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Review

Cryo-bioorganic chemistry: molecular interactions at low temperature

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Abstract. Freezing of aqueous or organic solutions plays a pivotal role in enhancement of rate and/or yield of biomolecular reactions. The smooth conditions of the frozen state at low temperature can also suppress racemization and side-product formation of the reactions. Molecular interactions in liquid undercooled solutions, on the other hand, offer the possibility to study enzyme activity mechanisms in vitro and a chance for survival of organisms in vivo. This review illustrates the differences between frozen and liquid conditions on several small and large biomolecules, together with the synthetic use of freezing. In relation to the freezing effect on enzyme activity, a peculiar phenomenon is discussed: 'cryo-oscillations' are temporal motions of trypsin activity in frozen solution in the presence of Mn^{2+} ion. The molecular basis of cold adaptation is also discussed, which points to mechanisms evolved by organisms living at subzero temperatures. The factors involved in the freezing effect are shown; i.e. the role of freeze-concentration and frozen solvent surface is demonstrated and elucidated using several examples.

Key words. Frozen; undercooled; aqueous/organic solution; cryo-reactions; cryo-oscillations; peptide synthesis; antifreeze proteins (AFPs); ice nucleation proteins (INPs).

Introduction

There is a growing demand to study molecular interactions in frozen and supercooled solutions because of their great importance from a biochemical, biophysical and biotechnological point of view. Furthermore, investigations in frozen, i.e. microheterogeneous, systems are also important theoretically, since reactions in such phases proceed in a different manner from the homogeneous ones. Although studies of reactions in frozen and supercooled solutions date back to the 1930s, real breakthroughs occurred only in the 1960s. A survey of reactions in frozen systems can be found in [1–6]. Because the early works were concerned with the cold storage of foods and it was realized that the role of enzymes is a decisive factor in food preservation, these studies concentrated mainly on enzyme action at low temperatures [7–10]. Comparison of frozen and undercooled solutions of enzyme systems revealed that below the freezing point of the reaction mixture there was a significant decrease of rate in the kinetics of frozen solutions compared with the undercooled ones or with the theoretical values obtained by extrapolation of the Arrhenius plots. Nevertheless, the newer systematic studies of different reactions showed in several instances an increasing rate and/or yield effect of freezing in aqueous and organic solutions as well. Storage of pharmaceuticals in frozen and liquid solutions also showed different and sometimes contradictory results. The objective of these types of studies was to enhance the shelf-life of the drug solutions [e.g. 11, 12]. Moreover, the suppression of racemization and side reactions by freezing could also be detected in some instances. Con-

cerning the rate- and/or yield-enhancing effects of freezing, it has generally been attributed to the concentration of solutes in the liquid cavities (vesicles) of the frozen systems. Beside this freeze-concentration effect, however, the role of other subtle factors has also been considered. Despite the published studies, it was not universally appreciated that freezing does not mean the dead end of a reaction in every case, as this misconception is demonstrated, e.g. by the notion that 'the transformation of water into ice brings to an end all chemical reactions' [13].

'Cryo' derived from the Greek word *kryo*, means icy cold or frost, and here it is used in the sense of low temperature. Nevertheless, low temperature is a relative concept that is different for a physicist and a cryochemist. In this review it is limited to the temperature range around freezing and of -10 to -30 °C below freezing of a given aqueous or organic solution, in frozen and liquid (undercooled) phase, respectively.

Reactions in frozen solutions

Bioorganic reactions

In an aqueous solution frozen in a temperature range wherein no eutectics are formed, the freeze-concentration effect means that all nonaqueous constituents are concentrated in a diminished liquid phase of the cavities. This two-phase system will persist until the eutectic temperature is attained. At or below this temperature only a solid-state reaction should occur. When freezing begins, grains of crystalline ice begin to grow, and the solutes are rejected from the ice and concentrated at the freezing interface of the growing ice crystals. As each single crystal of ice grows further, the solutes are more and more concentrated because they cannot escape from the solution surrounded by the walls of ice grains. Therefore, the concentration of the solutes becomes high in the solution and the reaction rate may be enhanced, in some instances as large as three orders [e.g. 14], compared with liquid systems. In other words, freezing is actually the equivalent of partial drying, because it means the removal of water (as ice) from the solution in a smaller or larger extent, and by this manner the freeze concentration can result in supersaturated solutions of the reactants in which the solutes can be concentrated even in 0.1% of the original liquid volume [5]. Freezing as partial drying means also some dehydration of the reactants, i.e. a less effective solvation of the solutes that results in activation (destabilization) of the reactants. When frozen to any given temperature, dilute and concentrated solutions will reach the same concentration with respect to the given solutes, which means that the molal concentration of the unfrozen phase in the vesicles will depend only on the temperature. Thus, at a given temperature an initially dilute solution will form a larger amount of ice than a solution of high initial concentration. Because of this colligative character, even a compound that is not involved in the reaction (e.g. NaCl) and has no effect on the reaction rate in unfrozen conditions can have a significant decreasing rate effect in the frozen state by increasing only the volume of the liquid phase and thereby diluting the concentration of the reactants of the bimolecular reaction. At low temperatures, furthermore, the influence of the decreased temperature on the energy and diffusion rate of the molecules should also be considered, as both of them counteract the rate enhancement and the freeze-concentration effect can be overlayed to a large extent. It is possible to determine separately the effects of low temperature and freeze concentration by (i) extrapolating the Arrhenius plot to subzero temperatures and (ii) undercooling the solution (practically possible to around -10 °C) and (iii) taking small samples of the aqueous solution emulsified in an inert carrier (e.g. in mineral oil), which then allows solutions to be undercooled as low as -40 °C. This method of Franks and coworkers has been demonstrated in ascorbic acid oxidation (see below).

Several kinetic studies of reactions in frozen systems suggest that beside the freeze-concentration effect, the ice crystals themselves can influence the success of reactions. These types of contributions are assigned to the specific properties of the ice [1], i.e. a possible catalytic effect of the ice crystals, the dramatically decreased relative permittivity (dielectric constant) as compared with liquid water (ε 3.3/-10 °C and 88/0 °C [15]) together with an accelerated mobility (0.19 cm s^{-1}/-10 °C) and transfer rate of protons [16, 17]. The role of high proton mobility and transfer rate in reactions of frozen solutions has been demonstrated in more instances, as has been shown, e.g., by an iodate-hydroxylamine oxidation, where the proton production is a slow-rate determining step [18]. The around 500-fold enhanced reaction rate in frozen conditions as compared with the undercooled one (at -5 and -10 °C) points to an important contribution of the ice itself to this pH-regulated oxidation. Fennema [4] reviewed the factors important for the freeze-mediated acceleration. These effects may be combined with freeze-induced pH changes, as has been detected in some buffer solutions [19]. To avoid the unexpected consequences of this pH change, it is worthwhile to test the solution in advance in the presence of a universal pH indicator. Marked changes in the colour of the frozen solution indicate a shift in pH, which may be unfavourable [20].

The results of reactions running in frozen organic solutions suggest that the effects of freeze-concentration and frozen surface exist in this case, too. In contrast to frozen aqueous solutions, however, a dramatic change

of polarity (relative permittivity) on freezing of an organic solution it cannot be expected. For example, the ε data from the liquid and frozen phases of dioxane are the same, both being 2.1 at $+20$ versus 0 °C [21]. Solvents and cosolvents which are most often used for cryo experiments are listed in table 1 according to their increasing polarity.

