Cryoenzymology in aqueous media: Micellar solubilized water clusters

(subzero temperatures/inverted micelles/cytochrome *c*/trypsin)

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ABSTRACT Amphiphilic compounds dissolved in nonpolar organic solvents form inverted micelles whose aqueous centers can solubilize relatively large amounts of enzymes. The solutions are homogeneous and optically transparent and have low viscosity and freezing points; they provide unique and favorable systems to perform the main cryoenzymologic studies previously carried out in mixed solvents. The preparation and properties of such micelles are described. The absorbance spectra of cytochrome *c* at various temperatures from -38°C to 20°C are given. The pH dependence of trypsin-catalyzed hydrolysis of benzoylarginine ethyl ester in inverted micelles, as well as kinetic properties and activation energy, is described. Some problems are analyzed.

Fluid aqueous-organic mixtures have been used extensively in studies of enzyme-catalyzed reactions to stabilize intermediates and to accumulate kinetic and thermodynamic information (1, 2). Although attractive, such procedures have problems associated with the presence of high concentrations of organic cosolvents, even when one can demonstrate that they modify neither the enzyme's specific activity nor the reaction pathway. They generally do affect the binding of substrates with the enzyme as well as reaction velocity. An ideal procedure would permit investigation of temperature effects on reactions in unmodified aqueous media in which the observed results would reflect only a "pure" temperature change of enzyme specific activity.

Recently (3-5), we have demonstrated the usefulness of water-in-oil emulsions to stabilize supercooled water droplets against freezing due to heterogeneous nucleation. The severe limitations to optical and stopped flow measurements resulting from abnormally high turbidity and viscosity prompted us to seek new procedures to provide highly fluid transparent micellar solutions. The principle adopted is rather simple: many amphiphilic surfactants form micelles in nonpolar organic solvents and in aqueous solutions. They are, however, "inverted" relative to the aqueous phase; the water-soluble head groups build the polar core of the micelle shielded by the apolar hydrocarbon tails. As a result the micelles contain relatively large amounts of water, and many water-soluble molecules, including enzymes, dissolve in the water droplets held by the nonaqueous oil-soluble surfactant solutions.

The present paper reports preliminary observations on various enzymes whose solutions remain homogeneous, optically transparent, and of low viscosity over a broad range of subzero temperatures. Cryoenzymologic experiments are performed satisfactorily, in spite of specific residual problems, which are analyzed briefly.

MATERIALS AND METHODS

Surfactants. Amphiphilic compounds possess two groups in the same molecule that differ greatly in solubility relationships. That is: (i) a hydrophilic group tending toward water-soluble and hydrocarbon-insoluble, and (ii) a lipophilic group tending to be hydrocarbon-soluble and water-insoluble. Amphiphilic properties are most marked in compounds with strong, and at least roughly matched, hydrophilic and lipophilic functions. The anionic surfactant bis(2-ethylhexyl) sodium sulfosuccinate (Aerosol OT, AOT), molecular weight 444.57, from Fluka (Buchs, Switzerland), has been used after purification and drying (6).

Several other surfactants of nonionic nature have been tried, including poly(oxyethylene)oleic alcohol of various degrees of oxyethylenation (Brij type, Sinulsol from Seppic, Paris, France).

Solvents. Normal heptane and silicone oils of very low viscosity [1 centipoise (cP), 1 mPa·s] (Rhodorsil from Rhone-Poulenc, Paris, France) with melting points of -90 and -50°C , respectively, were used to prepare dry solutions of surfactant by heating at $+60^{\circ}\text{C}$ for 15 min and cooling to room temperature.

Micellar Solution. The aqueous system (enzyme solution) was added to the dry solvent-surfactant solution with a microsyringe, and the solution was shaken, then treated in an ultrasonic bath at 4°C to ensure that the aqueous enzymes solution was taken up by the micelles. Homogeneous micellar solutions were tested spectroscopically at 350 nm to check for transparency (whether or not they were colorless). The testing was also continued as a function of temperature until the turbid region was reached.

Enzyme Assays. A number of enzymes have been investigated; observations with cytochrome *c* (Sigma, type VI) and trypsin (Sigma, type III) are presented as examples.

Absorbance spectra were recorded between $+30$ and -40°C with an Aminco DW 2 spectrophotometer. Light-scattering measurements of micellar solutions were made in both a Beckman Acta III spectrophotometer and an Aminco Bowman spectrofluorometer, each equipped with a thermostated cell compartment and a temperature control device described elsewhere (7).

Tryptic enzyme activity was recorded with the Aminco DW 2 in the dual wavelength mode between 255 and 290 nm using as substrate benzoyl-L-arginine ethyl ester (BzArgOEt, highest quality grade from Fluka). The reaction was initiated by mixing in the sample cuvette 50 μl of enzyme micellar solution and 950 μl of a substrate micellar solution. In standard experiments, the

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Abbreviations: AOT, Aerosol OT [bis(2-ethylhexyl) sodium sulfosuccinate]; cP, centipoise.

