CRYOENZYMOLOGY

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I. INTRODUCTION

Cryoenzymology refers to the study of enzymes at subzero temperatures using fluid cryosolvents.[1-4] Its greatest value lies in the potential to provide kinetic and thermodynamic information for individual elementary steps in enzyme catalysis, and to allow the individual enzyme-substrate intermediates to be stabilized for sufficient time periods to permit the acquisition of structural information. Lest it appear that cryoenzymology is the panacea for resolving enzyme mechanisms let us quickly add that the cost of such potential advantages is the necessity for a fluid solvent system, most commonly an aqueous-organic mixture. Consequently one is faced with the task of ascertaining the effects of such a solvent on the system under study. In this review we will be particularly concerned with the following two themes: (1) what have we learned from cryoenzymology, and what is its potential as a means of providing desirable information and (2) how relevant is the information obtained at subzero temperatures in cryosolvents to the mechanisms that occur under normal conditions?

It has long been known that enzyme-catalyzed reactions usually obey the Arrhenius relationship

$$k = Ae^{-Ea/RT}$$

where k represents the rate constant, A is a constant (the frequency factor), and Ea is the energy of activation, which is related to the enthalpy of activation by the expression $\Delta H^{\dagger} = E_a - RT$. Thus lowering the temperature will lower the reaction rate. The physical basis for cryoenzymology may be considered to be the fact that the different elementary steps in an enzyme-catalyzed reaction will normally have different enthalpies of activation; consequently, although each elementary step may have evolved to have a similar rate and free energy of activation under physiological conditions,[5] the temperature dependence of each step will vary, often very considerably. An additional premise is that by initiating the reaction at a sufficiently low temperature the interconversion of normally transient intermediates may effectively be stopped, and the intermediates may thus be accumulated and stabilized individually.[1]

The upper limit for the rate of an enzyme-catalyzed reaction is expected to be that of the diffusion-controlled complexation of the enzyme and substrate — typically about 10^7 to 10^8 M^{-1} sec^{-1}.[6] The most efficient enzymes do indeed have turnover numbers of such magnitude.[5] A noteable consequence of such an overall rate for an enzyme-catalyzed reaction is that each enzyme molecule must catalyze the transformation of about 10^7 substrate molecules to product per second. This in turn means that the lifetime of a given enzyme-substrate complex must be of the order of 10^{-7} sec. Further, since

enzyme-catalyzed reactions are known to involve several elementary steps and therefore to have several transient intermediate species present on the reaction pathway, the lifetimes of these intermediates must be $\leqslant 10^{-7}$ sec. Since most enzymes have turnover numbers in the 10^3 to 10^6 sec^{-1} range, most enzyme-substrate intermediates must have lifetimes of milliseconds or less!

It is this short-lived nature of the intermediates, coupled in many cases with very low concentrations, that has made the mechanistic dissection of enzyme-catalyzed reactions so refractory. Although a great deal of useful information has been obtained using a variety of rapid-reaction techniques, until recently it has been difficult to acquire detailed *structural* knowledge of the transient enzyme-substrate intermediates. With the advent of the technique of cryoenzymology in the past decade it has now become possible to effectively reverse much of the rate enhancement induced by the enzyme and to slow down the transformation of enzyme-substrate complexes to the point where many normally transient intermediates can be stabilized for relatively long time periods.

So far the methodology of cryoenzymology has been applied mostly to the detection, accumulation, and stabilization of very short-lived intermediate species in enzyme catalysis and related biochemical systems (e.g., protein-ligand binding[7]); to the purification and study of normally labile, unstable proteins,[8] to the study of events in the folding and unfolding of proteins;[9] and to the study of multifunctional protein and protein-nucleic acid complexes (e.g., ribosomes, chloroplasts[10]). However, the approach does appear to be of general applicability to biochemical systems involving transient species.

In this review we will restrict our discussion to systems in which *fluid* solvent systems are required in order to permit nonrate-limiting diffusion of ligand to protein, and we will not consider cases in which nonfluid glasses are used, as in the study of myoglobin-ligand association/dissociation, for example.[11] The scope of this article will encompass the inherent advantages and limitations of the technique; various aspects of the method in general, especially as they relate to solvent-protein interactions; and a review of a limited number of systems which have been investigated using cryoenzymology, both as a means of illustrating a variety of different features of the approach, and also to indicate the type of information which the method has made available and which in many cases could not have been obtained by other techniques presently available.

A comprehensive, detailed qualitative and quantitative understanding of enzyme catalysis has been a long-standing goal. A necessary, although not necessarily sufficient, requirement to achieve this goal is a detailed knowledge of all the intermediates and transition-state structures along the reaction pathway. The technique of cryoenzymology has the potential to provide much of this information. Several recent reviews covering various aspects of the technique are available.[1-4,12-17]

Cryoenzymology utilizes the following features of enzyme catalysis: the existence on the catalytic reaction pathway of several enzyme-substrate or enzyme-product intermediate species, typically separated by energy barriers with enthalpies of activation of 7 to 20 kcal/mol; and the fact that the energies of activation for the individual elementary steps are usually significantly different. Thus temperatures of $-100°C$ will result in reductions of the order of 10^5 to 10^{11} in the rates of intermediate transformation compared to 25 or 37° C.[1] The general experimental basis for the trapping of enzyme-substrate intermediates is as follows: if the reaction is initiated by mixing enzyme and substrate at a suitably low temperature only the initial noncovalent ES complex will be formed. This is a consequence of insufficient energy being available to overcome the energy barrier to the subsequent intermediate. If the temperature is gradually increased a point will be reached where ES is transformed into the following intermediate. Maintenance or reduction of this temperature will allow this intermediate to be trapped. Further raising of the temperature will result in transformation into a subsequent

intermediate and so on until the overall rate-limiting step is reached, at which point turnover will occur. Any intermediate whose rate of formation is faster than its rate of breakdown may be accumulated in this manner. Consequently an inherent limitation of the method is that not all intermediates are necessarily accessible to the technique. In some cases it is possible to run the catalytic reaction in both directions thus increasing the number of intermediates which can be trapped. This would be particularly essential in cases where the rate-limiting elementary step occurred very early along the reaction pathway.

A. Advantages and Limitations of Cryoenzymology

The major advantages of the method stem from the potential to accumulate and stabilize each intermediate on the productive reaction pathway individually, and the consequent possibility of obtaining structural as well as kinetic information from the reaction of *specific* substrates.

The application of the low temperature trapping technique to specific substrates eliminates the uncertainties present with nonspecific substrates, in which the much slower rate could be due to a different mechanism.[18] In fact with few exceptions current detailed *structural* knowledge regarding enzyme-substrate complexes or intermediates comes entirely from the use of pseudosubstrates or inhibitors. In either case the crystallographically determined structure reflects a *nonproductive* species. Consequently the relative positions and orientation of the "substrate" and the essential catalytic groups are unlikely to be those found in catalytically productive species. Since current high resolution structural techniques, such as X-ray diffraction, neutron scattering, and nuclear magnetic resonance, require samples of high concentration and long lifetimes, they are clearly unsuited for the study of transient intermediates in enzyme-catalysis under *normal* conditions. However, cryoenzymology permits the accumulation and stabilization of relative high concentrations of such intermediates for long time periods, thus allowing the application of such procedures.

A unique potential of cryoenzymology is that of permitting the investigation of a single elementary step at a time. In addition, the large temperature range over which experiments can be carried out has clearly demonstrated the possibilities of detecting species which are "unobservable" under normal conditions. For example, consider an intermediate which is formed in a step with an energy of activation of 10 kcal/mol, and which breaks down with an energy of activation of 15 kcal/mol. If the rate of formation and breakdown are identical at 25° C, then no significant build-up of the intermediate will occur. If the lifetime of the intermediate under these conditions is 1 msec, then it will be unobservable by stopped-flow techniques, for example. If the temperature were lowered to −100° C, however, the intermediate would now take about 15 min to form, and close to a week to break down! Under such conditions the intermediate would not only be readily detectable, but could be studied by a variety of conventional techniques. An example of such an intermediate is the acyl-enzyme formed in the reaction between elastase and N^{α}-carbobenzoxy-L-alanine *p*-nitrophenyl ester, discussed in detail subsequently.[19,20] Another way in which normally undetectable intermediates have been detected through cryoenzymological studies is in cases where the rate-determining step for an intermediate changes from being formation at higher temperatures to breakdown at lower ones.[21,22] Such a case is that of β-glucosidase, considered in more detail in a later section.

One of the most valuable attributes of cryoenzymology appears to be its widespread applicability, not only to all types of enzymes, but also in the sense of the variety of chemical, physical, and biophysical techniques which can be applied in the study of enzyme species at subzero temperatures. These range from pressure-relaxation kinetic studies to X-ray crystallography, for example.

The major limitations of cryoenzymology include the necessity for a fluid solvent system to avoid rate-limiting enzyme-substrate diffusion; the possibility that a different catalytic mechanism occurs under the subzero temperature conditions; the distinct possibility that the cryosolvent and the low temperatures may bring about changes in the structure of the protein, which may also lead to affects on catalysis; the necessity for specialized equipment; the possibility that the rate-limiting step in the overall reaction may be so positioned that very few intermediates can be detected; and the need for environment-sensitive reporter groups to monitor the interconversion of intermediates. A number of these aspects will be considered in detail below.

B. Versatility of Cryoenzymology

Some idea of the wide variety of biochemical systems which may be successfully studied using cryoenzymology is given by the following list of applications to which the approach has been used.

1. Purification of Labile Proteins

Douzou and co-workers in particular have worked on the development of modifications to standard enzyme purification procedures to allow their use at subzero temperatures.[23] The application of these methods to the purification of labile proteins is based on the enhanced stability of such compounds at lower temperatures All the frequently used purification methods have been successfully adapted to subzero temperatures; e.g., gel filtration,[24,25] ion-exchange chromatography,[26] affinity chromatography,[27,28] isoelectric focusing,[23] and electrophoresis.[23] Relatively unstable proteins such as nitrogenase and xanthine oxidase have been readily purified in this manner.[8]

An intriguing aspect of such an approach is the use of affinity chromatography in which the ligand attached to the support is the substrate for the enzyme being purified. This method can of course be used with nonlabile enzymes, and has been successfully used to purify elastase[27] and immunoglobulins.[28] Whether this will prove to be a generally useful method is not clear. The main problem is that cryosolvents based on mixed aqueous-organic solvents usually decrease the affinity of the enzyme for the substrate (see Section I.D). In the case of elastase this was circumvented by using a high salt concentration to depress the freezing point sufficiently to effectively stop turnover without significantly affecting the affinity of the enzyme for the substrate.[27]

2. Isolation of Normally Transient Intermediates

The application of procedures mentioned in the above section has permitted the isolation of enzyme-substrate intermediates which had been trapped at subzero temperatures. For example, an intermediate in the bacterial luciferase reaction was isolated at −20° C by gel filtration[24] and identified as a 4a-hydroperoxy adduct on the basis of its spectral properties.[30,31] Similarly, an acyl-enzyme in the reaction of chymotrypsin was isolated by gel filtration at −40° C.[25,32] In some cases favorable enzyme stability at extremes of pH has allowed the stabilization of an intermediate in aqueous solution at 0° C. Thus another acylchymotrypsin was isolated by gel filtration at 0° C at pH 2.5.[33]

3. Detection of Normally Undetectable Enzyme-Substrate Intermediates

The following are some cases in which the use of appropriately low temperature has permitted the detection of a normally unobservable species, either through a change in the rate-determining step or by a sufficient decrease in the rate of intermediate breakdown: a glucosyl-enzyme in β-glucosidase catalysis, discussed in detail subsequently;[34] an acyl-enzyme in the carboxypeptidase reaction with an ester substrate, also

Table 1
SOME ENZYMES AND RELATED PROTEINS STUDIED BY CRYOENZYMOLOGY

Enzyme	Cosolvent[a]	Ref.
Chymotrypsin	MeOH, DMSO	32, 101
Trypsin	DMSO	36, 61, 114
Papain	DMSO, EtOH	62, 111
Elastase	MeOH, DMSO	20
Subtilisin	DMSO, MeOH	182
Carboxypeptidase A	MeOH, EtGly	22
β-Glucosidase	DMSO	34
β-Galactosidase	DMSO, MeOH	78
Lysozyme	MeOH, DMSO	71, 86
Ribonuclease A	MeOH, EtOH	9
Alkaline phosphatase	MeOH, Gly	45
Myosin Sl	EtGly	172, 179
Alcohol dehydrogenase	DMSO, DMF	7, 173
Glyceraldehyde-3-phosphate dehydrogenase	MeOH/EtGly	183
Dihydrolysate reductase	MeOH, EtOH	184
Bacterial luciferase	EtGly	24, 31
D-amino acid oxidase	EtGly	77
Glucose oxidase	EtGly-MeOH	185
Cytochrome oxidase	EtGly	124
Xanthine oxidase	EtGly	174
Peroxidase	MeOH, Gly, EtGly, DMF	44, 82
Catalase	Gly	175
Hemoglobin	MeOH, EtGly	176
Cytochrome P450	Gly, EtGly	155, 177
Lactoglobulin	EtGly	81
Thermolysin	MeOH, DMSO	186
β-Lactamase	MeOH, DMSO	187
Malate dehydrogenase	MeOH	188
Glutamate dehydrogenase	MeOH	178
Leucine aminopeptidase	MeOH	189
Arginine kinase	EtGly	180, 181

[a] Abbreviations: MeOH, methanol; EtOH, ethanol; EtGly, ethylene glycol; Gly, glycerol; DMSO, dimethyl sulfoxide; DMF, dimethylformamide.

considered in detail below;[22] conformational isomers of liver alcohol dehydrogenase with bound NADH;[7] and noncovalent enzyme-substrate complexes preceding the tetrahedral adduct in the reaction of trypsin and elastase with *p*-nitroanilide substrates.[35,36]

4. Detection of Intermediates in Enzyme Catalysis

In virtually every enzyme-catalyzed reaction which has been studied at subzero temperature (Table 1) it has been possible to demonstrate the presence of a trapped intermediate species.

5. Determination of the Structure of Enzyme-Substrate Intermediates

The feasibility of preparing trapped enzyme-substrate intermediates in the crystalline state suitable for X-ray diffraction studies has been established.[19,20] The procedure has been applied to intermediates in elastase and ribonuclease catalysis,[13,19] and is considered in more detail subsequently.

6. Application to Multifunctional and Membrane-Bound Systems

A number of multifunctional enzyme complexes have been investigated using cryoenzymolgy. These include ribosomes,[194] cytochrome oxidase,[37] chloroplasts,[10] and cytochrome p-450.[38] Several of these systems are considered in Section III.

7. Detection of Intermediates in Protein Folding

Subzero temperatures and aqueous-organic cryosolvents have been used to detect intermediate species in the folding and unfolding of ribonuclease.[9]

C. Types of Cryosolvent

Several fluid media are now available for studying enzyme systems at low temperatures. These include aqueous-organic solvents of low freezing points,[39] supercooled water droplets suspended in nonmiscible solvents,[40,75] and inverted micelles with aqueous cores.[41] By far the most widely used cryoenzymological method at the present time involves the use of mixed aqueous-organic cryosolvents. Although enzymes have been dissolved in wholly organic solvents such as methylamine-dimethylamine-methyl hydrazine and have been shown to have retained activity upon evaporation of the solvent,[42] and mitochrondrial membrane proteins have had their physical properties evaluated in chloroform-methanol,[43] it seems unlikely that this class of solvent will be of general applicability. Solvent mixtures containing water appear to be far more useful, generally because they can have low-temperature properties very similar to those in which the particular enzyme under study is normally functional. The earliest reported investigations of enzyme catalysis at subzero temperatures in fluid cryosolvents are those of Maier and Tappel,[44,45] in which peroxidase and alkaline phosphatase catalysis were examined in 50% glycerol or methanol to temperatures as low as −30° C. However, it was not until the work of Douzou and his colleagues that a systematic study of the physicochemical properties of mixed solvents ensued. A knowledge of these parameters is essential if one is to optimize the conditions for enzyme activity and stability. A great deal of information is now available for many different cryosolvents, and their effects on enzyme integrity are presented below.[39]

Cosolvents are of three types: alcohols (methanol, ethanol), polyols (ethylene glycol, glycerol, 2-methyl-2,4-pentanediol), and aprotic solvents such as dimethyl sulfoxide and dimethyl formamide. The polyols are known to be relatively innocuous towards biological material since they are widely used in cryobiology to preserve living systems against fatal injuries due to freezing and thawing.[46] However, since there is a need to preclude rate-limiting enzyme-substrate diffusion, it is necessary to use fluid solvents of low viscosity at subzero temperatures. Consequently the ideal cryosolvents would be those containing methanol (which have much lower viscosities than those containing polyols or dimethyl sulfoxide even near their freezing points).[2,39] Unfortunately some enzymes are specifically denatured by even low concentrations of simple aliphatic alcohols. This problem may sometimes be overcome in two ways: (1) the synchronization of solvent addition and cooling may decrease the extent of denaturation by trapping the native state of the enzyme at subzero temperatures[47] and (2) ternary solvent mixtures containing a polyol and methanol can be used as a compromise.[48]

Consequently for the majority of biochemical systems it is relatively simple to find a fluid medium in which activity is retained at subzero temperatures. The choice of cosolvent is largely empirical at present since little can be predicted concerning the effect of a particular organic solvent on enzyme activity and structure. This is due to the complex and usually unknown structure of the solvent and its interactions with the protein. For instance there will be preferential attraction of the more polar component of the cryosolvent by solutes leading to clustering, and exclusion of the less polar

component[1] (see also Section I.E). Thus the composition of the solvent around the protein may be quite different from that in the bulk solvent.

Each enzyme system to be investigated at subzero temperatures must be tried in different cosolvents and at selected temperatures to test its catalytic activity, since the crucial issue of an enzyme-catalyzed reaction at subzero temperatures in aqueous organic solvents is whether the observed reaction pathway is the same as that under normal conditions and whether detected intermediates are on this pathway.[14] For instance, complications with hydrolase-catalyzed reactions in alcohol solvent systems can occur due to competition between water and alcohol for an enzyme-substrate intermediate.[1] In addition, cosolvents very often induce changes in the kinetic parameters of enzymatic reactions (K_m, k_{cat}), and even though these effects are fully reversible by infinite dilution and are independent of time, such changes may affect variations in the energy barriers corresponding to the formation of intermediates and transition states and introduce uncertainties into correlations with studies under normal conditions. Nevertheless good correlations have been generally found, and qualitative treatment suggests the net effect of subzero temperatures and cosolvents are small on hydrogen bonding and electrostatic forces, but may be more substantial for hydrophobic interactions.[1-4]

It is pertinent at this point to discuss the effect of cosolvents on physical parameters, such as pH and the dielectric constant of the medium, which directly affect enzyme activity. For the sake of clarity each parameter will be discussed separately.

*1. pH**

Since enzymatic reactions and enzyme stability are dependent upon the protonic activity of the medium, a measurement of the apparent pH (pH*) is necessary.[47] As the activity coefficient of the proton will vary with different solvent systems, each cryosolvent will have its own pH* scale.[2] Generally the pKa of weak electrolytes increases with the cosolvent concentration — sometimes as much as 3 pH units — since the extent of dissociation is dependent upon the dielectric constant of the medium.[39]

Douzou and co-workers have performed extensive and detailed studies on the cosolvent and temperature dependence of pH*.[39] It is possible to measure directly the pH* of a cosolvent-buffer system at any temperature using modified pH electrodes,[49] but generally an estimate of the pH* of a solution at subzero temperature is made from the linear dependence of pH* on temperature using ordinary electrodes above 0° C. The buffers most commonly used in cryosolvents are formate or chloroacetate (pH* 3 to 5), acetate (pH* 5 to 7), and cacodylate or phosphate (pH* 7 to 9). pH* vs. activity profiles for enzymes can shift due to the cosolvent effect on the pKa(s) of ionizing groups in the active site, as well as those of substrates: consequently the pH* must be adjusted accordingly if optimum conditions for catalysis are to be maintained.

Maurel and Douzou[50] have constructed enthalpy-entropy compensation patterns for the ionization of various catalytic groups at given temperatures for each of several solvent systems. Any exposed ionizable group would manifest a compensation pattern; the absence of a compensation pattern may be expected for buried groups located in a hydrophobic environment. It has been found that the compensation temperature is very different for neutral and cationic acids. The pKa of neutral acids increases with cosolvent concentration, and the pKa of cationic acids usually shows either little change or a decrease. Consequently a knowledgeable use of cryosolvents can throw light on the nature of the group involved in enzyme catalysis provided one is aware of the pitfalls of observed pKa assignments.[51]

2. Dielectric Constant

The dielectric constant (D) or electrical permittivity is the most fundamental

parameter of electrostatics involved in chemical laws (dissociation and solubility of macromolecules, acid-base equilibria, reaction rates), and it would be expected that addition of cosolvents affecting the dielectric constant of the medium would have a profound effect on enzyme activity and stability. While D normally decreases as the cosolvent concentration increases (markedly in the case of methanol and 2-methyl-2,4-pentanediol and less so for dimethyl sulfoxide and polyols),[4] it increases exponentially with respect to temperature (in mixed solvents). Consequently most cryosolvents have D values isodielectric with that of water (80 at 25° C) at subzero temperatures, usually well above their freezing points. However, a detailed study of enzyme reactions in different cryosolvents with different dielectric constants showed no correlation between the dielectric constant per se and observed variation in K_m and k_{cat}, and evidence suggested that electrostatic interactions were largely unaffected.[52] The effect of cosolvents on K_m appears to depend on a hydrophobic partitioning effect between the bulk solvent and the active site, when enzyme-substrate interactions are predominantly determined by hydrophobic interactions.[52] In practice the value of the dielectric constant appears to be of less importance than the specific inhibitory effect of certain cosolvents.

3. Viscosity

Viscosity is an important parameter of cryosolvent systems due to the inefficiency of mixing reactants at viscosities >50 cp, and the need to avoid conditions where diffusion controls the rate of reaction.[2] Essentially organic solvents change the network of hydrogen bonds in water, and an increase in the number of H-bonds, as in glycerol-water systems, will increase the viscosity over that for water in addition to the increase with decreasing temperature.[2] Ternary solvents are useful for decreasing solvent viscosity and some can be supercooled by 20° C. They normally consist of polyols and water, with methanol added at low temperatures to avoid the denaturing effect with methanol seen at higher temperatures.

4. Solubility and Ionic Strength

Generally neutral salts up to a concentration of 0.1 *M* (0.2 *M* for KI) are soluble in 50% organic solvents down to the freezing point of the solvent. Weak electrolytes present solubility problems in some cooled mixtures with high organic solvent content, but concentrations ≤0.01 *M* are generally satisfactory. The buffer salts most satisfactory with respect to solubility are chloroacetate, acetate, and cacodylate. The last of these can be used at a concentration of 0.1 *M*. Tris and phosphate both precipitate at low temperatures even at 0.01 *M* concentrations.

A major problem is that of enzyme solubility in cryosolvents. Quantitatively the addition of a protein to mixed solvents is equivalent to adding a salt. A low salt concentration favors the solubility of proteins in mixed solvents, whereas high salt concentrations decrease the solubility. This is in addition to the precipitating effect of organic solvents; consequently a balance must be sought between cosolvent and salt concentrations to maximize solubility at a given temperature. Solubility relates directly to temperature in mixed solvents, and both the solubility of salts and most proteins decrease as the temperature decreases. The underlying mechanism of solubility in mixed solvents may be largely electrostatic. Changing the dielectric constant can to some extent explain changes in solubility since adding a cosolvent to water decreases D. Generally the solubility of proteins increases as the pH* is removed from the isoelectric point, but it must be remembered that the isoelectric point may be different in mixed solvents to that in aqueous solution. The solubility of a particular protein is dependent upon five independent variables: temperature, pH*, cosolvent, salt, and protein concentrations. The best conditions for a particular concentration of enzyme to avoid precipitation at low temperatures can only be found empirically.

5. Polyelectrolytes

Douzou and Balny[53] have shown that water-soluble electrolytes at low ionic strengths develop strong electrostatic potentials so as to cluster molecules of water at the expense of the organic solvent and induce relative solvation of the enzyme system. This has a net effect of protection against cosolvent effects on kinetic parameters such as K_m. For instance, K_m for the hydrolysis of benzoyl-L-arginine ethyl ester by trypsin has a value of 2×10^{-4} *M* in 50% dimethyl sulfoxide, pH* 9.4, whereas the addition of microgram quantities of RNA lowered K_m to a value similar to that in aqueous solution.[53] The adverse effects of cosolvent on k_{cat} were also partially removed by the addition of polyelectrolytes. This rationale is also used to explain the protecting effect of RNA on ribonuclease in cryosolvents and of lysozyme, where the enzyme itself is a polycation.[54]

Low temperature procedures in the presence of organic solvents are not free from problems even when all the requirements to assure enzyme activity are fulfilled (namely pH*, D, ionic strength). These additional potential problems are associated with intrinsic effects of cosolvents such as a disruption of the long-range intermolecular order of water, and a change in the solvation and transport behavior of ions and bases. In part these drawbacks have spurred a search for conditions in which the only altered parameter from that in aqueous solution at ambient temperatures is temperature.

With water-in-oil emulsions[55] it is possible to stabilize supercooled water against freezing due to heterogeneous nucleation.[56] This extends the experimentally accessible temperature range to the onset of homogeneous nucleation (−40° C). Such emulsions have been prepared by dispersing water (containing buffer, enzyme, and substrates) in heptane, safflower oil, or silicones of low viscosity, supersaturated with a water-insoluble surfactant to prevent coalescence of water droplets, and microemulsifying to give water droplets with diameters from 1 to 5μm.[40,57] This cosolvent-free aqueous medium appears to correspond more closely to physiological conditions and has been applied to the preservation of single cells at subzero temperatures.[58] Although there are many technical difficulties associated with enzyme studies in this medium, results are encouraging.

Studies on various redox states of cytochrome P450 have shown that the spectra recorded are identical to those obtained under normal conditions at the same temperatures, and that the stability of oxyferro-cytochrome P450 was similar in water-in-oil emulsions and cryosolvents at −20° C.[57] Interestingly, the thermodynamics governing the spin-state equilibrium of camphor-bound cytochrome P450 were similar in supercooled water and under normal conditions, but markedly different in cryosolvents.[57] Douzou et al.[55] working on the recombination of horse radish peroxidase with carbon dioxide following photodissociation in supercooled water have shown the activation energy for the process in homogeneous and emulsified water is practically identical, whereas there is a marked difference in mixed solvents. These results appear to reflect the relatively unmodified nature of supercooled water in contrast to more significant effects on solvent structure observed with the use of cryosolvents. However, temperature effects will still operate in supercooled water. The dielectric constant will be 100 at −40° C, for instance, and temperature effects on the pKa(s) of weak electrolytes, e.g., buffers and enzyme residues, will still be present. The extent of this effect will depend on the type of ionizing group due to differences in the enthalpies of dissociation. Consequently there may be shifts in pH-activity profiles of enzymes as the temperature is decreased. Such an effect can, however, be corrected by suitable adjustments in the buffer employed.

The main drawbacks of water-in-oil emulsions are concerned with the very high viscosity and turbidity of such systems. While such features are necessary results of providing suitable stability and supercooling of water, the first precludes the rapid mixing of reactants and the second dictates the use of special optical instrumentation due to light scattering. In addition one cannot use proteins which are likely to be adsorbed at the oil/water interface such as proteins of membrane origin. Finally the addition of

polymeric solutes such as polyethylene glycol, which further depress the homogeneous nucleation temperature may give rise to unwanted cosolvent effects.

An alternative procedure to that of water-in-oil emulsions has been the use of inverted micelles with aqueous cores.[41,59] Amphiphilic surfactants (generally anionic such as sodium di-iso-octyl sulfosuccinate) in the presence of water in low viscosity silicone oils have been used to form reversed micelles which are homogeneous and give rise to transparent solutions of low viscosity. These media can be regarded as solutions of water in apolar solvents, the amphiphile acting as cosolvent. The great advantage of these solutions is the optical transparency to 250 nm and the very low viscosity ($\leqslant$1 cp at room temperature). Most of these solutions are of low freezing points ($-50°$ C) but show turbidity well before this point, and the lowest temperature usually obtainable without turbidity is $-40°$ C.[59] The higher the water content of the solutions, the higher the temperature at which turbidity occurs; however, lower concentrations often alter the absorption spectra of dissolved enzymes, possibly resulting from effects due to limited "free" water. It is known that entrapped water in reversed micelles is characterized by a microviscosity which is superior to that of bulk water, and increases as temperature decreases. Thus ice-like structures may be present in the water pools above the freezing point of the apolar solvent.