Ascorbic acid oxidation. The oxidation of ascorbic acid to dehydroascorbic acid has been carried out at pH 5.5 with molecular oxygen [25] or hydrogen peroxide [26, 27] in liquid and frozen solution, respectively. The oxidation followed pseudo-first-order kinetics in liquid water and in ice as well, indicating a significant rate increase of the frozen dilute solution (10^{-4} M ascorbic acid) compared with the liquid undercooled one. The theoretical values of the undercooled solutions could be obtained by Arrhenius plots, whereas the measured data are the results of tracing the reaction kinetics in undercooled droplets emulsified in mineral oil, according to Franks' method [28]. Although the frozen dilute solutions produced quite similar kinetic constants [25, 27], the results of the 23-times-more concentrated ones differed significantly. Namely, the rate of oxidation carried out with hydrogen peroxide excess in frozen solution was independent of the ascorbic acid concentration [27], whereas the reaction with molecular oxygen showed a rate decrease in concentrated conditions [25]. According to the explanation of Hatley and co-workers [27], the deviation between the two methods is rooted in the difference in the oxygen supply. Because the hydrogen peroxide excess means a constant availability of oxygen, and the ascorbic acid concentration of the frozen solution is determined only by the given subzero temperature (see above), it is evident that a similar rate can be obtained in dilute and concentrated frozen solutions as well. However, the molecular oxygen dissolved in the concentrated solution will be partly lost to the atmosphere on freezing. In contrast, the oxygen expelled from the dilute solution is unable to escape from the ice matrix as the amount of ice is much larger than that produced by the concentrated solution. The trapped oxygen provides a reservoir by diffusion into the liquid phase of the ice cavities. This implies that the dissolved oxygen is the limiting factor in this case.

Table 1. Physical constants of cryo-solvents and -cosolvents arranged with increasing polarity.

Solvent	E_T^{N*}	ε†	t_{mp}(°C)‡
p-Xylene	0.074	2.3	13.2
Benzene	0.111	2.3	5.5
1,4-Dioxane	0.164	2.2	11.8
N,N-Dimethylformamide	0.386	38.2	−60.4
Dimethylsulfoxide	0.444	47.2	18.5
Acetonitrile	0.460	36.6	−43.8
Formamide	0.775	111.6	2.5
Glycol	0.790	41.4	−13.0
Glycerol	0.812	46.5	18.2

*Empirical solvent polarity parameter, scale ranges from 0.000 for tetramethylsilane (TMS) to 1.000 for H_2O. Normalized $E_T(30)$ values, the excitation absorption energy of a pyridinium N-phenolate betaine solvatochromic dye [22]. †Relative permittivity for the pure liquid at 20 °C [23]. ‡Melting point [24].

Morpholinolysis. Bruice and Butler [29] observed a considerable acceleration of the morpholinolysis rate of two thiolactones (δ-thiolvalerolactone and γ-thiolbutyrolactone) in ice versus liquid water at -10 °C. What is more, it has been proven by kinetic evidence that the mechanism changes from third to second order. While in liquid solution morpholinolysis was catalyzed by morpholine and its conjugate acid, in ice it appeared as an unassisted nucleophilic displacement reaction; i.e. the general acid/base pathway was replaced by direct nucleophilic attack. It was suggested by the authors that, in the frozen case, the ice structure itself takes part in the proton transfer step via its high proton mobility. This suggestion has been supported by a kinetic isotope effect of deuterium.

Hydrolysis. Grant and coworkers [30] described an especially interesting effect: the imidazole- and base-catalyzed hydrolysis of the β-lactam bond of penicillin in frozen aqueous solution at -5 to -30 °C. For example, after rapid freezing of the samples to -78 °C and warming them to and storing them at -18 °C, caused a 78% destruction of the molecule with imidazole and 38% with pH 9.9 borate buffer. The reaction occurred in frozen conditions but not in and undercooled liquid system or at 38 °C/17 h. The presence of various solutes, e.g. acetone, ethanol, glycerol (5–10% v/v) and Na^+ ion, respectively, abolished the catalyzed hydrolysis in frozen conditions, possibly due to disrupting the structure of the ice to some extent and/or changing the dielectric properties of the ice. Although the authors did not exclude the role of some freeze-concentration effect in the generation of this hydrolysis phenomenon, they attributed the results mainly to a favourable substrate-catalyst orientation on the ice crystal surface. Nevertheless, the influence of high proton mobility has also been considered, as it may facilitate a fast proton transfer in the nucleophilic or general base catalysis in the β-lactam cleavage. A kinetic isotope effect could also be detected here, because the hydrolysis rate in ice was about twice that in frozen D_2O. However, the observed phenomenon is limited not only to the amide bond of β-lactam, but has also been shown by the trypsin molecule. Namely, the trypsin proteinase lost 80% of its tryptic activity on imidazole attack at -18 °C/42 h.

Hydroxylaminolysis. It was possible to inhibit the hydroxylaminolysis of some amino acid esters (e.g. phenylalanine, tyrosine, glutamic acid esters) by structural analogs in frozen aqueous solution at -18 °C [31].

The kinetics of these inhibitions resembled the competitive inhibition of enzymes and has been considered to be further proof of the catalytic-like activity of ice. However, Pincock argued the conclusion of enzyme-like specific inhibition of hydroxylaminolysis and related the results simply to a kinetic manifestation of the colligative freeze-concentration effect of a further solute beside the reactants [2], i.e. a dilution of the reactant concentrations by another molecule.

In further series of hydroxylaminolysis, Grant and Alburn found that the addition of dioxane to the aqueous solution of glutamine produced unexpected results in the reaction kinetics. Namely, the increasing concentrations of dioxane (from 0 to 4 M) resulted at maximum in an approximately sixfold increase of rate at +1 °C (liquid) and a similar decrease at −18 °C (frozen) [32]. A similar inverse type influence of dioxane on the reaction kinetics appeared in the hydroxylaminolysis of 2,5-diketopiperazine at the same temperatures. This surprising phenomenon can be attributed to the polarity (permittivity)-decreasing and ice structure-distorting/breaking character of dioxane. The decreased polarity causes a decreased solvation of the reactants in the dioxane-water mixture as compared with the water alone (ε: 58/4 M dioxane at 0 °C; 88/H_2O at +1 °C [21]), which in turn results in the destabilization of the reactants and by this manner the increase of the reaction rate. The effects acting in the frozen solution, on the other hand, can counteract each other, because the freeze-concentration of the solutes together with the rate-enhancing role of dioxane in the liquid voids of the frozen system improves the reaction rate, while—in addition to the low temperature—the elimination of some catalytic ice surfaces by dioxane acts against the rate enhancement. This means that diminution of the catalytic effect of ice together with the decreased temperature can overcompensate the rate-increasing influences.

Acetylation. The role of dioxane has been investigated in a quite different reaction: the acetylation of triglycine by 4-nitrophenyl acetate in liquid and frozen water and dioxane-water (75%, v/v) solutions [33]. The experiments were conducted around pH 8.3 and at temperatures between +20 and −15 °C. A significant rate increase could be observed in the frozen aqueous solutions as related to the undercooled ones (more than a 60-fold at −5 and −10 °C). Some rate enhancement generated by dioxane has also been found in experiments carried out in liquid water versus liquid dioxane-water solutions (three to seven times at +20 and −5 °C). However, in contrast with the results of hydroxylaminolysis, comparison of the rates in frozen and undercooled dioxane-water solutions indicated a slight rate increase (three times at −5 °C). It should be considered, however, that in this case the results of identical temperatures are compared.

Table 2. Esterification in liquid and frozen dioxane solutions.*

Acid	t_R †	Methylester yield (%)‡	
	(min)	+22 °C	−18 °C
Benzoic	3.5	17	80
Salicylic	2.3	27	31
3,5-Dinitrobenzoic	1.2	60	62

*Coupling of acid and methanol with DCC activation and DMAP catalysis (abbreviations: see in text). †Mean retention times of RP-HPLC, isocratic elution with 60%/40% (v/v) methanol/water. Peaks were monitored at 254 nm. ‡Yield (%): integrated areas of identified peaks.

