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Enzyme Catalysis in Water Pools

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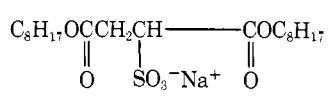
Abstract: α-Chymotrypsin catalyzes the hydrolysis of *N*-acetyl-L-tryptophan methyl ester in water pools consisting of 0.11 M Aerosol OT and 2.8 M H_2O (buffered at pH 7.0 with 0.01 M phosphate) in heptane. The k_{cat} and K_M(app) were found to be 0.63 s^{-1} and 2.5×10^{-4} M (compared with 28 s^{-1} and 9.5×10^{-5} M for the same reaction in bulk water). Titration of the enzyme inside the pools with *p*-nitrophenyl acetate showed that the reduced activity at pH 7.0 is not the result of protein denaturation. Instead, the α-chymotrypsin solubilized in 95% heptane experiences a 1.5-unit shift to the right in its sigmoidal rate-pH profile. This shift places pH 7.0 on the low-pH plateau of the profile, thus greatly diminishing the apparent rate. At higher pH values (where the enzymatic rates reach their maximum), pool-incorporated enzyme actually has a *larger* k_{cat} than does enzyme in bulk water. Enzyme activity is insensitive to the pool size, which was varied from much smaller to much larger than the enzyme. This suggests that α-chymotrypsin molecules "create" their own micelles in the heptane rather than occupy empty ones already present. Circular dichroism studies indicate no major conformational changes in the protein within the water pools.

"Water pools" refer to inverted micelles containing a large quantity of water.[1-3] They are formed, for example, by adding water to heptane solutions of certain ionic surfactants such as bis(2-ethylhexyl)sodium sulfosuccinate (called Aerosol OT or AOT).[4-6] Presumably, clusters of water in the heptane are encased by AOT so that the alkyl chains lie in the aprotic solvent, whereas the sulfonate groups dip into the aqueous regions. A homogeneous mixture of 10% water in heptane can be prepared with only 0.1 M AOT.



The recent discovery by Martinek, Berezin, and co-workers that enzymes dissolve and retain activity in water pools[7] prompts us to report now a detailed examination of α-chymotrypsin in water pool systems composed of 95–98% heptane. Apart from the inherent interest in determining how enzymes behave under these unusual conditions, we were motivated by the following questions: How does enzyme activity depend on the size of the water pools? What happens when the pool size becomes smaller than that of the enzyme? What is the pH-rate profile inside a pool? Does the enzyme experience pronounced conformational changes within the pool? How does substrate transport across the heptane-water boundary affect the enzyme kinetics? These questions concerning micellar chymotrypsin have not been previously addressed.

From a practical standpoint, pool-entrapped enzymes have potential utility in enzyme-mediated syntheses involving water-insoluble compounds. Incorporating enzymes into pools

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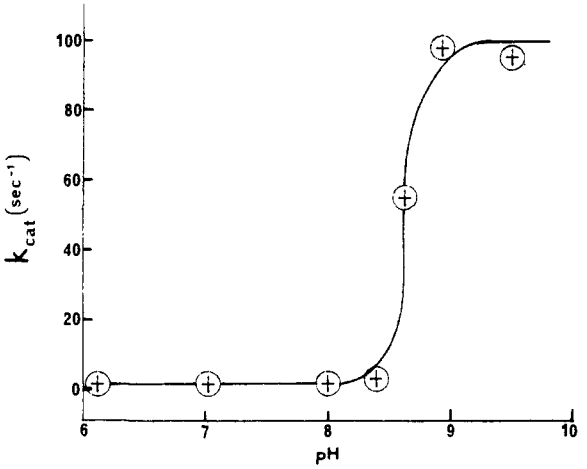


Figure 1. The rate-pH profile at 25.0 °C for the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan methyl ester in water pools (0.11 M AOT and 2.8 M H_2O in heptane). The inflection point for the corresponding curve in bulk water lies at pH 7.0.

also permits the study of interfacial biological reactions without the experimental difficulties that often accompany more than one phase.[8] A French group this year has successfully applied water pools to cryoenzymology.[9]

Experimental Section

Materials. Fisher Aerosol OT ("100% dry" solid) was purified by adding 30 g to 60 mL of warm methanol. The mixture was then cooled in ice and centrifuged while cold. After we discarded the sediment, the supernatant was stirred overnight with 3.0 g of Darco A-60 activated charcoal. The charcoal was separated by filtration, and the AOT was recovered by removing the solvent with a rotary evaporator and drying the residue over P_2O_5 for 12 h in an evacuated desiccator (0.1 mm).

