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SUPERCOOLED WATER AS MEDIUM FOR ENZYME REACTIONS AT SUBZERO TEMPERATURES

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Summary

A water-in-oil emulsion technique was employed to investigate enzyme-catalyzed reactions at sub-zero temperatures in the supercooled liquid state. The results obtained with a monooxygenase (bacterial cytochrome *P*-450) clearly indicate the potentialities as well as the technical problems of the procedure which might be successfully used to investigate enzyme systems sensitive to cosolvents.

Introduction

Cryo-enzymology

Recent years have witnessed an increasing interest in studies of enzyme catalyzed reactions at low temperatures. The detailed information which can be derived from investigations at ordinary temperatures is severely limited by the high reaction rates and labile nature of intermediates formed. Low temperatures should favour the temporal resolution of the contributing reactions and facilitate the detection and identification of intermediate species. Also of interest is the conformational stability of proteins at low temperatures. Indications are that proteins may well have a temperature of maximum stability and that both high and low temperatures progressively favour unfolding [1]. In practice the lower temperature limit for experimental investigations has been the equilibrium freezing point of the solution, since proteins, including enzymes, in partly frozen systems often exhibit behaviour which bears little resemblance kinetically or thermodynamically to that of the same system in solution at ambient temperatures [2].

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To overcome the low temperature limit, fluid aqueous-organic mixtures have increasingly been used in low temperature studies of enzyme reactions. The potential of such procedures was first shown by studies on peroxidase [3] and since then on a number of other enzyme systems, such as luciferases [4,5], cytochrome *P*-450 [6,7], hydrolytic enzymes [8—10], and more recently with crystals [11—13].

Low temperature procedures allow the accumulation of kinetic and thermodynamic information and permit stabilized intermediates to be analyzed by a variety of spectroscopic and chemical techniques. The value of such techniques in studies of enzyme mechanisms is now well documented and has recently been reviewed [14]. Attractive though such procedures may be, they are not free from problems, mainly associated with the presence of high concentrations of organic cosolvents, even if it can be demonstrated that the cosolvent does not affect the specific activity of the enzyme or the pathway of the reaction. Thus, it is well documented that the organic cosolvents used, e.g. methanol, dimethyl sulphoxide, *N*,*N*-dimethylformamide, or ethylene glycol, cause drastic changes in the peculiar long range intermolecular order which is the unique feature of liquid water [15]. As a result such organic co-solvents markedly influence the solvation and transport behaviour of ions and the dissociation equilibria of acids and bases [16]. Since the conformational stability of proteins in solution is governed by the balance of these interactions (and others, such as the hydrophobic interaction), it is to be expected that major changes in the medium composition will affect any process which depends on the solvation interactions of amino acid residues.

It is well established that the conformational and association equilibria of proteins are very sensitive to changes in pH, to concentrations of salts and the presence of organic co-solvents which are known either to bind specifically to certain sites, or which exert their influence through a strengthening or weakening of hydrophobic interactions [17].

Supercooled water

To avoid solvent effects and yet extend the subzero range of temperatures under conditions closer to the physiological ones, we have demonstrated the usefulness of supercooled water [18]. With the aid of water-in-oil emulsions it is possible to stabilize supercooled water against freezing due to heterogeneous nucleation. This extends the experimentally accessible temperature range to the onset of homogeneous nucleation (approx. —40°C) [19]. Such emulsions have been prepared by dispersing water in heptane or safflower oil, supersaturated with sorbitan tristearate as water-insoluble surfactant, with water droplet diameters from 1 to 5 μm [20]. Emulsions have been used to carry yeast cells into the supercooled state and returning them to room temperature, following which the emulsions could be broken and the cells plated and assayed for viability. We have applied this emulsification procedure to investigate enzyme-catalyzed reactions at sub-zero temperatures, and we here present the results on bacterial cytochrome *P*-450, a monooxygenase, already intensively studied in mixed solvents [6,7].