Reactions of benzoic acid derivatives. Interesting results of cryo reactions have been obtained also with two benzoic acid derivatives (both are metabolites of biochemical routes): (i) aqueous alkaline hydrolysis of 4-hydroxybenzoic acid esters, and (ii) esterification of benzoic acid and its substituted derivatives in dioxane on the other, were studied in liquid and frozen solutions. (i) Shija et al. [34] found a marked acceleration of the alkaline hydrolysis of 4-hydroxybenzoic acid methyl, ethyl and n-propyl esters, in frozen versus liquid conditions. The largest rate-enhancement effect occurred at −9 °C, where the methyl 4-hydoxybenzoate showed more than a 20-fold enlarged rate constant in 0.01 M NaOH solution. The rate constants followed pseudo-first-order kinetics equally in liquid and frozen conditions. The rate of hydrolysis in frozen solution was independent of the hydroxide ion concentration at given subzero temperature, according to the colligative character of solutes. Although the calculated and observed rate data followed similar trends, the observed values were frequently larger, with a 50% rate over the predicted ones. The calculations were based on a freeze-concentration model together with a rate reduction according to the Arrhenius equation. The difference between the observed versus calculated values indicates again that reactions in frozen solutions cannot be interpreted with the freeze-concentration conception alone. (ii) On the other side, the aim of esterification of benzoic acid and its substituted derivatives was to elucidate the role of frozen organic solvents of low polarity in chemical reactions. These esterifications have been carried out in our laboratory in dioxane solution with methanol reagent [35]. In order to acquire information on some subtle effects, methanol was chosen as being a weak nucleophile. Esterification of acids with methanol has been carried out by N,N′-dicyclohexylcarbodiimide (DCC)-activated and 4-dimethylaminopyridine (DMAP)-catalyzed coupling at +22 and −18 °C (frozen) temperature, respectively. The results of esterifications have been evaluated by reverse phase high-performance liquid chromatography (RP-HPLC) technique (table 2).

The various reaction times of the different acids were chosen to achieve maximal ester yields in frozen conditions. The data shown in table 2 show well that the yields of these bimolecular reactions in liquid solution depend on the polarization of the benzene ring by its substituents; i.e. the highest ester yield of the 3,5-dinitrobenzoate is based on its electron-withdrawing nitro substituents. Nevertheless, the esterifications conducted in frozen conditions reveal the catalytic role of the frozen dioxane surface of low polarity (table 1). This contribution is indicated by the benzoic acid as producing the highest yield in shortest time. Namely, this acid of the series can be bound most strongly to the frozen surface due to its most apolar unsubstituted benzene ring (ε 2.3). Consequently, the polar substituents diminish the binding ability of the molecules, and by this manner the catalytic role of the frozen surface also decreases. This tendency is reflected as well by the decreasing retention times of the molecules in the RP-HPLC analysis, according to their decreasing binding strength on the apolar C_{18} stationary phase. It appears, however, that on freezing only the benzoic acid esterification exhibited a significant change in yield. The similar yields of the other two acids in liquid versus frozen phases point to a balance between the counteracting positive and negative effects on reactivity. The freeze concentration together with a slight catalytic effect of the frozen surface represents positive influence, whereas the decreased energy at low temperature means the negative effect.

Poly-amino acid synthesis. The simplest oligo- and polypeptides are the monotonous poly-amino acids. This means that single amino acids are activated in some way and then coupled with peptide bonds to shorter or longer polymers. The first cryo example of this type was an oligo-amino acid built by opening the β-lactam ring of 6-aminopenicillanic acid (6-APA). The same authors who reported the hydrolysis of the penicillin β-lactam ring in frozen conditions described the intriguing imidazole-catalyzed polymerization of potassium 6-APA in frozen aqueous solution at -18 °C [36].

The analysis of the product showed only an oligomer of eight units. Although this polimer compound cannot be considered to be natural peptide model, the use of the β-lactam ring for polymerization is an interesting addition to the several activation methods involved in peptide bond formation. Another early contribution to this topic was the polymerization of N-carboxy-amino acid anhydrides (NCAs) in frozen dioxane [37]. Polymerization of NCAs of amino acids, e.g. D-phenylglycine, 1-amino-cyclobutanecarboxylic acid, 1-amino-cyclopentanecarboxylic acid and so on, has been investigated in liquid and frozen dioxane, where the polymerization proceeded at a low rate in liquid and an at least 10 times higher one in frozen solution between $+5$ to -26 °C. The number-average degree of polymerization varied between 13 and 23. According to the authors, the results suggest an alignment of the reactants in the frozen matrix, where the alignment (ligation) may occur in channels of molecular dimension of the frozen solvent. This phenomenon could resemble clathrate formation, where the solvent takes part as a template enclosing solute molecules.

Further striking results of freezing have been found by Liu and Orgel [38, 39] in work investigating the oligomerization of negatively charged α- and β-amino acids in aqueous solution. The oligomerization of α- and β-glutamic acids, aspartic acid, and α-amino and β-amino adipic acids was carried out with activation by water-soluble reagents at 25 and -20 °C (frozen) temperatures and then traced by HPLC analysis. The water-soluble reagents were carbonyl-diimidazole (CDI) and 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDAC). The dependence of oligomerization on the condensing agents and temperatures appears to be as follows: (i) α-amino acids are oligomerized efficiently by N-carboxyanhydride-forming reagents (CDI); (ii) β-amino acids are oligomerized by reagents which activate the carboxyl group directly (EDAC); (iii) oligomerization of β-amino acids proceeds very efficiently at -20 °C (under 'eutectic' freezing) even from very dilute solutions of the substrates. It was shown by HPLC-elution profiles that oligomerization of β-glutamic acid at -20 °C produced peptides in the range of 15–20 units (maximum: 45) with a yield of over 50%. The efficiency of polymerization and the length distribution of the oligomers was almost unaffected by the solute concentration over the broad range of 0.1–100 mM at -20 °C. This is in good agreement with the rule (see above) that only the temperature determines the solute concentration in the liquid vesicles of the partially frozen solution. This colligative character of the solute molecules is proven by the observation that at -20 °C the presence of NaCl, an indifferent solute, suppressed the polymerization reaction. The relevance of these oligomerization reactions to prebiotic chemistry has been discussed by the authors. According to these speculations, the EDAC reagent may model prebiotically plausible compounds such as cyanamide and cyanoguanidine, whereas the CDI-generated N-carboxyanhydrides can be related to reactions that involve activation of amino acids in the presence of carbon dioxide (bicarbonate ion). It is worth to note that Liu and Orgel also succeeded in polymerization of β-amino acids on the surface of minerals such as hydroxylapatite 'polymerization on the rocks' instead of ice [40]. This discovery of prebiotic chemistry together with cryo-polymerization in ice are significant steps toward eluci-

dating of the mechanism of polypeptide formation on the primitive earth.

Peptide synthesis. Although chemical syntheses of peptides in solid phase, in solution and recombinant technique are thoroughly elaborated methods, the advantage of reactions running in the smooth conditions of frozen solution is still an attractive challenge. Enhanced reaction rate and/or yield, diminution of racemization, as well as suppression of side reactions can be expected in frozen systems, and these possibilities substantially increase the importance of peptide coupling in frozen solutions. Chemical couplings in frozen dioxane and in other organic solvents have been initiated by us [41, 42], since (i) four protected dipeptides and (ii) a protected tripeptide have been synthetized in our laboratory. Terc-butyloxycarbonyl- (Boc) and terc-butyl- (Bu^t) groups have been used for N- and C-terminal protection of the -Gly-Ala-, -Phe-Ala-, -Gly-Phe- and -Phe-Phe- dipeptides and -Ala-Phe-Ala- tripeptide. (i) The dipeptide coupling reactions have been carried out with 4-nitrophenyl esters in dioxane, and their pseudo-first-order kinetics have been traced spectrophotometrically by following the 4-nitrophenolate product absorbance [41]. The data obtained demonstrate that the coupling rates in frozen dioxane at -18 °C exceed with about one order the rates in liquid solution at $+40$ °C. The values indicate also that the decreasing sequence of rates from -Gly-Ala- to -Phe-Phe- is similar in the frozen versus liquid phase. This points to some steric effect of the bulky phenyl group of the -Phe- residue at the active ester and amino nucleophile reactant sites, which may hinder the approach of the nucleophile to the reacting ester group. (ii) The DCC-mediated coupling of the tripeptide, on the other hand, has been carried out at -18 (frozen) and $+22$ °C (liquid) conditions, in solvents of different polarity (table 1), i.e. in dioxane, dimethylsulfoxide and formamide, as well as in solvent mixtures (90%/10%, v/v) of dioxane combined with acetonitrile, dimethylforamide, dimethylsulfoxide and formamide. The reactants were the following: Boc-Ala-Phe-OH and H-Ala-OBu^t, and the coupling reactions have been traced and evaluated by RP-HPLC analysis. The integrated areas under the RP-HPLC peaks compared with those of the authentic samples served for quantitative evaluation of the peptide yield, epimerization and N-dipeptidylurea side-product formation [42].