FIG. 1. Dependence of $\text{H}_2\text{O}/\text{AOT}$ ratios for AOT precipitation as a function of temperature. $\bullet$, 9%; $\blacksquare$, 3%; $\blacktriangle$, 1.5% (wt:vol) AOT. Aqueous phase, 100 mM Tris-HCl buffer, pH 9.0; nonpolar organic solvent, silicone oil (viscosity 0.65 cP). Measurements were carried out by light scattering at 350 nm.

AOT was 1.5% (wt:vol) in silicone oil (viscosity 0.65 cP) and the water phase 1% (vol:vol). The enzyme stock solution (0.1 mM) was made in 1 mM HCl and stored at $+4^\circ\text{C}$; the substrate was dissolved in the buffer. Stock solutions were prepared daily.

For the substrate measured in micellar solutions $\Delta\epsilon_{255-290\text{nm}} = 1700 \text{ M}^{-1} \text{ cm}^{-1}$. The kinetic parameters k_{cat} and $K_{\text{m (app)}}$ were determined from Lineweaver-Burk plots with a substrate concentration range from 10 to 100 mM in the aqueous phase.

RESULTS AND DISCUSSION

Effect of temperature on micellar solutions of enzymes

The micellar solutions containing AOT were examined as a function of temperature to determine the range over which they remain homogeneous. Of the three regions previously described (8)—colorless, blue translucent, and turbid—the first two are homogeneous and stable and represent the regions of water solubility; the third, appearance of turbidity, is dependent on the AOT content and the water/surfactant ratios. As shown in Fig. 1, the extent of these regions, particularly of the turbid one, are remarkably dependent on the concentration parameters; they depend also on the particular surfactant and solvent used. We found a remarkable difference in the solubility diagrams of anionic and neutral surfactants; the latter show colorless regions only at a higher temperature range ($>-15^\circ\text{C}$ under the best conditions), whereas AOT is suitable to temperatures as low as -45°C (see Fig. 1).

At subzero temperatures the water in micelles is certainly supercooled in the transparent region, as were water droplets of much larger size obtained with insoluble surfactants. Turbidity might result from a rearrangement of micellar aggregates, a change in the average number of particles, or an altered distribution of aggregates.

The presence of enzymes and of electrolytes (neutral salts, buffers) did not markedly affect the solubility of water in the nonaqueous solutions of AOT nor appreciably modify the minimum temperature of the transparent region. Fig. 2 shows that the absorbance spectra of cytochrome *c* can be recorded down to -40°C . The spectra demonstrate that cytochrome *c* is dissolved at room temperature as well as in a broad range of subzero temperatures; meanwhile ϵ values are dependent on the water/surfactant ratio. With decrease in water/surfactant ratio, ϵ values are higher and tend to become identical to the bulk water values at 6% (vol:vol) water and 9% (wt:vol) AOT. With the noticeable exception of myoglobin, the hemoproteins

FIG. 2. Absorbance spectra of cytochrome *c* in micellar solution at selected temperatures. The solution was 7.5 mM cytochrome *c*, 2% (vol:vol) H_2O (containing 200 mM sodium cacodylate buffer at pH 7.2), 9% (wt:vol) AOT, in silicone oil (viscosity 0.65 cP). —, $+20^\circ\text{C}$; ---, -10°C ; - - -, -30°C ; ·····, -38°C .

tested were in solution for investigation under these conditions. In spite of a weak absorbance by micelles below 350 nm, protein spectra can be recorded easily down to 250 nm. Thus controlled amounts of surfactant-entrapped water suspended in nonpolar solvents provide suitable media for spectroscopic investigation of enzymes at subzero temperatures. These conditions were therefore used to investigate enzymes over the normal temperature range (9, 10). More numerous studies have been devoted to the catalytic processes of polyatomic molecules (11, 12). Because, however, we are only at the beginning of the application of inverted micelles to enzymology and cryoenzymology, there is little documentation.

Numerous problems are presented by the behavior of surfactants in nonpolar solvents, and by the influence of added salts and experimental proteins. Even unrecognized impurities may affect the physical chemical properties of the micelles in, as yet, unpredictable ways. The addition of water, enzymes, or both causes the micelles to swell and to assume different shapes, which are still a matter of controversy even in the simplest conditions (13). The present results, obtained with selected enzymes, surfactants, and solvents in arbitrary ratios, cannot be generalized at this time, for all of the specific aspects of micellar enzyme solutions are not understood, let alone predictable.

The early results are, nevertheless, very encouraging. Numerous enzymes, ranging in mass from thousands to hundreds of thousands of daltons, can be solubilized in water regions and remain stable, homogeneous, and optically transparent over broad ranges of subzero temperatures.