There is still only limited knowledge of the actual physico-chemical properties of micromicellar solutions of enzymes such as the local pH conditions, and interface and partition effects. Also the adjustment of effective polarities, acidities, and ionic strength of these solutions is still largely empirical. Many enzymes are soluble and active in reversed micelles, depending upon a complex relationship between their molecular weights, the water content, the type of surfactant used and the water-to-surfactant ratio.[41] For instance, the absorption spectra of most enzymes are markedly influenced by the last variable, and the small size of the micelles, often only accommodating one protein molecule, undoubtedly leads to a more highly ordered water structure than normally found in bulk water. In addition the enzyme is not always protected from the denaturing effects of the bulk apolar solvent — often resulting in a total and progressive loss of activity. Studies with trypsin[41] and cytochrome P450[59] have shown that substrate affinity is greatly decreased in micromicellar solutions from that under normal conditions. This effect is probably due to a partitioning of enzyme, substrate, or both, between surfactant-entrapped water molecules and those occupying the core of the micelle, and differential interactions (electrostatic, hydrophobic, nucleophilic, and electrophilic) altering the free energy of activation of the overall catalytic process. In addition pH activity profiles have been shown to be shifted in micellar solutions due to the electrostatic effect of the surfactant head groups.[41]

Consequently, although many enzymes are active in water pools the available evidence shows that this unique reaction medium exerts a profound influence on such activity, resulting in sometimes large and unpredictable effects. More studies are clearly needed before the effects of changing the nature and concentration of surfactant, the concentration of water, and the type of nonpolar solvent can be evaluated. Nevertheless results clearly show that micromicelles can be successfully used to investigate enzyme systems at subzero temperatures, have extreme versatility, and are free of the problems of viscosity and turbidity associated with oil-in-water emulsions. It appears that no single low temperature system is going to be ideal in cryoenzymology; each should complement the other.

D. Cryosolvent Effects on Protein Structure and Enzyme Function

The relative merits of the various types of fluid solvents which have been used in cryoenzymological investigations have been considered above. By far the majority of the

extant studies have been carried out in mixed aqueous-organic solvent systems, rather than the various types of supercooled water systems.

The question regarding the effects that such cosolvents may have on both the protein structure and the enzyme function is obviously of critical importance if the results at subzero temperatures are to be considered applicable to the reaction mechanism under normal conditions. The underlying physical bases of any such effects are unlikely to be well understood, given our general poor understanding of liquid structure and of protein-solvent interactions. Pragmatically, however, what is of concern in cryoenzymological studies is whether any adverse solvent effects occur, and if they do, the manner in which they are manifested.

Since hydrophobic interactions play the major role in stabilizing the native conformation of proteins, and since aqueous-organic cryosolvents have considerably greater hydrophobicity than aqueous solvents,[52] concern about possible effects of the cosolvents on protein structure is quite justified, and in certain cases a definite problem. It is therefore essential that the first stage of a cryoenzymological investigation of a particular enzyme should demonstrate that the cryosolvent used does not have any adverse effects on the protein structure. This can be done in a number of ways.[14,60] Different approaches to this question involve techniques of differing resolution, and hence sensitivity, to solvent-induced effects on protein structure. At one extreme is X-ray crystallographic comparison of the crystalline protein in aqueous and cryosolvent,[19] at the other is UV difference or fluorescence-difference spectrophotometry.[32,61,62] The results which have emerged from a number of investigations of cosolvent effects on enzyme structure may be summarized as follows:

1. The native structure is destabilized relative to that in aqueous solution, i.e., the thermal denaturation occurs at lower temperature.[9,62]
2. Spectral signals associated with exposed aromatic residues are perturbed by the cosolvents.[14,32,61,62]
3. The native structures in the cryosolvents appear to be very similar to those in aqueous solution.[19] For example, Figure 1 shows the aromatic region of the high resolution (360 MHz) protein N.M.R. spectrum of ribonuclease A in aqueous and methanolic cryosolvents. Such similar spectra must reflect very similar underlying protein conformations.
4. The native structures in cryosolvents usually have very similar catalytic properties to those in aqueous solution (vide infra).
5. There is good evidence that the "hydration" shell about the surface of the native protein is not significantly perturbed by the cosolvent (see next section).

The effect of alcoholic cryosolvents on bovine ribonuclease A provides a good example to illustrate the lack of effect of cosolvent on protein structure. Both ethanol[9] and methanol[191] destabilize the native state of ribonuclease, as determined by the midpoint of the thermal denaturation transition. For example, as shown in Table 2 as the methanol concentration is increased from 0 to 80% the Tm decreases from 42.0 to 10.6° C. In addition the cooperativity of the transition decreases as the methanol concentration increases and the Tm decreases (Table 2). The most likely explanation of this observed decrease in cooperativity lies in the stabilization of partially folded intermediate states, which are not significantly populated under normal denaturation conditions.

At 0° C in cryosolvents in which the enzyme is in its native state, the catalytic properties toward 2′,3′-CMP in aqueous solution, 60% ethanol, and 70% methanol are quite similar both in terms of observed reaction rates and pH and temperature dependencies.[9] Since the catalytic activity of an enzyme is expected to be very sensitive to any significant

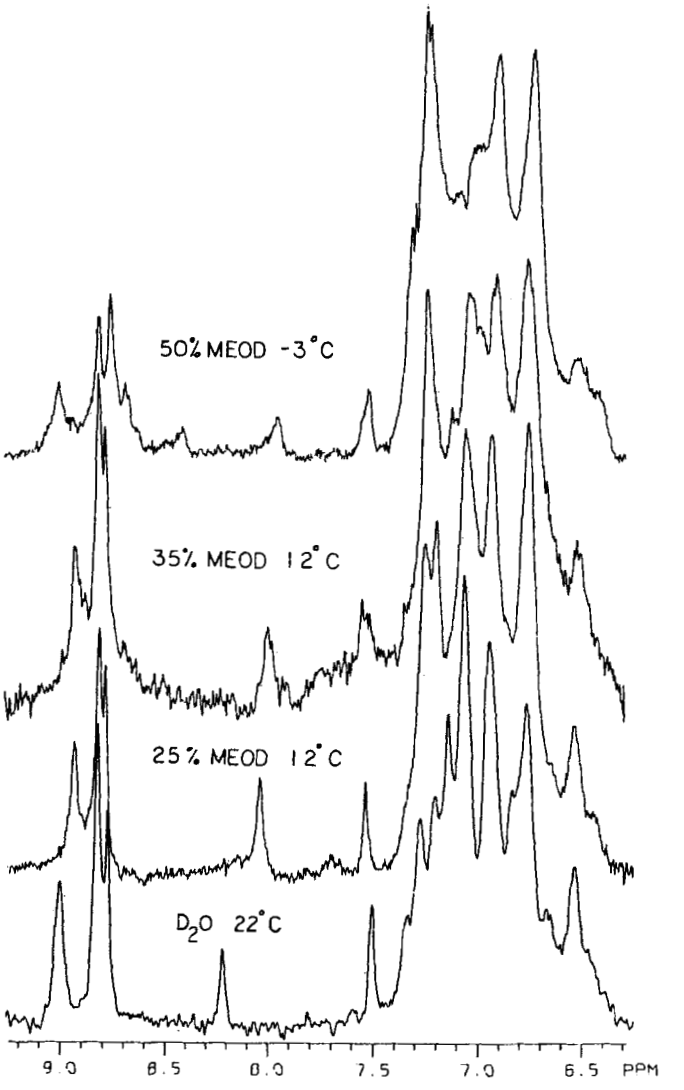

FIGURE 1. High resolution ^{1}H N.M.R. spectra of the aromatic region of native Ribonuclease A in aqueous and methanolic solutions pH* 3.0.

adverse structural perturbation, these findings indicate that the native state in alcohol cryosolvents must be very similar to that in aqueous solution. High-resolution ^{1}H N.M.R. experiments support this claim. As shown in Figure 1 the spectra of the native protein in methanol cryosolvents, up to at least 70% methanol, are essentially identical to those in aqueous solution at the same apparent pH. Such similarity in chemical shifts implies that the underlying native conformation of the enzyme must be essentially the same in aqueous and cryosolvent solution.

Further support in this regard comes from X-ray crystallographic studies which reveal that the overall structure of ribonuclease A in alcohol cryosolvents, both at room temperature and at −60° C, is the same as that in aqueous solutions.[192] Detailed studies on other enzymes, e.g., chymotrypsin,[32] trypsin,[61] and papain[62] support the generality that the *native* states of enzymes (i.e., at temperatures below the beginning of the thermal denaturation transition) in cryosolvents are very similar to those in aqueous solution.

Table 2
EFFECT OF METHANOL CONCENTRATION ON T_M AND THE COOPERATIVITY OF THE TRANSITION FOR RIBONUCLEASE A[191]

MeOH (%)	T_M (°C)	T (°K) 10% denatured/ T (°K) 90% denatured
0	44.0	0.964
9.9	42.0	0.970
19.9	39.9	0.960
29.9	37.3	0.966
39.8	32.0	0.952
49.8	28.8	0.934
59.5	23.7	0.926
69.5	21.0	0.908
74.7	17.6	0.919
79.4	10.6	0.881

E. Cosolvent-Protein Interactions

The fact that many enzymes have now been shown to essentially possess the same catalytic activity in mixed aqueous-organic cryosolvents at low temperatures as in aqueous solution means that the relatively more hydrophobic cryosolvents are not causing a significant change in the protein conformation. There are of course certain caveats associated with the above statement, e.g., the temperature must be low enough to be below the thermal denaturation transition (in many cases such temperatures are above 0° C), and corrections for cosolvent effects on the pK(s) of key ionizing groups which may shift the pH optimum must be taken into consideration, as must the possible effect of cosolvent on substrate binding. However, these are effects that do not perturb the gross conformation of the enzyme.

On the basis of the generally accepted role of hydrophobicity in the maintenance of the native state of proteins[63-65] it is surprising, and unexpected, that methanol, ethanol, dimethyl sulfoxide, and dimethyl formamide-based cryosolvents do not cause denaturation or altered conformations. It is apparent that the lack of cosolvent effect on protein structure must, at the molecular level, reflect a relatively intact hydration "shell" surrounding the protein in the cryosolvent. Some experimental support for this assumption comes from crystallographic observations on elastase and ribonuclease A, both in 70% aqueous methanol cryosolvents, which showed no evidence of cosolvent associated with the protein molecule.[192]

Recent additional evidence for this hypothesis, as well as a thermodynamic explanation for the phenomenon, comes from a study of the interaction of 2-methyl-2,4-pentanediol (MPD) with ribonuclease A by Pittz and Timasheff.[66] In this investigation light scattering, circular dichroism, and refractive index measurements were used to demonstrate preferential interaction of water to ribonuclease in aqueous-MPD solvent mixtures, i.e., an apparent repulsion of the MPD from the protein, as well as aggregation of the enzyme in the presence of the relatively hydrophobic cosolvent.

The results indicated that, as might be expected due to the hydrophobicity of the cosolvent, the presence of MPD causes thermodynamic destabilization of the whole system (MPD-water-ribonuclease). This thermodynamic destabilization, relative to an aqueous solution of ribonuclease A, will tend to drive the system to a new equilibrium state. This could occur through one, or a combination, of three ways: (1) a change in the protein conformation so as to minimize the thermodynamically unfavorable protein-solvent contacts, or through a reduction in protein-cosolvent interactions either by

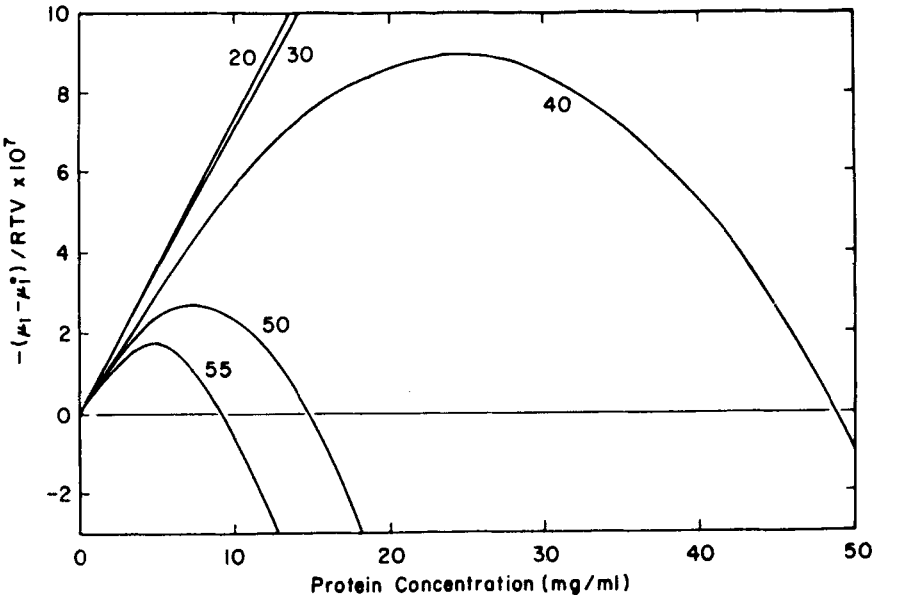

FIGURE 2. Effect of MPD on the chemical potential of water as a function of protein concentration, at pH 5.8, 25° C. The numbers on the curves refer to the volume percent of MPD in the system. (Reprinted with permission from Pittz, E. P. and Timasheff, S. N., *Biochemistry*, 17, 620, 1978. Copyright (1978) American Chemical Society.)

(2) preferential exclusion of cosolvent from the vicinity of the protein (i.e., preferential hydration) or (3) through protein aggregation (which involves less protein-cosolvent contacts).

The data for MPD (but not for 2-chloroethanol or ethylene glycol[67]) indicate that mechanism (1) does not occur, but that both (2) and (3) are operative. It seems reasonable that these findings are of general applicability to protein-aqueous-organic solvent systems. For reasons relating to the physical-thermodynamic properties of the cosolvent, some cosolvents will tend to promote protein conformational changes as a means of minimizing the inherent destabilization introduced by the presence of the organic cosolvent. Such solvent-protein interactions will be unfavorable as far as biological activity is concerned; hence such solvent systems will be unsatisfactory as cryosolvents. For example, the presence of a few percent of methanol causes total loss of activity of β-glucosidase.[21] In other cosolvent-protein systems the thermodynamic destabilization will be minimized either by preferential hydration or protein aggregation. Our observations suggest that cryosolvents found to be satisfactory for cryoenzymology studies will normally tend to operate by both such mechanisms, leading to the observed tendency of many proteins to aggregate in cryosolvents.

As shown in Figure 2 the chemical potential of ribonuclease is negative in 50% MPD up to 15 mg/mℓ protein at 25° C. This means that at this temperature, concentrations of the enzyme above 15 mg/mℓ will result in phase separation, i.e., aggregation and precipitation as observed.

This hypothesis provides an attractive rationale for many of the observations concerning protein-cosolvent interactions. The question arises, however, as to the nature of the underlying property responsible for determining whether the unfavorable potential protein-cosolvent interaction will be alleviated by protein structural changes, or by preferential hydration and aggregation. Since ΔG_{tr} (the free energy of transfer) of amino acid side chains from water to 50% MPD is similar to that for transfer to

Table 3
INCREASE IN K_M DUE TO THE PRESENCE OF ORGANIC COSOLVENT FOR A NUMBER OF ENZYME-SUBSTRATE SYSTEMS

Enzyme	Substrate	Cryosolvent[a]	Increase in K_M	Ref.
Chymotrypsin	*N*-acetyl-L-tryptophan *p*-nitrophenyl ester	DMSO, 65	500-fold	32
Papain	N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester	DMSO, 60	1000-fold	62
β-Glucosidase	*p*-Nitrophenyl-β-D-glucoside	DMSO, 50	10-fold	34
Lysozyme	Chitotriose	MeOH, 80	11.5-fold	71
Ribonuclease	RNA	MeOH, 30	0	52
Trypsin	N^{α}-benzyloxy carbonyl-L-lysine *p*-nitrophenyl ester	DMSO, 65	150-fold	61
Carboxypeptidase A	O-(trans-p-chloro cinnamoyl)-L-β-phenylacetate	EtGly, 50	0	100
β-Galactosidase	*o*-Nitrophenyl-β-D-galactoside	DMSO, 50	0	78
LADH	Ethanol	DMF, 50	300-fold	173

[a] Abbreviations: MeOH, methanol; EtGly, ethylene glycol; DMSO, dimethyl sulfoxide; DMF, dimethylformamide.

denaturing cosolvents,[68] the lack of denaturing effect of MPD must be due to its exclusion from the immediate vicinity of the protein and not to a less-favorable free energy of transfer.

An examination of the mole fraction of salt required to cause phase separation — i.e., "salting out" of the cosolvent in 50% aqueous MPD and 50% aqueous 2-chloroethanol — revealed that MPD was much more effective in promoting such phase separation.[66] These results indicate that aqueous MPD solvent systems become very thermodynamically destabilized in the presence of charged groups. Pittz and Timasheff thus conclude that it is the charged groups on the protein surface which are responsible for repelling MPD from the immediate vicinity of the protein. Physically this occurs by the "coating" of the charged groups on the protein by either water (preferential hydration) or protein (aggregation).

It has long been known that the presence of an organic solvent will usually cause an increase in the observed value of K_m in an enzyme-catalyzed reaction.[69] Empirically the relationship between solvent concentration and either K_m or K_i (for competitive inhibitors) is found to be log-linear.[32,52,62,70] Consequently enzyme-catalyzed reactions carried out in cryosolvents based on mixed aqueous-organic solvents systems exhibit this phenomenon. The observed increases in K_m vary with the nature of the cosolvent and its concentration, and the particular enzyme-substrate system (Table 3) and range from very small[22,71] to very large.[62] In early investigations these effects were attributed to the decreased dielectric constant of such mixed solvents; however, the results from several systems indicated that dielectric effects alone were insufficient to account for the observed effects.[72,73] Various alternative possibilities were considered, the most promising being a combination of dielectric constant effects on substrate partitioning, coupled with competitive inhibition by the cosolvent.[72] It should be noted that these investigations were made under rather restrictive conditions in that only a single cosolvent of reduced dielectric constant was used.

Maurel[52] has recently reported a detailed investigation of the effect of solvent dielectric constant and hydrophobicity on enzyme catalytic parameters. Solvent systems of reduced, similar, and increased dielectric constant relative to that of water were studied, using enzyme systems in which either electrostatic or hydrophobic interactions were

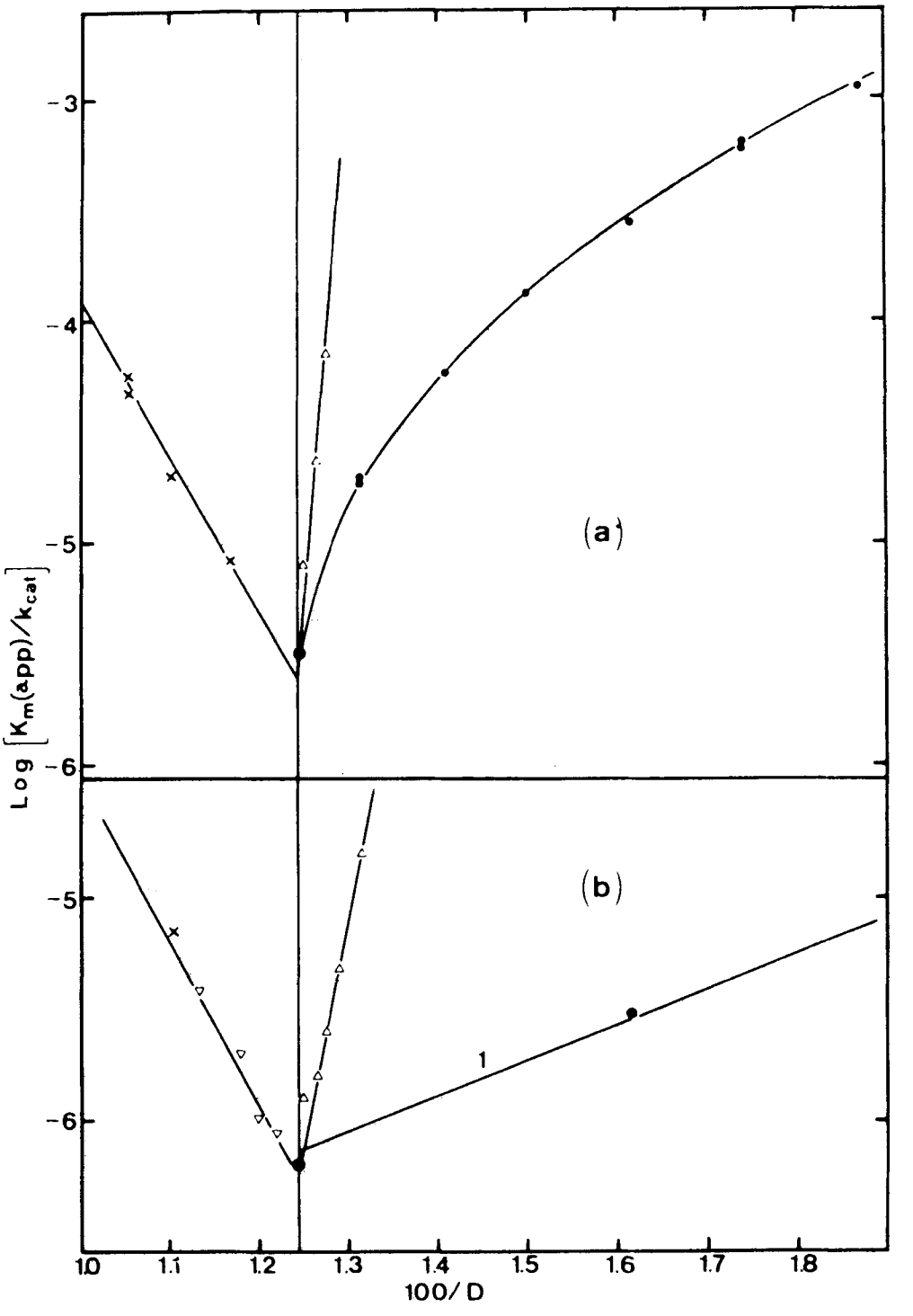

FIGURE 3. Plots of log [$K_{m(app)}$ / k_{cat}] against the reciprocal of dielectric constant for various water-organic solvent mixtures. (a) α-Chymotrypsin/ *N*-acetyl-L-tryptophan ethyl ester reaction; (b) trypsin/benzoyl-L-arginine ethyl ester reaction. Dioxane, ●—●; dimethylsulfoxide, Δ—Δ; formamide, x—x; N-methylformamide, ∇—∇. Straight line 1, results in water/dioxane (70:30) from Reference 73 for comparison. (From Maurel, P., *J. Biol. Chem.*, 253, 1680, 1978. With permission.)

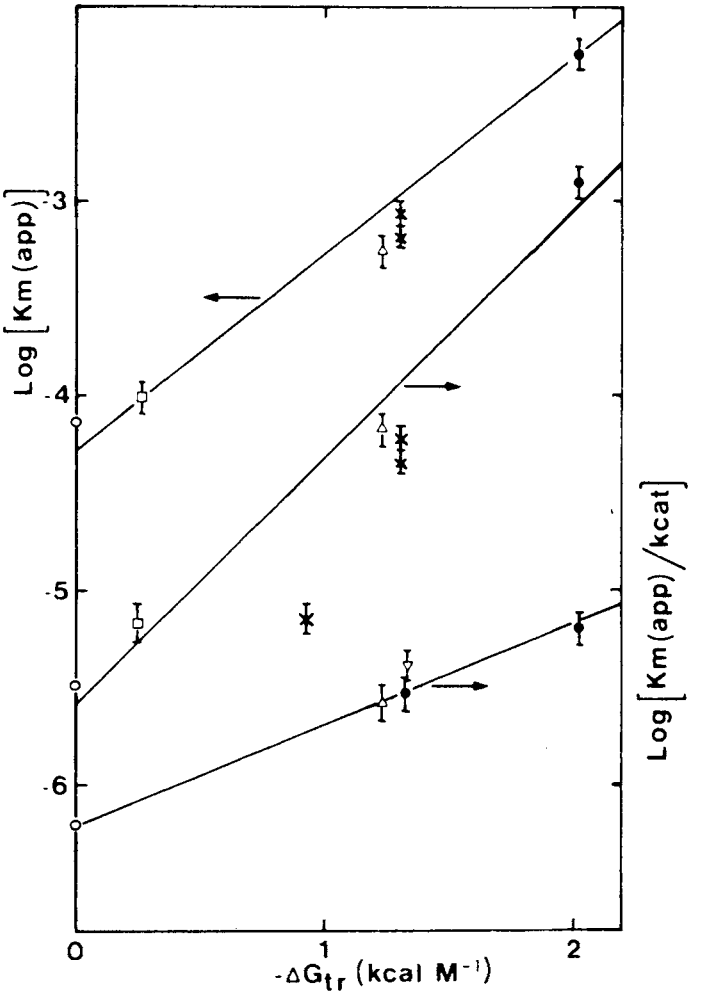

FIGURE 4. α-Chymotrypsin and trypsin reactions. Plots of log [$K_{m(app)}$] and log [$K_{m(app)}$ / K_{cat}] against Δ G_{tr} in pure water and the various water-organic solvents (70:30 if not specified). Upper lines — α-chymotrypsin; ○—○, water; ●—●, dioxane; △—△, dimethylsulfoxide; x—x, formamide; □—□, glycerol. Lower line — trypsin: ○—○, water; ●—●, dioxane, △—△, dimethylsulphoxide (20 and 30%); x—x, formamide (20%); ▽—▽, *N*-methylformamide (25%). (From Maurel, P., *J. Biol. Chem.*, 253, 1682, 1978. With permission.)

paramount in substrate binding. The parameters measured were k_{cat}, K_m, and their ratio. For both chymotrypsin (involving predominantly hydrophobic interactions in substrate binding) and trypsin (involving mixed hydrophobic and electrostatic interactions) no correlation between solvent dielectric constant and k_{cat} / K_m was found (Figure 3). For the ribonuclease-RNA system (in which electrostatic interactions predominate), variation in the solvent dielectric constant had very little effect on K_m,[52,54] On the other hand, a good correlation between solvent hydrophobicity (as measured by the G_{tr} — i.e., solubility — of *N*-acetyl-tryptophan ethyl ester) and K_m was found for both chymotrypsin and trypsin for several aqueous-organic solvents of widely different dielectric constant (Figure 4). The conclusion to be drawn from these results is that it is the solvent hydrophobicity, and not its dielectric constant, that is more responsible for the frequently observed increase in

K_m in cryosolvents. Furthermore these results suggest that for protein interactions in general, be it with small ligands or other proteins, it is the hydrophobicity of the solvent system, and not its dielectric constant, which will potentially cause changes in the nature of the interaction in the cryosolvent compared to that in water. Additional effects due to competitive inhibition by the cosolvent may occur. For example the curvature in the plot of k_{cat}/K_m for chymotrypsin in dioxane (Figure 3) has been attributed to the known binding of dioxane in the active site.[52]

From the Debye-Huckel theory the free energy of electrostatic interaction of two ions in solution involves contributions from both ion-ion interactions and the ionic atmosphere (the Debye-Huckel constant) which change in opposite directions as the dielectric constant varies. In addition the dielectric constant at the protein-solvent interface is likely to be considerably different from the bulk dielectric constant, and also to vary from cosolvent to cosolvent (see preceding section).

From studying the effect of cosolvent concentration on the affinity of an enzyme for a competitive inhibitor[52,70] it has been shown that the effects on K_i are parallel to those on K_m, indicating that the effect of the cosolvent is on affinity of the enzyme for the ligand. The phenomenon may be viewed as an hydrophobic effect on the partitioning of the substrate between bulk solvent and the active site. Those enzyme systems in which hydrophobic interactions make a significant contribution to the binding will thus be most affected: the substrate will partition less favorably to the more hydrophobic active site in the presence of the mixed aqueous-organic solvent system than in aqueous solution, due to the greater hydrophobicity of the solvent in the former case.

F. Cosolvent and Temperature Effects on the Free Energies of Proteins

The presence of the organic cosolvent in a cryosolvent causes significant changes in the structure and physical properties as compared to aqueous solution; in particular the dielectric constant and the hydrophobicity of the solvent will be different. In addition, of course, the actual structure of the solvent will be changed; e.g., different hydrogen bonding patterns. The nature of the solvent-protein interactions will therefore be different. In particular one expects effects on the pK(s) of ionizable groups, changes in the nature of electrostatic interactions within the protein, and possible differences in solvation of exposed groups. It is also apparent that one will find differences in the absolute Gibbs free energies of the protein itself, as well as any intermediates and transition states present during the catalytic reaction. Since it is conceivable, perhaps even likely, that the nature of the interactions between the protein and the solvent will be somewhat different in different intermediates and transition states, one would expect that the relative Gibbs free energies for each enzyme species (i.e., free enzyme and enzyme-substrate complex or intermediate, or transition state) may be different in a cryosolvent as compared to aqueous solution under otherwise similar conditions, e.g., the same temperature and apparent pH. The significance of this aspect is that the values of parameters such as ΔH_o, $\Delta H^{\dagger}$, $\Delta G^{\dagger}$ etc. will be expected to be somewhat different in the cryosolvent compared to their values in aqueous solution.