The peaks of the tripeptide epimers of LLL versus LDL configurations could be resolved without difficulty by using a C_{18} column and acetonitrile-trifluoroacetic acid/H_2O (0.1% v/v) solvent mixture in a linear gradient elution. The reactions conducted in dioxane and dioxane/acetonitrile mixture resulted in the highest tripeptide yields. The results in neat solvents indicate decreasing reactivity with increasing polarity of the solvent, as there was practically no coupling in the most polar formamide. This means an increasing inhibiting effect of the solvation shell around the reactant molecules with the polarity enhancement of the solvents. To understand the polarity effect of the solvent mixtures, on the other hand, is difficult because of more specific interactions. It can be concluded by comparison of the data obtained from experiments in frozen and liquid solutions that freezing of the reaction mixture resulted in every case in significant suppression of the N-dipeptidylurea side-product formation together with a slight diminution of tripeptide epimerization. The coupling yields were similar in the frozen versus liquid phases in all cases. Because the yields of the experiments of frozen versus liquid conditions relate to data obtained at $-18/+40$ °C for the dipeptide and $-18/+22$ °C for the tripeptide, a significant yield-increasing effect of freezing can be expected to correlate with the values the same temperature of -18 °C, i.e., the frozen solution results compared with the presumably decreased yields of liquid solutions at -18 °C.

Enzymic reactions

Cold and freezing effects on proteins. The contributions to in vitro protein stability are hydrophobic, hydrogen-bonding, salt bridge-forming and van der Waals effects, where the pivotal role is played by the hydrophobic interactions. This is evident from the general findings that $\sim 50\%$ of the residues of globular proteins are apolar and that these residues are much more highly conserved than are polar ones [43].

In discussion of cold and freezing effects on proteins (enzymes), a distinction must be drawn between (i) low temperature per se and (ii) freezing. (i) Cold-induced partial changes of protein structure and activity (denaturation in some extent) are fully reversible, and they are exerted by low temperature through changes in the physical properties of the aqueous solvent, e.g. diffusion rate, acid/base ionization and hydrogen bond energies [5]. Furthermore, the hydrophobic interactions weaken on cooling since they have a positive temperature coefficient. (ii) Freezing, on the other hand, can lead to reversible and irreversible denaturation of proteins, due to the freeze concentration of solutes together with the dramatic removal of water as ice. According to the investigations of Strambini and Gabellieri [44], in many instances the freezing of globular proteins results merely in reversible partial denaturation. They investigated the ice-induced partial unfolding of proteins by detecting the phosphorence lifetime of tryptophan residues buried in the compact rigid core of globular proteins (one Trp/subunit). The selected proteins were monomers: azurin and ribonuclease T_1; dimers: alcohol dehydroge-

nase and alkaline phosphatase; tetramers: glyceraldehyde-phosphate dehydrogenase and lactic dehydrogenase. The authors found a loosening of the native fold, i.e. some loss of secondary and tertiary structures. However, the phenomenon appears to be fully reversible on thawing for the monomeric and dimeric enzymes and partly restores the original structure and catalytic activity for the tetrameric ones. The results are attributed to the adsorption of the protein molecules onto the surface of ice (see below). This new interpretation also involves the stabilizing action of cosolutes as decreasing the adsorption affinity of the proteins to the ice surface partly coated by the cosolutes. (Some other effects are also considered.)

Change in enzyme activity with temperature. In this section enzyme reactions of different specificity are given in which the freezing of the aqueous solutions has some effect on the enzyme catalysis. In 1939 Lineweaver [9] studied proteolysis rates in frozen and supercooled liquid conditions of chymotrypsin with casein substrate at -5 °C and of cathepsin with hemoglobin substrate at -2.8 °C. Freezing of the solutions caused a 20-fold and 3-fold decrease of rates, respectively. Similar effects have been obtained by Sizer and Josephson [10], who investigated the kinetics of hydrolyses catalyzed by lipase, trypsin and invertase enzymes over the temperature range from 50 to -24.5, -15 and -18 °C. The substrates were the followings: tributyrin, casein and sucrose. The log rate of hydrolysis versus 1/T plots followed the Arrhenius equation for all three enzymes with a sharp break, however, between 0 and -2 °C, which are the freezing points. The plots revealed a lower activation energy above this critical temperature and a four to five times higher activation energy of the frozen solutions below this point. Nevertheless, it has been proven by the authors that there is no irreversible inactivation of the enzymes, even at very low temperatures. Namely, the storage of these enzymes for 27 days at -70 °C did not diminish their hydrolytic activity as subsequently measured at 30 °C.

Table 3. Mean amplitudes of trypsin activity oscillations at different temperatures.*

T (°C)	Interval (h)	*n*	s/$\bar{x}$† (%)
Experiment	00.0-00.0	00 00	00.0 00.0
Experiment			
-10	23.6–93.4	70 74	49.1 55.9
0	3.0–29.0	51	13.1
	2.5–29.3	43 43	12.3 13.3
25	0.8–4.2	65	10.7
Control			
-10			
Ca^{2+}	0.5–97.0‡	162	6.7
trypsin	— #	38	6.2

*Conditions as given in figure 1. †Coefficient of variation: represents the relative mean amplitude. The numbers in two columns refer to parallel experiments. ‡Treatment in the presence of Ca^{2+} ion instead of Mn^{2+}. # Started with active trypsin and then all samples quenched after a 1-h treatment at -10 °C in frozen solution.

