ADVANCES IN ENZYMOLOGY

AND RELATED AREAS OF MOLECULAR BIOLOGY

Founded by F. E. NORD

Edited by ALTON MEISTER

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CRYOENZYMOLOGY IN AQUEOUS MEDIA

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CONTENTS

I. Aqueous Media Withstanding Subzero Temperatures: Unfreezable Water

While fast kinetic techniques are an essential and definitely established part of enzyme studies, allowing experiments to be performed in increasingly shorter time periods, lack of information about what they mean on a molecular level is responsible for the current interest in attempting to carry out a number of reactions in more accessible time periods at lower temperatures. Enzyme studies at subzero temperatures have been reviewed in this series and elsewhere (1–4). Up to now, they have consisted mostly of the investigation of enzyme reactions that normally occur in seconds and milliseconds. These are slowed to the time scales of hours, minutes, or seconds with the stabilization and determination of many intermediates and occasionally with kinetic studies of elementary steps.

In spite of an increasing number of successful cryoenzymologic studies, two main problems characterize and rule this new area, namely, (1) the presence of high concentrations of organic solvents used as "antifreeze" that might influence reaction pathways and (2) the absolute necessity of finding a way of circumventing the technical barrier caused by the difficulty of mixing two solutions of high viscosity in a time period in the millisecond range, a difficulty encountered with most mixed solvents at subzero and sometimes at normal temperatures.

A series of suitable tests are used to check whether detected intermediates in aqueous–organic solvents are on the actual catalytic pathway observed under normal conditions with fast kinetic techniques, but in spite of very encouraging results, it appears that cosolvents can reversibly, but significantly, change the respective values of substrate affinity, heat of formation, and activation energies through changes in substrate partitioning between the active site and the bulk solvent, in protein conformation,

and so on. Changes in $K_{m\,(app)}$ and k_{cat} values might be responsible for changes in the rate-determining steps such that some intermediates could become more fleeting and others could accumulate, and therefore for artifacts that definitely should be investigated and, when found, used as "tools" to investigate the characteristics of reaction pathways (4).

In a number of cases, uncertainties raised by the effects of mixed solvents on enzyme activity could be resolved if control experiments could be carried out in fluid aqueous media at subzero temperatures. Water-in-oil emulsions make it possible to reach such a goal and to investigate down to −40 and −60°C, but we see later that these media are not free from problems. Two main types of water-in-oil emulsions have been used, each one presenting some advantages and disadvantages: depending on the surfactant used, emulsions contain water droplets micrometers in dimension that accommodate numerous enzymes without noticeable perturbations but are highly turbid and viscous, or they involve water droplets nanometers in dimension and are homogeneous, optically transparent, and of very low viscosity but are not suitable for any enzyme system or for a number of enzyme reactions. In spite of problems and limitations, the last type of emulsion makes it possible to undertake fast kinetic studies, that is, rapid mixing of reactants at subzero temperatures. Thus it is possible both to record events in a short time and to initiate chemical changes in an equally short time, a prerequisite for carrying out fast kinetics that is not met in most mixed solvents at lower temperatures. On the other hand, reactions normally occurring in the microsecond range and then only accessible to relaxation techniques could be slowed in principle to the time scale of milliseconds and then studied by rapid mixing, with all the advantages offered by this technique, were it not for the fact that the relatively limited concentrations in enzyme accommodated by the water pools would often jeopardize the detection of the reactions.

Bulk water rarely supercools more than a few degrees below its melting point and the crystallization of ice can be subdivided into two phases: the nucleation of the ice crystal and its subsequent growth, which is influenced by temperature. When conditions are such that the rate of loss and the rate of gain of water molecules by the crystal are equal, so that the crystal has an equal possibility of vanishing or growing depending on subsequent events, this crystal is said to be of critical size, and nucleation characterizes the development of a critical-sized nucleus through the random aggregation of water molecules. At low temperatures, the bom-

barding water molecules are less energetic, reducing the loss of molecules from the crystal surface, so that the critical size is smaller than at higher temperature. Theoretical relationships involved in homogeneous nucleation have been developed (5) and indicate the number of degrees below the melting point at which spontaneous homogeneous nucleation takes place. On the other hand, it has been repeatedly found that water droplets with dimensions in micrometers generally freeze well below the melting point (6); the maximum temperature to which water can be supercooled appears to be in the range of −40 to −50°C. This would be suitable to carry out cryoenzymological studies.

