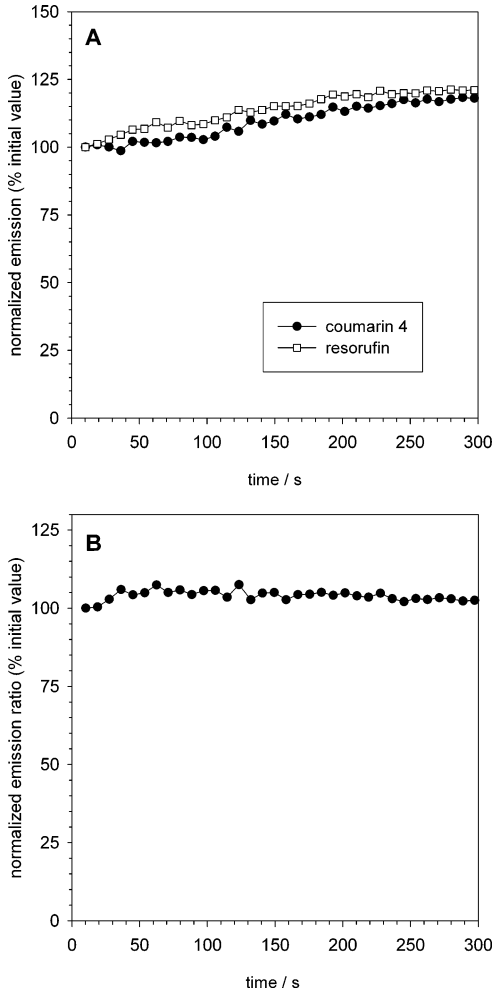

Figure 4. Fluorescence emission intensity versus time for a 15-μL levitated droplet containing 8 μM coumarin 4 and 200 nM resorufin. Each trace was normalized so that the initial value was 100%. (A) Coumarin 4 emission at 475 nm and resorufin emission at 581 nm. (B) Internally standardized by taking the coumarin 4-to-resorufin emission ratio from (A).

What is of particular importance is that, despite the instabilities, the coumarin 4 and resorufin emission track each other well. Thus, resorufin, or another fluorophore that is easily resolved from the analyte, can serve as an internal standard. By holding the concentration of resorufin constant, the ratio of coumarin 4-to-resorufin emission can serve as an analytical signal that it is independent of both droplet size and position. Figure 4B, which shows the ratio of the two signals shown in Figure 4A, demonstrates the improved stability of the analytical signal over a 5-min period. A typical internally standardized linear calibration at 22 °C gave $R^2 = 0.998$ and calibration at −6 °C gave $R^2 = 0.94$.

Kinetics of AP at Room Temperature. Under the conditions described here, the AP-catalyzed reaction is consistent with the Michaelis−Menten kinetic mechanism[20] with a $K_M \approx 10$ μM (unpublished results). At a substate concentration of 200 μM, the reaction takes place under saturated conditions at a maximal rate determined solely by the AP concentration. Over the 300-s time period of these experiments, there is no significant decrease in substrate concentration so the rate of the reaction will be constant.

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Table 1. Rate of AP-catalyzed MUP Hydrolysis

temperature (°C)	volume	rate ± s (nM s^{-1})	mean $R^2 \pm s$	no. of meas
22 ± 2	~1 mL	11.8 ± 0.4	0.99946 ± 0.00005	5
22 ± 2	15 μL	12.0 ± 0.7	0.959 ± 0.075	5
−6 ± 2	12 μL	2.1 ± 0.2	0.617 ± 0.049	3

Table 1 summarizes data from a number of measurements of the reaction rate at 22 ± 2 °C in a 15-μL levitated droplet, in ~1 mL of solution in a quartz cuvette, and in a 12-μL levitated droplet at −6 ± 2 °C. The rate of the reaction was determined by linear regression; mean values of the square of the correlation coefficient (R^2) and its standard deviation (s) are also listed in Table 1. The rate of coumarin 4 formation in the levitated droplet (12.0 ± 0.7 nM s^{-1}) was in excellent agreement with the rate observed the cuvette (11.8 ± 0.4 nM s^{-1}). Under the saturating conditions used here, the rate of the reaction is diffusion-limited.[21] Streaming flow around the levitated droplet[22] can cause circulation or agitation of the fluid within the droplet. This circulation might result in an elevated rate for this diffusion-limited reaction if it significantly increased the rate of mass transfer within the droplet. However, the consistency of the reaction rate in the cuvette and the levitated droplet at 22 ± 2 °C indicates that the circulation is negligible and that the levitation process does not perturb the kinetics of the reaction.