Methods

Emulsification procedure

Two suitable carrier fluids, permitting emulsification and being at the same time innocuous towards proteins, were used: corn oil and safflower oil. These oils possess a high ratio of polyunsaturated linoleic acid to saturated fatty acids, respectively 5 : 1 and 9 : 1. Their solidification occurs at temperatures below −35°C.

Numerous emulsification trials were carried out to determine the optimum conditions for achieving highly disperse water-in-oil emulsions. A surface active agent, sorbitan tristearate (commercially available as Span 65) was added to the oil phase at 2.5% w/v and dissolved by gentle heating, after which the solution was cooled to room temperature. The aqueous enzyme solution, containing a buffer of suitable pH and the necessary additives (salts, substrates, etc.) was mixed with the Span/oil phase in the proportion of 3 : 7 v/v. Prolonged manual shaking followed by high speed micro-emulsification in a standard blender were absolutely necessary to obtain a stable emulsion of the right particle size distribution.

Examination of emulsions can be carried out by light microscopy, revealing droplet sizes from 1 to 5 μm diameter, and differential thermal analysis provides a means of determining the freezing of water droplets [20]. We set up a simpler, but efficient method, i.e. the recording of fluorescence intensity of the water-soluble dye 1-anilino-naphthalene-8-sulphonate which has a very low (0.001) fluorescence quantum yield in liquid solutions [21], changing dramatically by up to 20-fold upon freezing, the emission maximum being shifted by 25—35 nm.

This fluorometric assay permits recordings equivalent to the classical thermograms, whereas the slope of the fluorescence enhancement gives a clear indication of the homogeneity of the emulsion. It also affords a check that supercooling can be maintained for hours at temperatures as low as −40°C, so that investigation of enzyme kinetics by absorption spectroscopy can be carried out in the metastable fluid.

Spectroscopic recordings of enzyme reactions and intermediates

The monitoring of enzyme-catalyzed reactions in supercooled water-in-oil emulsions by uv or visible spectrophotometry meets with serious technical difficulties due to the high turbidity of the samples and requires spectrophotometers suitably adapted for extremely turbid suspensions. We used an 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. Using cuvettes of 2—5 mm depth with thermostatically temperature-controlled cell-holders absorption spectra were recorded.

Preliminary spectroscopic assays were carried out with cytochrome c and myoglobin in their oxidation states (ferri, ferro, carboxy-ferro) 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.

Results and Discussion

Absorption spectra of cytochrome P-450 at sub-zero temperatures

Optical spectra are shown in Fig. 1 of various redox states of the bacterial cytochrome *P*-450 in the supercooled emulsified phase. This monooxygenase, which hydroxylates camphor is chosen as an example of the possibilities and limitations of a sub-zero 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.

Temperature effects on the spin state equilibrium of substrate bound bacterial cytochrome P-450

The binding of substrate (camphor) to the ferric cytochrome *P*-450 induces a change in its spin state from essentially low spin to essentially high spin, as previously observed under normal conditions [22]. Actually Fe_S^{3+} is in a temperature-dependent spin state equilibrium which has been studied in water above 0°C [23] as well as below 0°C in a 1 : 1 (v/v) mixture of ethylene glycol and aqueous buffer. These two sets of experiments gave quite comparable results, in that an increase of the low-spin to high-spin ratio was observed as the temperature was decreased. However, definite solvent effects on the equilibrium constant at room temperature and on the enthalpy of the transformation