α-Chymotrypsin (three times crystallized) was obtained from Worthington. *N*-Acetyl-L-tryptophan methyl ester (U.S. Biochemicals) and *N-trans*-cinnamoylimidazole (Sigma) were recrystallized from ethyl acetate and *n*-hexane, respectively. Both were stored in a refrigerated desiccator. *p*-Nitrophenyl acetate (Aldrich) was recrystallized twice from benzene-hexane. Aldrich "Gold Label" spectrophotometric grade heptane was used as received, as were the reagent-grade buffer components. Deionized water was used throughout.

Kinetic Measurements. In a typical run, 10-mm cuvettes were filled with a 3.00-mL heptane solution consisting of 0.11 M AOT, 2.8 M H_2O (buffered at pH 7.00 with 0.01 M phosphate), and 5.0×10^{-4} M *N*-acetyl-L-tryptophan methyl ester. After thermostating the stoppered curvette at 25.0 ± 0.1 °C for 15 min in an Acta II UV spectrophotometer set at 300 nm, the enzyme-catalyzed ester hydrolysis was initiated by adding 100 μL of 6.8×10^{-5} M α-chymotrypsin dissolved in the above water pool system but lacking substrate. The absorbance was then traced as a function of time. Initial velocity data from this run and six others at different ester concentrations (all performed in duplicate) were analyzed in the usual Lineweaver-Burk manner (assisted by an HP9830 calculator-plotter and a linear least-squares program). Changes in absorptivity ($\Delta\epsilon$) required for calculation of the Michaelis-Menten parameters had to be determined for each set of conditions (pH, pool size, etc.). Values of $\Delta\epsilon$ ranged from 90 to 230.

Results and Discussion

The size of a water pool in heptane depends primarily on the $[H_2O]/[AOT]$ ratio (hereafter designated "*R*").[10] For example, when $R = 2.8$ the average aggregation number of AOT is 31; this increases to 324 when $R = 22$. It is thus possible to alter the size of water pools continuously up to an *R* of about 50, above which phase separation occurs. Most of our work was carried out with [AOT] = 0.11 M and $[H_2O]$ = 2.8 M in heptane where the pools are in the "medium size" category ($R = 25$). Optically clear solutions of α-chymotrypsin were secured by hand shaking heptane-AOT-water over the solid enzyme (pure heptane being totally unable to dissolve the protein). The stability of α-chymotrypsin solubilized in heptane varies with *R*. We observed no turbidity after 50 h at room temperature with an 8.1×10^{-5} M enzyme solution having an *R* of 48; a slight turbidity was evident with an $R = 25$ solution after 25 h.[11] In our hands, the activity of enzyme dissolved in pools of pH 7.0 and $R = 25$ decreased about 15% in 24 h at 25 °C. Enzyme solutions were, consequently, always prepared immediately prior to the kinetic runs.[12] Since 0.11 M AOT was in 10^3-10^5 M excess over the enzyme, the pools would not be expected to contain more than one enzyme molecule each.

Initial runs with enzyme in pools buffered at pH 7.0 led to a $k_{cat} = 0.63$ s^{-1} and K_M(app) $= 2.5 \times 10^{-4}$ M at 25.0 °C for the hydrolysis of *N*-acetyl-L-tryptophan methyl ester (*N*-ATME). This compares with $k_{cat} = 28$ s^{-1} and K_M(app) $= 9.5 \times 10^{-5}$ M for the same reaction in bulk water[13] at pH 6.98 and 25.0 °C. The reduced k_{cat} for pool-incorporated α-chymotrypsin could signify 100% enzyme with 2% bulk-water activity, 2% enzyme with 100% activity (the balance of the enzyme being denatured), or some intermediate situation. This type of uncertainty (unresolved in the two other recent kinetic investigations of solubilized enzymes[7,9]) can be removed only by analyzing the levels of inactive enzyme in the pool solutions. We thus attempted to isolate the enzyme from the water pool media at reduced temperatures and to titrate it in bulk water according to known procedures.[14] Unfortunately, our efforts to recover any active enzyme from the relatively large amounts of surfactant were unsuccessful. We did, however, manage to titrate α-chymotrypsin while the enzyme was solubilized in heptane-AOT-water at pH 8.9. Our method was similar to one described in the literature for the titration of α-chymotrypsin with *p*-nitrophenyl acetate in bulk water.[15] The titration showed that about 74% of the weighed enzyme was active immediately after preparing the pool solutions (compared to 84% in bulk water). Hence, the 50-fold decrease in k_{cat} represents a general reduction in enzyme reactivity, *not* 98% enzyme denaturation.