Kinetics of AP in Supercooled Solution. Measurements on the rate of the reaction in supercooled water have also been made. At −6 ± 2 °C, the rate of coumarin 4 formation was found to be 2.1 ± 0.2 nM s^{-1} (Table 1). At −6 °C, the levitated droplets were not as stable in the levitator as droplets at 22 °C. This is evident in the lower R^2 value, which indicates more scatter in the data. There is a 6-fold slowdown in the rate of the reaction going from 22 to −6 °C. At −6 °C, however, the pH is not 8.5, since the K_a of tris is temperature-dependent. While we cannot directly determine the temperature of the levitated droplet, three lines of evidence indicate that the droplet is indeed supercooled. First, some levitated droplets were observed to freeze rapidly (<1 min) in the levitator, presumably caused by heterogeneous nucleation. This indicates that cooling of the droplet in the levitator is rapid. Second, when freezing occurred, the droplet became opaque and the fluorescence emission spectrum was overwhelmed by scattering of the excitation light. Visual inspection of the droplets used to measure the rate at −6 ± 2 °C did not indicate freezing, nor was light scattering observed in the emission spectrum. Finally, there was no evidence of a slowdown in the rate of coumarin 4 formation over the course of the reaction as would be expected if the droplet cooled slowly. Bovine intestinal AP has already been shown to be active down to much lower temperatures, but only in the presence of significant amounts of antifreeze: Champion et al.[23] observed AP activity in concentrated sucrose solutions (10−58 wt %) down −24 °C using the substrate *p*-nitrophenyl phosphate, and Bragger et al.[24] observed AP activity down to −100 °C in 60% DMSO/20% ethylene glycol/20% water with MUP as the substrate.

Refining the Prototype Instrument. There are some limitations to this preliminary study on AP using our prototype instrument that need to be corrected. The most significant is the limited degree of supercooling. While we have attempted to measure AP activity in supercooled water at lower temperatures, the stability of the droplet in the levitator became poor. This may have been caused by temperature instability within the transducer itself since the resonant frequency is temperature-dependent. This process has a complex feedback since the transducer will draw less current off-resonance causing it to cool slightly. Trinh[18] described an acoustic levitator with automatic feedback control of the reflector position, transducer frequency, and signal amplitude that functioned over the −40 to 150 °C temperature range. Incorporation of such feedback control will greatly improve the stability of the levitated droplet and enable levitation at lower temperatures. A second refinement that is necessary is a direct determination of the droplet temperature. This has already been reported in levitated droplets using Eu^{3+}(EDTA) fluorescence emission[25] and La_2O_2S·Eu phosphorescence lifetime[26] measurements. Another necessary improvement is that the pH of the supercooled droplet must be determined accurately. While the temperature-dependence of the K_a values of many buffer systems are known in the temperature range of liquid water, it is not clear if these data could be extrapolated for use with deeply supercooled water. Spectrometric determination of pH using a fluorescent indicator presents a conundrum since both the K_a of the indicator and the weak acid form of the buffer will be unknown in the supercooled solution as both are temperature-dependent. However, Douzou[27] described a stepwise method of establishing a pH scale in supercooled liquids starting with 0.01 M HCl. Since 0.01 M HCl is fully dissociated, the proton activity can be estimated using extended Debye−Hückel theory. This HCl solution then serves as an accurate standard on which to establish a supercooled pH scale. An alternating series of indicators and buffers with increasingly basic pK_a values is then used to calibrate the pH scale. Finally, droplet evaporation limits the time scale of the experiments and causes the concentration of the reactants to increase over time. This can be overcome by raising the humidity inside the chamber or by adding microdroplets of water to the levitated droplet over the course of the experiment.[12]

CONCLUSIONS

For a number of years, the use of supercooled water and aqueous solutions has been of interest in the field of enzymology. Acoustic levitation permits the preparation of supercooled aqueous solutions without the need to resort to antifreeze, water-in-oil emulsions, or narrow capillaries. We have described here an acoustic levitator apparatus and reported its first use in a kinetic study on an enzyme. The rate of the AP-catalyzed hydrolysis of MUP under saturating conditions (i.e., high substrate concentration) at 22 ± 2 °C in a levitated droplet was found to be same as in bulk solution, indicating that the levitation process does not disrupt the enzyme kinetics. A 6-fold decrease in the rate was

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observed when the reaction was carried out in supercooled aqueous solution at −6 °C.

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SUPPORTING INFORMATION AVAILABLE

Derivation of a two-wavelength mixture analysis method for overlapping emission spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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