Enzyme activity

Enzyme solutions known to accumulate characteristic intermediates—for example, the peroxidatic process of horse radish peroxidases—have been shown clearly to be stabilized and observable at subzero temperatures in reversed micelles (14).

We have also shown that several enzyme-catalyzed hydrolytic reactions—e.g., trypsin hydrolysis of BzArgOEt, are well accommodated at room temperature in selected conditions of medium. The pH-activity profiles of the tryptic hydrolysis of the BzArgOEt in micellar solutions and in bulk water are presented in Fig. 3. The conditions are defined in the legend. Micellar solutions show a marked shift in the pH profile. This shift very probably arises from the influence on the enzyme of the assembly of negatively charged polar heads of the polyanionic

FIG. 3. pH dependence of $k_3/k_{3\max}$ for tryptic hydrolysis of BzArgOEt at +22°C. —, In micelles formed with 1% (vol:vol) aqueous phase in silicone oil (viscosity 0.65 cP) containing 1.5% (wt:vol) AOT. Buffers are 125 mM sodium acetate, sodium cacodylate, Tris-HCl, and ethanolamine-HCl. Substrate, 25 mM; trypsin, 5 μ M. - - -, In homogeneous buffered water, ionic strength 10 mM (17).

environment resulting from AOT. Such data present additional strong implications of the effects of polyelectrolytes on enzyme catalysis (15, 16).

Relatively high buffer concentrations, ≈ 100 mM, are required to provide a suitable pH for enzyme activity. Activity measurements are possible over a broad temperature range, as shown in the Arrhenius plot, Fig. 4. The activation energy [11.5 ± 0.5 kcal/mol (48.1 ± 2.1 kJ/mol)] is essentially identical to the value obtained in bulk water over the narrower temperature range of 30° to 4°C.

These satisfying results are somewhat clouded by the substantial effects observed on substrate affinity as compared to those found in bulk water and in the cosolvent 1:1, vol:vol ethylene glycol/water. The respective K_m (app) values are 3.5 mM for the micelles [concentrations in the water phase, 1% (vol:vol) water], 4 μ M in bulk water, and 84 μ M in cosolvent (17); the $k_{\text{cat}}(V_{\text{max}})$ values are: 8.7 s⁻¹ in bulk water, 11.0 s⁻¹ in mixed solvent (ethylene glycol/water 1:1 vol:vol), and 9 s⁻¹ in micellar solution.

In fact, such changes should be expected when one considers the number of parameters that can influence enzymes and substrates in reverse micellar systems. Because rates of numerous organic reactions are also affected by inverted micelles (11, 12), much attention has been paid to specific rate effects (18) and to factors influencing the rates. Still, very little is known of the details of mechanisms contributing to rate enhancement or retardation. One may cite as probable mechanisms the partitioning of enzyme, substrate, or both between surfactant-trapped water molecules and those occupying the core of the micelle and differential interactions (electrostatic, hydrophobic, nucleophilic, electrophilic) altering the free energy of activation of the overall process as well as K_m and V_{max} values.

The magnitude of rate effects could be drastically altered upon changing the enzyme system and, for a given enzyme, upon changing the nonpolar solvent, the nature and concen-

FIG. 4. Arrhenius plot of k_3 for the trypsin-catalyzed hydrolysis of BzArgOEt in micellar solution. 1% (vol:vol) aqueous phase containing 200 mM Tris-HCl buffer, pH 9.0; 1.5% (wt:vol) AOT; in silicone oil (viscosity 0.65 cP).

tration of the surfactant, and the concentration of added water. The quantitative observations now available are insufficient to establish suitable relationships among factors affecting reaction rates; thus the procedures remain largely empirical.

The results with trypsin do not show an alteration in the free energy of activation when compared to values obtained in bulk water. The substantial rate alterations observed may have resulted from unfavorable substrate and enzyme partitioning and from orientation, which could be at least partially corrected by changing the surfactant. On the other hand, the type of non-polar organic solvent used (heptane or silicone oils) has been found to influence results significantly.

Stopped-flow experiments show that the exchange of dissolved solutes between micelles occurs within the millisecond range. This result is very encouraging for further fast kinetic studies. It includes evidence of a very mobile structure and a characteristic capacity of micelles to dissociate very rapidly upon local changes in conditions—e.g., in concentration upon mixing of two solutions.

CONCLUSION

Reversed micelles clearly present good potential for use in cryoenzymology. Homogeneous and optically transparent solutions of low viscosity and freezing point should permit investigation of many enzyme-catalyzed reactions in aqueous environments, possibly by rapid mixing of the reactants. Most enzymes assayed to date enjoy stability in these very mobile structures and are able to function in a broad range of subzero temperatures.

The procedure is, however, far from free of specific and still challenging problems due to the unusual conditions the medium imposes on the enzyme systems. These problems deserve careful investigation both to improve present performance and to decide whether observed results reflect the “normal” functioning of enzymes.

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