AOT has little effect on the k_{cat} for the α-chymotrypsin-catalyzed hydrolysis of *N*-ATME in *bulk water;* thus, 1.2×10^{-4} M AOT in pH 7.0 buffer (close to saturation) gave the same k_{cat} as did pH 7.0 buffer without AOT. Diamylsodium sulfosuccinate, a more water-soluble surfactant than AOT, lowered the bulk-water k_{cat} by a factor of less than 2 at concentrations of 5.0×10^{-2} M. Pool systems with three different AOT concentrations (0.055, 0.11, and 0.20 M) gave the same k_{cat} (0.64, 0.63, and 0.65 s^{-1}) at constant pH and *R* (7.0 and 25). These experiments made it seem unlikely that the impaired enzyme activity in pools of pH 7.0 stemmed solely from an AOT-induced unfolding of the enzyme.

The origin of the apparent decrease in enzyme activity in neutral pools became apparent upon our elaborating the entire rate-pH profile (Figure 1). The sigmoidal curve for the *N*-ATME hydrolysis resembles that of the corresponding bulk-water reaction[16] except for a 1.5-unit shift to more basic pH values. Since this shift places pH 7.0 on the low-pH plateau of the profile, the observed rate in the pool is greatly diminished. At high pH values, where the pool reaction reaches its fastest velocity, the enzyme has a $k_{cat} = 98$ s^{-1}, which is actually 2 times *greater* than the maximum k_{cat} for the same reaction in bulk water.

The pH values used in Figure 1 are those of the aqueous buffers from which the water pool solutions were prepared. A question arises as to the relationship between these numbers and the actual proton activity within the pools. When the pH electrodes were immersed directly into a heptane-AOT-water solution, the meter response agreed fairly closely with the pH of the original buffer provided that the water content in the

Table I. Apparent pHs of Water Pools Prepared with Borax or Phosphate Buffers in Heptane as a Function of R[a]

R	0.01 M borax, pH 9.80	0.01 M phosphate, pH 7.00	deionized water, pH 6.13
4.1	6.8		
13.9	9.2		
25.2	9.7	6.7	5.4
51.3	9.8		

[a] [AOT] = 0.11 M.

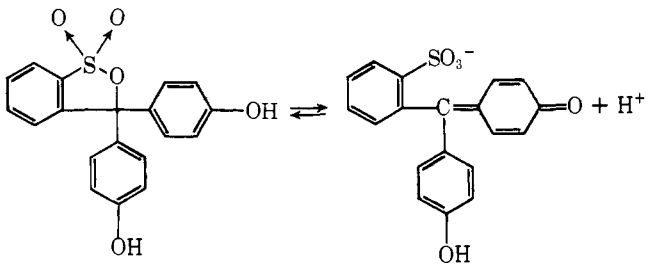
Table II. Effect of R Values on the Michaelis-Menten Parameters for α-Chymotrypsin-Catalyzed Hydrolyses of N-ATME in Water Pools[a]

R	k_{cat}, s^{-1}	K_M(app) $\times 10^4$, M
12	(0.93)[b]	(2.3)[b]
15	0.82	2.1
25	0.63	2.5
40	0.58	3.2
50	0.48	3.5

[a] Buffered at pH 7.0 with 0.01 M phosphate; 25.0 °C; [AOT] = 0.11 M in heptane. [b] Enzyme solutions became turbid after 2 h.

heptane was sufficiently high (Table I). For example, pool systems with R values of 25 prepared with a pH 9.80 borax buffer or with a pH 7.00 phosphate buffer gave meter readings of 9.7 and 6.7, respectively. Only when R values were small did large deviations appear between the buffer and pool. Thus, a pool solution of $R = 4$ containing a pH 9.80 buffer displayed an apparent pH of only 6.8. This discrepancy is hardly surprising in view of the trace quantities of water (<1%) available to the electrodes. In any event, the data for the larger pools ($R \geq 25$) indicate that the 1.5-unit shift is not an artifact but arises from a bona fide pK_a change at the active site.