In a similar fashion one would expect that differences in temperature would also result in different protein-solvent interactions, with consequent effects on the thermodynamic parameters of the system. Thus even if supercooled water is used as the cryosolvent one should not be surprised to find differences in the activation parameters. Rather surprisingly there have been very few detailed investigations of the effect of varying environmental parameters on the thermodynamic properties of proteins. Privalov[74] has calorimetrically measured the thermodynamic properties of native and denatured lysozyme as a function of pH and temperature. This work may be considered a prototypical study of the temperature dependence of a given solvent variable — in this

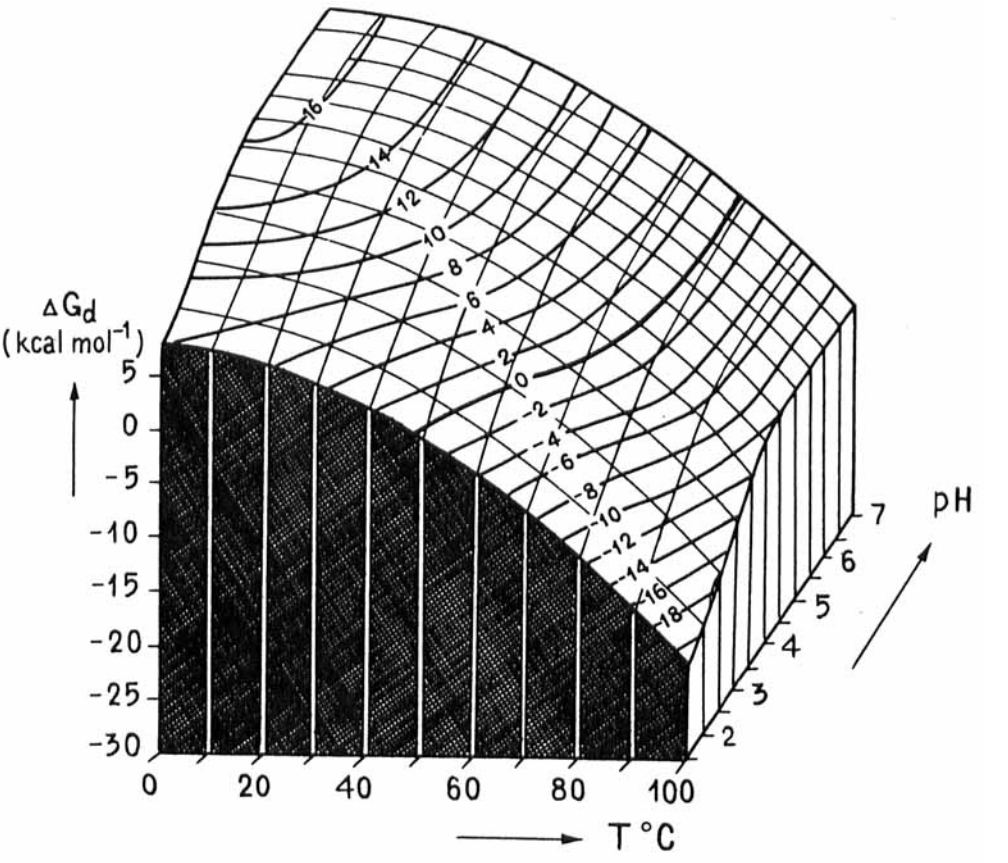

FIGURE 5. Denaturational Gibbs energy change ΔGd vs. temperature and pH for lysozyme. (From Pfeil, W. and Privalov, P. L., *Biophys. Chem.*, 4, 41, 1976. With permission.)

case pH. The results (Figure 5), e.g., show that as pH is varied at a given temperature, there is considerable change in the free energy of the particular state of the protein. Similarly if the pH is held constant, the free energy of the species changes as the temperature changes. One would expect similar three-dimensional energy surfaces if other variables, such as cosolvent concentration, are used in place of pH in the above investigation.

There are several pieces of data in the literature to support this idea. If there are relative changes in the enthalpy of the ground state of a particular intermediate and its transition state on going from aqueous to cryosolvent, then a change in the observed energy of activation is expected. The following are some examples of systems which show both large and small solvent effects on energies of activation. Figure 6 shows a comparison of the Arrhenius plot for the autooxidation of bacterial cytochrome P450 in the oxy-ferro state, without substrate in aqueous buffer (1′) and 50% ethylene glycol (2′). The energies of activation differ by 5 kcal mol^{-1}. In the presence of the substrate (lines 1 and 2), however, the energies of activation are the same within experimental error.[4] The data suggest that the differences in enthalpy of the protein in the ground and transition states in the presence of the substrate are essentially the same in both aqueous and cryosolvent solutions, whereas in the absence of the substrate either the ground state structure, or that of the transition state, or both, are significantly different.

Another case in which substantial changes in energy of activation occur on changing

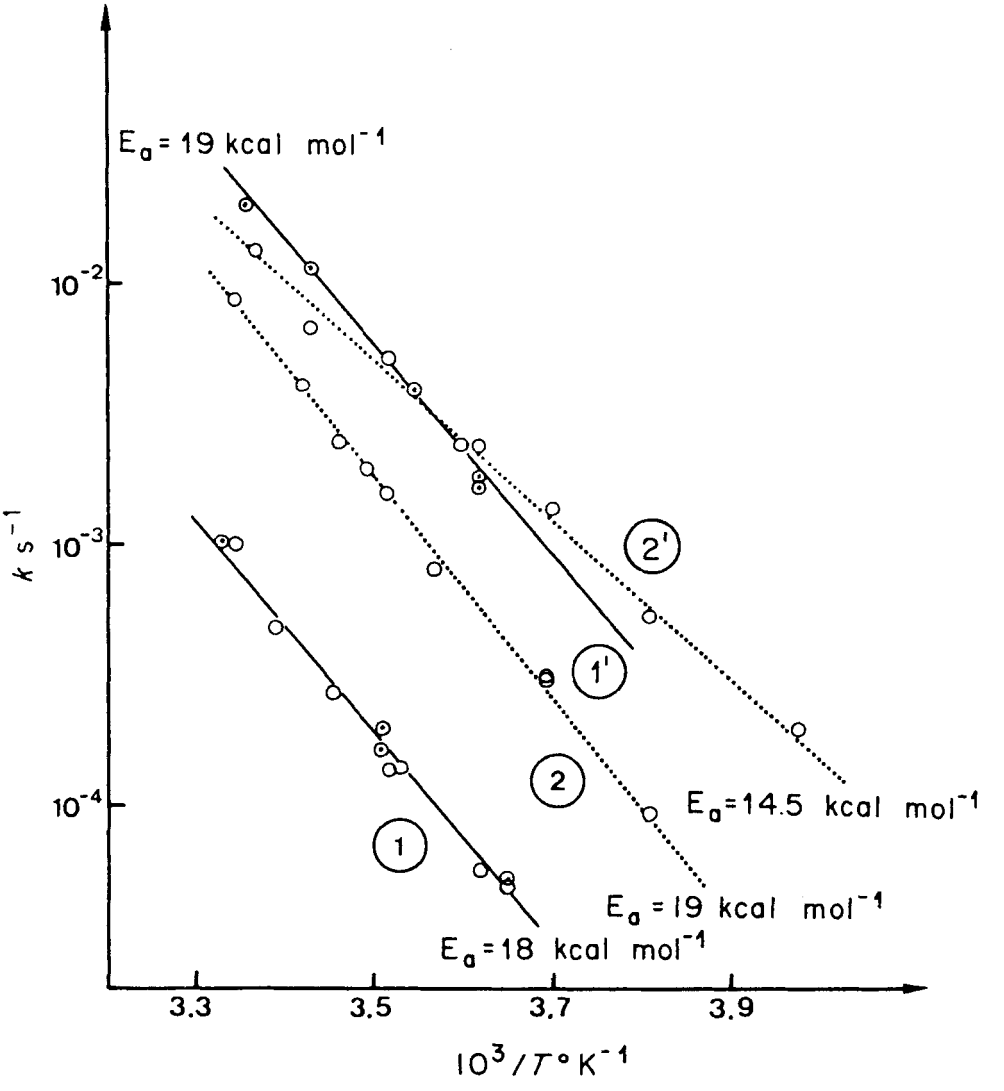

FIGURE 6. Arrhenius plots of the autoxidation rate constants of $m_{\bar{o}_2}^{\pi}$ and $m_{\bar{o}_2}^{l}$ in aqueous buffer and EGOH-buffer mixtures (volume ratio 1:1): (1) $m_{\bar{o}_2}^{\pi}$ in 0.05 *M* phosphate buffer containing 3 m*M* camphor and 0.1*M* KCl (pH 7.4, pH 7); (2) $m_{\bar{o}_2}^{\pi}$ in the 0.1 *M* phosphate buffer (pH 7)-EGOH mixture (pa_H = 7.4 at 20°C) containing 3 m*M* camphor and 0.1 *M* KCl; (1') $m_{\bar{o}_2}^{l}$ in 0.5 *M* phosphate buffer (pH 7.4); (2') $m_{\bar{o}_2}^{l}$ in the 0.1 *M* phosphate buffer (pH 7)-EGOH mixture (pa_H = 7.4 at 20°C). (From Douzou, P., *Advances in Enzymology*, 45, 231, 1977. With permission.)

solvent is the recombination of CO to horseradish peroxidase after photodissociation.[55] In this system the energies of activation for the reaction are the same in aqueous and supercooled water solutions, and considerably larger in 50% ethylene glycol.

Thus the observation that the energy of activation, or the pH dependence, of an enzyme-catalyzed reaction is different in a cryosolvent does not necessarily imply that there is any change in the reaction pathway or mechanism. Rather, such changes reflect the expected effects of the different solvent environment on the physical properties of the protein.

Table 4
THE TEMPERATURE DEPENDENCE OF THE APPARENT pK$_a$ OF THE IONIZATION-CONTROLLING DISSOCIATION OF β-LACTOGLOBULIN DIMERS IN 50% ETHYLENE GLYCOL[81]

Temperature (° C)	20	10	0	−10	−20	−30	−40
pK apparent	7.7	8.1	8.4	8.7	9.05	9.3	9.55

G. Effects of Low Temperatures on Oligomeric Proteins

Although most proteins are more stable at lower temperatures, some enzymes exhibit loss of activity as the temperature is decreased. These proteins are oligomeric in nature, and are commonly called cold-labile.[76] It has generally been assumed that the loss in activity is due to dissociation of the subunits to the inactive monomeric form and is caused by the weakening of hydrophobic interactions maintaining the quaternary structure. The basis for these assumptions lies mostly in the fact that hydrophobic interactions are entropy driven, with very little contribution from enthalpy.[63-65] Consequently, the strength of the hydrophobic interaction will decrease with decreasing temperature. In addition, it has generally been assumed that the major attractive force involved in subunit interactions is hydrophobic. One would therefore predict that low temperatures, and particularly subzero temperatures, would tend to cause oligomeric proteins to dissociate into monomers. In fact since hydrophobic interactions also play a major, if not the major, role in stabilizing the folded structure of the subunit itself, one might also expect that low temperatures would destabilize the native conformation.

Given the anticipated loss of quaternary structure to be expected at subzero temperatures, one is faced with an apparent paradox in that a number of oligomeric enzymes (see Table 1) have been found to be catalytically active at subzero temperatures, and in some cases to show no evidence for any such dissociation at temperatures as low as −60° C.[7,34,77,78] Similarly it is clear, as previously discussed, that at least most proteins that are monomeric do not show any evidence of unfolding in the subzero temperature range accessible to cryoenzymology studies. How can we account for this apparent discrepancy between observed and thermodynamically expected behavior? Bock and Frieden[76,79] have presented evidence to indicate that in many cases, possibly all, the low temperature-induced dissociation of oligomers leading to cold lability is in fact due to a pH effect. That is the change in temperature perturbs the pK of an ionizable group involved in the quaternary structure, so that it is really the change in ionization state that is responsible for the dissociation. Thus it is the shift in the apparent pK responsible for the pH-dependent inactivation that is the cause of the loss of activity at low temperatures.

Douzou and co-workers[2,80,81] have carried out a detailed investigation of the effects of temperature and solvents on the state of association of β-lactoglobulin. It was known that the protein existed as a dimer of nonidentical units at pH 7, 25° C, and that lowering the temperature to 0° C resulted in dissociation to the monomeric subunits. The presence of 50% ethylene glycol did not affect this process. A detailed investigation revealed that at 20° C the dissociation phenomenon was dependent on an apparent pK of 7.7, i.e., pH values below 6 favored the dimer, whereas higher values resulted in the reversible dissociation. By studying the reaction in 50% ethylene glycol it was possible to determine the effect of decreasing temperature on the pK* of the ionization controlling the dissociation. As may be seen from Table 4 the pK* moves to progressively higher values as the temperature is decreased (at −40° C the pK* having risen 2 units to 9.5). The net effect of this temperature-dependent ionization is that at subzero temperatures, e.g.,

−40° C, the dimer will be the stable species at pH* values as high as 8.5. Therefore in the appropriate pH* range one can observe that at 20° C the protein is dissociated, but as the temperature is decreased the monomers associate.[2]

Obviously the quaternary structure of β-lactoglobulin is controlled by a key ionization suggesting that the controlling element is an electrostatic, rather than a purely hydrophobic interaction. The fact that in at least many cases of cold lability in proteins the effect is actually a pH-dependent one (see above) suggests that key ionic interactions as observed with β-lactoglobulin may be quite common in quaternary interactions.

Another case in which lowering the temperature causes enhanced association appears to be that of β-glucosidase.[21] In this instance the enzyme is predominantly dissociated at 25° C in 50% dimethyl sulfoxide at high pH*. On lowering the temperature to −25° C or below the associated molecule is the stable species. Again a pH-dependent ionization appears to be involved.

II. THE DETECTION, ACCUMULATION, AND STABILIZATION OF INTERMEDIATES OF SOLUBLE ENZYMES AT SUBZERO TEMPERATURES

The feasibility of using subzero temperatures to stabilize normally transient intermediates in enzyme catalysis was first demonstrated by Douzou and his co-workers using horse-radish peroxidase nearly 10 years ago.[2,82] Intermediate complex I, formed by reaction of peroxidase and hydrogen peroxide, could be obtained in 98% yield and was quite stable at temperatures below −40° C. The addition of a hydrogen donor then resulted in transformation to complex II which could also be stabilized at such temperatures. The spectral (absorption, circular dichroism, e.s.r.) and kinetic properties of these intermediates were consistent with data obtained in aqueous solution using rapid-flow techniques.[2]

In subsequent sections various aspects concerning the study of intermediates at subzero temperatures will be considered. The general strategy used in such experiments has been to initially demonstrate the lack of adverse effects of the chosen cryosolvent on the catalytic and structural properties of the enzyme.[14] Next attempts are made to detect intermediates using appropriate means of monitoring probes in either the enzyme or substrate structure at subzero temperatures, and to characterize detected intermediates kinetically. Finally studies to determine the detailed structures of trapped intermediates are necessary.

The temperature necessary to effectively stop the turnover reaction varies greatly depending on the particular enzyme-substrate system, the cryosolvent, the pH*, and the enzyme concentration. In some cases where the energy of activation of the rate-limiting step is large, as in the glycosidases, temperatures as high as −20° C at the pH optimum may be sufficient; in other cases, e.g., ribonuclease, temperatures below −70° C may be necessary. By choosing a nonoptimal pH* the turnover reaction for most enzymes can be brought to a halt at −50° C or higher. Some representative data are given in a recent review.[14]

A particularly useful subzero temperature experiment is to measure the enzyme active-site concentration using the "burst titration" approach. This procedure is especially applicable for most hydrolytic enzymes, and involves measuring the concentration of released product under nonturnover conditions. For example, the active-site concentrations of several proteases have been determined at subzero temperatures in aqueous-organic cryosolvents using p-nitrophenyl ester substrates.[20] These experiments indicate close agreement between the enzyme concentrations at subzero temperatures with those determined under normal conditions.

The number of extant cryoenzymology studies precludes detailed discussions of all the systems which have been investigated. Consequently, the scope of this section is selective and not comprehensive.

A. Cryoenzymology of Lysozyme and Glycosidases

Cryoenzymological investigations of these enzymes have led to a number of valuable contributions. We will consider a number of interesting aspects of these systems. In contrast to lysozyme which has been extensively studied[83] and whose structure has been determined at high resolution by X-ray diffraction, β-galactosidase,[84] and particularly β-glucosidase, are less well understood, and no crystallographic data are currently available. In part these features reflect the fact that lysozyme is a very small monomeric enzyme, whereas the glycosidases are much larger oligomers. However, the limited amount of mechanistic data available for β-glucosidase and β-galactosidase[84,85] suggests that the catalytic mechanisms are probably similar to that of lysozyme.

Lysozyme has been investigated under cryoenzymology conditions both by Douzou et al.[54,86] and Fink et al.,[71] the major interest being to determine the role of ground-state strain and distortion in the catalytic mechanism; the ultimate answer being expected from X-ray crystallographic investigation of low temperature-trapped enzyme-substrate intermediates.[87] In fact, for many years lysozyme has been considered the archetypical example of an enzyme whose mechanism involved critical ground-state distortion. However, the existence of such strain has recently been questioned on both experimental[88] and theoretical grounds.[89]

β-Galactosidase and β-glucosidase have been studied at subzero temperatures,[21,34,78] both as subjects for determining the effects of cryoenzymological conditions on oligomeric hydrolases and also to shed more light on their mechanisms of action.

Lysozyme has long been known to be particularly stable in high concentrations of organic solvents. For example, Hamaguchi and co-workers[90–92] have reported that the enzyme shows no spectral evidence of structural perturbations at room temperature up to 50% methanol or 60% dimethyl sulfoxide when examined using absorbance or circular dichroism. Detailed studies of the effects of methanol and dimethyl sulfoxide on hen egg-white lysozyme have been reported by Fink et al.[71] Using the enzyme's intrinsic fluorescence to detect structural effects, and the catalytic activity, both toward cell-wall and oligosaccharide substrates and inhibitors, they demonstrated that at subzero temperatures the enzyme exhibited no adverse effects in up to as much as 90% methanol and 65% dimethyl sulfoxide.

In a series of studies using various approaches, including rapid-reaction techniques, Rupley and co-workers[93] have shown that under normal conditions the rate-limiting step in the lysozyme-catalyzed hydrolysis of the hexasaccharide of *N*-acetylglucosamine is the formation of the putative oxocarbonium ion, and that this step is preceded by several isomerizations of the ES complex. The high energy of activation of the rate-determining step in this reaction results in the turnover reaction becoming effectively negligible at temperatures below −25° C.[86] The presence of three tryptophan residues in the active-site cleft of HEW lysozyme[83] provides a convenient probe for monitoring the binding of inhibitors (such as *N*-acetylglucosamine and its trisaccharide), as well as the catalytic reaction.

Douzou et al.[86] utilized the strong temperature dependence of lysozyme catalysis to obtain the UV-difference spectra of the trapped enzyme-substrate complex (Figure 7). In addition, under steady-state conditions, they showed that the UV-difference spectrum of the enzyme + hexasaccharide against enzyme alone was essentially identical in both aqueous solution and several cryosolvents. This observation provides strong evidence that the major intermediate present during turnover is the same in both aqueous and

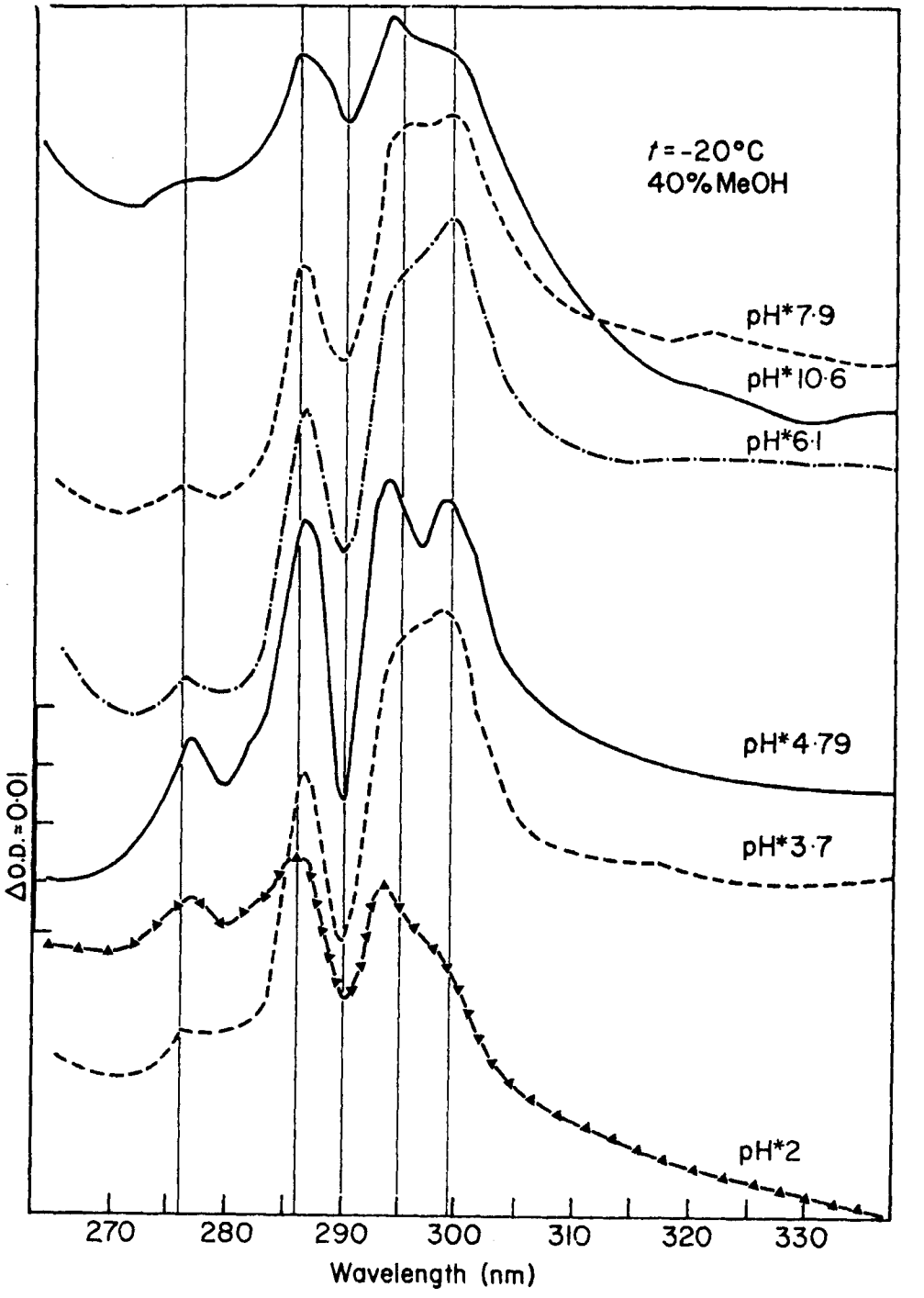

FIGURE 7. Difference spectra of lysozyme plus $(GlcNA_c)_6$ against lysozyme at various values of pH* in 40% methanol at −20° C. (With permission from Douzou, P., Hui Bon Hoa, G., and Petsko, G. A., *J. Mol. Biol.*, 96, 367, 1976. Copyright by Academic Press Inc. (London) Ltd.)

cryosolvent systems. Interestingly the different spectra were very similar for enzyme + hexasaccharide in both 50% methanol and in 5 *N* NaCl, both at +20 and −20° C. This provides further support for the lack of effect of solvent on the catalytic reaction.

Whereas the pH*-rate profile for oligosaccharide substrates in the cryosolvents was found to be normal, with an optimum around pH 5 it was observed that when a suspension of cell walls was used as substrate the pH optimum was shifted significantly. This observation was subsequently followed with a detailed investigation[54] of the role of the electrostatic potential developed by the highly charged cell walls. Application of the theory of polyelectrolytes[94] allowed satisfactory explanation of the experimental observations.

A detailed investigation of the interaction of lysozyme with oligosaccharides of *N*-acetylglucosamine has been carried out by Fink et al.[71] using the intrinsic fluorescence emission as a probe. Binding of the inhibitor chitotriose was observed to be biphasic over the 0 to −110° C range, in the pH*-optimum region. From the variation in rate of the faster phase with inhibitor concentration (which exhibited saturation kinetics) it was concluded that a pre-equilibrium reaction must occur prior to the observed reaction. Therefore the binding of the trisaccharide must involve two isomerizations after the initial complexation. The binding of the trisaccharide had previously been found to be biphasic; however, no saturation of the faster phase had been observed.[95] The fluorescence difference spectra of the final enzyme-trisaccharide complex as well as their pH dependence were very similar in both aqueous and cryosolvent systems, leading to the conclusion that the reaction and structure of the complexes were also not affected by the cryosolvent.

When the reaction with the hexasaccharide substrate was examined in a similar manner, three reactions were detected over the 0 to −90° C range.[71] The change in fluorescence emission at 345 nm as a function of time is shown in Figure 8. At −70° C the relative rates for the three reactions were in the ratio ≥300:20:1. Since the trisaccharide is known to bind to the A-C subsites of the active site,[83,96] it provides a convenient model for nonproductive binding of the hexasaccharide, i.e., binding to only the A-C subsite region. In addition, the displacement of the dye Biebrich Scarlet, which is believed to bind in the E-F subsite region,[96] can be used to distinguish between productive and nonproductive binding of the hexasaccharide. Such distinction is necessary because in aqueous solution — 20° C — the hexasaccharide binds in a 1:1 ratio between the productive and nonproductive modes.

From comparison of the interaction of the tri- and hexasaccharide it was shown that at subzero temperatures in methanolic cryosolvents, the productive binding mode of chitohexose was preferred by about 6:1 to the nonproductive mode.[71] This finding is of course very important to subsequent investigations of the catalytic mechanism using the hexasaccharide substrate; however, it was also somewhat surprising since the results observed under normal conditions[93] suggested that lower temperatures would favor the nonproductive mode of binding. One reason why the observed low temperature data are in disagreement with the extrapolated behavior stems from the fact that the Van't Hoff and Arrhenius plots for both binding and kinetics are not linear (see Figure 6 in Reference 71). The breaks in the plots for the binding constants occur around 20° C, a temperature at which a structural transition has previously been reported for lysozyme.[97] The Arrhenius plots also indicate that changes in the enthalpy of activation for the earlier steps in the reaction (both catalysis and trisaccharide binding) must occur in the vicinity of −70° C.

Since it is to be expected that the combination of cosolvent and subzero temperatures will affect the free energy of different enzyme-substrate complexes and transition states differently, it is not so surprising that there are differences of the sort discussed above between the low temperature data and those obtained under normal conditions. In general, however, the results reported for the reaction of lysozyme with oligosaccharides at subzero temperatures are in remarkably good agreement with those obtained under normal conditions. In fact, this accord between the data obtained at subzero

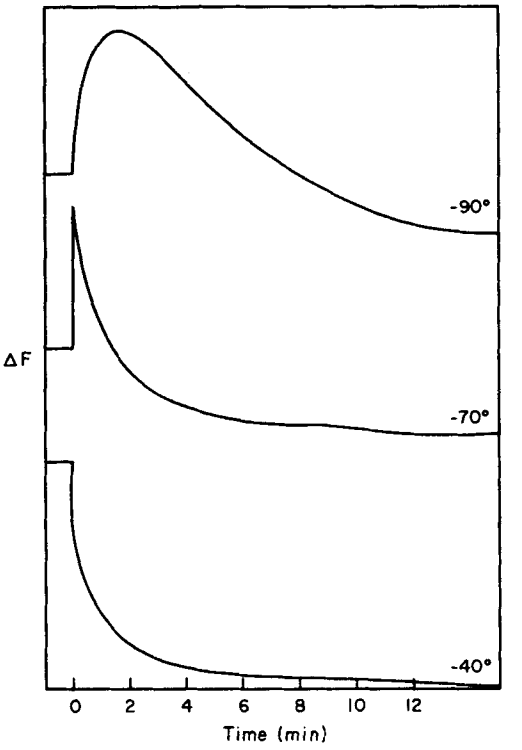

FIGURE 8. Tracings of time-dependent changes in fluorescence emission in the reaction of lysozyme with the hexamer of *N*-acetyl-D-glucosamine. The runs at −90 and −70° C were in 80% aqueous methanol; that at −40° C was in 50% methanol, pH* 7.1; excitation at 280 nm; emission at 345 nm. $[E]_o = 3 \times 10^{-7}$ *M*. Substrate was added at time = 0. (Reprinted with permission from Fink, A. L., Homer, R., and Weber, J. P., *Biochemistry*, 19, 815, 1980. Copyright (1980) American Chemical Society.)

temperatures and those found under normal conditions is one of the noteworthy aspects of the cryoenzymology studies on lysozyme. As previously alluded to the rather bizarre nature of the typical cryoenzymology experiment compared to the normal physiological conditions mandates that cryoenzymologists must, as much as is possible, demonstrate the consistency between the low temperature observations and those under normal conditions.