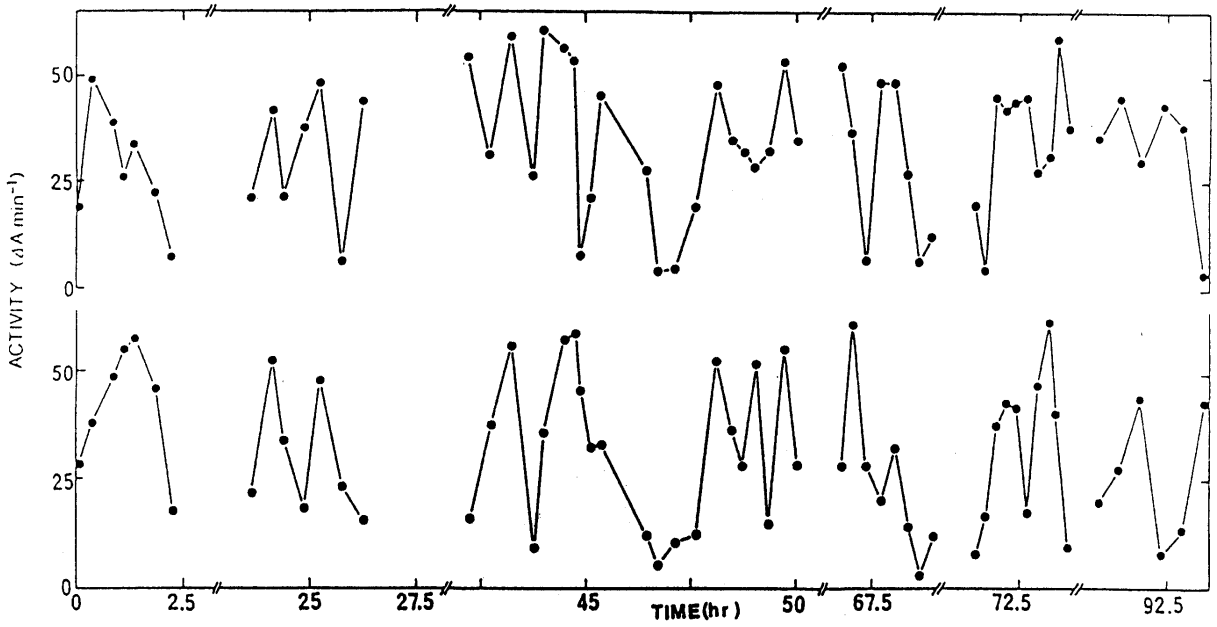

Figure 1. Trypsin activity oscillations in frozen aqueous solution at -10 °C and pH 8.4 in the presence of Mn^{2+} ion. Treated in separate frozen samples of trypsinogen activated with trypsin and sampled in duplicate. Activities were monitored spectrophotometrically on N^2-benzoyl-L-arginine-methyl ester substrate.

Cryo-oscillations: trypsin activity. Far-from-equilibrium systems of nonlinear reactions can exhibit structuring in time and space, resulting in temporal and/or spatiotemporal oscillations (see e.g. [45–48]). A nonlinear reaction refers to two simultaneous counteracting mechanisms, e.g. a feedback catalytic reaction step together with an inhibiting one of different rates in forward and backward directions. Among of several examples of oscillations, some oscillating enzyme reactions have been described [49–52]. Temporal enzymic oscillations have been observed in homogeneous and pseudo-homogeneous conditions. Nevertheless, this type of periodicity can also be generated by compartmentalizing a system; e.g. a papain enzyme membrane has been described as being capable of exhibiting pH oscillations in the course of its substrate hydrolysis [53], whereas oscillations in the activity of phosphofructokinase were observed under conditions where the enzyme was separated from the bulk solution by an inert membrane [54]. These sustained oscillations are consequences of coupling between a feedback enzymatic reaction and the diffusion of substrate, metabolite and reagent, respectively. Instead of using artificial membranes, however, a frozen aqueous solution can be considered as a compartmentalized system potentially capable of generating oscillations. This possibility has been demonstrated for the first time by us, as discovering the phenomenon of temporal motions of trypsin activity in frozen aqueous solution at -10 °C and pH_0 8.4 in the presence of Mn^{2+} ion [55, 56]. Manganese ion has been successfully used as a calcium ion substitute in the autocatalytic activation of trypsinogen into trypsin as well as in stabilization of the active trypsin enzyme structure [57–59]. The important role of Mn^{2+} ion in oscillations of trypsin activity has been shown by the observation that this phenomenon does not appear with Ca^{2+} ion (table 3). In addition to the oscillatory behaviour of trypsin, temporal oscillations in aqueous solution structured by freezing have been detected only in one other case, namely in the electron spin resonance (ESR) tracing of Mn^{3+}/Mn^{2+} changes of the classical Belousov-Zhabotinskii reaction [60].

Random aperiodic trypsin activity motions (oscillations) of parallel experiments are shown in figure 1. Two types of control experiments have been done to reveal the signal/noise ratio, namely (i) the experiment was started with active trypsin instead of trypsinogen activation and following 1 h treatment at -10 °C (frozen) all samples were quenched simultaneously, and (ii) the treatment was carried out in the presence of Ca^{2+} instead of Mn^{2+} ions also at -10 °C. The first parts of the graphs of figure 1 correlate to the autocatalytic activation of trypsinogen, and therefore these parts were not considered in the evaluations. For quantitative comparison of the different experiments the coefficient of variation ($s/\bar{x}$; s: standard deviation and $\bar{x}$: mean value of activity data) has been used as a relative mean amplitude. It can be seen from table 3 that the oscillations versus control runs are definitely significant ($F < 1\%$). The deviations between the data of parallel sampling are also in the range of experimental error. Table 3 shows that the experiments carried out without stirring at 0 and 25 °C, respectively, produced oscillations of smaller but also significant relative mean amplitudes [56].

The conditions of trypsin activity motions are sufficient for the decisive two criteria of a system to exhibit oscillations, namely the far-from-equilibrium condition combined with a kinetic nonlinearity. The frozen solution of trypsin (activated trypsinogen) and Mn^{2+} ions can be considered to be heterogeneous, microscopically dissipative structure (not isolated from its microenvironment), where the chemical events run in the liquid part of the microcompartments (cavities, vesicles). In the given case, the kinetic nonlinearity can arise from the combination of a feedback type chemical reaction with a mass transport process. In other words, the obtained aperiodic oscillations are the result of delicate coupling between the chemical and diffusional controls in the frozen heterogeneous system. The chemical control means that there is conformational control of the substrate binding site of trypsin due to the redox-regulation of the -S-S- bridge of the binding site by an Mn^{3+}/Mn^{2+} couple. The diffusional factor takes part, on the other hand, in the accumulation of enzyme-bonded versus free Mn^{3+}/Mn^{2+} ions around the protein, i.e. in the aperiodical formation of a reductive and oxidative milieu. Among the six disulfide bridges of the trypsin proteinase, the Cys191–Cys220 has the largest effect on trypsin activity because both residues are parts of the substrate binding site (specificity pocket). Furthermore, the Cys191 amino acid residue is located in the neighbourhood of the Asp189 carboxylate, responsible for the primary substrate specificity of the enzyme [61]. The reductive opening of the -S-S- bridge results in a conformational change of the specificity pocket, which means a decrease in the productive substrate binding ability. According to our hypothesis, the reductive effect of the Mn^{2+} ion bounded at the very neighbourhood of the -S-S- bridge may be mediated by a Mn^{2+}...N...S-S electron donor-acceptor relay system being in a sterically suitable position. Although the stabilization of an Mn^{3+} ion and cysteine residue cannot be expected in an aqueous solution, nevertheless; Mn^{3+} ion bounded to a protein may exist because of the nature and geometry of ligands around the metal ion can dramatically lower its redox potential (e.g.: Mn^{3+}/Mn^{2+} versus Mn^{3+}/Mn^{2+}-hemato-porphyrin and Cys-/CysH; $E^{o'}|V| + 1.09$ versus -0.342 and -0.340, respectively [62]. Moreover, some superoxide dismutase enzymes form stable complexes of high-spin

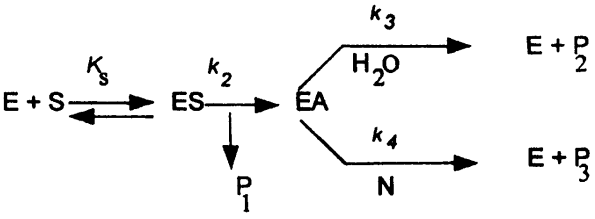

Figure 2. Scheme of proteinase-catalyzed peptide synthesis. E, enzyme; S, ester substrate; ES, Michaelis complex; EA, acyl-enzyme; N, amino-nucleophile; P_1, alcohol; P_2, hydrolysis; and P_3, peptide products.

trivalent manganese ions [63]. Leaving the trypsin molecule, the generated Mn^{3+} ion becomes an oxidizing agent for cysteines, raising the possibility of reconstitution the original bridge. The Mn^{2+}-induced appearance of trypsin activity oscillations as well as their disappearance for Ca^{2+} ions is an indirect indication of the reality of the suggested mechanism.