The sigmoidal rate-pH profile of α-chymotrypsin-catalyzed reactions originates from the ionization of an imidazolium ring at the active site. Apparently, the negatively charged water pool interface, created by the sulfonate groups, stabilizes the imidazole conjugate acid so that it is 1.5 pK_a units less acidic. If this is true, then phenolic compounds should be likewise stabilized and thus less acidic in pools than in bulk water. Such was found to be the case for phenol red. The dye in a pool of $R = 15$ has a pK_a of 8.6 compared to 7.9 in bulk water (Figure 2). In summary, α-chymotrypsin in pools of $R = 25$ seems to behave rather normally aside from a pK_a shift that also manifests itself with a nonprotein acid.



An AOT pool with $R = 11$ has a radius of about 20 Å (too small to house the ellipsoidal α-chymotrypsin molecule of dimensions 40 × 40 × 51 Å).[17] As R increases from 11 to 44, the pools enlarge to sizes greatly exceeding that of the enzyme. Two possibilities presented themselves with regard to the behavior of the enzyme in small pools: (1) The enzyme could compress, distort, or otherwise accommodate to pools smaller than itself in which case a reduced enzyme activity was likely. (2) The pools could build around the enzyme so as to satisfy

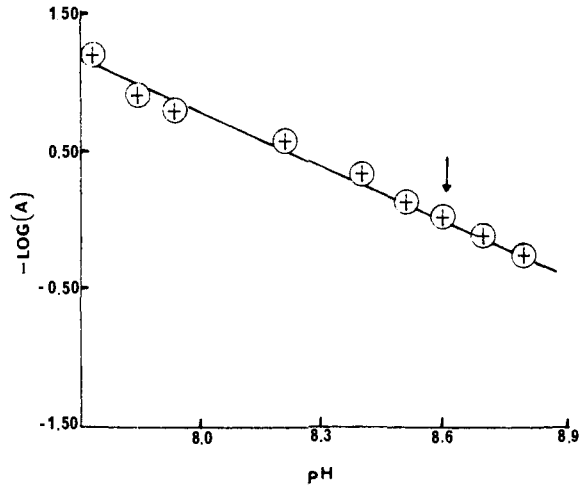


Figure 2. Spectrophotometric pK_a determination of phenol red in water pools ($R = 15.4$; [AOT] = 0.11 M; [phenol red] = 7.41×10^{-6} M). Parameter A represents the observed absorbance at 560 nm divided by the difference between the maximum possible and observed absorbance at 560 nm. The arrow indicates the pK_a of the pool-incorporated dye.

the solvation needs of the protein. In this event, k_{cat} should be relatively insensitive to R. The data in Table II show that k_{cat} varies but slightly with R. Thus, k_{cat} equals 0.93, 0.63, and 0.48 when R is 12, 25, and 50 (pH 7.0 at 25.0 °C). It seems, therefore, that enzyme molecules create their own pools regardless of the R value and the size of the neighboring empty pools. Since low levels of additives are capable of promoting "normal" micelles in bulk water,[18] protein-induced formation of inverted micelles appears reasonable. Of course, the possibility exists that even at small R values sufficient numbers of large pools are present to incorporate the protein. This rationale, however, would require pool solutions to be much less monodisperse than is generally believed.

Substrate must partition into the water pools prior to binding at the active of the enzyme. If an insignificant fraction of the substrate dissolves in the intermicellar heptane, then the value of K_M(app) would not be greatly affected by the partitioning preequilibrium. Such is the case for N-acetyl-L-tryptophan methyl ester, which is more than two orders of magnitude more soluble in water than in heptane and which has a rather normal K_M(app) of 2.5×10^{-4} M (Table II). On the other hand, another excellent α-chymotrypsin substrate, N-cinnamoylimidazole, reacts sluggishly in pool systems owing to a less favorable affinity for water. (This property made it impossible to use N-cinnamoylimidazole for titrating the enzyme.[14]) Pool systems are currently being utilized to study transport processes across interfaces under homogeneous conditions.