As mentioned above, one of the end goals of low temperature experiments with lysozyme is to obtain a crystalline intermediate in which ground-state distortion would be present, if such strain does exist, and which is stabilized for sufficient time so that crystallographic structural determinations may be made. One complication in this regard with lysozyme is that essentially all the known crystal forms of the enzyme involve partial occlusion of one active site by an adjacent molecule in the crystal lattice, with the consequence that productive binding of true substrates is precluded. There are two possible means of circumventing this problem. One involves the fact that the energy of activation for lysozyme catalysis is quite high, and that therefore only relatively high subzero temperatures are needed to stop the turnover reaction and thus accumulate an intermediate. It might therefore be possible to find conditions where the intermediate is formed in solution, the temperature then being dropped to a value where the intermediate is stable for an indefinite period, and also where the solubility limit is exceeded, so that the intermediate will crystallize. Preliminary data reported by Fink et al.[71] suggest that

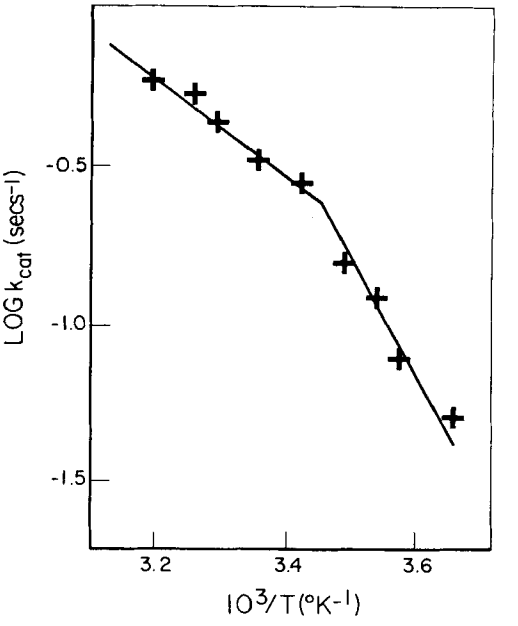

FIGURE 9. Arrhenius plot of the turnover reaction in aqueous solution for the catalysis of *p*-nitrophenyl-β-D-glucoside by β-glucosidase at pH 6.7. (After Weber, J. P., Ph.D. thesis, University of California, Santa Cruz, 1980.)

this approach may work in that it is at least possible to crystallize the enzyme at temperatures in the −40 to −80° C range.

The alternative approach is to find a form of lysozyme which crystallizes in a suitable manner so that the active sites are not occluded. Only time will tell if such a crystal form will be found; an additional complication is the relatively large size of productive substrates of lysozyme, which also necessitates that the crystals must have large solvent channels in order to allow the substrate to diffuse.[13]

Glycosidases represent an important class of enzymes, e.g., a number of metabolic disorders involve their malfunction. It has been hoped that an understanding of their mode of action will be very useful in many medical applications. The simple carbohydrate glycosidases thus represent useful models for a wider family of enzymes, since it seems likely that a common type of catalytic mechanism exists. There has been considerable interest regarding the existence of an enzyme-glucose intermediate in β-glucosidase catalysis. Investigations by Legler[98] at 37° C failed to detect a presteady-state burst of *p*-nitrophenol in the hydrolysis of *p*-nitrophenyl-β-D-glucoside, suggesting that either there was no such intermediate or that the rate-limiting step preceded it. Fink and Good[34] investigated the same reaction, but at subzero temperatures using dimethyl sulfoxide cryosolvent, and reported evidence for a stoichiometric burst of *p*-nitrophenol, implying the rate-limiting breakdown of an enzyme-glucose intermediate (either covalently or noncovalently attached glucose) as indicated in Equation 1.

$$E + G\text{-}P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.G\text{-}P \xrightarrow{k_2} \begin{matrix} E.G \\ + \\ P \end{matrix} \xrightarrow{k_3} E + G \qquad (1)$$

A hint of the reason for the apparent discrepancy between the results from the experiments in aqueous solution at 37° C and those at subzero temperatures came with Takahashi's report[99] that a burst of *p*-nitrophenol could be detected in stopped-flow

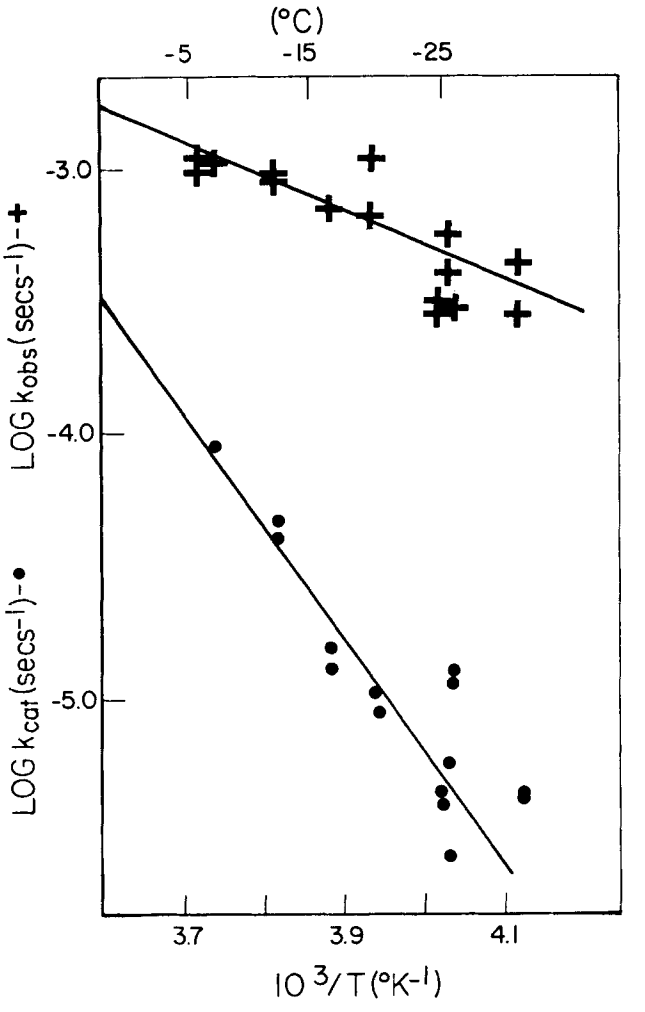

FIGURE 10. Arrhenius plots for k_{cat} and k_{obs} (rate for the "burst" reaction) for the β-glucosidase-catalyzed hydrolysis of *p*-nitrophenyl-β-D-glucoside at subzero temperatures in 50% dimethylsulfoxide. pH* = 8.0 (After Weber, J. P., Ph.D. thesis, University of California, Santa Cruz, 1980.)

experiments at 15°C in aqueous solution, suggesting that a change in the rate-determining step may occur between 37 and 15° C, from formation of the enzyme-glucose intermediate at higher temperatures to rate-limiting breakdown of the intermediate at lower temperatures.

This temperature-dependent change in the rate-determining step has subsequently been confirmed[21] under both aqueous and cryoenzymological conditions. For example in aqueous solution, the Arrhenius plot (Figure 9) shows a break at 20° C, with energies of activation of 17 ± 1 and 6.7 ± 1 kcal mol^{-1}, respectively, for the lower and higher temperature range. In 50% dimethyl sulfoxide the reaction involving the release of *p*-nitrophenol and presumably concomitant formation of the enzyme-glucose intermediate is readily detected at temperatures below −5° C (Figure 10). The Arrhenius plot for this reaction has an energy of activation of 6.0 ± 1 kcal/mol, which may be compared with that for turnover (19 ± 2 kcal/mol) under these conditions. A change in rate-determining step is calculated to occur at 24 ± 5° C in the cryosolvent.[21]

This system serves to dramatically illustrate one of the advantages of cryoenzymology,

namely the ability to detect and study intermediates which are effectively "undetectable" under normal conditions. A similar situation has been reported for carboxypeptidase A in which a key intermediate has rate-limiting breakdown at subzero temperatures, and rate-limiting formation under normal conditions.[22,100] A similar situation may also exist in the case of β-galactosidase, since a "burst" of *p*-nitrophenol can be detected at subzero temperatures,[78] but has not been reported under normal conditions.

B. Cryoenzymology of Serine Proteases

Detailed cryoenzymological investigations of the serine proteases have been carried out by Fink and co-workers to probe the basis of enzyme catalytic efficiency at the most fundamental level possible. The small size, lack of regulatory control, and relatively simple reaction of these enzymes makes them attractive candidates for such studies. The goal of these experiments has been to obtain a detailed series of "time-lapse pictures" of the transformation of substrate to product, including both structural details of each intermediate at atomic resolution as well as the kinetics and energetics of the intermediate interconversions.

It has become apparent that the trypsin family of enzymes all respond very similarly to cryosolvents and cryoenzymological conditions, and apparently have a common mechanism resulting in similar experimental observations in similar experiments with similar types of substrates. Such commonality is not surprising given their assumed common ancestory and three-dimensional homology. As a result of their similarities it seems justified for the present purpose to consider, except where noted otherwise, that phenomena noted for one member of the group will also occur with the others.

Detailed investigations of the effect of dimethyl sulfoxide and methanol cosolvents on chymotrypsin,[32,70] trypsin,[61] and elastase[35] have demonstrated the lack of adverse effects of such solvents on both structural and catalytic properties of the mammalian serine proteases at subzero temperatures. Ethylene glycol has also been found to be a satisfactory cosolvent for chymotrypsin[102] and trypsin.[103]

The existence of acyl-enzyme intermediates in chymotrypsin catalysis has been demonstrated under normal conditions by their chromatographic isolation at low pH (<5) from chromophoric *nonspecific* substrates such as *N*-(*trans*)-cinnamoyl imidazole[104] and by spectral detection with specific substrates,[105] as well as by the common values of k_{cat} for substrates with common acyl moieties (reflecting rate-determining breakdown of the common acyl-enzyme).[106] Confirmation that acyl-enzymes occur and can be trapped and isolated at subzero temperatures has been provided by Fink and co-workers.[25,32,33]

p-Nitrophenyl ester substrates are well suited for studies of acyl-enzyme formation and breakdown in the serine proteases since the release of the chromophoric nitrophenol group provides a simple means of monitoring the acylation reaction. The smaller rate constant and larger energy of activation for the deacylation step in most cases mean that at appropriately low temperatures not only is the rate of acylation much faster than that of deacylation, but also that the latter can be essentially zero. The acylation and deacylation reactions for elastase, trypsin, and various forms of chymotrypsin with specific *p*-nitrophenyl ester substrates were studied by Fink and Ahmed[20] using both dissolved and crystalline enzymes. For the enzymes in solution in the −40 to −70° C range, the rates of acylation were orders of magnitude faster than those of deacylation. From the amount of *p*-nitrophenol liberated in the acylation reaction it was possible to measure the fraction of active enzyme under these low temperature conditions in comparison with that under normal conditions.

For example, using the specific substrate *N*-acetyl-L-tryptophan *p*-nitrophenyl ester, in which the rate-limiting step is hydrolysis of the acyl-enzyme, it is possible to monitor the rate of acyl-chymotrypsin formation by the rate of release of *p*-nitrophenol. Under

nonturnover conditions (e.g., −40° C, pH* 5.5) a "burst" of *p*-nitrophenol is released, the concentration of which gives the concentration of acyl-enzyme and of available active sites.[20,32] If the reaction mixture at the completion of the acylation reaction is then subjected to gel filtration on Sephadex LH-20 at −40° C, eluting with the same dimethyl sulfoxide cryosolvent, it is possible to isolate the acyl-enzyme which has a half-life of 10 hr under these conditions.[25] In more recent studies it was found possible to isolate the acyl-enzyme formed from the reaction of chymotrypsin with *N*-2(furylacryloyl)-L-tryptophan methyl ester in aqueous solution at 1° C, pH 2.5 by gel filtration.[33]

The significance of these results is threefold. The experiments demonstrate that the basic catalytic mechanism is not perturbed by the combination of cryosolvent and subzero temperature, that gel filtration methods can be used at such temperatures to separate enzyme-substrate intermediates from other compounds, and that *p*-nitrophenyl substrates (or similar species) can be used to determine the active-site concentration of enzymes under cryoenzymological conditions. Typically 80 to 100% of the active-site concentrations determined by burst assays in aqueous solution are found in cryoenzymology experiments.[20]

From such experiments it was shown that the acyl-enzyme, *N*-carbobenzoxy-alaninyl-elastase, in 70% methanol at pH* 5.7, broke down to products at a rate of less than half a percent per day at −70° C. This intermediate is thus quite stable enough for structural elucidation experiments requiring long periods of data collection, such as X-ray diffraction. But, as the name implies, X-ray crystallographic experiments require the sample to be crystalline. As discussed in detail below it was found possible to make crystalline acyl-enzymes by allowing the low-molecular weight substrates to diffuse into crystals of the enzyme in cryosolvent at appropriate temperature and pH*.[19,20] With the crystalline enzymes the reaction rates were found to be much slower than in the corresponding experiments with dissolved enzyme due to diffusional constraints.[13] The deacylation rates in the −50° C region were found to be effectively zero, allowing high concentrations of crystalline acyl-enzymes to be accumulated. The only exception to this was the case of α-chymotrypsin, in which only a 3% yield of acyl-enzyme was obtained. This particular experiment served as an excellent control since α-chymotrypsin is known to crystallize as a dimer in which neighboring molecules occlude or block the active site, thereby preventing substrate binding.

By using the UV-transparent substrate *N*-acetyl-L-phenylalanine methyl ester, Fink and Wildi[107] utilized the intrinsic fluorescence emission of chymotrypsin to monitor the catalytic reaction at subzero temperatures. Triphasic kinetics were observed and attributed to substrate binding, acylation, and deacylation, based on their pH and temperature dependence. A major decrease in fluorescence emission and a blue shift in λ_{max} was associated with the formation of the acyl-enzyme, and suggested a significant perturbation of a tryptophan, probably Trp-215, located close to the active site.[107] Similar studies with trypsin and *N*-acetyl-L-lysine methyl ester failed to detect significant changes in the fluorescence, but did show absorbance changes.[61]

The effect of cosolvent concentration and temperature on the pH dependence of trypsin-catalyzed hydrolysis has been investigated by Maurel et al.,[103] and has been discussed previously.

The existence of tetrahedral intermediates in the enzyme-catalyzed reactions of esters and amides has long been a question of concern in mechanistic enzymology. That such intermediates are on the reaction pathway has been predicted on the basis of their existence in corresponding nonenzymatic systems[108] and the presence of "oxyanion holes" (detected crystallographically) that can stabilize the incipient tetrahedral oxyanion by hydrogen bonding.[109] *p*-Nitroanilide substrates are expected to be particularly well suited for the detection of intermediates during the dynamics of catalysis, especially tetrahedral adducts, due to their chromophoric properties.[35,110] For

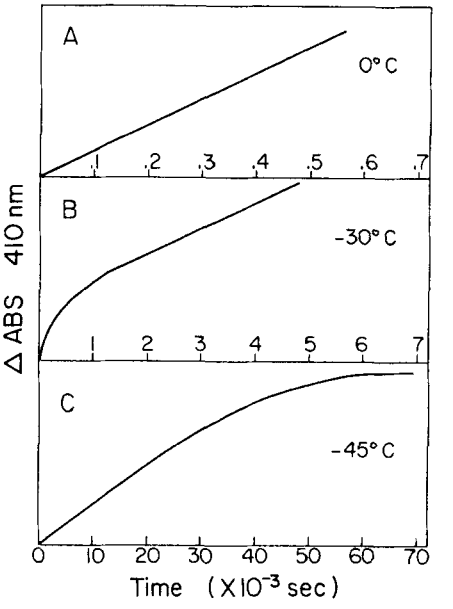

FIGURE 11. Traces of the time-dependent changes in absorbance at 410 nm corresponding to (A) turnover, (B) "burst" kinetics, and (C) tetrahedral intermediate formation found for both elastase and trypsin with specific anilide substrates.

example, having the chromophore involved in the bond being cleaved in the catalytic reaction should provide a very sensitive probe of events at the active site. In addition, the electronic properties of the *p*-nitroaniline moiety are expected to stabilize the putative tetrahedral adduct.

The interaction of elastase with di- and tripeptide *p*-nitroanilide substrates has been investigated in 70% methanol at subzero temperatures.[35] The *p*-nitroanilide substrates have λ_{max} values around 320 nm, the product, *p*-nitroaniline, has a λ_{max} around 385 nm, and the tetrahedral intermediates have λ_{max} values around 360 nm.[111] It is therefore quite easy to distinguish between the turnover reaction, tetrahedral intermediate formation, or preceding intermediates. When elastase was mixed with *p*-nitroanilide substrate at progressively lower temperatures as a function of pH*, a total of three different reactions could be detected corresponding in reverse order, to turnover, tetrahedral adduct formation, and a preceding reaction resulting in the formation of a substrate-like species.[35,36] Time-dependent changes in the absorbance spectrum similar to those in Figure 11 were observed for the formation and breakdown of the putative tetrahedral intermediate. The tetrahedral intermediate was identified on the basis of its spectrum, with a λ_{max} of 359 ± 2 nm, and its pH* dependence (pK* = 7.0 at −40° C). Of note is the fact that the rate of formation of the tetrahedral intermediate is essentially negligible below pH* 6, at − 40° C. Under such conditions the preceding intermediate can be accumulated.[35] One measure of the accord between the data obtained at subzero temperatures and that under normal conditions comes from the fact that the tetrahedral intermediate formed in the reaction of elastase with AcAlaProAla *p*-nitroanilide can also be detected under normal conditions using stopped-flow methods.[112] Excellent agreement between the observed rates at 20° C and those extrapolated from subzero temperatures was found.[35] The pH* dependence of the tetrahedral intermediate concentration in the cryoenzymology studies was investigated. Maximum concentrations were found only at high (>9) values of pH*.

Very similar results have been obtained in cryoenzymological investigations of the

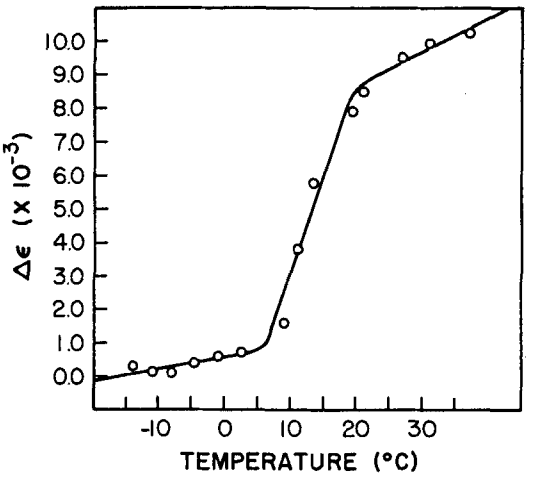

FIGURE 12. The thermal denaturation of papain in 7.65 *M* dimethylsulfoxide, pH* 3.5, $E_o = 1.4 \times 10^{-5}$ *M*. (Reprinted with permission from Fink, A. L., and Angelides, K. J., *Biochemistry*, 15, 5290, 1976. Copyright (1976) American Chemical Society.)

reaction of trypsin with *p*-nitroanilide substrates.[36] These cryoenzymology experiments have provided the first good experimental support for the existence of tetrahedral adducts as discrete intermediates (as opposed to transition states) in protease catalysis. In addition they have demonstrated the presence of a preceding, noncovalent enzyme-substrate complex whose existence was not previously suspected. Since both tetrahedral intermediate and enzyme-substrate complex can be stabilized for long time periods these investigations demonstrate the major value of cryoenzymology.

C. Thiol Protease Studies

Recent studies concerning papain illustrate both the means of ascertaining the effects of cryosolvents on the catalytic and structural properties of the enzyme, and the use of chromophoric groups, either in the substrate or intrinsic to the enzyme, to monitor the dynamic events during catalysis. Preliminary experiments demonstrated that papain was stable at 0° C in high concentrations of dimethyl sulfoxide and ethanol, but not methanol.[62] Detailed investigations of the effect of these cosolvents on the catalytic properties of the enzyme were carried out using the specific substrate N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester. As the dimethyl sulfoxide concentration was increased from 0 to 60% the value of k_{cat}, at 0° C, fell in direct proportion to the decrease in water concentration, as would be expected. On the other hand, the value of K_m increased exponentially due to the hydrophobic effect on substrate binding[52] (see Section I.D). When this reaction was monitored in 60% dimethyl sulfoxide over the 0 to −45° C range a linear Arrhenius plot was obtained. Correction for the effect of cosolvent on K_m and extrapolation to 25° C yielded a value in excellent agreement with that reported in the literature.[62] These observations indicate that there is no change in the rate-determining step in that temperature range, nor is there a temperature-induced conformational change which affects the essential catalytic residues. The pH*-rate profile for the turnover reaction in 60% dimethyl sulfoxide at 0° C was quite similar to that in aqueous solution.[62]

The possible effects of the cosolvent on the structural properties of the enzyme were investigated as follows. The thermal denaturation transition of the enzyme in 60% dimethyl sulfoxide was found to have a midpoint of 12° C, at pH* 3.5. The transition was

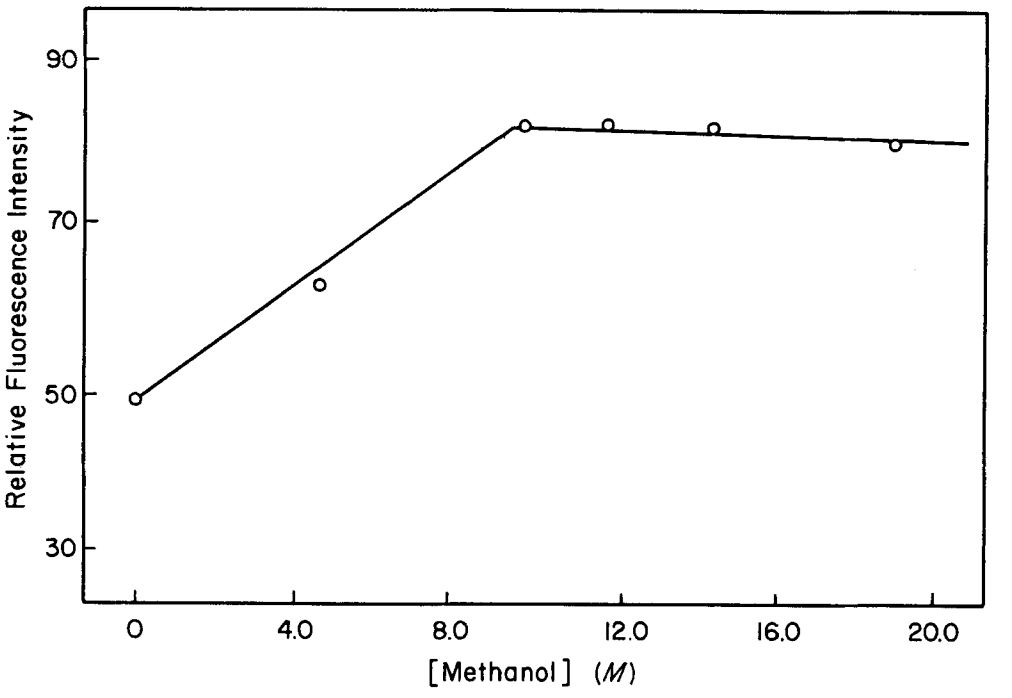

FIGURE 13. The effect of methanol on the intrinsic fluorescence emission of papain, as monitored using Tyr → Trp energy transfer. Conditions were 0° C, pH* 7.0. Excitation was at 260 nm, emission at 330 nm (λ_{max} for Trp emission). The sharp break at 8.4 *M* methanol signifies a structural rearrangement.[190]

quite sharp (Figure 12), and at temperatures below 5° C the enzyme was fully in its native state.[62] As a means of detecting possible conformational effects the intrinsic spectral properties of papain were monitored as a function of increasing cosolvent concentration. Fluorescence, UV absorbance, and circular dichroism spectra all showed linear or smooth monotonic changes as the cosolvent concentration was increased at 0° C. These changes reflect solvent effects on the exposed residues. The lack of sharp breaks in such curves signifies the absence of structural perturbations. Such sharp breaks in plots of this sort were observed at higher than 60% concentrations of dimethyl sulfoxide, at 0° C, and with methanol as cosolvent (Figure 13).

The lack of adverse effects of the cosolvent on the structural and catalytic properties demonstrated in the above series of experiments indicates that 60% dimethyl sulfoxide is a satisfactory cryosolvent for studying papain catalysis.

At temperatures below −45° C, pH* 6 to 7, the reaction of papain with N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester appears as a first-order release of *p*-nitrophenol, corresponding to the formation of the acyl-enzyme, followed by no further release of *p*-nitrophenol for a time period of hours. In other words the acyl-enzyme has been stabilized by these conditions. Furthermore the concentration of *p*-nitrophenol released indicates that essentially stoichiometric amounts of the acyl-enzyme are formed, if excess substrate is present.[62]

There is a strategically placed tryptophan residue in the active site of papain, and since the great majority of the fluorescence emission of the enzyme comes from this particular residue it is especially well suited as a probe of active-site events. Advantage of this phenomenon was taken to study the interaction of the enzyme with the relatively UV-transparent substrate N^{α}-carbobenzoxy-L-lysine methyl ester.[113]

Maximal changes in the 276-nm region were detected during the reaction of N^{α}-

carbobenzoxy-L-lysine methyl ester with papain under nonturnover conditions. Monitoring the reaction at this wavelength revealed triphasic kinetics at temperatures sufficiently low that no turnover occurred. The two faster phases could also be detected by changes in the fluorescence emission of the enzyme. The first reaction observed was very fast, even at −65° C, and was attributed to the initial enzyme-substrate complexation. The subsequent reaction had a pK* of 3.4 at −40° C. The amplitude of the fluorescence change associated with this second reaction was found to be pH dependent. The slowest of the three observed reactions had a pK* of 4.8 at −17° C, with a heat of ionization of 8 kcal/mol. The same ionization appeared responsible for the pH dependence of the fluorescence change of the second reaction and the rate of the third reaction. The data were interpreted as indicating that the second reaction involved a substrate-induced conformational change, and the third reaction corresponded to the formation of the acyl-enzyme. Control reactions using various inhibited forms of the enzyme proved useful in interpreting the results. A catalytic reaction mechanism was proposed to account for the subzero temperature observations, as were many of the apparently contradictory previous pK assignments.[113] Further support for this mechanism was obtained in a subsequent study that shall be considered next.

Angelides and Fink[110,111] investigated the reaction of papain with N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide at subzero temperatures, with the idea that the chromophoric *p*-nitroaniline moiety would function as a sensitive probe of events occurring during the actual bond-breaking/making step(s) as well as possible changes in the environment of the leaving-group site in the enzyme during the catalytic process. Their findings are particularly noteworthy since they demonstrate clearly the advantages of cryoenzymology in yielding otherwise difficult-to-obtain mechanistic details. In this instance the interpretation of the low temperature results also allowed clarification of previous long-standing confusion regarding the basis of the pH dependence of many papain reactions.