Water can supercool when prevented from forming macroscopic crystals, and a droplet technique has been developed in which water droplets are suspended in an insoluble (nonpolar) carrier and prevented from coalescing with a surfactant (sorbitan tristearate) (7,8). The carrier is saturated with 5% (w/w) sorbitan tristearate by gentle heating; the emulsion is formed by blending water with the cooled, supersaturated carrier in a Waring Blendor or a smaller motor-driven emulsifier. These water-in-oil emulsions are stable at subzero temperatures. The droplet diameters are micrometers in dimension. It is easy to generate 10–100 ml of emulsion containing as much as 50% (w/v) water. Emulsions are highly turbid and viscous.

An alternative and very useful medium for investigation of fluids at subzero temperatures is provided by amphiphilic surfactants. Polar groups of such surfactants are concentrated in the interior of their aggregate while their hydrophobic moieties extend into, and are surrounded by, the bulk nonpolar carrier. The nonpolar carrier dissolves the amphiphilic surfactant by gentle heating and the emulsion is formed by blending water with the cooled solution under sonication. A considerable amount of water [as much as 10–20% (w/v)] can be solubilized by such "reversed" or inverted micelles, the droplet diameters being nanometers in dimension. Reversed micellar solutions are homogeneous, optically transparent, and of very low viscosity ($\leq$1 cP), and some of them are stable at subzero temperatures.

They might be quite suitable for investigating "solubilized" membrane-bound enzymes that are inserted in media much closer to physiological conditions than they would be in bulk solvents.

Both types of emulsions present respective advantages and disadvantages for enzyme studies. They are respectively analyzed here from a physical-chemical point of view and are examined with solubilized pro-

teins, while the success and failures of their preliminary applications are reported and compared to previous applications in mixed solvents, the main goal of the present work being to check whether these media can be proposed to enzymologists as possible substitutes or probatives of mixed solvents.

II. Water-in-Oil Emulsions with Water-Insoluble Surfactant

These emulsions eliminate the transformation of the metastable supercooled states into macroscopic crystals. We see later that supercooled water can be stabilized at subzero temperatures depending on subsequent conditions of emulsion and temperature. Because of this stabilization, there is now sufficient information available on water in the supercooled state and data suggest that supercooled water has a tendency toward a more open packing of the molecules, possibly an increased similarity to ice. Studies on the nucleation and freezing of small droplets of water (1 μm to 1 cm in diameter) show freezing only at subzero temperatures and that, if a sample of pure water that normally supercools to −20°C has its thermodynamic equilibrium freezing point lowered by solutes to −2°C, it will then supercool to −22°C. It has been shown that amino acids, peptides, and proteins are efficient nucleators of supercooled water only when crystalline and are ineffective in solution (9). Thus supercooling should be unaffected by solubilized proteins, an expectation that has been repeatedly confirmed in this laboratory.

A. EMULSION PROCEDURE

Various emulsification formulae can be used: all involve sorbitan tristearate (Span 65) as a water-in-oil surface-active agent to which water is added. Oil carriers are supersaturated with Span 65, one of a number of commercially available water-insoluble surfactants; supersaturation is obtained with 5% (w/w) Span 65. Different oil carriers can be used: the most popular is *n*-heptane, but we prefered to use silicone oils (Rhodorsil from Rhône-Poulenc). Safflower or even corn oil is also suitable carrier medium that is harmless toward proteins, while oil phases containing 75% (w/w) liquid paraffin and 25% (w/w) lanolin as the surfactant have been used in physical-chemical studies of water droplets (10). All these carriers present a low freezing point ($\leq -50°C$).

The emusification carrier fluid, Span 65 in any of the carriers listed above, is prepared by dissolving the surfactant in heated oil, after which

the solution is cooled to room temperature. For good water-in-oil emulsification [in the presence of 30–50% water (w/v)], prolonged manual shaking prior to high-speed refining is necessary, presumably because air bubbles entrapped by shaking facilitate oil encapsulation of water droplets, or yet because they permit air–water–oil interfaces to form such that the oil would encapsulate the water to reduce the water–air interfacial energy.