The reproducibility of activity motions is well demonstrated by the similar shapes and sizes of curves of experiments sampled in duplicate (fig. 1). Nevertheless, the activity motions do not exactly reproduce either themselves or each other. All are similar, but never identical; i.e. they show similar shapes in the given curve and between the different curves, independent of the time scale of sampling. This independence of oscillations points to a fractal-like structure that is similar at any scale of magnification, i.e. a self-similar type [64]. The aperiodic, irregularly shaped motions suggest a high sensitivity of the phenomenon on the local conditions; the actual solute concentrations, pH values and diffusion rate in the given microenvironment. This sensitivity is also indicated by the slight deviations between the parallel sampled curves. We conclude therefore that the appearance of the phenomenon depends to a large extent on the heterogeneity of the system.

It should be noted, however, that the peculiar phenomenon of trypsin activity motions was strongly criticized and questioned in a comment [65], which has been answered and discussed in our reply [66].

Proteinase-catalyzed synthesis of peptides. Continuing their previous work of amino acid ester hydroxylaminolysis in ice, Grant and Alburn [67] studied the same transfer reaction, with trypsin catalysis in frozen (-23 °C) and liquid ($+1$ °C) aqueous solutions. The reactions have been carried out at pH 7.5 with a large excess of the hydroxylamine nucleophile. The authors found that freezing also accelerated the rate in this serine enzyme-catalyzed hydroxylaminolysis. Freezing changes the relative rates of hydrolysis versus hydroxylaminolysis of the acyl-trypsin intermediate in favour of aminolysis. As can be seen from the scheme (fig. 2), the water and hydroxylamine (N), i.e. weak and strong nucleophiles, compete for the acyl-enzyme (EA). The freeze-concentration of the nucleophile together with the dramatic reduction of the liquid water by freezing can increase the hydroxylaminolysis rate together with the suppression of the competitive hydrolysis of the acyl-enzyme. Based on this discovery, Schuster and co-workers [68] were the first to apply this phenomenon to kinetically controlled peptide synthesis in ice catalyzed by serine and cysteine proteases. Thus the acyl donor substrates were amino acid derivatives specific for the given enzyme, and the amino component nucleophiles were amino acids, their derivatives, and dipeptides, as well. Although it has been shown that reasonable yields can be obtained also at -60 °C temperature, the optimal temperatures have been found at -10 to -25 °C, where the nucleophiles were applied in excess. Jakubke and co-workers published several successful enzyme-catalyzed peptide syntheses in frozen aqueous systems, where a yield-increasing effect of freezing could be detected in most cases (for reviews see [69–71]). The scope of the technique is limited by the enzyme specificity; nevertheless, to overcome this problem the authors used proteinases of different substrate specificity (e.g. α-chymotrypsin papain). Furthermore, it has been shown by the synthesis of N-maleyl-Phe-Leu-NH_2 that solubility problems can also be eliminated with the addition of some water-miscible cosolvent (table 1) up to 10%, as in this example a mixture of water/dimethylsulfoxide ($\leq 10\%$, v/v) has been used. The authors have also proven that the freeze concentration is not the only source of peptide yield enhancement. Namely, the amount of unfrozen water of the microinclusions in the frozen reaction samples has been determined by the nuclear magnetic resonance (^{1}H-NMR) relaxation time technique at subzero temperatures [72].

According to these findings of a diminished amount of unfrozen water in the cavities, enzyme-catalyzed syntheses have been carried out at room temperature in solutions of high-solute concentrations. That these highly concentrated reactant solutions did not produce at room temperature the yields of the dilute frozen ones, i.e. they could not fully simulate the conditions of frozen systems (-15 °C), suggests that the freeze concentration is not the only source of the yield-increasing effect [69, 72]. In contrast to this conclusion, however, Tougou [73] studied the same effect in a similar manner in frozen and liquid solutions at -18 and $+20$ °C, and they concluded that the concentrated solutions match the conditions of the unfrozen liquid phase in ice. This means that the authors do not consider any other effects beside the freeze concentration. It seems, therefore, that to reveal factors other than freeze concentra-

tion required further investigations. Whatever the real mechanism, however, the following advantages of proteinase-catalyzed peptide synthesis in frozen solution can be concluded: (i) freezing the reaction mixture significantly increases the peptide yield together with the suppression of some competitive reactions; (ii) there is no need for side-chain protecting/deprotecting strategies; (iii) coupling can be carried out also with N^{α}-un protected acyl donors and free amino acid nucleophiles, but the results depend strongly on the reaction conditions; (iv) there is no danger of racemization during the coupling reaction; (v) using of D-amino acid derivative nucleophile is also possible as catalyzed with α-chymotrypsin and clostripain, and (vi) cosolvent ($\leq 10\%$,v/v) can be given to the aqueous solution to increase the solubility of the given solute.

The freeze-effect on syntheses of different types catalyzed by glycosidases and ribonuclease, respectively, has also been studied. Hänsler and Jakubke [70] obtained a lower yield of the β-galactosidase-catalyzed synthesis of O-β-D-galactopyranosyl-N-Z-serine methyl ester (Z-: benzyloxycarbonyl-) in the frozen state compared with the liquid one, as being 18% at -5 and 44% at 25 °C. Similar poor results were found in the α-glucosidase-catalyzed synthesis of the α-D-glucopyranosyl-derivative of N-Z-serine methyl ester. A further interesting observation is described in the same article concerning the ribonuclease A-catalyzed synthesis of cytidyl-(3′-5′)-uridine. According to the freeze-concentration model, it has been shown that at low reactant concentrations the freezing improved the yield, whereas starting with higher substrate concentrations, the result was just the opposite. In the given case, the yields are at low reactant concentrations, 32% at -10 °C and 20% at 0 °C, whereas at higher concentrations the yields are 19% at -10 °C and 28% at 0 °C.

After the pioneering work of Jakubke's group, similar peptide synthesis experiments also began in other laboratories [73, 74].

Enzymic peptide synthesis in liquid. Besides the freeze mediated reduction of the amount of liquid water, addition of organic cosolvents to the aqueous reaction mixture also fulfils the reduction of the relative water concentration. This in turn means that the competitive hydrolysis of the acyl-enzyme can be suppressed by this technique. Nilsson and co-workers [75] followed this route by combining the low water content (2.5–5%, v/v) with subzero temperature. This method produced almost no hydrolytic side reaction in the α-chymotrypsin-catalyzed synthesis of the Ac-Tyr-Ala-NH_2 dipeptide, as carried out by the reaction in acetonitrile/water (95%/5%, v/v) solution at -22 or -35 °C. It should be noted that instead of the large excess of nucleophile, as used in the frozen syntheses, only equimolar amounts of the reactants have been applied; i.e. 0.1 M Ac-Tyr-OEt and 0.1 M H-Ala-NH_2. Considering the hydrolytic side reaction of this coupling at 70% ester consumption, the ratio of aminolysis/hydrolysis (%) increased from 8.7% at $+22$ °C to about 97% at -35 °C. A dipeptide yield of about 82% could be obtained in N,N-dimethylformamide/water (50%/50%, v/v) solution at -22 °C. The authors suggest a different temperature dependency of the aminolysis versus hydrolysis reactions; i.e. lower temperatures decrease the water activity to a larger extent than they influence the reactant activities, dissolved in the organic phase. According to a similar conception, Iborra and co-workers [76] used salts at subzero temperatures to control the water activity of the solutions. By this method the authors realized the α-chymotrypsin-catalyzed synthesis of kyotorphin (Tyr-Arg). Summing up, it can be concluded that these methods are a powerful tool in enzyme-catalyzed peptide synthesis, even in liquid conditions at subzero temperatures.