Comparison of the steady-state kinetic parameters revealed that the rate-limiting step for the *p*-nitroanilide substrate was in the acylation half of the reaction.[111] The large effect of the dimethyl sulfoxide cosolvent used in these studies on K_m (see above) meant that under nonsaturating concentrations of substrate very substantial rate reductions occurred in experiments at subzero temperatures. A total of four different reactions could be detected spectrophotometrically prior to the turnover reaction when the interaction of papain with the *p*-nitroanilide substrate was monitored as a function of pH* and subzero temperature. The slowest of these four processes was identified as formation of the tetrahedral intermediate, in part on the basis of the spectrum of the product of this reaction (Figure 14), which has a λ_{max} of 365 nm, in contrast to the λ_{max} of 320 nm for the substrate and 380 nm for the product. Each of the four elementary reactions was characterized with respect to its pH*, temperature, and concentration (enzyme or substrate) dependence. The unusual, inverse hyperbolic relationship between rate of the second observed process and substrate concentration, under conditions of excess substrate, led to the conclusion that there must be a preequilibrium between two forms of the enzyme prior to substrate binding.[111] Thus the first two reactions observed were ascribed to the binding of substrate to free enzyme, the slower process reflecting rate-limiting enzyme isomerization to the substrate-binding form. Corroboration that the observed reactions reflected interaction between substrate and the active site was obtained in a variety of control experiments in which the active site was blocked with bulky groups such as the *N*-tosyl-L-lysinechloromethyl ketone adduct, or the very small S-methyl group.[111]

The isomerization between the two forms of the enzyme was postulated to involve rotation of the side chains of His-159 and Asp-158 so as to change from a situation at high pH in which the imidazole is hydrogen bonded to Asn-175 (pK_{Im} = 4, pK_{SH} = 8), to

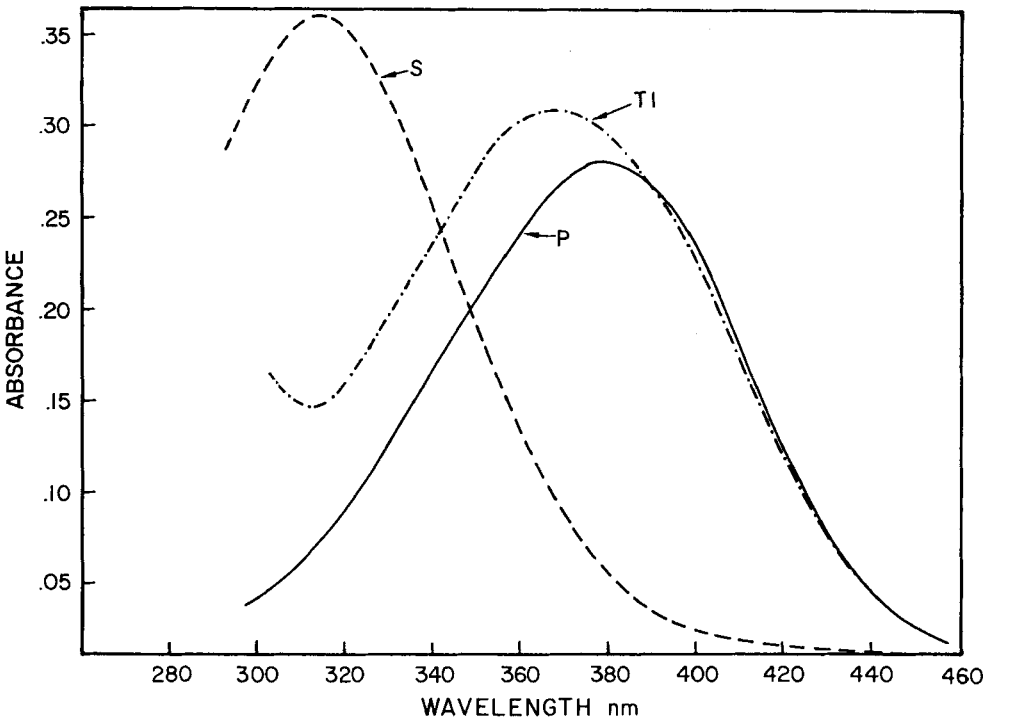

FIGURE 14. Spectrum of the tetrahedral intermediate (TI) formed from the reaction of papain with N^{α}-carbobenzoxyl-L-lysine *p*-nitroanilide in 60% dimethylsulfoxide at −40°C, pH* 6.1. The spectra of the substrate (S) and product *p*-nitroanilide (P) are included for comparison. (After Angelides, K. J. and Fink, A. L., *Biochemistry*, 18, 2355, 1979.)

one in which the imidazole has rotated so as to now interact electrostatically with the carboxylate of Asp-158 and the thiolate of Cys-25 ($pK_{Im} = 8$, $pK_{SH} = 4$). It was further postulated that steric hindrance would occur in the binding of most substrates to the latter form, so that binding would only occur significantly to the high-pH form. However, the "catalytically active" form involves the imidazole in the alternate position. Thus an isomerization must occur after substrate binding, and it is this isomerization which is responsible for the third observed process. More data from both cryoenzymology experiments, as well as those carried out under normal conditions, support the proposed hypothesis. An interesting feature of the catalytic reaction is the observation that the tetrahedral adduct is the most stable intermediate on the reaction pathway.[111]

A number of interesting aspects relating to the tetrahedral intermediate were found in this investigation. The rate of formation exhibited bell-shaped pH* dependence, with pK*(s) in the vicinity of 4 and 8. The concentration of the accumulated tetrahedral adduct was also quite pH* dependent, with 3% of the enzyme being present as adduct at pH* 3, and gradually increasing to 100% at pH* 9.3[110]

Evidence that the catalytic mechanism observed at subzero temperatures was consistent with that occurring under normal conditions was found in the observation that the formation of the tetrahedral intermediate could be detected using stopped-flow spectrophotometry at 20°C, aqueous solution, at the expected rate calculated from extrapolation of the low temperature data.[110] Since there is only a very narrow range of conditions under which the tetrahedral adduct can be detected at above-zero temperatures, the knowledge about the reaction provided by the low temperature studies

was necessary to find them. This raises the general point that the often-substantial variation in pH and temperature of the microscopic rate constants in enzyme catalysis can mean that a particular intermediate is detectable under only quite restrictive conditions.

D. Carboxypeptidase A

Carboxypeptidase is probably the most studied of the metallo proteases. Considerable uncertainty still exists regarding the mechanistic details, however, especially the role of the zinc. Cryoenzymological methods have been used by Makinen and co-workers to study the mechanism of action of carboxypeptidase A in the hydrolysis of the substrate *o-trans-p*-chlorocinnamoyl-L-β-phenyllactate (ClCPL).[22,100] Cryosolvents used were ternary mixtures of ethylene glycol-methanol-water, the methanol being added at low temperatures to avoid enzyme precipitation caused by the high methanol concentrations required to reduce the viscosity of the medium. The kinetics of hydrolysis of the substrate were followed down to $-40°$ C with enzyme in excess to avoid inhibition by the product, L-β-phenyllactate. Hydrolysis was found to be biphasic below $-10°$ C, indicating two consecutive unimolecular reactions, and signaling the appearance of an intermediate not detectable at ambient temperatures. The rate of formation of this intermediate was some 40 times faster than its breakdown at $-60°$ C, allowing spectral analysis over a period of 90 min.[22] Significantly, partial denaturation of the enzyme intermediate with urea had little effect on its spectrum, as did addition of the tightly binding inhibitor, L-benzyl-succinate. However, the spectrum of the intermediate was not observed if substrate was added after the addition of inhibitor at $-60°$ C. These results indicate that a conformational change in the enzyme was not responsible for the observed spectrum, and that it was probably due to a covalent intermediate, presumably a *p*-chlorocinnamoyl enzyme formed prior to the rate-limiting step.

The biphasic kinetics observed below $-10°$C were therefore attributed to rapid acylation (k_f) followed by hydrolysis of the acyl-enzyme as the slow step (k_s). At high temperatures ($>-10°$ C), in mixed solvents only, deacylation was found to proceed faster than acylation, giving monophasic kinetics and no indication of the intermediate. Extrapolation of k_f to 25° C from the Arrhenius plot gave a value in good agreement with k_{cat} in the same cryosolvent. From the rate of hydrolysis of the denatured covalent intermediate at $-30°$ C it was concluded that a mixed anhydride with the γ-carboxylate oxygen of Glu-270 was formed as the covalent intermediate with *p*-chlorocinnamate, L-phenyllactate being formed on acylation. In addition the spectrum of the intermediate (with an increase in absorbance at ~310 nm) was taken to indicate a retention of the α,β conjugated aromatic structure of the cinnamoyl portion. This would be incompatible with formation of a tetrahedral carbonyl carbon. The activation energy for deacylation (20.6 kcal mol^{-1}) is considerably higher than that for the hydrolysis of model anhydrides (11.0 kcal mol^{-1}), and there was a large positive entropy of activation taken to indicate a covalent intermediate more highly constrained and ordered than the products and/or due to a disordering of structural water, as in the replacement by a ligand of a metal ion coordinated water molecule.[22]

Subsequent studies[100] have shown that the native zinc enzyme is noncompetitively inhibited by both methanol and ethylene glycol, and confirmed that enzyme kinetics with ClCPL do not follow the same rate law in mixed solvents as in aqueous solution. For both the zinc enzyme and the substituted cobalt enzyme k_s (down to $-45°$ C) measured in ethylene glycol-methanol-water cryosolvents extrapolates to k_{cat} measured in aqueous solution at 25° C, whereas k_f for the zinc enzyme extrapolates to k_{cat} at 25° C in the cryosolvent.

For the zinc and cobalt enzymes the rate-determining step changes from deacylation

(k_s) to acylation (k_f) in going from aqueous solution to cryosolvent media at room temperature. As a result the kinetic isotope effect seen in aqueous solution with D_2O is not seen in cryosolvents at room temperature (for both the zinc and cobalt enzymes). For the substituted cobalt enzyme deviation from a linear Arrhenius plot (corresponding to k_s) down to $-40°$ C in ethylene glycol-methanol-water cryosolvents is observed only at low pH and is too small for accurate measurement.

The overall effect of these rate changes is that the reaction catalyzed by both the zinc and cobalt enzymes is characterized by the same rate-limiting step in aqueous solutions at ambient temperatures as in cryosolvent media at subzero temperatures. Thus results obtained at subzero temperatures permit deduction of the nature of the rate-limiting step (deacylation) of the enzyme-catalyzed reaction in aqueous solution. It has been suggested that at ambient temperatures in cryosolvents the change in the rate law arises as a result of binding of a noncompetitive inhibitor (i.e., the cosolvent), altering the availability of the Michaelis complex for further reaction, and that nevertheless the catalytic steps of the reaction are identical in aqueous and cryosolvent media.[100]

A study of the pH dependence of k_s for the zinc and cobalt enzymes has provided evidence for the ionization of a group associated with deacylation of the putative covalent acyl-enzyme intermediate. Temperature dependencies for the pKa values of 7.25 and 6.08 for zinc and cobalt enzymes, respectively, at $-25°$ C yields ΔH_{ion} values of 7.2 and 6.2 kcal mol^{-1}. Using arguments of Douzou and co-workers,[50] it was concluded that the parallel behavior of the pH dependence of both k_s and ΔH_{ion} for both enzymes, independent of solvent composition, indicated a very similar environment for this ionizing group and that it was buried in an interior region inaccessible to bulk solvent. The metal ion-dependent pKa shift of this group indicated that it was coordinated to the metal ion, and since the amino acid ligands coordinating the metal ion (His-196, His-69, Glu-72) are sterically inaccessible for direct participation in the catalytic process,[115] the only residue which could be responsible for the observed pH dependence of k_s would be the metal-coordinated water molecule.

This conclusion is consistent with the known ΔH_{ion} of 6 to 8 kcal mol^{-1} for the ionization of a water ligand in model 5-coordinated Co^{2+} and Zn^{2+} complexes.[116] The temperature dependence of pKa extrapolates to values of 6.1 and 4.9 for the zinc and cobalt enzymes, respectively, at room temperature. Independent results from steady-state kinetic studies of pH vs. log k_{cat}/K_m profiles have indicated that the ionization of two groups with pKa values of 6.4 and 9.3 are involved in productive binding of ClCPL[117] and peptide substrates[118] by the zinc enzyme, and that substitution of zinc by cobalt changes the former pKa to 5.0, at least for peptide hydrolysis.[119] These results lend support to the notion that a metal-bound water molecule is involved in the catalytic rate-determining step of peptide hydrolysis in carboxypeptidase A. It should be noted, however, that previous studies have suggested that productive binding of substrate involves displacement of water, and the carbonyl oxygen of the susceptible peptide bond is then coordinated to the metal ion as a fourth ligand.[115] This new scheme therefore differs with respect to the catalytic role of the metal ion-bound water molecule. The minimal catalytic sequence for *o*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate hydrolysis can be summarized as follows:

1. Binding of the free carboxylate group of the substrate by Arg-145 and the aromatic side chain by the hydrophobic pocket
2. Nucleophilic attack on the carbonyl carbon of the substrate by Glu-270 to give a mixed anhydride, probably via a tetrahedral intermediate
3. Deacylation of the mixed anhydride with a metal-bound hydroxide group (this could occur via direct nucleophilic attack on the carbonyl carbon atom or via indirect general base catalysis by an intervening water molecule H-bonded to the

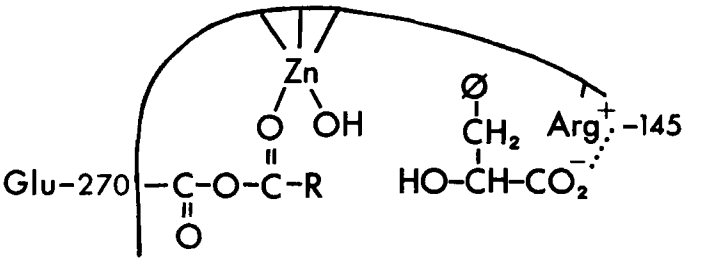

FIGURE 15. Schematic drawing of the structure of the metal ion environment in the acyl-enzyme intermediate of carboxypeptidase A formed with ClCPL as determined by EPR studies. The structural relationships of the ionized metal-bound water molecule and of the carbonyl oxygen of the scissile bond of the substrate acyl moiety are shown coordinated to the metal ion as required for deacylation. (From Makinen, M. W., personal communication. With permission.)

metal bound hydroxide group); however, it is not known whether the catalytic roles of Glu-270 and metal-bound water are different in peptide hydrolysis.

The use of a good substrate and cryoenzymological methods has given results at variance with those derived from X-ray diffraction studies with inhibitors and pseudosubstrates. In the latter case coordination to the metal ion is by the carbonyl oxygen of the scissile bond of the substrate following displacement of the liganded water molecule. This is taken to represent a nonproductive spatial relationship which cannot be a catalytically productive configuration in ester hydrolysis and consequently does not represent the true relationships required for conversion of substrate to product.

The pH dependence of ClCPL hydrolysis in cryosolvents implies that a metal-hydroxide group is the nucleophilic agent in the hydrolysis reaction, and structural identification of this metal-bound water molecule in the acyl-enzyme intermediate has been achieved by the use of both water and ClCPL selectively enriched with ^{17}O, in the latter case in the carbonyl oxygen of the scissile bond.[120] The first derivative epr spectrum of the cobalt-substituted enzyme is identical in aqueous or cryosolvent systems, while there is a noticeable decrease in the microwave saturation behavior in the presence of ^{17}O water arising from a spin-spin interaction of ^{17}O-enriched water with the metal ion. This change in saturation behavior is abolished in the presence of glycyl-L-tyrosine, consistent with the proposal that bound water is displaced by coordination of the carbonyl oxygen of the inhibitor to the cobalt ion.

The spectrum of the mixed anhydride acyl-enzyme of ^{17}O ClCPL and the cobalt enzyme formed at $-70°C$ in ethylene glycol-methanol-water shows a change in saturation behavior compared to that of the intermediate derived from normal (^{17}O free) substrate. In addition the saturation behavior of the acyl-enzyme is influenced by the presence of ^{17}O-enriched water. These results indicate that the coordination environment of the Co^{2+} ion of carboxypeptidase A in the catalytically productive configuration has both O-donor ligands (water and the carbonyl oxygen of the substrate) coordinated to the Co^{2+} — in other words a pentacoordinate Co^{2+} ion. It is likely that Zn^{2+} is also pentacoordinate, since the identical ionization is observed in rate-limiting deacylation. A schematic illustration of the acyl-enzyme intermediate is shown in Figure 15.

This latter investigation, in particular, also serves as an excellent example of the potential power of cryoenzymology in the elucidation of enzyme mechanisms.

III. INTERMEDIATES IN THE CATALYTIC REACTIONS OF MEMBRANE-BOUND SYSTEMS

One might expect that the properties of biological membranes would make them rather unsuitable for investigations in the presence of mixed solvents and at subzero temperatures. Surprisingly, however, a number of membrane-bound systems have been successfully studied under cryoenzymological conditions. These include chloroplasts,[10] microsomes,[121] mitochondria,[122,123] cytochrome oxidases,[37,124] and respiratory particles from *E. coli*.[125] The anticipated undesirable effects stem from both the possible adverse effects of the more hydrophobic cryosolvent on the lipid bilayer and its hydrophobic components, as well as the effect of temperature on the phase transition of the membrane, including the fact that at subzero temperatures the membrane is likely to be "locked" into its crystalline state.[126]

A. Chloroplasts

The chloroplast membrane (thylakoid) contains the photosynthetic electron transport chain consisting of two photosynthetic centers connected by a series of electron transport components. This electron transport system can reduce acceptors, naturally ferredoxin and ultimately NADP, as well as artificial acceptors, at the expense of water. In addition, illuminated chloroplasts take up protons from the external medium. This is usually considered to be the result of the vectorial arrangement of the electron transport chain in the thylakoid membrane.

As described by Cox[17] cryoenzymology may be of particular advantage in the study of complex systems such as chloroplasts where complicated and specialized equipment is usually necessary to study the reactions of interest due to the nature of the system under study. Lowering the temperature sufficiently to slow individual reaction steps down to the point where they can be followed using high sensitivity, dual wavelength spectrophotometers provides many benefits.

Several studies have shown that the electron transport system of chloroplasts is functional in 50% ethylene glycol at temperatures as low as −40° C.[10,127-129] These include the demonstration of the reduction of added electron acceptors;[127] the fact that compounds which are normally inhibitors or uncouplers of this system such as 3(3′,4′-dichlorophenyl)-1,1-dimethylurea are also functional at subzero temperatures with fluid suspensions of chloroplasts,[130] as well as the ability of chloroplasts to evolve oxygen in 50% ethylene glycol at subzero temperatures, indicating that water is indeed the oxidized species.[128]

The use of a fluid medium in the study of chloroplast reactions has obvious advantages compared to frozen aqueous solutions or suspensions in glasses. For example, one can diffuse in small ligands such as electron acceptors or inhibitors. At above-zero temperatures 50% ethylene glycol causes a 50% reduction in the rate of acceptor reduction compared to aqueous solution (Figure 16). The energy of activation for the rate of reduction of artificial electron acceptors in aqueous and 50% ethylene glycol solutions is very similar, in both cases exhibiting a break in the Arrhenius plot around 5° C (Figure 16), probably due to a membrane phase change.[131] The reduction of oxidized 2,3,5,6-tetramethyl-*p*-phenylenediamine is biphasic, this system being another example where lowering the temperature results in increased resolution of two adjacent reactions — the energy of activation of the slower phase being significantly larger than that for the faster phase, resulting in greater resolution of the two phases at lower temperatures.[127] Previously the biphasicity could only be detected with rapid reaction techniques. Higher concentrations of inhibitors were found necessary to bring about complete inhibition in

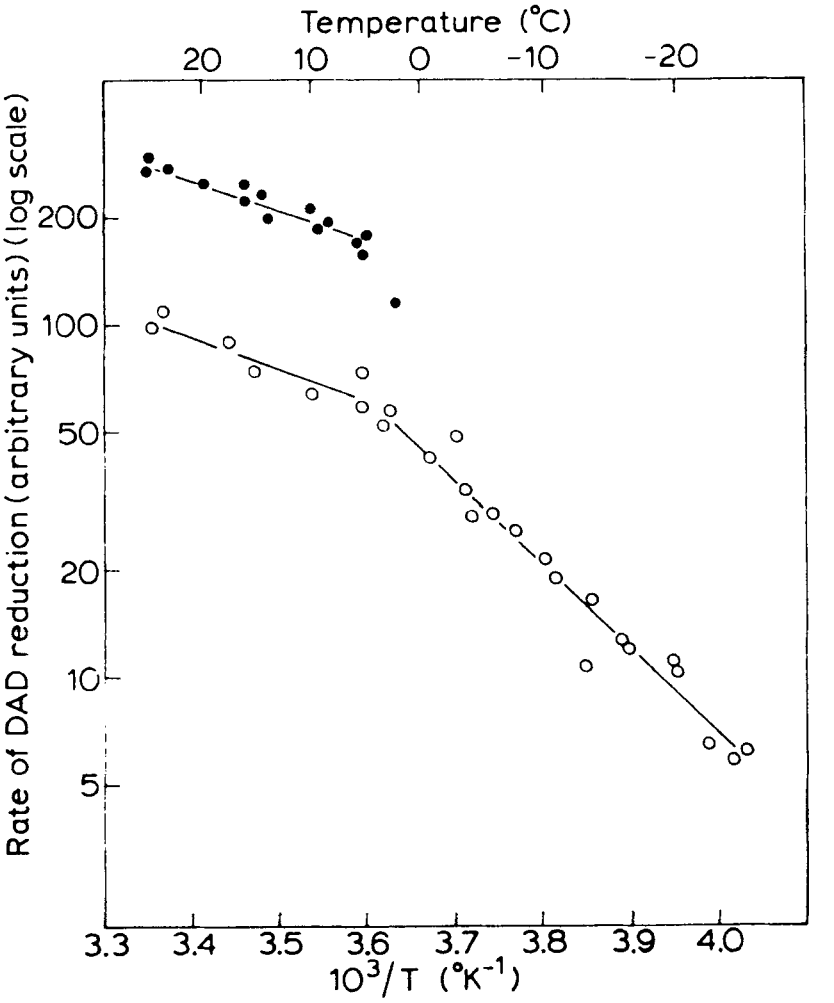

FIGURE 16. Arrhenius plot for the reduction of oxidized 2,3,5,6-tetramethyl-*p*-phenylenediamine by chloroplasts suspended in a medium containing 40% ethylene glycol (○) and in aqueous solution (●). (From Cox, R. P., *Biochim. Biophys. Acta*, 387, 593, 1975. With permission.)

the cryosolvent compared to those required in aqueous solution. This observation probably reflects the previously discussed hydrophobic partitioning effect, perhaps in this case reflecting partitioning to the membrane phase rather than the receptor sites themselves.

Although the chemiosmotic theory as applied to chloroplasts has received substantial support, there are a number of unresolved questions. These include the number of protons transported per turnover of the electron transport chain, and the rates of the proton exchange relative to the electron transfer reactions. Both the electron transfer reactions and the proton translocation are rapid at room temperature which results in difficulties in accurately determining the exact events occurring. Consequently the ability to monitor the reaction at the much slower rates found at subzero temperatures has allowed detailed studies to be carried out on the proton transport system.[130] Olsen and Cox[130] found that the uptake of protons by chloroplasts in the presence of light and their subsequent efflux in the dark could be readily followed at −16° C using suspensions of

chloroplasts in 50% ethylene glycol. Under single turnover conditions 3 protons were taken up using methyl viologen as the electron acceptor. This may be compared with values of 2 to 4 protons reported by different investigators under normal conditions using fast-reaction techniques.[132-134] The low temperature experiments revealed that flash-induced proton uptake was monophasic with a half-life of 3 sec at −16° C, and the proton release into the chloroplast interior was biphasic with half-lives of <0.1 and 3 sec, respectively. These data may be compared with those reported by Auslander and Junge[132] of 60 msec for proton uptake and 0.6 msec for plastoquinone reduction. It is of note that a proton ratio of 3 per electron is expected if plastoquinone participates in a protonmotive quinone cycle as suggested by Mitchell.[135]

The data on the rates of proton uptake and release at subzero temperatures suggest that reduction of plastoquinone occurs faster than uptake of protons from the external aqueous phase as was found in aqueous solution[132,133] at room temperature. The latter observations prompted the suggestion that a barrier to proton diffusion between the external aqueous phase and the site of quinone reduction exists. Such a barrier would explain the fact that only one phase of proton uptake is observed, both at room temperature and at subzero temperatures.[130,133]

The rate-limiting step of the electron transport chain is the oxidation reduction of plastoquinone. During steady-state illumination components before the rate-limiting step, including plastoquinone, are reduced, and those after it, including cytochrome *f* and the reaction center chlorophyll of photosystem I (P700), are oxidized. Measurement of the reduction of cytochrome *f* and P700 in the dark following illumination is therefore a way of studying the rate-determining step.[19] The arrangement of the electron transport components between the two photosystems is still unclear. The value of low temperature investigations in sorting out the different components and their roles in this part of the electron chain has been made clear by Cox's studies on cytochrome *f* and P700. Using subzero temperatures to −35° C and 50% ethylene glycol as cryosolvent, Cox[10] investigated the reduction of P700 in the dark following a period of illumination, which would reduce the plastoquinone pool and oxidize components on the donor side of photosystem I. The reaction kinetics could be readily followed at temperatures below −10° C using a standard spectrophotometer. First-order kinetics were found with an energy of activation similar to that observed in the reduction of the electron acceptor methyl purple. The reaction could be inhibited by dichlorophenyldimethylurea. Furthermore the cycle of oxidation and reduction could be repeated many times without any change in the observed kinetics.

These experiments indicated that there was no evidence for any qualitative change in the behavior of the chloroplasts down to −35° C, and that electron flow from the donor side of photosystem II to the acceptor side of photosystem I occurs at this temperature. Evidence of additional complexity was noted at temperatures below −35° C. It is also of interest that the rates of reduction of cytochrome *f* and P700 were increased by uncoupling reagents, indicating that the membrane was sufficiently intact to maintain a proton gradient under these conditions. The oxidation and reduction of cytochrome *f* were also observed under similar conditions. The activation energy for cytochrome *f* was similar to that for P700, but the rate differed by a factor of three times faster.[10] These observations are consistent with Haehnel's[136] conclusions based on studies at room temperature — that cytochrome *f* is not a component lying on the reaction pathway between plastoquinone and P700. The investigations which have been carried out so far on this system demonstrate the potential of cryoenzymology in the dissection of such complex systems. It seems reasonable to expect considerable further advances in our understanding of photosynthetic mechanisms to occur through future low temperature investigations.

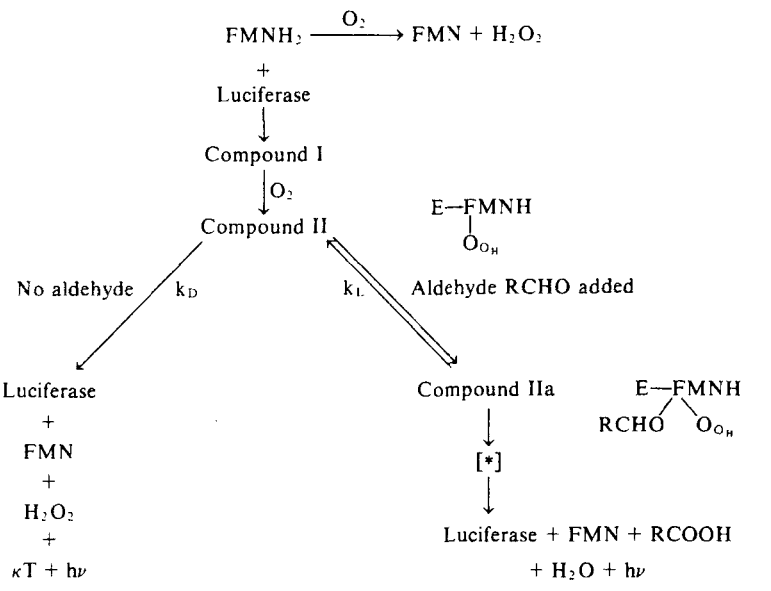

FIGURE 17. Scheme depicting the pathways and intermediates in the luciferase-catalyzed oxidation of $FMNH_2$ by molecular oxygen. (From Douzou, P., *Advances in Enzymology*, Meister, A., Ed., 45, 217, 1977.)

B. Bacterial Luciferase

Bacterial luciferase is a flavoprotein consisting of two subunits, alpha and beta, of molecular weight 79,000 which catalyses the oxidation of FMN by molecular oxygen.[137,138] One of the products of this reaction is light and it is now well established that the emitting species of this bioluminescent system is an enzyme complex protected in some way against the various types of energy conversion that would lower quantum yields. The reaction pathways and intermediates thought to be involved in the bacterial reaction are given in Figure 17. Since the turnover rate leading to bioluminescence in the presence of long chain aldehydes is 4 min^{-1} at 20° C and the initial steps in the reaction are rapid, a long-lived intermediate, II, is formed with a lifetime of tens of seconds. However, in spite of its relatively long lifetime, characterization had not been possible, mainly due to contamination from nonenzymatically oxidised FMN. Hastings[24,30,31] has employed low temperature techniques to purify and study intermediate II, formed during the catalytic cycle of the luciferase from *Achromobacter fischeri.*

Catalytically reduced FMN was incubated with luciferase and molecular oxygen in 50% ethylene glycol/pH* 7.0 (a cryosolvent in which the enzyme has been shown to be catalytically active) for the shortest period to allow intermediate II formation at 4° C. Dilution with an equal volume of cryosolvent at −40° C trapped the intermediate, which could then be separated from free oxidized flavin and H_2O_2 by chromatography at −20° C in a specially designed column and cold chamber. At −20° C the intermediate is stable for hours and has been extensively studied by spectroscopic methods.

The results are best summarized with reference to the postulated in vitro pathway of Figure 17. First, addition of a long chain aldehyde to purified intermediate II and warming to room temperature results in bioluminescence with kinetics which are precisely exponential and independent of enzyme concentration. Calculations based on the quantum yield indicate one FMN molecule bound per luciferase molecule, as previously reported, and a specific activity of the intermediate equal to that of pure luciferase.