All emulsions are characterized by a low freezing point, high turbidity and high viscosity, and water droplet diameters in the range of 1–5 μm are readily achieved as shown by light microscopy.

We currently use silicone oils as insoluble carriers. These oils are chemically inert and are characterized by a high oxygen solubility. Their intrinsic viscosities range from 0.65 to several hundred centipoises at room temperature. A low-viscosity oil (1 cP at 25°C) increased in viscosity to 4 cP at −50°C.

Emulsions obtained with silicone oils of low viscosity (<10 cP) are quite stable. However, the presence of the surfactant gives rise to a high viscosity (>50 cP) at room temperatures. Such a viscosity confers stability to the emulsions but precludes rapid mixing of reactants and therefore kinetic studies. As we see later in more detail, the intense light scattering by these emulsions raises problems in spectrophotometric recording.

Thus use of water in oil emulsions in the presence of a water-insoluble surfactant meets a number of severe limitations that are further examined when we consider applications to enzyme studies.

B. PHYSICAL-CHEMICAL PROPERTIES

1. *Supercooling and Freezing of Water Droplets*

Supercooling is to be expected in emulsions where the water phase is highly dispersed into spherical droplets whose granulonutric distribution is very narrow, the mean diameter being 3–5 μm depending on emulsion procedure. For the sake and reproducibility of investigations of supercooling and freezing, various techniques can be used and later we describe calorimetric determinations as well as fluorescence probing, which has been set up in this laboratory and might be adopted in most of biochemists laboratories.

a. Calorimetric Determinations. The total heat capacity of a calorimeter chamber containing the emulsion can be determined by conventional drift calorimetry and the differences in heat capacities

between supercooled water and ice are calculated over selected temperature intervals. Data can also be obtained by differential scanning calorimetry (DSC), a voltage proportional to the heat capacity of the sample being recorded continuously during a rapid temperature scan. The accuracy of the two methods appears to be comparable, but most works have been carried out with DSC. The DSC experiments are usually carried out by means of a power-compensating automatic calorimeter (Perkin-Elmer DSC 18, or DSC 2). Using this apparatus, it is possible to record, as a function of the temperature T_p of the sample holder, the variation of d_q/d_t, which represents the difference between the power supplied to the cell containing the sample to be studied and the power supplied to the reference cell.

It has been shown that d_h/d_t, which represents the heat exchanged per time unit by the sample itself, can be determined from d_q/d_t, whose values are obtained from the thermal signal given by the recorder of the calorimeter when a change of state occurs within the sample. In the same way, the actual temperature T_s of the sample can be deduced from the temperature T_p of the sample holder after taking into account the slight variation resulting from the change of state occurring within the sample. For instance, in the case of an emulsion of water characterized by a value of P equal to 0.25 and submitted to a steady cooling at the rate of $T_p = -1.25°K/min$, the analysis of the experiment shows that the variation of d_q/d_t with time is virtually identical to that of d_h/d_t and that the discrepancy between T_s and T_p is less than 0.5°K. According to the specifications given by the designer of the DSC 2, the extent of supercooling can be determined within 0.5°K. Consequently, the values of T_p directly recorded by the apparatus are also virtually identifical to those of T_s.

DSC recordings permit the determination of the most probable freezing temperature (T^*) of a population of water droplets dispersed within an emulsion and thermograms corresponding to the breakdown of supercooling are currently reported in literature. Such thermograms are schematized in Figure 1.

This theoretical thermogram indicates that all the droplets in the emulsion supercool to about −40°C, following which all the droplets nucleate and crystallize. Other interesting observations can be made concerning the investigation of the evolution with time of a population of water droplets held at subzero temperatures higher than T^* in order to determine the distribution in time of the breakdowns of supercooling.