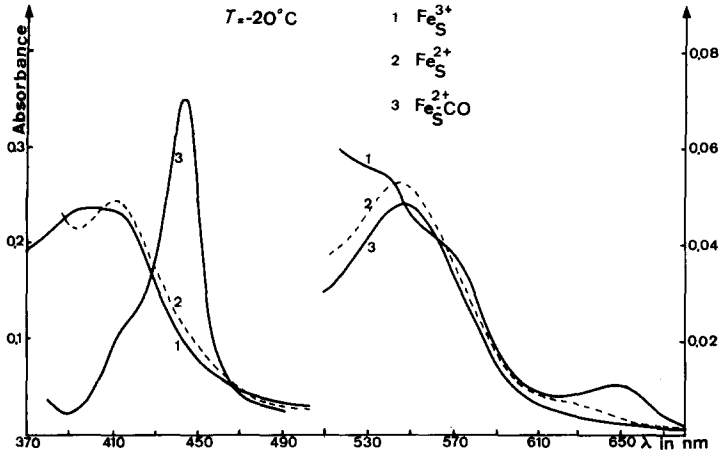

Fig. 1. Optical spectra of various redox states of bacterial-substrate-bound cytochrome *P*-450 in a water-in-oil emulsion at sub-zero temperatures. A stock solution of bacterial cytochrome *P*-450 prepared in the Laboratory of Dr. I.C. Gunsalus in 100 mM phosphate buffer (pH 7) containing 400 μM camphor, was diluted 20 times in 50 mM phosphate (pH 7) containing 100 mM KCl and saturated with camphor ($6 \cdot 10^{-3}$ M). 1.5 ml of this solution (at 10°C) was added to 3.5 ml of corn oil in which 2.5% (w/v) Span 65 had been dissolved. Emulsification was performed by prolonged manual shaking followed by high speed microemulsification in a commercial blendor. The emulsion was then transferred to the sample cuvette of an Aminco-Chance DW2 spectrophotometer and maintained at −20°C. The reference cuvette was filled with an identical emulsion containing no cytochrome *P*-450. 1. Oxidized, substrate-bound cytochrome *P*-450 at −20°C (Fe_S^{3+}). 2. Same suspension after addition of 10 μl of a 10^{-1} M dithionite solution and re-emulsification at +10°C. Spectrum recorded at −20°C. 3. Same suspension after addition of carbon monoxide. The longer wavelength part of the spectra (500 nm to 700 nm) is recorded with a higher sensitivity (right hand absorbance scale).

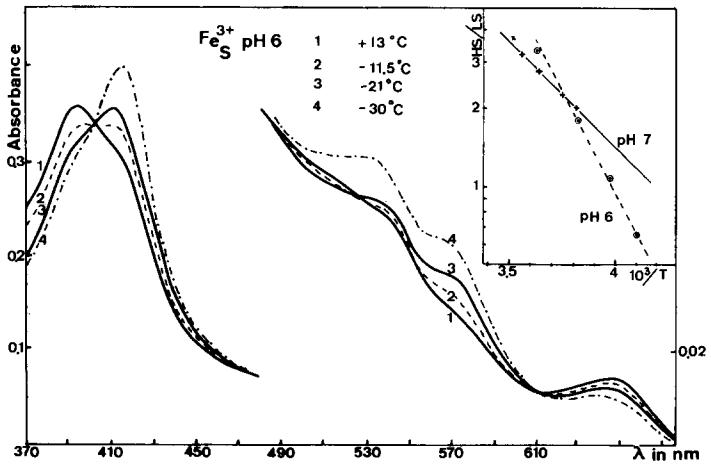

Fig. 2. Spectra of ferric substrate-cytochrome *P*-450 (Fe_S^{3+}) in emulsified water droplets at different temperatures. Same experimental procedure as in Fig. 1 except that the aqueous buffer containing camphor and KCl is at pH 6. High spin and low spin concentrations are calculated from the absorbance values at respectively 392 and 417 nm [21] corrected for the temperature effect on ϵ values [23]. 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 (unpublished data).