Second, in the absence of aldehyde, warming of intermediate II results in little or no light emission, yielding FMN, luciferase, and H_2O_2. Stoichiometric formation of H_2O_2 was shown by the potential for chemiluminescence in the presence of luminol. Similarly H_2O_2 was shown to be absent following decomposition of intermediate II via the "light" pathway. Both the "light" and "dark" pathways yielded the expected proportions of recyclable FMN and luciferase.

Third, it had long been deduced that intermediate II contained bound oxygen since bioluminescence occurs subsequent to the removal of free oxygen once this intermediate has been formed.[4] Purified intermediate II has an absorbance maximum at 370 nm and a fluorescence maximum at 485 nm (close to the wavelength of bioluminescence at 490 nm). Concomitant with bioluminescence is the formation of free oxidized flavin with an absorption maximum at 450 nm and fluorescence maximum at 530 nm. It was therefore concluded that in intermediate II, the bound flavin is not in the oxidized form. Later studies have shown that when oxidized FMN is bound to luciferase it is actually nonfluorescent.[139] The reported spectrum for intermediate II is consistent with the formation of an oxygen adduct at position 4a on reduced FMN (Figure 17), and low molecular weight models have supported this proposal.[124,140,141]

Fourth, the reaction scheme of Figure 17 postulates the reversible binding of aldehyde to intermediate II, and thus the existence of at least two species at this stage, namely II and IIa. Difference spectra of intermediate II and intermediate II plus aldehyde has shown a spectral red shift associated with IIa. This spectral shift was not seen when aldehyde was replaced by the corresponding alcohol. However direct isolation of this intermediate IIa has not been possible, even by column chromatography at −30° C, since it breaks down at a much lower temperature than intermediate II.

Fifth, the combination of luciferase with reduced flavin has been postulated to form a complex I that has only a transient existence in the presence of O_2. Mixtures of reduced flavin and luciferase prepared in the absence of molecular oxygen and examined spectrally between +24° and −24° C showed no absorption band in the 300- to 400-nm range, indicating that the absorption at 370 nm identified with intermediate II cannot be attributable to a possible luciferase-reduced flavin complex, intermediate I.

The isolation and characterization of the long-postulated intermediate II, using low temperature techniques, has provided support for the proposed reaction scheme of Figure 17 and spectral evidence for its chemical nature as an oxygenated intermediate containing a flavin peroxide. Moreover, although there is a spectral similarity between intermediate II and the bioluminescence emission spectrum, it seems almost certain that intermediate II cannot be the emitting species since a subsequent reaction with aldehyde is required to populate an excited state (* of Figure 17). Consequently it is probable that an intermediate or product is formed whose excited state is populated during the reaction. The nature of this intermediate is, as yet, unknown. It is clear that oxidized FMN cannot be the emitter due to the spectral dissimilarities. It has been found that dilution of intermediate II at −20° with an aqueous solution of oxidized cytochrome *c* ($Fe^{3+}p$) leads to thermal decomposition of the intermediate yielding reduced cytochrome ($Fe^{3+}p$).[2]

Such a reaction can be explained by the production of superoxide anions and it is possible that the production of such ions may be responsible for bioluminescence.[4] However, it has been shown that there is only one binding site for FMNH per luciferase molecule, and intermediate II does not require an additional molecule of oxygen to yield bioluminescence.[4] In addition it has been shown that long-chain aldehydes have a very marked stimulatory effect on light emission and that the aldehyde is converted into the corresponding fatty acid[142] (Figure 17). That the oxidation of a single FMNH molecule would not provide sufficient energy for photon emission at 490 nm implicates aldehyde

oxidation as a necessary feature of bioluminescence. The overall reaction for the catalytic oxidation of reduced FMN then becomes

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{luciferase}} FMN + H_2O + RCOOH$$

Clearly further work is required before all the features of the bioluminescent reaction can be reconciled. The situation is further complicated by the finding that the fluorescence emission spectrum of intermediate II at −30° C indicates the presence of two components, one with a maximum at 485 nm and one with a maximum above 500 nm (but not oxidized FMN). With continued irradiation at 370 nm, the maximum at 485 nm increases fivefold. Samples both before and after irradiation have the same excitation spectrum, form the same amount of FMN on warming, and have the same bioluminescence potential on warming in the presence of aldehyde. The emission spectrum of bioluminescence has been recorded during warming in the presence of aldehydes and spectrally it seems to be identical to the fluorescence emission spectrum of intermediate II following irradiation at 370 nm. It is not known whether the component formed following irradiation is also a normal intermediate in the pathway of bioluminescence.

The reaction of bacterial luciferase is closely analogous to certain flavoprotein hydroxylases and oxidases, and in all systems a reduced flavin-enzyme intermediate can react with oxygen even in the absence of a second substrate (e.g., aldehyde) to give an oxygenated form which may be designated as the active oxidizing species.[137] These active oxidizing species have similar spectral properties in all the above systems, and optical and chemical evidence strongly suggests a flavin C4a-peroxide structure for the oxygen adduct. Recent work has shown conclusively, at least for the bacterial luciferase system, that the oxygen-containing intermediate II is a C4a-substituted adduct of reduced FMN. Using ^{13}C-enriched FMN at position 4a Ghilsa et al.[143] have shown that at −15° C in 20% ethylene glycol the ^{13}C-N.M.R. spectra of luciferase with bound oxidized and reduced FMN, and reduced FMN with oxygen, have chemical shifts at 137, 103 ppm, and 74 ppm, respectively. The appearance of one resonance at 74 ppm, paralleling the formation of the oxygenated intermediate, can confidently be assigned to the enriched C-4a carbon of FMN. Upon warming to room temperature the 74-ppm resonance disappeared paralleling the oxidation of FMN.

Work on model compounds has shown that the ^{13}C chemical shift for hydroxyl group-substituted compounds at C4a is the same as that for the oxygenated luciferase-FMNH complex. Kemal and Bruice[144] have shown that 4a-flavin hydroperoxides undergo a chemiluminescent reaction in the presence of aldehyde with many parameters of the process similar to those of bioluminescence:

1. The absorption and fluorescence emission spectra of 4a-flavin hydroperoxides were similar to those of intermediate II.
2. 4a-flavin hydroperoxides react with aldehydes to form what is postulated to be a species whose further reaction is accompanied by light emission.
3. Chemiluminescence occurs equally well in aerobic or anaerobic conditions.
4. The time course of light emission is characterized by an initial increase in light intensity followed by an exponential decay similar to that observed with luciferase.
5. Aldehydes are converted to their corresponding acids in the chemiluminescent reaction.
6. In the absence of aldehyde, 4a-flavin hydroperoxides form H_2O_2.
7. As is implied in (3), the reaction of oxygen with reduced flavin is irreversible, as is the reaction of oxygen with the luciferase-reduced flavin intermediate.[12]

Although these results do not allow a detailed mechanism of bioluminescence to be proposed, the use of cryoenzymological methods to temporally resolve and characterize intermediates in the bioluminescent reaction has permitted a more detailed study of the pathways involved.

C. Cytochrome P450 (Bacterial)

Bacterial cytochrome P450, a heme protein involved in the hydroxylation of camphor, has been studied by Douzou and co-workers at low temperatures. They have observed the formation[38] and subsequently purified oxygenated intermediates,[145,146] and have studied its interactions with camphor.[147]

Hui Bon Hoa et al.[147,148] have studied camphor binding to cytochrome P450 at temperatures as low as −40° C in 50% ethylene glycol by both stopped-flow and conventional spectrophotometry. The dissociation kinetics were followed by trapping free ferric cytochrome with metapyrone in which dissociation of the cytochrome P450-camphor complex is rate limiting. The effect of ethylene glycol on the interaction of camphor and cytochrome P450 is to decrease both the association (k_1) and to a lesser extent the dissociation (k_{-1}) rate constants. If it is assumed that the driving force for camphor binding is the removal of camphor from an aqueous environment to a hydrophobic region of the protein 149, then the higher hydrophobicity of ethylene glycol as compared to water might be expected to decrease the free energy of solvation of camphor, thus descreasing the driving force for association. Both k_1 and k_{-1} had similar temperature dependencies in the presence and absence of ethylene glycol, suggesting that the solvent does not effect the conformation of the hydrophobic pocket of cytochrome P450. The mechanism of oxygen activation and camphor hydroxylation by cytochrome P450 must rely on very special structural features of the heme pocket. These features appear to be profoundly affected by binding of substrate, as reflected in the shift of the spin-state equilibrium from mostly low spin for the free ferric enzyme (Fe^{3+}), to mostly high spin for the ferric enzyme-substrate complex ($Fe^{3+}\cdot RH$).

Changes in the high spin (hs)/low spin (ls) equilibrium constant (K_{stat}) for the camphor-bound cytochrome have been followed spectroscopically at saturating concentrations of camphor.[148] It has been found that 50% ethylene glycol cryosolvent displaces the spin equilibrium to practically 100% hs from that in aqueous medium, while lowering the temperature increasingly favors the low spin form of $Fe^{3+}\cdot RH$. Van't Hoff plots of K_{stat} were linear between 0 and −40° C. The pH* dependence of the rate constants k_1 and k_{-1} between +5 and −20° C has been interpreted as implying that in the physiological range of pH* there is a fast equilibrium between two ionization states of both free and camphor-bound cytochrome P450. Considerations of two apparently distinct ionization constants implicates a single histidine residue which following camphor binding has its pKa lowered by one unit from 7.3 to 6.25 at 0° C, presumably by modification of the electrostatic potential of its environment. While $\Delta H^{\dagger}$ and $\Delta S^{\dagger}$ for k_1 were independent of pH* in the physiological pH* range, the same parameters for k_{-1} varied considerably, indicating the dissociation mechanism is very sensitive to the ionization of the probable histidine residue. A plot of k_1/k_{-1} vs. pH* gave a maximum in the 6.7 to 7.4 pH* range, in contradiction to the idea that affinity of camphor is governed by hydrophobic interactions alone. pH*-dependent H-bonding may also be important.

Since the camphor binding site of cytochrome P450 is very close to the heme group it was postulated that an ionization-induced conformational change of the $Fe^{3+}\cdot RH$ camphor binding site would disturb the geometry of the heme group and hence modifiy K_{stat} of $Fe^{3+}\cdot RH$. Both ΔH_{stat} and ΔS_{stat} varied as a function of pH* in the range where there is a change in the dissociation mechanism of cytochrome P450 and camphor. It was concluded that camphor binding causes a change in the electrostatic potential at the

camphor binding site. Under this improved electrostatic potential the state of ionization of a specific protein residue is affected and in turn might govern the energetic electron distribution of the Fe^{3+} ion in the physiological pH* range. It should be stressed that the combined effect of temperature and solvent lowered both k_1 and k_{-1} sufficiently to allow direct measurements of their dependence upon temperature and pH*, which is not possible using conventional techniques.

Further studies have been aimed at shedding light on the structural basis of the mechanism of the spin conversion.[150,151] Whereas at room temperature only small changes can be observed when parameters such as ionic strength and pH are changed, at subzero temperatures in mixed organic solvents changes from nearly 100% low spin to nearly 100% high spin can be induced and studied. Using cryosolvents of ethylene glycol/water 1:1 with a range of buffers at −40° C, it has been found that increasing pH* favors the high-spin form. Potassium chloride has the same effect although acting independently to lower the apparent pKa of the transition in a linear fashion. Other cations have a similar though less pronounced effect.[150] Van't Hoff plots indicated that at high pH* and KCl concentrations ΔH_{stat} was lowered whereas the presence of organic solvents and high camphor concentration increased ΔH_{stat}. Concentrations of camphor greater than 1 m*M* decreased the fraction of enzyme in the high-spin form, and subsequent experiments have indicated that a second camphor molecule binds to cytochrome P450 with a K_d of 2 m*M*, probably acting as a spin modulator of $Fe^{3+} \cdot RH$.

It has been suggested that the shift in the apparent pKa of the spin transition due to ionic strength can be explained by the polyanionic nature of at least a part of the protein. Goldstein et al.[94] have shown that the pH in an electrostatic field of a polyelectrolyte approaches the pH of the medium at high ionic strengths. A similar relationship exists between the apparent pK and intrinsic pK of an ionizable group located in an electrostatic potential. The group influencing the low- to high-spin transition can best be thought of as being in a polyelectrolyte environment, and this proposal is supported by the linear relationship between its apparent pKa and the logarithm of the ionic strength.

Although these and other results show that the cosolvent neither denatures nor destroys enzyme activity, it does have a pronounced (although reversible) effect on the camphor binding mechanism and K_{stat} and ΔH_{stat} for the spin equilibrium. It is not clear at the present time whether the cosolvent, and the other factors, affect the heme structure governing the spin rate directly, or indirectly by interacting with some remote amino acid residues.

1. Preparation of Transient Intermediates of Cytochrome P450

The purification and isolation of the ternary oxy-ferrous camphor-bound intermediate, $Fe^{2+}_{o_2} \cdot RH(m^{rs}_{o_2})$, which is the real starting species leading to substrate hyroxylation, has been achieved by column chromatography on Sephadex LH20 at −20° C in 50% ethylene glycol. The protocol involved initially forming the reduced, substrate-bound cytochrome P450 (m^{rs}) by proflavin-mediated photosensitized reduction in the presence of camphor at +10° C in an anaerobic environment. The oxygenated intermediate was then formed at +10° C and rapidly cooled to −40° C in order to prevent its conversion to the ferric form m^{os}. Column chromatography in the presence of a high concentration of camphor afforded complete separation of the intermediate from both proflavin and H_2O_2 formed from the rapid reoxidation of proflavin by excess oxygen. The intermediate could be stored at −35° C without appreciable reoxidation. The cosolvent used did not affect either the rate or the energy of activation for the reaction process compared to normal conditions.[38,146] In addition no denaturation nor changes in hydroxylating ability were found for any of the redox states of cytochrome P450 (m^o, m^{os}, m^{rs}, $m^{rs}_{o_2}$) in the mixed solvent.

Similarly the binary, substrate-free, oxy-ferrous cytochrome P450, Fe^{2+}-O_2 ($m^r_{o_2}$) was purified using the same cryosolvent. Although the ternary complex $m^{rs}_{o_2}$ was initially formed, subsequent chromatography on Sephadex in the absence of camphor allowed both the $m^{rs}_{o_2} \rightarrow m^r_{o_2}$ conversion to occur and the separation of $m^r_{o_2}$ from both the exogenous redox components and camphor. Although the substrate-fee complex is less stable than the corresponding substrate-bound complex, appreciable decay did not occur at 77° K and storage was possible. The nonenzymatic autooxidation rates of $m^{rs}_{o_2}$ and $m^r_{o_2}$ in aqueous organic solvents at subzero temperatures are very low, with half-lives of 48 and 2.5 hr, respectively, at −30° C. Although the activation energy for the process is the same in aqueous and cryosolvent media for $m^{rs}_{o_2}$, there is a lowering of the corresponding activation energy for $m^r_{o_2}$.[145] The autooxidation kinetics of the two complexes retain distinct characteristics, presumably reflecting differences in protein structures, since it is known that camphor binding protects the enzyme during autooxidation, possibly due to an effect on the heme pocket configuration.

The multistep oxygen reduction and hydroxylation sequence of camphor has been studied in fluid media at subzero temperatures, exploiting differences in activation energies, and has allowed resolution of the overall process into two univalent reductions of P450 by the natural electron donor putidaredoxin (Pd^-). These constitute the initial reduction of the cytochrome P450-substrate complex to give $Fe^{2+}\cdot RH$, and the second electron transfer to the oxy-ferrous substrate complex $Fe^{2+}_{o_2}\cdot RH$ to give products.[152] All studies were performed in the cryosolvent ethylene glycol/phosphate buffer 1:1. The initial reduction of cytochrome P450 in the presence of camphor by Pd^- was found to be a reversible bimolecular reaction.[153]

$$Fe^{3+}\cdot RH + Pd^- \rightleftharpoons Fe^{2+}\cdot RH + Pd^0$$

Analysis of the kinetics from −10 to −40° C indicated that the primary rate-limiting event is formation of a dienzyme complex, Pd^--$Fe^{3+}\cdot RH$ followed by reversible intermolecular electron transfer. The reduction of ferrous oxycytochrome was achieved by initially reducing photochemically $Fe^{3+}\cdot RH$ and Pd^0 in an anaerobic environment of 15° C to give the equilibrium

$$Fe^{2+}\cdot RH + Pd^- \rightleftharpoons Pd^-\text{-}Fe^{2+}\text{-}RH$$

The oxyintermediate was then formed by bubbling with oxygen after cooling to −40° C in the presence of camphor. The subsequent decomposition into Fe^{3+} and product ROH followed. The rates were found to be monophasic, first order, with half-lives dependent only on temperature. The product yield followed a saturation curve, and by analogy to the first reduction process it was proposed that an initial dienzyme complex was formed:

$$Pd^- + FeO_2^{2+}\cdot RH \rightleftharpoons (Pd^-\cdot FeO_2^{2+}\cdot RH) \rightleftharpoons Pd^0 + Fe^{3+} + ROH + H_2O$$

By effectively starting from the preformed dienzyme complex, Pd^--$Fe^{3+}\cdot RH$, the decomposition of the oxygenated complex at low temperatures is uncoupled from subsequent recycling ($Fe^{3+} \rightarrow Fe^{3+}\cdot RH \rightarrow Fe^{2+}\cdot RH \rightarrow FeO_2^{2+}\cdot RH$) and from spontaneous nonhydroxylating decay ($FeO_2^{2+}\cdot RH \rightarrow Fe^{3+}\cdot RH + O_2 + e^-$). This was possible because at subzero temperatures the half-life of putidaredoxin binding and the reduction rate by the first electron are much slower than the decomposition process. In addition the oxy-ferrous complex in the absence of putidaredoxin is relatively stable below −20° C. Consequently one is allowed a clear view of the decomposition process not

possible at room temperature. The monophasic, [Pd^-]-independent kinetics of the reaction were taken to indicate that the whole process is rate limited by the decomposition of an unknown intermediate, which could not be detected using low temperature techniques.

In summary, the mechanism of hydroxylation by bacterial monooxygenase has been resolved to show four main steps: (1) binding of the substrate camphor (RH) by free ferric cytochrome Fe^{3+}; (2) reduction of this E-S complex by an iron-sulfur protein, putidaredoxin (Pd^-); (3) binding of molecular oxygen to give a ternary oxyferrous compound; and (4) uptake of a second electron initiating the reactions leading to the hydroxylated substrate (ROH) with restoration of free enzyme (Fe^{3+}). In the complete native system both a flavoprotein and putidaredoxin, act as electron donors and effectors that complete the cytochrome.

It is pertinent at this point to draw the reader's attention to experiments performed on bacterial cytochrome P450 in water-in-oil emulsions[57] and inverted micelles.[59] Although the results generally agree with those found in mixed solvents there are a few notable exceptions. For instance the cosolvent effect on the thermodynamics of the spin-state equilibrium of camphor-bound cytochrome P450 was not seen in supercooled water. On the whole, however, the use of cryosolvents appears to be preferred, due to unresolved technical difficulties with alternative low temperature systems.

D. Cytochrome P450 (Liver)

In addition to bacterial cytochrome P450, low temperature techniques have been used to explore the well-known multienzyme systems performing drug hydroxylation in the liver endoplasmic reticulum of rats. This system involves two flavoproteins — NADPH-cytochrome P450 reductase and NADH-cytochrome b_5 reductase — and two cytochromes — cytochrome b_5 acting as an electron carrier and cytochrome P450, which is the hydroxylating system.

It should briefly be pointed out that the purification of microsomal cytochrome P450 has been achieved by column chromatography at −20° C in a glycerol/phosphate buffer cryosolvent on DEAE cellulose.[154] Separation of cytochrome P450 from the other enzymes of the hydroxylation system, as well as from the inactive isomer cytochrome P420, was readily achieved. Studies on purified enzyme in 50% ethylene glycol have shown that over a broad range of temperatures (+20 to −40° C), the activation energy and the dissociation constant for the binding of CO do not vary.[155] It was concluded that cytochrome P450 did not undergo any temperature-dependent structural change, and that both ethylene glycol and glycerol had no adverse effects on the enzyme.

Studies have been aimed at defining conditions for the spectrophotometric study and activity assays of each membrane-bound and solubilized enzyme of the hydroxylating system at various temperatures, and if possible, to observe the activity of the whole system.[156] Observations at room temperature of microsomal suspensions in ethylene glycol cryosolvents showed that the rates of reduction of endogenous cytochrome P450 and exogenous cytochrome *c* by NADPH-cytochrome *c* reductase markedly decreased as the concentration of added ethylene glycol increased. A similar decrease was seen when cytochrome *c* was reduced by soluble NADH-cytochrome *c* reductase. In both cases the solvent effect was explained in terms of the accessibility of membrane cytochromes (P450 and b_5) to the solvent, since electron transfer from NADPH-cytochrome *c* reductase to cytochrome P450 and exogenous cytochrome *c* was influenced to the same extent by the solvent. However, the solvent effect on the reduction of cytochrome *c* was similar when NADPH-cytochrome *c* reductase was bound to the membrane or solubilized.

The effect of temperature on the enzyme system was such that the reduction rate of cytochrome *c* by both NADPH- and NADH-cytochrome *c* reductase was practically 0 at 0° C, and the enzymic reduction of P450 was unobservable below −5° C; the reduction of cytochrome b_5 by NADH- and NADPH-cytochrome b_5 reductases could be detected down to −25° C. This indicated that the interruption of the reduction of cytochrome P450 at −5° C was due to a rate-limiting electron transfer from NADPH-cytochrome *c* reductase to cytochrome P450 which resulted in the accumulation of reduced cytochrome b_5 in the presence of fully oxidized cytochrome P450. These preliminary studies of the individual activities of the various enzymes of the assembly in mixed solvents between +20 and −30° C showed the normal sequence of events, and the hydroxylating membrane-bound function was preserved.

Later studies by Debey et al.[157] concentrated on the production of the oxidizing species O_2^- and H_2O_2 by the hydroxylating system to determine whether such species are normally released by NADH-cytochrome P450 reductase or by cytochrome P450 itself. It was shown that following enzymic reduction of cytochrome P450 and the whole microsomal system at room temperature under anaerobic conditions in ethylene glycol/Tris buffer and cooling to −30° C (which inhibits enzymatic electron transport activity of the microsomal system) that subsequent warming in the presence of oxygen and luminol caused luminescence with a maximum light emission at −16° C. Numerous controls, including the observed luminescence sensitivity to cytochrome P450 inhibitors, and the variation of the luminescence intensity with time and temperature suggested that the formation and decomposition of some unstable species was responsible — namely Cyt $P450^{2+}$-O_2. (It should be noted that a second luminescent peak is obtained between 0 and 20° C corresponding to the steady-state concentration of this intermediate once the electron flow of the microsomal system is resumed above 0° C.) Similar studies revealed spectroscopically the reaction of oxygen with microsomal cytochrome P450 reduced by the substrate hexobarbitol between −20 and −45° C.[127] At −45° C the spectra observed corresponded to Complex I which was progressively converted into Complex II. This rapid transformation of Complex I to II suggested the influence of other components of the electron transport chain (such as cytochrome b_5) on the stability of complex I. It was suggested that the observed luminescence with luminol between −20 and −10° C could be due to the decomposition of Complex II. By analogy with the normally stable bacterial oxy-ferrous complex $Fe^{2+}\cdot RH\text{-}O_2$ at −40° C which is quickly destroyed by Pd^-, it was suggested that the microsomal cytochrome P450-O complex could have a similar fate in the presence of electron donors such as cytochrome b_5 or NADH reductase.[121] Estabrook et al.[158] identified an oxy-ferro intermediate during the steady-state oxidation-reduction cycle by which hepatic microsomal cytochrome P450 hydroxylates natural and foreign compounds. Recent work has allowed the formation and stabilization of such an oxy-ferro intermediate of highly purified microsomal cytochrome P450 from rabbit liver in glycerol/aqueous buffer at −30° C.[159] The spectrum (and presumably structure) was very similar to that obtained for the bacterial cytochrome P450 intermediate and the reported observations give support for the existence of an oxy-ferro complex during the turnover of intact microsomes.

One of the many involved problems of the hydroxylating system of rat liver microsomes is the nature and function of the membrane environment. Leon et al.[160] have suggested that the enzymic reduction of cytochrome b_5 by NADH could serve as an intrinsic probe of membrane fluidity. Solubilized cytochrome b_5/b_5-reductase system exhibits monophasic reduction kinetics over the temperature range +15 to −20° C in aqueous ethylene glycol, whereas in intact microsomes the process becomes increasingly heterogeneous below 0° C, reflecting heterogeneities in membrane structure observable as a distribution in reaction rates and activation energies. The reduction of microsomal

cytochrome b_5 in aqueous ethylene glycol, glyercol, or propylene glycol was followed by stopped-flow spectrophotometry and the kinetics could be interpreted as representing bimolecular protein-protein interactions related to their diffusibility in the membrane plane and affected by a progressively modified membrane structure at low temperatures. At temperatures greater than 0° C phospholipid exchange and protein relaxation rates are faster than electron transfer. Below 0° C the temperature slows down uniformly the relaxation process and reveals the heterogeneity of the microenvironment, reflected by a broad spectrum of rate constants. These results illustrate the potential usefulness of cryoenzymology for the investigation of lipid-mediated, protein-protein interactions.

In view of the importance of the hydroxylating system in drug metabolism it is necessary to point out that many problems, such as the function of each individual component and their interactions, and the molecular mechanism of oxygen activation and hydroxylation are still unresolved. The electron transfer reactions leading to the hydroxylated substrate are too fast and intermediates too fleeting to be properly characterized, and contamination by recycling precludes any consistent knowledge of the nature and reactivity of intermediates occurring beyond the oxy-ferrous intermediate. Recent experiments have coupled the decomposition of this oxy-ferrous intermediate, followed spectroscopically, with the formation and concomitant fluorescence of 7-OH-ethoxycoumarin from the substrate ethoxycoumarin.[161] This result gives support to the suggestion that light emission in the presence of luminol is linked to a single redox cycle of cytochrome P450. A reactive species with a higher oxidation state than ferric has been suggested as following the oxy-ferrous intermediate.

In summary, cryoenzymological studies on both the bacterial cytochrome P450 and the liver microsomal hydroxylating systems have shown that the use of cryosolvents and subzero temperatures can allow the resolution of elementary steps and accumulation of intermediates without altering the reaction pathways normally involved. The most valuable results are related to temperature-controlled uncoupling of reactions and the conditions for single turnover, as well as the isolation of the oxy-ferrous intermediates.

E. Cytochrome *c* Oxidase

Cytochrome *c* oxidase has been the subject of many studies because of its unique role as the donor of electrons to molecular oxygen in the respiratory chain. In particular, reactions of this enzyme with oxygen have been probed by low temperature experiments aimed at elucidating the features of ligand binding and electron transport.[37,162-164]

Chance and his colleagues have used a triple-trapping procedure to study the functional intermediates in the reaction of cytochrome oxidase with oxygen.[165] Essentially carbon monoxide-inhibited, fully reduced, mitochondrial or soluble cytochrome oxidase preparations are cooled in the dark to −15 to −30° C in ethylene glycol cryosolvent. Under these conditions the velocity of dissociation of CO from the heme of cytochrome oxidase is markedly decreased; thus oxygen does not combine with the enzyme to any significant extent. Oxygen is added to the preparation by stirring or by H_2O_2 decomposition in the presence of catalase. The second step is to rapidly freeze the mixture at −80° C. This traps the reduced components of the respiratory chain, the CO-inhibited cytochrome oxidase, and O_2 in a stable state. The sample is then equilibrated at the desired temperature, and the reaction of cytochrome oxidase with O_2 initiated by laser flash photolysis. Once the desired stages of the reaction are reached samples are quenched at −196° C in liquid nitrogen or a mixture of chilled isopentane and propane where they can be studied spectroscopically.