These studies are of interest mainly because of the possible applications

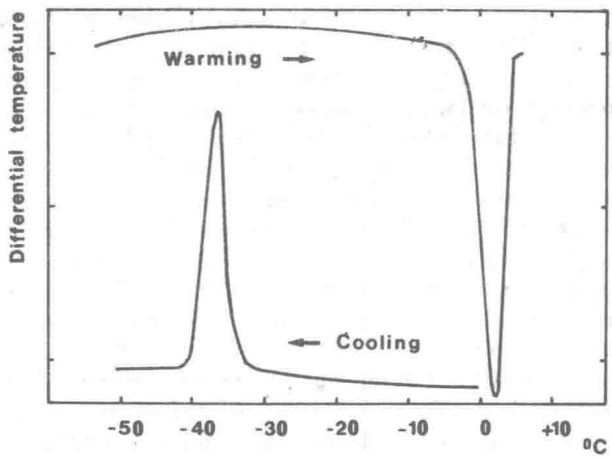

Fig. 1. Thermograms obtained in water-in-oil emulsions.

in the field of cryoenzymology. It has been shown (11) that the freezing of individual droplets is distributed in time, the time taken to achieve total freezing of the emulsion becoming shorter as temperature comes closer to T^*.

Determination of supercooling and of its evolution with time can be made by a more direct and simple method that has been used in this laboratory, namely, fluorescence probing.

b. Fluorescence Probing. The technique of fluorescence can be used to obtain information about supercooling and freezing of water-in-oil emulsions when the compound 1,8-anilinonaphtalenesulfonate (ANS) is selected as the fluorescent probe. This compound displays a strong affinity for water droplets and its properties, for example, quantum yield, lifetime, and position of the fluorescence maximum are extremely sensitive to the polarity of the microenvironment (12). This behavior may be used to test the effective polarity of water droplets and their supercooling and freezing.

The very low fluorescence quantum yield recorded in liquid solutions changes dramatically upon freezing, and fluorescence recordings so obtained resemble the DSC thermogram. An example of such behavior is shown in Figure 2.

The slope of the fluorescence enhancement curve at subzero tempera-

tures gives a clear indication of the homogeneity of the emulsion, as well as a good estimate of the supercooling range. The inflection at −38.8°C is close to what is usually regarded to be the homogeneous nucleation temperature of water, that is, the temperature at which water freezes spontaneously in the absence of impurities that normally act as nuclei for freezing. Recordings of fluorescence intensity as a function of time at selected subzero temperatures permit a check on the stability of the supercooled state. Data collected during numerous trials clearly show that with the techniques employed it is impossible to predict a freezing point for any given emulsion. For these reasons, checks should be carried out with each emulsion system prior to using it in a kinetic run. Such fluorometric recordings are easy to perform on very small aliquots (200–300 μl) of the emulsions. It has been established that the results are in good agreement with those obtained by DSC and can be used to determine the range of temperature and time over which supercooling occurs.

2. *Physical-Chemical Properties of Water Droplets*

Previous investigations with mixed solvents have clearly established that temperature variations in the subzero temperature range produce

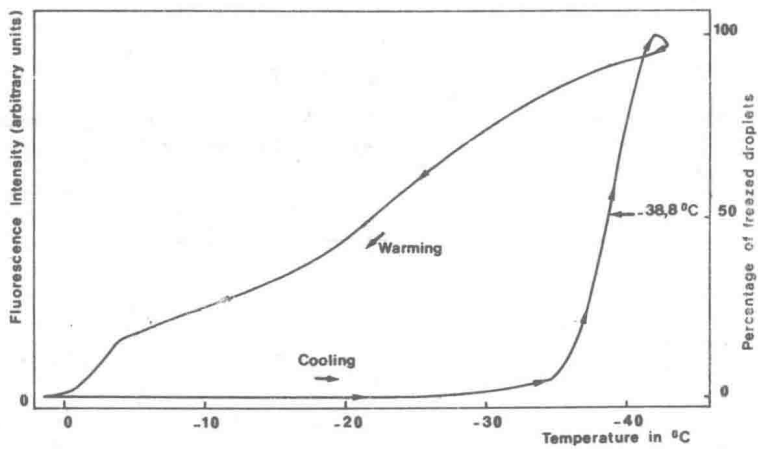

Fig. 2. Fluorescence intensity, as a function of temperature, of ANS (10^{-3} *M*) in a 10% water emulsion in silicone oil (viscosity 0.65 cP at +25°C) containing 2.5% (w/v) sorbitan tristearate (Span 65). Cooling speed = 2.5°C/min, freezing temperature of water droplets = −38.8°C; λ_{exc} = 350 nm, λ_{em} = 485 nm. Fluorescence apparatus from Aminco-Bowman.

changes in a number of physical-chemical properties directly relevant to enzyme specific activity (4), and such changes are to be expected with supercooled water.