were noticed. Fig. 2 shows that a similar high spin to low spin transition occurs when the temperature of the supercooled water-in-oil emulsion is decreased. Furthermore the clear indication of an isosbestic point suggests that the observed spectral changes reflect a two-state equilibrium. The van't Hoff plots of equilibrium constants are shown in the inserts of Fig. 2 at two different pH values. Corresponding ΔH values in emulsified water droplets are respectively $\Delta H_{pH6.0} = 30 \pm 2$ kJ · mol^{-1} and $\Delta H_{pH7.0} = 14 \pm 2$ kJ · mol^{-1}. These values are similar to a value obtained in the standard aqueous medium above 0°C (ΔH_{pH7} = 10.3 kJ · mol^{-1}) [23] but differs significantly from ΔH obtained with ethylene glycol-water mixtures (1 : 1 v/v), i.e. $\Delta H_{pH6.0} = 42$, and $\Delta H_{pH7.0}$ = 30 kJ · mol^{-1} [24]; these results clearly show the co-solvent effect on the thermodynamics governing the spin state equilibrium. In both types of media, however, a similar ΔH dependence on pH is observed*, suggesting an identical mechanism for the high spin-low spin conversion of this hemoprotein in homogeneous solution as a function of temperature.

Several events of the cytochrome *P*-450 reaction cycle (substrate binding, redox potential changes, putidaredoxin binding, product release) are characterised by changes in the spin state equilibrium which appear to be delicately balanced [23,24]. The achievement of sub-zero temperatures is a prerequisite to detailed kinetic studies of the reaction. Also the thermodynamic study of the iron spin state equilibrium over a more extended temperature range should shed light on the heme structure and the heme-linked oxygen activation.

* The exact definition of pH in aqueous/organic solvent mixtures and its proper measurement are frequently glossed over in such comparative studies.

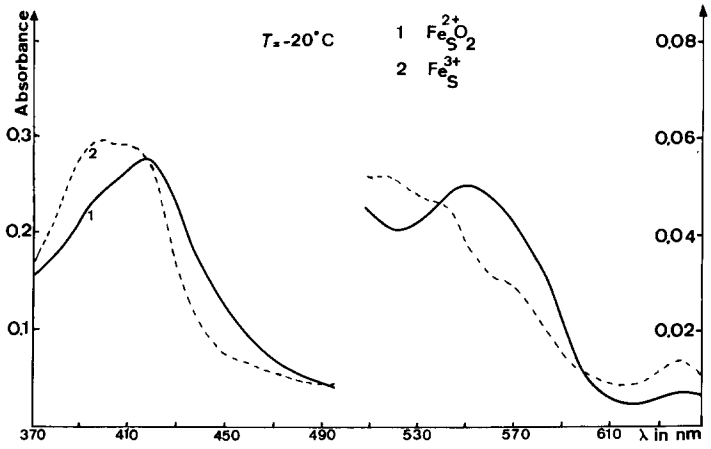

Fig. 3. Spectrum of the oxy-ferro cytochrome *P*-450 ($Fe^{2+}_{S} \cdot O_2$) stabilized at −20°C in supercooled emulsified water droplets. 10% (v/v) of an aqueous solution of bovine liver catalase (Sigma, 10 units final) had previously been emulsified in the oil phase and allowed to stand 1 h at room temperature before being cooled to 0°C. This procedure was found necessary to destroy peroxides in the oil and to decompose the oxygenated compound. Cytochrome *P*-450 solution in a 50 mM phosphate containing camphor and KCl was reduced at room temperature by limited amounts of dithionite and then thermostatted to +1°C; oxygen was vigorously bubbled through, in order to allow a complete oxidation of the excess dithionite and formation of the oxy-compound. This solution was then emulsified as quickly as possible into the oil pretreated with catalase, and transferred to the precooled cuvette of the spectrophotometer. The whole procedure lasted approx. 5 min. 1. Spectrum after stabilization of the temperature at −20°C. This spectrum is stable for at least 30 min. 2. Spectrum taken at −20°C after heating to 20°C for 40 min.