This procedure has permitted a detailed study of the intermediates formed after flash photolysis for the following reasons. First, intermediates accumulate because the energies of activation of the subreactions of the respiratory chain increase with the

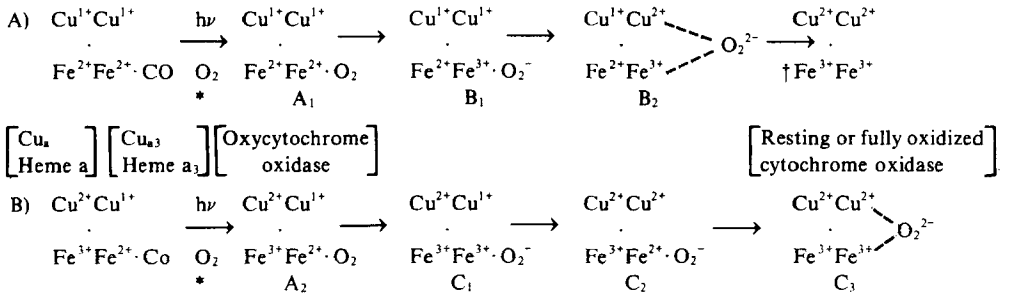

FIGURE 18. Summary of the intramolecular electron transfer reactions of cytochrome oxidase with oxygen. (A) From fully reduced enzyme. (B) From mixed valence-state enzyme. (*) Denotes ligand exchange reaction, (†) intermolecular electron transfer reactions. (After Chance, B. and Leigh, J. S., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 4777, 1977.)

sequence of steps in the chain. The initial reactions with oxygen are of lower energy of activation than later steps, hence lowering the temperatures makes them relatively faster than the oxidation of later components in the chain. Second, at low temperatures complications involved in the function of the total respiratory chain are minimized and the activation of electron transfer appears to be limited to cytochrome oxidase and its nearest neighbors, the electron donor cytochromes *a*, *c*, c_1, and an iron-sulfur protein. Third, the reactions are confined to the vicinity of the heme site of cytochrome oxidase by freezing of the surrounding medium. Thus one can separate those aspects of the kinetics that depend upon the nature of the heme site from those that depend upon the solvent or membrane environment. Finally, it is necessary to mention the demonstrated tolerance of mitochondrial preparations and of the electron transfer kinetics to low temperatures and the mixed solvents used.

The use of low temperature procedures with cytochrome oxidase from higher eukaryotic cells have shown the existence of three classes of functional intermediate compounds of cytochrome a_3 and oxygen. Compounds of type A are formed between −125 and −150° C. They do not involve electron transfer to O_2, and include oxy compounds of the type $Cu^{+}a_3^{2+}-O_2$ where cytochrome oxidase is in the same redox state as that prior to CO dissociation. They are essentially the results of a ligand exchange reaction. Compounds of type B are formed between −95 and −60° C and involve oxidation of the heme and copper components of cytochrome a_3 by a two-electron transfer to oxygen to form the peroxy compounds $Cu^{2+}a_3^{3+}$-O_2^{2-} or $Cu^{2+}a_3^{2+}$-O_2H_2. Compounds of type C are formed above −100° C from mixed valence states of the oxidase obtained by ferricyanide pretreatment, and might involve either a higher valency state or iron in a peroxy intermediate (or more likely a mixed valence state binuclear complex with heme a_3 reduced and its associated copper in an oxidized state, with both interacting with bound and partially reduced oxygen). These components act as electron acceptors for the respiratory chain and as functional intermediates in O_2 reduction. Compounds of type B above −60° C serve as electron acceptors for cytochromes *a*, *c*, and c_1. The rates of formation of compounds of type A, B, and C at very low temperatures can be extrapolated with consistency to agree with rates of electron transfer in cytochrome oxidase as measured at room temperature in the solubilized and membrane-bound enzymes.[162,166]

Assignment of oxidation states to the copper and iron atoms (Figure 18) during the reduction of molecular oxygen has come largely from near-IR observations (740 to 940 nm) and absorbance changes in the Soret (430 to 463 nm and (590 to 630 nm) bands, as well as epr measurements.[37,162,163,167] For instance there are no significant IR

(A) At −80° C

$$\text{cyt } C^{2+} + a_3^{3+} - OO^{2-} - Cu_{a3}^{2+} + 2H^+ \rightarrow C^{3+} + H_2O + a_3^{3+} - O^{2-} - Cu_{a3}^{2+}$$
$$(B_2)$$

(B) At −50 to −20° C

$$2c^{2+} + a^{3+}Cu^{2+}a \rightleftharpoons 2c^{3+} + a^{2+}Cu_a^{1+}$$

(C) At > −20° C

$$a_3^{3+} - O^{2-} - Cu_{a3}^{2+} + a^{2+}Cu_a^{1+} + 2H^+ \rightleftharpoons a_3^{3+}Cu_{a3}^{1+} + a^{3+}Cu_a^{2+} + H_2O$$

FIGURE 19. Intermolecular electron transfer reactions of cytochrome oxidase. (A) This reaction may proceed via a prior intramolecular oxidation-reduction reaction leading to formation of ferryl ion and subsequent reaction with cytochrome *c* to give the μ-oxo or resting form of cytochrome oxidase. (B) aCu_a participates in the electron transfer reaction. (C) There is a rapid reduction of $a_3^{3+} - O^{2-} - Cua_3^{2+}$ and oxidation of a Cu_a in the reaction that keeps pace with the oxidation of cytochrome *c*. (After Chance, B. and Waring, A., in *Cytochrome Oxidase,* Chance, B., King, T. E., Okonuki, K., and Orii, Y., Eds., Elsevier/North-Holland, Amsterdam, 1979, 353.)

absorbance changes associated with the formation of compounds of type A (although there are changes in the α region) indicating no significant electron transfer from copper or iron, whereas there is a broad absorbance change in the IR at 790 to 800 nm corresponding to partial oxidation of the copper in the formation of compound B_2. Complete oxidation of cytochrome oxidase above −62° C yields a peak at 820 to 830 nm corresponding to the fully oxidized state of copper found at room temperature. Compound C_2 has a peak at 740 to 750 nm. This band has been identified with the copper associated with heme a_3, while a second band at 609 nm has been associated with a charge-transfer interaction between heme a_3 and its associated copper and a superoxide anion. Since there is no Soret band at 655 nm in compound C_2 (although such a band is present in compound B_2), it is thought that heme a_3 remains in a reduced and/or liganded state, whereas heme a remains in an oxidized state. In addition, the electron spin resonance signal in compound C_2 has been assigned to a special state of cupric copper associated with heme a_3 or a bound superoxide anion. Compound C contains two types of oxidized copper — one is associated with heme a and is oxidized by ferricyanide, and one with heme a_3 and oxidized as the second step in the reaction with O_2. This qualifies the heme a_3-associated copper as being a better electron donor than iron, in contrast to the equality of donors in the configuration of compound B_2. Consideration of the absorbance bands for compound B_2 suggests a bridged or unbridged peroxy intermediate with a ferric-peroxide-cupric copper complex.

A summary of the intramolecular electron transfer reactions of cytochrome oxidase thought to occur with O_2 is given in Figure 18. These reactions are based on the assumption that heme a_3 and its associated copper are the principal reaction centers at low temperatures. The cytochrome oxidase of a mixed valency state with heme a and its associated oxidized copper gives a final configuration or reaction with oxygen similar to that of resting cytochrome oxidase, but differing in that the valence state of heme a_3 and its associated copper may be mixed and the iron may be liganded to partially reduced oxygen. Compound B_2 can oxidize cytochrome *c* at −60 to −70° C (monitored at 550 to 540 nm) without appreciable steady-state oxidation of cytochrome *a,* while at −30 to −40° C cytochrome *a* is oxidized as rapidly as cytochrome *c* (Figure 19).[168] These low temperature experiments have served to identify intermediates in oxygen reduction by cytochrome oxidase and in addition have revealed new characteristics of the electron transfer to cytochrome oxidase not hitherto revealed. Oxygen binding and intramolecular electron transfer to O_2 by Cyt a_3 and Cu a_3 (Figure 18) has been temporally distinguished from intermolecular electron transfer from cyt c and a Cua to compound B (Figure 19).[168]

Specific findings have been (1) the intermediate compounds of cytochrome oxidase in which only heme a_3 and its copper Cua_3 are involved (B_2 and C_2); (2) the identification of Cua_3 as a reactive and functional component; and (3) the hitherto unrecognized ability of a_3 Cua_3 to accept electrons from cytochrome *c*, where at very low temperatures, no change in heme aCua can be detected. This identifies a_3 Cua_3 (B_2) as a much more important electron transfer component of cytochrome oxidase function. Cytochrome oxidase can now be thought of as having two functional portions: a_3 Cua_3 dealing exclusively with oxygen reduction reactions and cytochrome *c*, plus aCua functioning to maximize a_3^{2+} Cu a_3^{1+} and to minimize intermediate compounds. a_3 Cua_3 deals with O_2 binding, O_2 reduction, intermediate compound formation, and short-range electron transfer reactions, while cytochrome *c* and aCua deal with electron transfer reactions. The mechanisms postulated emphasize the function of cytochrome *c*, c_1, and aCua in affording rapid reductants for a_3^{2+} Cua_3^{2+} and for compound B_2 at low temperatures and presumably at physiological temperatures as well. This has the effect of maximizing the concentration of reduced states and thereby maximizes the rate of reaction of cytochrome oxidase with oxygen.

The question of the catalytic activity of compound C_2 is still of considerable interest. The state of cytochrome a_3 must be a very special one that does not permit formation of a fully oxidized state in spite of the presence of oxidants on both sides of the oxidase itself (Figure 18). Compound C_2 does not at present qualify as an inactive intermediate in the electron transport chain.[169] Prior to these low temperature studies the existence of functional oxygen compounds of cytochrome oxidase had been a matter of controversy for a number of years. An understanding of the cytochrome oxidase function can only be perceived through the study of its functional intermediates. Thus in contrast to room temperature studies where the addition of oxygen to reduced oxidase reveals only the formation of the oxidized form, the use of the low temperature triple-trapping procedure has revealed the existence of the intermediate types A, B, and C. Furthermore, low temperature techniques have provided an opportunity to study these reactions to the exclusion of other spectroscopically detectable components of the respiratory chain.

Sharrock and Yonetani[169,170] have independently studied the low temperature kinetics of CO binding to cytochrome a_3 in cytochrome oxidase following flash photolysis. It has been found that although reassociation was approximately exponential there was a deviation from the simple Arrhenius relationship, especially at low CO concentrations in the frozen state. This was explained as being due to three binding regions between which CO could move. At low CO concentrations there is competition between sites, one of which becomes preferentially occupied at high temperatures. Since there were large entropy changes seen on the reassociation of CO and presumably therefore protein structural changes, it was postulated that one site was a preliminary step in the binding of CO to the heme. This site could be an amino acid residue, which then transfers CO to the heme site. It is possible that the binding of oxygen by cytochrome oxidase also proceeds through an intermediate state similar to that proposed for CO.

IV. X-RAY CRYOENZYMOLOGY

At present the most powerful method available for obtaining detailed structural information about proteins or their derivatives is that of X-ray diffraction. Unfortunately it is also essentially a static technique, long time periods (typically several days) being required to collect the data. Thus a time-averaged structure is obtained. Since events during the processes of enzyme catalysis occur on a much shorter time scale (< seconds), it is impossible to determine the detailed structures of enzyme-substrate intermediates at room temperature with the existing methodology. As a consequence

until very recently the practice has been to obtain the structures of complexes of the enzyme with inhibitors or pseudosubstrates, i.e., noncatalytically productive species from which mechanistically useful information could be obtained by inference. Since these stable species involve nonproductive complexes they are obviously much less satisfactory than a productive enzyme-substrate intermediate structure would be.

Several investigations have demonstrated that many crystalline enzymes are catalytically active under normal experimental conditions, probably reflecting in part the large concentration of solvent typically found in protein crystals. Petsko[87] has shown that if crystals of enzymes are transferred to suitable cryosolvents they may yield high resolution diffraction patterns at temperatures as low as −120° C. This work demonstrated that it is feasible to determine protein structures at subzero temperatures in aqueous-organic cryosolvents. It has also been shown that several enzymes are catalytically active in the crystalline state in cryosolvents at subzero temperatures.[21]

There are two general approaches which can be used to form a trapped crystalline intermediate at subzero temperature. One involves trapping the intermediate in the solution state, and then adjusting conditions so as to cause crystallization. This technique is of limited utility due to the relatively slow rate of crystal growth compared to that of intermediate breakdown, however, preliminary experiments with lysozyme indicate its feasibility in this case.[71] The more general procedure is to transfer crystals of enzyme to the cryosolvent, adjust conditions of pH* and temperature appropriately, and add the substrate allowing it to diffuse and form the desired intermediate.

As recently discussed by Fink and Petsko[13] there are a number of requirements which must be satisfied before cryoenzymological crystallographic experiments can be undertaken on trapped enzyme-substrate intermediates. These include demonstration of catalytic activity in both microcrystals (intrinsic catalytic activity of the crystalline enzyme) and diffractometer-size crystals, and in both mother liquor and cryosolvent. Additionally a successful means of transferring crystals from mother liquor to cryosolvent without loss of diffraction properties must be found, and finally the rate of formation and breakdown of the desired intermediate in the crystals must be determined. A detailed discussion of the problems involved in X-ray cryoenzymology has been presented elsewhere.[13]

Of major concern in such studies are the diffusional constraints placed on the substrate by the solvent pores of the crystal. In order to have the necessary high active-site occupancy it is essential that the substrate be able to reach the innermost molecules of the enzyme in the crystal. Furthermore the diffusion rate must exceed the rate of formation of the desired intermediate. A preliminary theoretical, experimental treatment of this diffusion-limitation problem has been given by Fink and Petsko.[13] Key factors include the solvent viscosity, size of substrate, size of the solvent channels in the crystal, nature of the substrate-pore-lining interactions, and crystal size.

So far the most extensive investigations have been carried out using proteases. As mentioned previously, acyl-enzymes, formed from the reaction of specific *p*-nitrophenyl esters with mammalian serine proteases, can readily be trapped at subzero temperatures using dissolved enzyme due to the rate-limiting deacylation.[20,32,61,62] The release of the chromophoric *p*-nitrophenyl moiety provides a very useful means of monitoring both the rates of acyl-enzyme formation and breakdown, as well as the active-site occupancy in cases of stabilized acyl-enzymes.[20,62] In a similar manner it has been shown that acyl-enzymes from α-chymotrypsin, trypsin, and elastase can be trapped in the crystalline state.[20]

The rates of the catalytic reactions were found to be significantly slower in the crystalline enzyme than in the dissolved state due mostly to diffusional limitations (as manifested, e.g., in the slower rates for larger crystals). For example, for elastase which

has been shown to have an intrinsic reactivity in the crystalline state of at least 40% that of the dissolved state, the reactivity with *N*-carbobenzoxy-L-alanine *p*-nitrophenyl ester with crystals of approximately 0.2-mm thickness is slowed down 30-fold.[20] Active-site occupancies in the 50 to 90% range were found for these acyl-enzymes.

A. Results with Elastase

Comparison of the native structure of elastase at room temperature in either aqueous or 70% methanol cryosolvent with that in the cryosolvent at −55° C indicates that there are few, if any, *significant* changes in the peptide backbone and interior of the molecule. There were, however, substantial changes in the electron density of some of the surface residue side chains reflecting both decreased mobility and occasionally changed positions of these groups.[19] The positions of the residues in the active site were unchanged; additionally, confirmation that the "hydration shell" surrounding the protein is not significantly penetrated by methanol was also obtained in these experiments. More recent data at 1.8 Å resolution show a water molecule hydrogen bonded to the active-site serine hydroxyl at subzero temperatures.[193]

The rate of formation of the acyl-elastase derived from *N*-carbobenzoxy-L-alanine *p*-nitrophenyl ester could be followed by monitoring changes in the intensity of selected reflections.[19] Excellent agreement with the rate determined by monitoring the rate of release of *p*-nitrophenol spectrophotometrically was found. Examination of the structure of the acyl-elastase at 3.5 and 2.5 Å resolution revealed the following major features: the carbonyl group of the acyl moiety in covalent bonding distance to the oxygen of Ser-195; no evidence for the presence of the *p*-nitrophenyl group; the alanine side-chain methyl in Van der Waals contact with the side chain of Val-216; no detectable changes in the position of the imidazole of His-57 compared to its location in the native enzyme; and no significant changes in the positions of other parts of the enzyme.[19] The most significant observation in this investigation is the fact that the position of the enzyme groups in the acyl-enzyme are identical, essentially, to those in the free enzyme. Since there is evidence that movement of the active-site imidazole, e.g., occurs during the formation of the acyl-enzyme, the implication is that such conformational changes have "relaxed" in the acyl-enzyme.[13,19]

Preliminary studies on the trapped, noncovalent complex formed between elastase and *N*-carbobenzoxy-L-alanine amide reveal that the active-site imidazole appears to move 1 to 2 Å closer to the side chain of Ser-195 in this intermediate.[13] This observation is consistent with results obtained using dissolved enzyme in which spectroscopic evidence for intermediates involving conformational changes in the ES complex prior to tetrahedral adduct formation has been obtained.[35,36] Therefore, the current picture for the acylation half of elastase catalysis is as follows. In the native protein the positions of Ser-195 and His-57 are such that there is no hydrogen bond between them. On binding of the substrate a conformational change is induced which results in movement of the imidazole toward the serine, resulting in formation of a hydrogen bond. This change may be facilitated by the displacement of the water molecule, which is hydrogen-bonded to the serine in the native enzyme by the substrate. The imidazole is now in a position to act as a base catalyst to facilitate the attack of the serine hydroxyl on the substrate carbonyl to form the tetrahedral adduct. Collapse of this intermediate to the acyl-enzyme occurs with acid catalysis by the imidazolium, and concurrent return to the original positions of the key active-site groups.[13]

The importance of knowing the kinetics of intermediate formation and breakdown in X-ray cryoenzymology, both in the dissolved and crystalline state, has been documented by Fink and Petsko[13] who detail the likely consequences of working "in the dark". Preliminary results of subzero temperature studies of ribonuclease and its catalytic

intermediates in the crystalline state have been reported.[13] These results again demonstrate the potential of the approach, as well as the difficulties.

V. CONCLUSIONS

It is clear that attainment of the major goal of most cryoenzymological investigations, namely the resolution of elementary steps and the stabilization of normally transient intermediates, is of quite general feasibility. Furthermore the method permits the use of the most specific (best) substrates, as well as essentially all the normally used physical-chemical and analytical techniques for studying biochemical reactions. However, the major price for such advantages is the necessity for a fluid solvent system at subzero temperatures. Many proteins have now been shown to be stable and functional in such solvents. With either mixed aqueous-organic cryosolvents or supercooled water systems the bulk solvent structure is considerably different from the normal aqueous milieu of most proteins. Current studies indicate that at least in some cases the native states of proteins are very similar in aqueous and cryosolvent media, due mostly to preferential hydration of the protein. On the other hand it is apparent that many enzyme-substrate systems do undergo some changes, especially in their kinetic properties, on going to subzero temperatures and cryosolvents. However, even in these cases as long as the cryosolvent is a "satisfactory" one, the rate enhancement, compared to the nonenzyme-catalyzed reaction is still of the order expected for enzyme catalysis, and there is little evidence to indicate significant mechanistic differences.

Numerous investigations have now shown that cryoenzymology can be used to achieve "single turnover", actually nonturnover conditions, even in cycling systems such as cytochrome P450. In more complex, multienzyme systems the technique may be used to bring about temperature-controlled uncoupling of coupled reaction systems.

Extant studies have demonstrated that intermediates which cannot be observed under normal conditions even with rapid-reaction techniques may become detectable at subzero temperatures, in some instances due to changes in the rate-limiting step and longer lifetimes and higher concentrations attainable with cryoenzymology.

An important aspect is the good correlation between observed properties, especially kinetic, of intermediates detected at subzero temperatures, and those observed in aqueous solution at ambient temperatures using stopped-flow methods. The presence of such accord lends support to the notion that the catalytic mechanism operating under cryoenzymological conditions is indeed similar to that under normal conditions.

Since the feasibility of trapping enzyme-substrate intermediates in the crystalline state at subzero temperatures and the determination of their structures by X-ray diffraction has been demonstrated, we can soon expect to obtain the high resolution (>1.5 Å) structures necessary to reveal intimate details of changes between one intermediate and the next on the catalytic pathway. The significance of this approach is that these trapped intermediates are productive complexes, not complexes involving inhibitors or pseudosubstrates. For example, the cryoenzymological studies discussed in Section II.D on carboxypeptidase A have given rise to findings at variance with those obtained from investigations of inhibitor complexes. That the latter findings had been extrapolated to cover substrate binding and reaction clearly indicates the potential pitfalls in studying nonproductive complexes, and the necessity, where possible, for the use of specific substrates. That cryoenzymology has now made the detailed study of enzyme-substrate interactions a reality is a testimony to the importance of the technique.

In conclusion the majority of the existing cryoenzymological studies concerning catalytic mechanisms have indicated consistency with known or expected schemes based on conventional studies in aqueous solution. However, an increasing number are both

leading to additional mechanistic insights into well-studied systems and allowing analysis of systems not hitherto available for study through conventional approaches.

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REFERENCES

1. **Fink, A. L.,** The use of subzero temperatures and fluid solutions in the study of enzyme mechanisms, *J. Theor. Biol.,* 61, 419, 1976.
2. **Douzou, P.,** *Cryobiochemistry: An Introduction,* Academic Press, New York, 1977.
3. **Fink, A. L.,** The study of enzyme mechanisms at subzero temperatures, *Acc. Chem. Res.,* 10, 233, 1977.
4. **Douzou, P.,** Enzymology at sub-zero temperatures, *Adv. Enzymol.,* 45, 157, 1977.
5. **Albery, W. J. and Knowles, J. R.,** Evolution of enzyme function and the development of catalytic efficiency, *Biochemistry,* 15, 5631, 1976.
6. **Gutfreund, H.,** Kinetic analysis of the properties and reactions of enzymes, *Prog. Biophys. Mol. Biol.,* 29, 161, 1975.
7. **Geeves, M. A. and Fink, A. L.,** The binding of NADH to horse liver alcohol dehydrogenase at subzero temperatures: a three step reaction, *J. Biol. Chem.,* 255, 3248, 1980.
8. **Tsopanakis, A. and Shall, S.,** The use of subzero temperatures and aqueous/organic solvent system to increase the stability of labile enzymes, *Biochem. Soc. Trans.,* 6, 1282, 1978.
9. **Fink, A. L. and Grey, B. L.,** Studies on the refolding and catalysis of ribonuclease at subzero temperatures, *Biomol. Struct. Funct.,* 471, 1978.
10. **Cox, R. P.,** The properties of cytochrome F and P700 in chloroplasts suspended in fluid media at subzero temperatures, *Eur. J. Biochem.,* 55, 625, 1975.
11. **Austin, R. H., Beeson, K. W., Eisenstein, L., Frauenfelder, H., and Gunsalus, I. C.,** Dynamics of ligand binding to myoglobin, *Biochemistry,* 14, 5535, 1975.
12. **Makinen, M. W. and Fink, A. L.,** Reactivity and cryoenzymology of enzymes in the crystalline state, *Ann. Rev. Biophys. Bioeng.,* 6, 301, 1977.
13. **Fink, A. L. and Petsko, G. A.,** X-ray cryoenzymology, *Adv. Enzymol.,* 52, 177, 1981.
14. **Fink, A. L.,** Enzyme catalyzed reactions in unfrozen, noncellular systems at subzero temperatures, *Adv. Chem.,* 180, 35, 1979.
15. **Douzou, P.,** The use of subzero temperatures in biochemistry, *Methods Biochem. Anal.,* 22, 401, 1974.
16. **Douzou, P.,** Enzymology at subzero temperatures, *Mol. Cell. Biochem.,* 1, 15, 1973.
17. **Cox, R.,** Cryoenzymology: the use of fluid solvent mixtures at subzero temperatures for the study of biochemical reactions, *Biochem. Rev.,* 6, 689, 1978.
18. **Rand-Meir, T., Dahlquist, F. W., and Raftery, M. A.,** Use of synthetic substrates to study binding and catalysis by lysozyme, *Biochemistry,* 8, 4206, 1969.
19. **Alber, T., Petsko, G. A., and Tsernoglou, D.,** Crystal structure of elastase-substrate complex at −55°C, *Nature,* 263, 297, 1976.
20. **Fink, A. L. and Ahmed, A.,** Formation of stable crystalline enzyme-substrate intermediates at subzero temperatures, *Nature,* 263, 294, 1976.
21. **Weber, J. P.,** Ph.D. thesis, University of California, Santa Cruz, 1980.
22. **Makinen, M. W., Yamamura, K., and Kaiser, E. T.,** Mechanism of action of carboxypeptidase A in ester hydrolysis, *Proc. Natl. Acad. Sci., U.S.A.,* 73, 3882, 1976.
23. **Douzou, P. and Balny, C.,** Protein fractionation at subzero temperatures, *Adv. Prot. Chem.,* 32, 77, 1978.
24. **Hastings, J. W., Balny, C., Le Peuch, C., and Douzou, P.,** Spectral properties of an oxygenated luciferase-flavin intermediate isolated by low temperature chromatography, *Proc. Natl. Acad. Sci. U.S.A.,* 70, 3468, 1973.
25. **Fink, A. L.,** The isolation of *N*-acetyl-L-tryptophanyl-alpha-chymotrypsin, *Arch. Biochem. Biophys.,* 155, 473, 1973.
26. **Balny, C., Le Peuch, C., and Debey, P.,** Low temperature column chromatography: application to microsomal hydroxylating system, *Anal. Biochem.,* 63, 321, 1975.

27. **Balny, C., Le Doucen, C., and Douzou, P.,** Affinity chromatography at subzero temperatures. A model study with porcine pancreatic elastase, *J. Chromatog.,* 168, 133, 1979.
28. **Andersson, K., Benjamin, Y., Douzou, P., and Balny, C.,** Organic solvents and temperature effects on desorption from immuno absorbant. DNP-BSA anti-DNP as a model, *J. Immunol. Meth.,* 23, 17, 1978.
29. **Perrella, M., Heyda, A., Mosca, A., and Rossi-Bernadi, L.,** Isoelectric focusing and electrophoresis at subzero temperatures, *Anal. Biochem.,* 88, 212, 1978.
30. **Hastings, J. W. and Balny, C.,** The oxygenated bacterial luciferase-flavin intermediate, reaction products via the light and dark pathways, *J. Biol. Chem.,* 250, 7288, 1975.
31. **Balny, C. and Hastings, J. W.,** Fluorescence and bioluminescence of bacterial luciferase intermediates, *Biochemistry,* 14, 4719, 1975.
32. **Fink, A. L.,** The alpha-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan *p*-nirophenyl esters in dimethyl sulfoxide at subzero temperatures, *Biochemistry,* 12, 1736, 1973.
33. **Fink, A. L., Feldman, R., and Zehnder, J.,** Reinvestigation of the reaction of chymotrypsin with *N*-furylacryloyl-tryptophan derivatives at acidic pH, *Biochem. J.,* 181, 733, 1979.
34. **Fink, A. L. and Good, N.,** Evidence for a glucosyl-enzyme intermediate in the β-glucosidase-catalyzed hydrolysis of *p*-nitrophenyl-β-D-glucoside, *Biochem. Biophys. Res. Commun.,* 58, 126, 1974.
35. **Fink, A. L. and Meehan, P.,** Detection and accumulation of tetrahedral intermediates in elastase catalysis, *Proc. Natl. Acad. Sci. U.S.A.,* 76, 1566, 1979.
36. **Compton, P. and Fink, A. L.,** The detection, accumulation and stabilization of a tetrahedral intermediate in trypsin catalysis, *Biochem. Biophys. Res. Commun.,* 93, 427, 1980.
37. **Chance, B., Saronia, C., and Leigh, J. S.,** Functional intermediates in reaction of cytochrome oxidase with oxygen, *Proc. Natl. Acad. Sci. U.S.A.,* 72, 1635, 1975.
38. **Eisenstein, L., Debey, P., and Douzou, P.,** P450 CAM: oxygenated complexes stabilized at low temperatures, *Biochem., Biophys. Res. Commun.,* 77, 1377, 1977.
39. **Douzou, P., Hui Bon Hou, G., Maurel, P., and Travers, F.,** Physical chemical data for mixed solvents used in low temperature biochemistry, *Handbook of Biochemistry and Molecular Biology,* Fasman, G., Ed., Chemical Rubber Co., Cleveland, 1976.
40. **Douzou, P., Debye, P., and Franks, F.,** Enzymology in supercooled water, *Nature,* 268, 466, 1977.
41. **Douzou, P., Keh, E., and Balny, C.,** Cryoenzymology in aqueous media: micellar solubilized water clusters, *Proc. Natl. Acad. Sci. U.S.A.,* 76, 681, 1979.
42. **Freed, S.,** Chemical-biochemical signal and noise, *Science,* 150, 576, 1965.
43. **Curtis, P. J.,** Physical characteristics of mitochondrial membrane protein dissolved in chloroform-methanol., *Biochim. Biophys. Acta,* 255, 833, 1972.
44. **Maier, V. P. and Tappel, A. L.,** Measurement of enzyme reaction velocities at low temperatures, *Anal. Chem.,* 26, 564, 1954.
45. **Maier, V. P., Tappel, A. L., and Volman, D. H.,** Reversible inactivation of enzymes at low temperatures. Studies of temperature-dependence of phosphatase- and peroxidase-catalyzed reactions, *J. Am. Chem. Soc.,* 77, 1278, 1955.
46. **Luisi, P., Bonner, F. J., and Pallegrini, A.,** Micellar solubilization of proteins in aprotic solvents and their spectroscopic characterization, *Helv. Chim. Acta,* 62, 740, 1979.
47. **Hui Bon Hoa, G. and Douzou, P.,** Ionic strength and protonic activity of supercooled solutions used in experiments with enzyme systems, *J. Biol. Chem,* 248, 4649, 1973.
48. **Travers, F., Douzou, P., Pederson, T., and Gunsalus, I. C.,** Ternary solvents to investigate proteins at subzero temperatures, *Biochimie,* 57, 43, 1975.
49. **Larroque, C., Maurel, P., Balny, C., and Douzou, P.,** Practical potentiometric determinations of proton activity in hydroorganic solvents at subzero temperatures, *Anal. Biochem.,* 73, 9, 1976.
50. **Maurel, P. and Douzou, P.** Solvent-temperature perturbations of ionizable groups as a tool for the investigation of the active site of enzymes, *J. Biol. Chem.,* 250, 2678, 1975.
51. **Knowles, J. R.,** The intrinsic pK values of functional groups in enzymes: improper deductions from the pH-dependence of steady-state parameters, *CRC Crit. Rev. Biochem.,* 4, 165, 1976.
52. **Maurel, P.,** Relevance of dielectric constant and solvent hydrophobicity, *J. Biol. Chem.,* 253, 1677, 1978.
53. **Douzou, P. and Balny, C.,** Cryoenzymology in mixed solvents without cosolvent effects on enzyme specific activity, *Proc. Natl. Acad. Sci. U.S.A.,* 74, 2297, 1977.
54. **Maurel, P. and Douzou, P.,** Catalytic implications of electrostatic potentials: the lytic activity of lysozyme as a model, *J. Mol. Biol.,* 102, 253, 1976.
55. **Douzou, P., Balny, C., and Franks, F.,** New trends in cryoenzymology. I. Super-cooled aqueous solutions, *Biochimie,* 60, 151, 1978.
56. **Ramussen, D. H. and MacKenzie, A. P.,** Clustering in super-cooled water, *J. Chem. Phys.,* 59, 5003, 1972.