While solubilities of buffers and neutral salts to ensure suitable conditions of medium raise no particular problems in emulsions, temperature effects on the pK's of weak electrolytes, that is, buffers and ionizing groups located at the active site and on the protein surface, should play an important role. These effects have been studied with mixed solvents and it has been shown that they are independent of the solvent composition but depend on the type of ionizing group. Data collected over several years are available elsewhere (3,4) and we only briefly mention general trends. In this connection the most important factor is the influence of temperature on p$K\omega$, describing the ionization equilibrium of liquid water; this is given by

$$pK\omega = \frac{3108}{T} + 3.55$$

where T is the temperature in °K. At −40°C, therefore, p$K\omega$ = 16.89 and pH = 8.5. This is reflected in the pK values of buffer systems, over and above the effect produced by the organic cosolvent (see Table I).

TABLE I

Typical Variations of pa_H of Buffers ($10^{-2}M$) in the Temperature Range +20 to −40°C in Fluid Media Ethylene Glycol–Water (50:50, v/v)

The pH value of each buffer, in pure water, has been arbitrarily chosen near the pK values; $pa_H = -\log a_H$, a_H being the protonic activity in nonaqueous media. The ionization enthalpy is not strongly different from that in pure water.

	pH, 20°C	pa_H, 20°C	pa_H −20°C	pa_H, −40°C
Anionic buffers				
Chloracetate	2.45	3.00	3.10	3.20
Acetate	4.55	5.05	5.25	5.35
Cacodylate	6.60	7.00	7.20	7.35
Phosphate	6.90	7.50	7.80	8.00
Cationic buffers				
Histidine	6.1	5.85	6.55	6.90
Bis-Tris	6.80	6.55	7.75	8.20
Bistrispropane	6.80	6.55	7.75	8.20
Tris	8.25	8.10	9.35	9.85

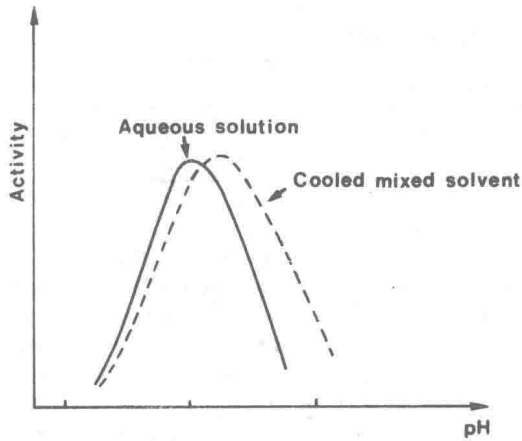

Fig. 3. Changes in pH–activity profiles in cooled mixed solvents as a consequence of changes in pK of ionizing groups at the active site of the enzyme.

It is important to select buffers and buffer concentrations such that the desired pH value at selected temperature is achieved.

The pK's of ionizing groups at the active site of the enzyme are affected according to their enthalpies of dissociation, and pH-dependent enzyme-catalyzed reactants are affected, giving rise to shifts in the pH–activity profiles, as schematized in Figure 3.

In principle, it is possible to correct for such an effect by making the necessary adjustments in pH. However, the optimum enzyme activity could continue to be affected by changes resulting from temperature effects on the degree of dissociation of ionizing groups on protein surface or yet from effects due to surfactant "walls."

Careful investigation must be devoted to any effect that might result from surface phenomena rather than "bulk" water, and such surface effects are of primary importance in micromicellar solutions (water-in-oil emulsions involving water-soluble surfactants) studied in Section II.B.

The dielectric constant (ϵ) of bulk aqueous and aqueous–organic solutions increases almost linearly with decreasing temperature (13) and an extrapolation of ϵ values between +20 and 0°C indicates that ϵ reaches about 100 at −30°C (Table II). Such a marked increase is likely to modify electrostatic interactions between enzymes and ionic residues on ligands, which would in turn modify kinetic parameters.