The examples concerning enzyme-catalyzed hydrolases and syntheses show that a positive effect of freezing on rate and/or yield is not a predictable general phenomenon but depends very much on the nature of the enzyme, reactants and conditions (concentrations, buffer, pH etc.). Furthermore, considering that freezing also means a partial dehydration of the solute molecules due to removal of water as ice, it can cause some structural and flexibility changes, i.e. activity modification of the enzyme molecules in the liquid microinclusions of the partially frozen solution. (see above). Nevertheless, Batyuk and co-workers [77] derived equations as the basis for quantitative description of the influence of physicochemical property alterations during freezing on the kinetics of enzymic reactions. A peroxidase-catalyzed oxidation was given as an example under conditions of directed crystallization. The authors suggest that these alterations may cause some cryodestruction of biological systems.

Reactions in liquid solutions at low temperature

Douzou and co-workers [78, 79] elaborated a sophisticated technique: the kinetics of cryoenzymology to study the detailed mechanisms of enzyme activity, i.e. means enzyme-catalyzed reactions dramatically slowed down at subzero temperatures in water-organic solvent (cosolvent) mixtures. This method opens up the possibility to investigate the intermediates of various steps of an enzyme-catalyzed pathway and the conformational changes of the enzyme accompanying each activation step. It also makes possible detection of some steps of the reaction process which were possibly hidden under normal conditions. According to the large rate reduction, reactions occurring in the microsecond range and then accessible only to relaxation techniques can be reduced to a time scale allowing rapid mixing (milli-

second range), whereas reactions in the millisecond range and normally requiring stopped-flow techniques can be reduced to a time scale of seconds and then studied by spectrophotometry. Furthermore, reactions normally occurring in a few seconds or minutes could be sufficiently reduced in rate to permit enzyme-substrate intermediates to be stabilized and isolated, and then studied by spectroscopic techniques, including X-ray diffraction. Namely, the enzyme crystals suspended in cryoprotective liquors are suitable for establishing the three-dimensional structure of stabilized enzyme intermediates in the presence of effectors. Cooled fluid mixtures allow the diffusion of several low-molecular-weight effectors into the crystals. This means that direct observation of productive enzyme-substrate intermediates is possible in such conditions, providing useful information about any conformational change induced in the enzyme-intermediate by effectors. For example, the acyl-enzyme intermediate of elastase, a serine proteinase, which was stable for more than one week at -55 °C, was successfully studied by X-ray crytallography [80–82]. Nevertheless, such investigations are restricted to effectors 2which are able to diffuse into the crystals and to situations in which interaction between the effectors and enzyme intermediates do not trigger further transformations on the reaction pathway.

It is important to use cryosolvents of low viscosities because otherwise they can reduce the reaction rate with a factor of $A = A_o/\eta$, where A is the preexponential term in the Arrhenius equation and η is the viscosity. Moreover, whereas in most cases the specificity of the enzyme is not changed by cryosolvents, the catalytic parameters are generally influenced. Namely, the k_{cat} parameter decreases and the $K_{m(app)}$ markedly increases on the solvent effect. The change in k_{cat} reflects the influence of the organic solvent on protein conformation, and the alteration in $K_{m(app)}$ points to the effect of the cryosolvent on the enzyme-substrate interaction. The cosolvents may also change the actual pH and ionic strength of the solution, which can be compensated by accurate concentration of the buffer and by addition of neutral salts to recreate the optimal conditions for enzyme catalysis. All of these circumstances emphasize the importance of control of the actual pathway obtained in mixed solvent and its comparison to that occurring in normal conditions of medium and temperature. Douzou and his group had proven the usefulness of the method on several enzyme systems. Later, the technique was also applied in other laboratories (see below).

Horseradish peroxidase catalysis. Horseradish peroxidase (HRP), containing ferric heme as a prosthetic group, catalyzes the oxidation of various substrates utilizing hydrogen peroxide. It has been established that this enzymatic reaction proceeds through the following mechanism:

$$\underset{\text{(ferric)}}{Fe^{III}\text{-heme}} \rightarrow \underset{\text{(compound I)}}{Fe(O)^{IV+\bullet}\text{-heme}} \rightarrow \underset{\text{(compound II)}}{Fe(O)^{IV}\text{-heme}}$$

where compounds I and II represent the oxyferryl intermediates. The formation of the oxidized enzyme, i.e. the catalytically active compound, I is produced by a ferric/H_2O_2 reaction together with the reduction of H_2O_2 to H_2O. This oxyferryl radical ion is reduced back to the ferric resting state either by two sequential one-electron-transfer processes from peroxidase substrates (e.g. guaiacol) via compound II intermediate or directly by two-electron oxidation with oxygen transfer to the substrates (e.g. thioethers). The formation of compound I was investigated by Baek and Van Wart [83] in methanol/phosphate buffer (50%, v/v), between the $+25$ to -35 °C temperature range. Ethyl hydroperoxide, tert-butyl hydroperoxide and peracetic acid were the applied substrates. In contrast, Watanabe and co-workers [84] studied the same process on polyethylene glycolated enzyme (PEG-HRP) in aqueous buffer as well as in organic media down to -20 °C. In this case the substrates were tert-butyl hydroperoxide, guaiacol, thioanisole and styrene. The polyethylene glycol modification of a protein provides it with good solubility in aqueous and also in organic solvents without the distortion of the active site [85]. In both laboratories low-temperature stopped-flow equipment combined with a rapid-scan spectrometer was used. According to the results obtained, the formation of compound I consists of two sequential steps, i.e. a fast and a slow step, where the first fast step indicates the formation of a labile precursor of compound I. Watanabe's group showed that the catalytic cycle of the PEG-HRP enzyme runs in the same manner in organic solution as in aqueous buffer. They also succeeded in stabilizing the labile precursor as well as the compound I intermediate at -20 °C in chlorobenzene, where the transient labile precursor has been assigned as a neutral peroxy-iron complex. Furthermore, it seems likely that a histidine residue in the heme pocket functions as a general acid-base catalyst in formation of the compound I intermediate.

Peptide synthesis in liquid. Two examples of conducting reactions at low temperatures and liquid conditions in solvent mixture and in saline environment, respectively, were discussed above, as considering Nilsson's [75] and Lozano's [76] methods of enzyme-catalyzed peptide synthesis.

Molecular basis of cold adaptation

Psychrophilic enzymes

Psychrophilic enzymes are products of psychrophilic organisms, which live at low-temperature environments such as polar and alpine regions or deep-sea waters and are also able to grow at subzero temperatures (for reviews, see [86–89]). Psychrophilic enzymes have to cope

with the reduction of reaction rate at low temperature; i.e. they have to improve their catalytic efficiency. Optimization of catalytic parameters can originate from a highly flexible structure which provides enhanced abilities to undergo conformational changes during catalysis. However, conformational flexibility involves thermal instability, too, and this suggests that the working enzyme structure has to achieve a balance between stability and flexibility at its physiological temperature. In improving the flexibility-activity behaviour of psychrophilic enzymes, the following destabilizing factors are considered. (i) Solvent effect: an increase in the number of interactions between the solvent water and the molecule can cause destabilization of the enzyme. These improved interactions are consequences of very hydrophilic surfaces, and they result in some reduction of the compactness of the protein's external shell (increase in flexibility). (ii) H-bonds: a lack of or reduction in the number of polar hydrogen bond-forming amino acid residues (Asn, Gln, Ser, Thr) may contribute to destabilization of psychrophilic enzymes. (iii) Hydrophobic effect: substitution of amino acids of hydrophobic side chains within the protein core for more polar ones can weaken the stability. (iv) Aromatic interactions: numerous interactions mediated by aromatic rings (Phe, Tyr, Trp) are missing in the psychrophilic homologues of the mesophilic enzymes. (v) Ionic interactions: the lack of salt bridges and the low occurrence of arginine residues on the protein surface contribute significantly to enzyme destabilization. (vi) Conformational freedom: proline residues in loops and turns of the proteins mean reduction in the degree of conformational freedom. However, they were not found in most of the psychrophilic enzymes studied.