57. **Douzou, P., Debey, P., and Franks, F.,** Supercooled water as medium for enzyme reaction at subzero temperatures, *Biochim. Biophys. Acta.*, 523, 1, 1978.
58. **Rasmussen, D. H., Macaulay, M. N., and MacKenzie, A. P.,** Supercooling and nucleation of ice in single cells, *Cryobiology*, 12, 328, 1975.
59. **Balny, C. and Douzou, P.,** New trends in cryoenzymology. II. Aqueous solutions of enzymes in apolar solvents, *Biochimie*, 61, 445, 1979.
60. **Fink, A. L. and Geeves, M. A.,** Cryoenzymology: the study of enzyme catalysis at subzero temperatures, *Meth. Enzymol.*, 63, 336, 1979.
61. **Fink, A. L.,** The trypsin-catalysed hydrolysis of *N*-alpha-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester in dimethyl-sulphoxide at subzero temperatures, *J. Biol. Chem.*, 249, 5027, 1974.
62. **Fink, A. L. and Angelides, K. J.,** Papain-catalyzed reactions a subzero temperatures, *Biochemistry*, 15, 5287, 1976.
63. **Kauzmann, W.,** Some factors in the interpretation of protein denaturation, *Adv. Prot. Chem.*, 14, 1, 1959.
64. **Tanford, C.,** Protein denaturation, *Adv. Prot. Chem.*, 23, 121, 1968.
65. **Tanford, C.,** Protein denaturation. III. Theoretical models for denaturation, *Adv. Prot. Chem.*, 24, 1, 1970.
66. **Pittz, E. P. and Timasheff, S. N.,** Interaction of ribonuclease A with aqueous 2-methyl-2,4-pentanediol at pH 5.8, *Biochemistry*, 17, 615, 1978.
67. **Timasheff, S. N.,** Protein-solvent interactions and protein conformation, *Acc. Chem. Res.*, 3, 62, 1970.
68. **Pittz, E. P. and Bello, J.,** Studies on bovine pancreatic ribonuclease A and model compounds in aqueous 2-methyl-2,4 pentanediol. I. Amino acid solubility, thermal reversibility of ribonuclease A, and preferential hydration of ribonuclease A crystals, *Arch. Biochem.*, Biophys., 146, 513, 1971.
69. **Singer, S. J.,** The properties of proteins in nonaqueous solvents, *Adv. Prot. Chem.*, 17, 1, 1962.
70. **Fink, A. L.,** Effect of dimethyl sulfoxide on the interaction of proflavine with alpha-chymotrypsin, *Biochemistry*, 13, 277, 1974.
71. **Fink, A. L., Homer, R., and Weber, J. P.,** Resolution of the individual steps in the reaction of lysozyme with the trimer and hexamer of *N*-acetyl-D-glucosamine at subzero temperatures, *Biochemistry*, 19, 811, 1980.
72. **Clement, G. C. and Bender, M. L.,** The effect of aprotic dipolar organic solvents on the kinetics of alpha-chymotrypsin-catalyzed hydrolysis, *Biochemistry*, 2, 836, 1963.
73. **Mares-Guia, M. and Figueiredo, A. F. S.,** Trypsin-organic solvent interaction. The simultaneous operation of competitive inhibition and dielectric effect, *Biochemistry*, 11, 2091, 1972.
74. **Pfeil, W. and Privalov, P. L.,** Thermodynamic investigations of proteins. III. Thermodynamic description of lysozyme, *Biophys. Chem.*, 4, 41, 1976.
75. **Douzou, P., Debey, P., and Franks, F.,** Supercooled water as a medium for enzyme reactions at subzero temperatures, *Biochimie*, 60, 151, 1978.
76. **Bock, P. E. and Frieden, C.,** Another look at the cold lability of enzymes, *Trends Biochem. Sci.*, 3, 100, 1978.
77. **Shiga, T., Layani, M., and Douzou, P.,** Low temperature spectrum of D-amino acid oxidase, in *Flavins and Flavoproteins*, Yagi, K., Ed., University of Tokyo Press, Tokyo, 1968, 40.
78. **Fink, A. L. and Angelides, K. J.,** The β-galactosidase catalyzed hydrolysis of *o*-nitrophenol -β-D-galactoside at subzero temperatures: evidence for a galactosyl enzyme intermediate, *Biochem. Biophys. Res. Commun.*, 64, 701, 1975.
79. **Bock, P. E., Gilbert, H. R., and Frieden, C.,** Analysis of the cold-lability behaviour of rabbit-muscle phosphofructokinase, *Biochem. Biophys. Res. Commun.*, 66, 564, 1975.
80. **Hui Bon Hoa, G., Guinand, S., Douzou, P., and Pantaloni, C.,** Alkaline-induced conformational changes of β-lactoglobulin in water-alcohol mixtures at low temperatures, *Biochimie*, 55, 269, 1973.
81. **Guinand, S., Hui Bon Hoa, G., and Pantaloni, C.,** Spectroscopic study of the thermal perturbations of β-lactoglobulin at low temperature in different solvents, *Biochimie*, 56, 863, 1974.
82. **Douzou, P., Sireix, R., and Travers, F.,** Temporal resolution of individual steps in an enzymic reaction at low temperatures, *Proc. Natl. Acad. Sci. U.S.A.*, 66, 787, 1970.
83. **Imoto, T., Johnson, L. N., North, A. C. T., Philips, D. C., and Rupley, J. A.,** Vertebrate lysozymes, *The Enzymes*, 7, 665, 1972.
84. **Wallenfels, K. and Weil, R.,** β-Galactosidase, *The Enzymes*, 7, 617, 1972.
85. **Legler, G.,** Active-site-directed inhibitors and the mechanism of action of glycosidases, *Mol. Cell. Biochem.*, 2, 31, 1973.
86. **Douzou, P., Hui Bon Hoa, G., and Petsko, G. A.,** Protein crystallography at subzero temperatures: lysozyme-substrate complexes in cooled mixed solvents, *J. Mol. Biol.*, 96, 367, 1976.
87. **Petsko, G. A.,** Protein crystallography at subzero temperatures. Cryoprotective mother liquors for protein crystals, *J. Mol. Biol.*, 96, 381, 1976.

88. **Schindler, M., Assaf, Y., Sharon, M., and Chipman, D. M.,** Mechanism of lysozyme catalysis, *Biochemistry,* 16, 423, 1977.
89. **Warshel, A. and Levitt, M.,** Theoretical studies of enzymic reactions: dielectric, electrostatic and steric stabilization of the carbonium ion in the reaction of lysozyme, *J. Mol. Biol.,* 103, 227, 1976.
90. **Hamaguchi, K.,** Structure of muramidase (lysozyme), VII. Effect of alcohols and related compounds on the stability of muramidase, *J. Biochem.,* 56, 432, 1964.
91. **Ikeda, K. and Hamaguchi, K.,** Interaction of alcohols with lysozyme. I. Studies on circular dichroism, *J. Biochem.,* 68, 785, 1970.
92. **Shimaki, N., Ikeda, K., and Hamaguchi, K.,** Interaction of alcohols with lysozyme. II. Studies on difference absorption spectra, *J. Biochem.,* 68, 795, 1970.
93. **Banerjee, S. K., Holler, E., Hess, G. P., and Rupley, J. A.,** Reaction of *N*-acetylglucosamine oligosaccharides with lysozyme, *J. Biol. Chem.,* 250, 4355, 1975.
94. **Goldstein, L., Levin, Y., and Katchalsky, E.,** A water-insoluble polyanionic derivative of trypsin. II. Effect of the polyelectrolyte carrier on the kinetic behavior of the bound trypsin, *Biochemistry,* 3, 1913, 1964.
95. **Halford, S. E.,** Stopped flow fluorescence studies on saccharide binding to lysozyme, *Biochem. J.,* 149, 411, 1975.
96. **Holler, E., Rupley, J. A., and Hess, G. P.,** Productive and unproductive lysozyme-chitosaccharide complexes. Equilibrium measurements, *Biochemistry,* 14, 1088, 1975.
97. **Jolles, P., Saint-Blancard, J., Allary, M., Perin, J-P., and Cozzone, P.,** Lysozymes: binding of *N*-acetylglucosamine and its short polymers to hen lysozyme at physiological temperatures, *FEBS Lett.,* 55, 165, 1975.
98. **Legler, G.,** The mechanism of action of glycosidases, *Acta Microbiol. Acad. Sci. Hung.,* 22, 403, 1975.
99. **Takahashi, K.,** Kinetic studies of the hydrolysis of *p*-nitrophenyl-β-D-glucoside catalysis by β-glucosidase by means of the stopped-flow method, *J. Sci. Hiroshima Univ Ser. A,* 39, 237, 1975.
100. **Makinen, M. W., Kuo, L. C., Dymowski, J. J., and Jaffer, S.,** Catalytic role of the metal ion of carboxypeptidase A in ester hydrolysis, *J. Biol. Chem.,* 254, 356, 1979.
101. **Fink, A. L.,** Cryoenzymology of chymotrypsin: the detection of intermediates in the catalysis of a specific anilide substrate, *Biochemistry,* 15, 1580, 1976.
102. **Kraicsovits, F. and Douzou, P.,** Chymotrypsin-catalyzed hydrolysis at subzero temperatures. Stabilization of ES complexes, *Biochimie,* 55, 107, 1973.
103. **Maurel, P., Hui Bon Hoa, G., and Douzou, P.,** The pH-dependence of the tryptic hydrolysis of benzoyl-L-arginine ethyl ester in cooled mixed solvents, *J. Biol. Chem.,* 250, 1376, 1975.
104. **Bender, M. L., Schonbaum, G. R., and Zerner, B.,** Spectrophotometric investigations of the mechanism of alpha-chymotrypsin-catalyzed hydrolyses. Detection of the acyl-enzyme intermediate, *J. Am. Chem. Soc.,* 84, 2540, 1962.
105. **Miller, C. G. and Bender, M. L.,** The direct observation of an acyl-enzyme intermediate in the alpha-chymotrypsin-catalyzed hydrolysis of a specific substrate at neutral pH, *J. Am. Chem. Soc.,* 90, 6850, 1968.
106. **Kezdy, F. J., Clement, G. E., and Bender, M. L.,** The observation of acyl-enzyme intermediates in the alpha-chymotrypsin-catalyzed reactions of *N*-acetyl-L-tryptophan derivatives at low pH, *J. Am. Chem. Soc.,* 86, 3690, 1964.
107. **Fink, A. L. and Wildi, E.,** Conformational change associated with the acylation of alpha chymotrypsin by *N*-acetyl-L-phenylalanine methyl ester, *J. Biol. Chem.,* 249, 6087, 1974.
108. **Bender, M. L.,** Mechanisms of catalysis of nucleophilic reactions of carboxylic acid derivatives, *Chem. Rev.,* 60, 53, 1960.
109. **Robertus, T. P., Kraut, J., Alden, R. A., and Birktoft, J. J.,** Subtilisin: a stereochemical mechanism involving transition state stabilization, *Biochemistry,* 11, 4293, 1972.
110. **Angelides, K. J. and Fink, A. L.,** Mechanism of thiol protease catalysis: detection and stabilization of a tetrahedral intermediate in papain catalysis, *Biochemistry,* 18, 2363, 1979.
111. **Angelides, K. J. and Fink, A. L.,** Mechanism of action of papain with a specific anilide substrate, *Biochemistry,* 18, 2355, 1979.
112. **Hunkapillar, M. W., Forgac, M. D., and Richards, J. H.,** Mechanism of action of serine proteases: tetrahedral intermediates and concerted proton transfer, *Biochemistry,* 15, 5581, 1976.
113. **Angelides, K. J. and Fink, A. L.,** Cryoenzymology of papain: reaction mechanism with an ester substrate, *Biochemistry,* 17, 2659, 1978.
114. **Maurel, P., Hui Bon Hoa, G., and Douzou, P.,** The pH dependence of the tryptic hydrolysis of benzoyl-L-arginine ethyl ester in cooled mixed solvents, *J. Biol. Chem.,* 250, 1376, 1975.
115. **Quiocho, F. A. and Lipscomb, W. N.,** Carboxypeptidase A: a protein and an enzyme, *Adv. Prot. Chem.,* 25, 1, 1971.
116. **Woolley, P.,** Models for metal ion function in carbonic anhydrase, *Nature,* 258, 677, 1975.

117. **Suh, J. and Kaiser, E. T.,** pH-dependence of the nitrotyrosine-248 and arsanilazotyrosine-248 carbopeptidase A-catalyzed hydrolysis of *0-(trans-p*-chlorocinnamoly)-L-Beta-phenyllactate, *J. Am. Chem. Soc.,* 98, 1940, 1976.
118. **Auld, D. S. and Vallee, B. L.,** Kinetics of carbopeptidase A. pH and temperature dependence of tripeptide hydrolysis, *Biochemistry,* 10, 2892, 1971.
119. **Auld, D. S. and Vallee, B. L.,** Kinetics of carboxypeptidase A. The pH dependence of tripeptide hydrolysis catalyzed by zinc, cobalt, and manganese enzymes, *Biochemistry,* 9, 4352, 1970.
120. **Makinen, M. W., Kuo, L. C., and Koch, T. R.,** Coordination environment of the metal ion of carboxypeptidase A in ester catalysis, personal communication.
121. **Debey, P., Leon, M., Balny, C., and Douzou, P.,** Investigation of microsomal hydroxylating system at subzero temperatures, *Microsomes and Drug Oxidations,* Pergamon Press, New York, 1977, 23.
122. **Erecinska, M. and Chance, B.,** Studies on the electron transport chain at subzero temperatures: electron transport at site III, *Arch. Biochem. Biophys.,* 151, 304, 1972.
123. **Balny, C., Debey, P., and Douzou, P.,** Reversible suspension of metabolism in subcellular organelles in "antifreeze" media at subzero temperatures, *Cryobiology,* 9, 465, 1972.
124. **Chance, B., Graham, N., and Legallis, V.,** Low temperature trapping method for cytochrome oxidase oxygen intermediates *Anal. Biochem.,* 67, 552, 1975.
125. **Pudek, M. R. and Bragg, P. D.,** Trapping of an intermediate in the oxidation, reduction cycle of cytochrome d in *Escherichia coli, FEBS Lett.,* 62, 330, 1976.
126. **Melchior, D. M. and Stein, J. M.,** Thermotropic transitions in biomembranes, *Ann. Rev. Biophys, Bioeng.,* 5, 205, 1976.
127. **Cox, R. P.,** The reduction of artificial electron acceptors at subzero temperatures by chloroplasts suspended in fluid media, *Biochem. Biophys, Acta,* 387, 588, 1975.
128. **Cox, R. P.,** Oxygen evolution at subzero temperatures by chloroplasts suspended in fluid media, *FEBS Lett.,* 57, 117, 1975.
129. **Pulles, M. P. J., Kerkhof, P. L. M., and Amesz, J.,** Photoreduction of plastoquinone to plasto-semiquinone in spinach chloroplasts at −40°C, *FEBS Lett.,* 47, 143, 1974.
130. **Olsen, L. F. and Cox, R. P.,** Light induced proton transport by chloroplasts suspended in fluid media at subzero temperatures: kinetics and stoichiometry, *Eur. J. Biochem.,* 95, 427, 1979.
131. **Shneyour, A., Raisin, J. K., and Smillie, R. M.,** The effect of temperature of the rate of photosynthetic electron transfer in chloroplasts of chilling-sensitive and chilling-resistant plants, *Biochim. Biophys. Acta,* 292, 152, 1973.
132. **Auslander, W. and Junge, W.,** The electric generator in the photosynthesis of green plants. II. Kinetic correlations between protolytic reactions and redox reactions, *Biochem. Biophys. Acta,* 357, 285, 1974.
133. **Fowler, C. F. and Kok, B.,** Determination of H^+/e^- ratios in chloroplasts with flashing light, *Biochim. Biophys. Acta,* 423, 510, 1976.
134. **Saphon, S. and Crofts, A. R.,** Protolytic reactions in photosystem II: a new model for the release of protons accompanying the photo-oxidation of water, *Z. Naturforsch,* 32c, 810, 1977.
135. **Mitchell, P.,** Chemiosmotic coupling in oxidative and photosynthetic phosphorylation, *Biol. Rev.,* 41, 445, 1966.
136. **Haehnel, W.,** Light-induced absorbance changes of plastocyanin *in situ* and its functional role in chloroplasts, *Proc. 3rd Int. Congr. Photosyn.,* 1, 557, 1975.
137. **Gibson, Q. H., Hastings, J. W., Weber, G., Duane, W., and Marsa, J.,** *Flavins and Flavoproteins,* Slater, E. C., Ed., Elsevier, Amsterdam, 1966, 341.
138. **Hastings, J. W. and Gibson, Q. H.,** Intermediates in the bioluminescent oxidation of reduced flavin mononucleotide, *J. Biol. Chem.,* 238, 2537, 1963.
139. **Baldwin, T. O., Nicoli, M. Z., Becvar, J. E., and Hastings, J. W.,** Bacterial luciferase, binding of oxidized flavin mononucleotide, *J. Biol. Chem.,* 250, 2763, 1975.
140. **Massey, V., Palmer, G., and Ballou, D.,** *Oxidases and Other Related Redox Systems,* King, T. E., Mason, H. S., and Morrison, M., Eds., University Park Press, Baltimore, 1973.
141. **Ghisla, S., Massey, V., Lhoste, J. M., and Mayhew, S. C.,** Fluorescence and optical characteristics of reduced flavins and flavoproteins, *Biochemistry,* 13, 589, 1974.
142. **McCapra, F. and Hysert, D. W.,** Bacterial bioluminescence: identification of fatty acid as a product, its quantum yield and a suggested mechanism, *Biochem. Biophys. Res. Commun.,* 52, 298, 1973.
143. **Ghisla, S., Hastings, J. W., Favaundon, V., and Lhoste, J. M.,** Structure of the oxygen adduct intermediate in the bacterial lucerferase reaction: ^{13}C nuclear magnetic resonance determination, *Proc. Natl. Acad. Sci. U.S.A.,* 75, 5860, 1978.
144. **Kemal, C. and Bruice, T. C.,** Simple synthesis of a 4a hydroperoxy adduct of a 1,5-dihydroflavin: preliminary studies of a model for bacterial luciferase, *Proc. Natl. Acad. Sci. U.S.A.,* 73, 995, 1976.

145. **Balny, C., Debey, P., and Douzou, P.,** The subzero temperature chromatographic isolation of transient intermediates of a multi-step cycle: preparation of the substrate free oxy-ferrous cytochrome P450, *FEBS Lett.*, 69, 236, 1976.
146. **Debey, P., Balny, C., and Douzou, P.,** The subzero temperature chromatographic isolation of transient intermediates of a multi-step cycle: purification of the substrate bound oxy-ferrous cytochrome P450, *FEBS Lett.*, 69, 231, 1976.
147. **Hui Bon Hoa, G., Bernard, M., Debey, P., and Gunsalus, I. C.,** Low temperature kinetic studies of camphor binding by *Pseudomonas putida* cytochrome P450, *Biochem. Biophys, Res. Commun.*, in press.
148. **Lange, R., Hui Bon Hoa, G., Debey, P., and Gunsalus, I. C.,** Ionization dependence of camphor binding and spin conversion of the complex between cytochrome P450 and camphor, *Eur. J. Biochem.*, 77, 479, 1977.
149. **Griffin, B. W. W. and Peterson, J. A.,** Camphor binding by *Pseudomonas putida* cytochrome P450. Kinetics and thermodynamics of the reaction, *Biochemistry*, 11, 4740, 1979.
150. **Lange, R., Bonfils, C., and Debey, P.,** The low spin, high spin transition of camphor bound cytochrome P450, *Eur. J. Biochem.*, 79, 623, 1977.
151. **Lange, R., Debey, P., and Douzou, P.,** Mixed solvents as tools to study the structural aspects of cytochrome P450 in solution at subzero temperatures, *Croat. Chem. Acta.*, 49, 277, 1977.
152. **Hui Bon Hou, G., Begard, E., Debey, P., and Gunsalus, I. C.,** Two univalent electron transfers from putidaredoxin to bacterial cytochrome P450 at subzero temperature, *Biochemistry*, 17, 2835, 1978.
153. **Sligar, S. and Gunsalus, I. C.,** A thermodynamic model of regulation: modulation of redox equilibria in camphor mono-oxygenase, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 1078, 1976.
154. **Balny, C., Le Peuch, C., and Debey, P.,** Column chromatography: application to microsomal hydroxylating systems, *FEBS Lett.*, 63, 321, 1975.
155. **Debey, P., Hui Bon Hou, G., and Douzou, P.,** Low temperature studies of microsomal cytochrome P450. I. Stopped flow experiments, *FEBS Lett.*, 32, 227, 1973.
156. **Debey, P., Balny, C., and Douzou, P.,** Enzyme assay in microsomes below zero degrees, *Proc. Natl. Acad. Sci. U.S.A.*, 70, 2633, 1973.
157. **Begard, E., Debey, P., and Douzou, P.,** Subzero temperature studies of microsomal cytochrome P450. Interaction of Fe^{2+} with oxygen, *FEBS Lett.*, 75, 52, 1977.
158. **Estabrooke, R. W., Hildebrandt, A. G., Baron, J., Netter, K. J., and Leibman, K. C.,** New spectral intermediate associated with cytochrome P-450 function in liver microsomes, *Biochem. Biophys. Res. Commun.*, 42, 132, 1971.
159. **Bonfils, C., Debey, P., and Maurel, P.,** Highly purified microsomal P450: the oxyferro intermediate stabilized at low temperature, *Biochem. Biophys. Res. Commun.*, 88, 1301, 1979.
160. **Leon, M., Bonfils, C., and Debey, P.,** Cytochrome b_5/cytochrome b_5 reductase interaction in microsomes: kinetics at subzero temperatures, *Arch. Biochem. Biophys.*, 191, 216, 1978.
161. **Debey, P., Gunsalus, I. C., and Douzou, P.,** Testing cosolvent cryoenzymology on multi-enzyme systems, *Mol. Cell. Biochem.*, 26, 33, 1979.
162. **Chance, B., Saronia, C., and Leigh, J. S.,** Functional intermediates in the reaction of membrane bound cytochrome oxidase with oxygen, *J. Biol. Chem.*, 250, 9226, 1975.
163. **Chance, B. and Leigh, J. S.,** Oxygen intermediates and mixed valence states of cytochrome oxidase: infrared absorption difference spectra of compounds A, B, and C of cytochrome oxidase and oxygen, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 4777, 1977.
164. **Poole, R. K., Waring, A. J., and Chance, B.,** Evidence for a functional oxygen-bound intermediate in the reaction of *Escherichia coli* cytochrome *O* with oxygen, *FEBS Lett.*, 101, 56, 1979.
165. **Chance, B. and Ingledew, W. J.,** States of copper in cytochrome oxidase, *Fed. Proc.*, 36, 727, 1977.
166. **Clore, G. M. and Chance, E. M.,** Low temperature kinetics of the reaction of fully reduced membrane bound cytochrome oxidase with oxygen in the soret, and near infrared regions, *Biochem. J.*, 177, 613, 1979.
167. **Chance, B. and Waring, A.,** The role of peroxidase like intermediates in the enzymatic function of cytochrome oxidase over a variety of temperatures, in *Cytochrome Oxidase*, Chance, B., King, T. E., Okonuki, K., and Orii, Y., Eds., Elsevier/North-Holland, Amsterdam, 1979, 353.
168. **Chance, B., Saronia, C., and Leigh, J. S.,** Compounds C2, a product of the reaction of oxygen and the mixed valence state of cytochrome oxidase, *Biochem. J.*, 177, 931, 1979.
169. **Sharrock, M. and Yonetani, T.,** Study of cytochrome oxidase cobinding site using low temperature flash photolysis, *Biochim. Biophys. Acta*, 434, 333, 1976.
170. **Sharrock, M. and Yonetani, T.,** Low temperature flash photolysis studies of cytochrome oxidase and its environment, *Biochim. Biophys. Acta*, 462, 718, 1977.
171. **Balny, C. and Hastings, J. W.,** Fluorescence and bioluminescence of bacterial luciferase intermediates, *Biochemistry*, 14, 4719, 1975.

172. **Trenthan, D.,** The adenosine triphosphate reaction of myosin and actomyosin and relation to energy transduction in muscle, *Biochem. Soc. Trans.,* 5, 5, 1977.
173. **Geeves, M. A., Koerber, S. C., Dunn, M. F., and Fink, A. L.,** The effect of cryosolvents on the spectral and catalytic properties of liver alcohol dehydrogenase., *Biochemistry,* in press.
174. **Sireix, R., Canva, J., and Douzou, P.,** Thermal control of the luminescence emitted by Xanthine Oxidase, *C. R. Acad. Sci. Ser.,* 270, 557, 1970.
175. **Strother, G. and Ackerman, E.,** Physical factors influencing catalase rate constants, *Biochem. Biophys. Acta.,* 47, 317, 1961.
176. **Banerjee, R., Douzou, P., and Lombard, A.,** Kinetics of association of carbon monoxide with hemoglobin at low temperatures, *Nature (London),* 217, 23, 1968.
177. **Debey, P. and Douzou, P.,** Photodissociation of iron(2+)-carbon monoxide by continuous irradiation, *FEBS Lett.,* 39, 271, 1974.
178. **Bradley, B. A., Cohen, A. H., and Fisher, H. F.,** The effect of methanol on the glutamate dehydrogenase reaction at 0°C, *Biochem. J.,* 25, 555, 1979.
179. **Travers, F. and Hillaire, D.,** Cryoenzymological studies on myosin subfragment 1, *Eur. J. Biochem.,* 98, 293, 1979.
180. **Travers, F., Bertrand, R., Roseau, G., and Van Thoai, N.,** Cryoenzymologic studies on arginine kinase: solvent, temperature and pH effects on the overall reaction, *Eur. J. Biochem.,* 88, 523, 1978.
181. **Barman, T. E., Travers, F., Bertrand, R., and Roseau, G.,** Transient-phase studies on the arginine kinase reaction, *Eur. J. Biochem.,* 89, 243, 1978.
182. **Fink, A. L. and Tsai, A.,** unpublished results.
183. **Fink, A. L., Geeves, M. A., and MacGibbon, A.,** unpublished results.
184. **Fink, A. L., Russel, M. E., and Howard, A.,** unpublished results.
185. **Fink, A. L. and Ahmed, A. I.,** unpublished results.
186. **Matthews, B.,** personal communication.
187. **Cartwright, S. J. and Fink, A. L.,** unpublished results.
188. **Wolfe, R.,** personal communication.
189. **Van Wart, H.,** personal communication.
190. **Fink, A. L. and Angelides, K. J.,** unpublished data.
191. **Biringer, R. G., Painter, B., and Fink, A. L.,** in preparation.
192. **Petsko, G. A.,** personal communication.
193. **Xuong, N. G., Cork, C., and Howard, A.,** personal communication.
194. **Douzou, P. and Hui Bon Hoa, G.,** personal communication.