TABLE II
Dielectric Constant of Water as a Function of Temperature

	Measured values			Estimated values			
Temperature, °C	+20	+10	0	−10	−20	−30	−40
D	80.4	84.2	88.1	92	96	100	104

We have seen that the properties of the droplets in the metastable supercooled states indicated a tendency toward a more open packing of water molecules, possibly an increased similarity to ice. Thus the micropolarity of such droplets could be markedly different from that of bulk water in the test tube and further ANS fluorescence analysis is needed to decide whether such a difference actually exists.

A last, but interesting, property of water-in-oil emulsions is that molecular oxygen can undergo unrestricted diffusion.

C. SOLUTIONS OF PROTEINS

Many enzymes in aqueous solutions have been successfully investigated in emulsion. It has been shown that they do not act as ice nucleators and are not denatured (14,15).

Recording of spectral characteristics of absorbing enzymes and the monitoring of their catalyzed reactions in supercooled water-in-oil emulsions by UV or visible spectrophotometry meets with serious technical difficulties because of the high turbidity of the samples and requires spectrophotometers suitably adapted for extremely turbid suspensions, such as the Aminco-Chance DW2 spectrophotometer, which combines a high stability with a high sensitivity, and an optical design (diffuser, unique photomultiplier, dual wavelength mode) suitable for the measurement of scattered light. Absorption spectra were recorded on several hemoproteins in cuvettes of 2–5 mm depth with thermostatically temperature-controlled cell holders.

Preliminary spectroscopic assays were carried out with cytochrome *c* and myoglobin in their various oxidation states (ferri, ferro, carboxyferro) to ensure that identical absorption spectra were obtained from water-in-oil emulsions and homogeneous aqueous solutions at room temperature. Temperature cycling did not produce any changes in the spectral intensities.

D. APPLICATIONS

1. *Studies of High Spin–Low Spin Conversion*

As an example of the kind of investigation that can be carried out under such conditions, let us mention the recording of absorption spectra of cytochrome P_{450} at subzero temperatures. Optical spectra of various redox states of the bacterial cytochrome P_{450} in the supercooled emulsified phase are shown in Figure 4. This monooxygenase, which hydroxylates camphor, is chosen as an example of the possibilities and limitations of a subzero temperature study of enzyme intermediates in supercooled water. As the temperature was dropped, the spectra showed no changes other than the well-known band sharpening.

Another interesting investigation was the spectrophotometric study of temperature effects on the spin-state equilibrium of substrate-bound

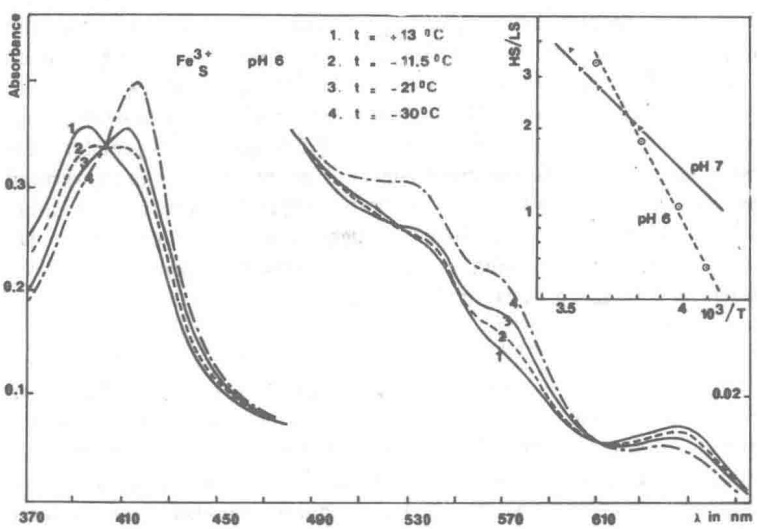

Fig. 4. Spectra of ferric substrate–cytochrome P_{450} (Fe_S^{3+}) in emulsified water droplets at different temperatures. The aqueous buffer containing camphor and KCl is at pH 6. High-spin and low-spin concentrations are calculated from the absorbance values at, respectively, 392 and 417 nm corrected for the temperature effect on ϵ values. Insert shows the van't Hoff plots of K_{eq} = HS/LS as calculated from the same experiment and from a similar experiment at pH 7.