The kinetic results prove that α-chymotrypsin solubilized in 95% heptane suffers no major conformational change impairing catalysis.[19] Circular dichroism data also point to the conformational stability of the enzyme.[20] Thus, CD spectra from 200 to 250 nm in aqueous buffer (pH 7.0) and in water pools (pH 7.0 and 8.6) are superimposable within experimental error. Small molar ellipticity differences in the higher wavelength region (e.g., $\theta_{pool} - \theta_{buffer} = 64$ at 270 nm) indicate only minor side-chain perturbations.[21]

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(20) CD spectra from 200 to 250 nm were performed on a Jasco J-20 spectrophotometer using 0.1-mm cells containing 9.0×10^{-5} enzyme in water pools where [AOT] = 0.11 M and $R = 25$. Spectra from 250 to 300 nm were carried out with the same solutions but with 5-mm cells. We thank Professor A. J. Sophianopoulas for his assistance with the CD experiment.
(21) Note Added in Proof: We call attention to the related work of Professor P. L. Luisi in Zurich which will be published (*Biochem. Biophys. Res. Commun.*).

Rate of Exchange of Water from the Active Site of Human Carbonic Anhydrase C

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Abstract: The rate constant for the exchange of ^{18}O between CO_2 and water and the rate constant for the exchange of ^{18}O between ^{12}C- and ^{13}C-containing species of CO_2 have been measured in the range of pH 6 to 8 in the presence of human carbonic anhydrase C. Solving the kinetic equations for the distribution of isotopes, we express these data as the rates of two independent steps in the catalysis: R_1, the rate at equilibrium of the catalytic interconversion of CO_2 and HCO_3^-, and R_{H_2O}, the rate of exchange from the enzyme of water containing the oxygen abstracted from bicarbonate. R_1 is generally independent of buffer, whereas R_{H_2O} increases with the concentration of the buffer imidazole or 2,4-lutidine, then levels off near 10 mM buffer. In the presence of 50 mM imidazole or 2,4-lutidine, R_{H_2O} when plotted against pH is bell-shaped with a maximum at pH 6.8. These results are consistent with an internal proton transfer between two ionizing groups of the enzyme $E(^{18}OH)BH \rightleftharpoons E(^{18}OH_2)B$, which becomes rate limiting for R_{H_2O} at larger buffer concentrations. The data suggest a pK_a near 6.8 for each ionizing group with a rate constant for proton transfer of $3.5 \times 10^6\ s^{-1}$. In the absence of external buffers, the ^{18}O content of E(OH)B will be greater than that of E(OH)BH because of the possibility of internal proton transfer converting E(OH)BH to $E(OH_2)B$ from which exchange of water can occur. Hence, the presence of external buffer enhances ^{18}O exchange by causing rapid interconversion between E(OH)B and E(OH)BH.

The zinc-containing metalloenzyme carbonic anhydrase catalyzes the hydration of CO_2 to produce bicarbonate and a proton,[1]

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \quad (1)$$

The turnover number for the catalyzed hydration of CO_2 by bovine and human C forms of carbonic anhydrase is very large (about $10^6\ s^{-1}$) so that the catalysis can be limited in rate by the transfer of the proton between the enzyme and external buffers in solution, a transfer which is necessary to regenerate the form of the enzyme active in hydration:

$$EH + B \rightleftharpoons E^- + BH^+ \quad (2)$$

The proton transfer of eq 2, in which B indicates a buffer that is not part of the enzyme, becomes rate limiting in the catalyzed reaction at buffer concentrations less than about 5 mM, as shown by initial velocity studies[2] and measurements of the rate of ^{18}O exchange between CO_2 and H_2O at chemical equilibrium.[3]

At high concentrations of external buffers the proton transfer of eq 2 is not rate limiting; however, measurement of the solvent isotope effects in H_2O and D_2O on maximal steady-state turnover numbers for bovine carbonic anhydrase[4] and human carbonic anhydrase C[5] suggests that an intramolecular proton transfer is rate limiting. It has been proposed that the intramolecular transfer involves a proton transfer group near the active site and the catalytic group.[5] Measuring the line widths of the ^{13}C resonances of a CO_2–HCO_3^- equilibrium mixture in the presence of human carbonic anhydrase C, Simonsson et al.[6] have determined that this intramolecular proton transfer is not involved directly in the catalytic conversion of CO_2 to HCO_3^-. Studies of the inhibition of esterase activity of human carbonic anhydrase C by equilibrium mixtures of CO_2 and HCO_3^- have also suggested the presence of a rate-limiting intramolecular proton transfer.[7]

The ^{18}O exchange between CO_2 and H_2O catalyzed by carbonic anhydrase is a complement to these studies. It has

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