Low temperature stabilisation of short-lived complexes in supercooled water

An example of the stabilisation of intermediate species in a purely aqueous medium is provided by the unstable oxygenated compound of cytochrome *P*-450 ($Fe^{2+}_{S} \cdot O_2$). which normally decays into Fe^{3+}_{S} [18]: this compound was formed at 0°C in buffer, quickly emulsified at the same temperature, and then transferred to the spectrophotometer cuvette which had been precooled to −20°C. The absorption spectrum at −20°C (Fig. 3) clearly shows the typical maxima at 418 nm and 545 nm; the shoulder at 392 nm is due to a small proportion of oxycompound autoxidized during the emulsification and transfer. Repetitive spectra recorded over a period of 1 h at the same temperature showed no further autoxidation. Thus the complex could be perfectly stabilized at −20°C in supercooled water. After heating for 20 min at 25°C the compound was totally transformed into Fe^{3+}_{S}, as shown by the absorption spectrum recorded after once again cooling to −20°C (spectrum 2, Fig. 3). All spectra recorded in supercooled water were identical to those obtained under normal conditions, except for a temperature-induced sharpening of the peaks and the spin state transition described above.

In preliminary trials the oxy-compound was reoxidized during the emulsification procedure due to the presence of contaminating peroxides in the oil; such a decomposition was avoided by treatment of the carrier with catalase, as described in the legend of Fig. 3.

Further attempts to form the oxy-compound directly in the emulsion were carried out using commercially available silicone oils of very low viscosity

(0.65—1.0 cP with solidification points of −65 and −87°C respectively). Under these conditions it was difficult to prevent phase separation, even at subzero temperatures. However, such trials indicate the usefulness of silicone oils of medium viscosity which give stable emulsions and permit the diffusion of reactants, rapidly enough for the build up of sufficient concentrations of intermediates and for the recording of kinetic data. Additional advantages of silicone oils are the higher oxygen solubility and their low tendency to autoxidation.

Conclusions

Emulsification procedures should permit the investigation of enzyme reactions in unmodified aqueous media, so that the observed results would reflect 'pure' temperature effects on enzyme specific activity; any possible interference due to the presence of a co-solvent could thus be circumvented. Comparative investigations in both types of media, such as exemplified by the study of the high spin-low spin equilibrium of the substrate-ferric cytochrome *P*-450, could be employed to correlate reaction mechanisms in pure water and in mixed solvents. Also the stabilities of native states are sensitively attuned to the solvation interactions of proteins [17]. The accessibility of a more extended temperature range should therefore make it possible to probe such hydration interactions under conditions where the intermolecular order in water becomes even better defined than it is just above the normal freezing point.

The emulsification procedure can be improved by use of different liquid carriers and other water insoluble surfactants. The accessible temperature range could be extended by the addition of polymeric solutes such as polyvinylpyrrolidone or polyethylene glycol which further depress the homogeneous nucleation temperature [19], always provided that such additives do not give rise to the 'co-solvent effects' previously mentioned. Of course, the water-in-oil emulsion procedure is not free from problems and lacks the facility of the mixed solvent technique. Thus, the turbidity of the emulsions precludes analytical techniques which rely on optical transparency. However, recent exploratory studies using EPR on supercooled emulsions of hemoproteins show promise (Franks, F., unpublished data), and in principle the same should apply to NMR based techniques.

The emulsion method cannot at present easily be applied to reactions which proceed rapidly at 0°C or in conjunction with proteins which are likely to be adsorbed at the oil/water interface (e.g. proteins of membrane origin). Problems of slow diffusion of reactants into water droplets could be overcome by starting at room temperature from pH conditions unfavourable to the reaction, but becoming favourable as the temperature is lowered.

Uncertainties raised by the effects of aqueous-organic solvents on enzyme conformation and activity can be solved by control experiments. However, values of kinetic or thermodynamic quantities obtained under such conditions should be compared with data from aqueous solution at the same temperature, and the procedure here described permits such a direct comparison.

Since the mixed solvent procedure is much easier to perform and is particularly well fitted to kinetic analysis, it would be wise to use emulsification,

wherever possible, as a comparative test of the co-solvent effects on enzyme specific activity, and to decide on the basis of the results obtained whether a suitable co-solvent can be found for any given cryo-enzymologic kinetic study.

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