Protein interactions with ice

Over the past decade, significant progress has been made in characterizing several types of proteins and polypeptides capable of interactions with ice. These include two functionally opposite classes of proteins. That is, (i) antifreeze glycoproteins and antifreeze proteins/polypeptides, which inhibit ice crystal growth, and (ii) ice nucleation proteins, which provide a proper template to initiate and promote ice growth. The acronyms AFGP, AFP and INP, respectively, are commonly given for these molecules [90].

Antifreeze proteins and polypeptides. Freezing is detrimental to most organisms. Many organisms of subzero environments ranging from fishes to plants and bacteria have evolved mechanisms to minimize freezing injury due to extracellular ice formation. This goal is served by the use of specialized antifreeze proteins and polypeptides [90–93]. These proteins appear to rely on a specific molecular structure and 'ice-lattice matching' to bind to particular ice crystal planes and modify ice crystal growth habits. Based on their primary structure, as well as the presence or absence of carbohydrates, these antifreeze molecules can be classified into two major groups: antifreeze glycoproteins (AFGPs) and antifreeze proteins/polypeptides (AFPs). Although these proteins and polypeptides have been isolated from several insects, spiders, bacteria and plants, the most studied ones are those of fish origin [90, 92, 93]. The four fish blood serum antifreeze types (AFGP and AFP I-III) consist of sets of closely related proteins and polypeptides resolvable by HPLC into 2–12 independently active components. Although there are major structural differences among these molecules, some understanding has been gained concerning their mode of action.

These protein and polypetide molecules become attached preferentially to specific planes of the growing faces of developing ice crystals and inhibit addition of water molecules to the ice lattice on the regular way. They produce the hexagonal bipyramidal ice crystals instead of hexagonal plates. This binding action results in a nonequilibrium freezing point depression, and consequently the ambient temperature must be lowered further before ice crystal growth can resume. Crystal growth inhibition and the resulting freezing point depression are considered to be the major means by which AFPs protect the body fluids of fishes from freezing. This freeze depressing effect is a noncolligative one; i.e. it is not based simply on the number of bound antifreeze molecules. On the other hand, the effect of AFPs on the melting point is an equilibrium effect and a purely *colligative* one. That means a considerable difference between the nonequilibrium freezing and melting points of solutions containing AFPs, and this disparity of temperatures is called thermal hysteresis. Thus these antifreezes are also called as thermal hysteresis proteins (THPs). The level of this noncolligative activity is high; e.g. for some species the observed freezing temperature lowering can be as high as $500\times$ that of the colligative salts on a molal basis [92].

The AFP type I of winter flounder origin is the most extensively studied antifreeze from which two major components have been isolated by HPLC techniques: HPLC-6 and HPLC-8. Both of them are rich in Ala (~ 65 mol%) and contain 37 amino acid residues. Moreover, their sequences are highly conserved, differing only in two residues at the same position. Asn, Asp and Thr residues of the sequences play the pivotal role in ice binding through their dipolar and hydrogen bond interactions. Sicheri and Yang [94] recently studied the X-ray crystal structure of the HPLC-6 component at 1.5 Å. They determined predominantly linear α-helical structure except the final peptide unit which shows a 3_{10}-helix conformation. The chain has cap structures at its N- and C-terminal ends which contribute greatly to stabilization of the α-helix structure in solution. The

ice-binding mechanism has been attributed to the regularly spaced polar residues matching the ice lattice along a pyramidal plane. This means four repeating ice-binding motifs, the side chains of which are rigid or restrained by pair-wise side-chain interactions to form a flat binding surface as shown in figure 3. The flatness maximizes the accessibility of binding groups to an ice surface. The authors suggest that the flat and rigid ice-binding surface may be a general feature of all AFPs. The X-ray findings have been supported by molecular dynamics simulations [95]. In contrast, however, the rigid side-chain model has been argued according to the results of two-dimensional NMR studies [96]. These NMR analyses proved that the conformation of the ice-binding side chains becomes less mobile only after an H-bonding network is established with the ice surface; i.e. a preformed ice-binding structure is not present in solution.

The L- and also D-enantiomers of type I AFPs have been chemically synthesized by Wen and Laursen [97, 98], who found that the mixture of these polypeptides has the same thermal hysteresis activity as either polypetide by itself. These results have been interpreted as showing the formation of homogeneous L- and D-AFP patches on the ice surface. Davies and co-workers [99] argued this interpretation, because the noninterference of the enantiomers could also be explained if each AFP molecule is acting independently. This independence has been proven by the authors with antifreeze mixtures (AFP I–III), where the activity was independent of the proportions of the different AFP types.

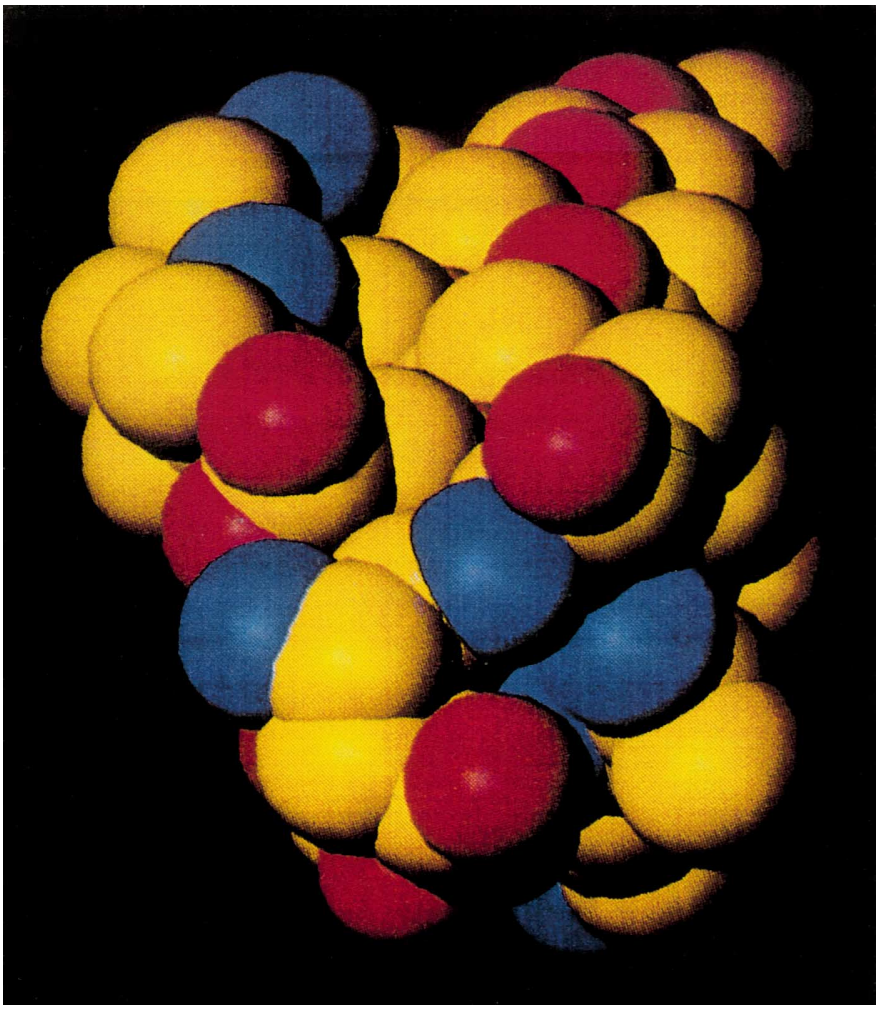

Figure 3. N-terminal axial view of AFP ice-binding surface (AFP type I HPLC-6). The N terminus towards the reader is tilted down 5° from the horizontal with the ice-binding surface facing upwards. C, N, O atoms are coloured yellow, blue and red, respectively. (Reprinted with permission from the the authors and Nature [94], © 1999, Macmillan Magazines Ltd, London).

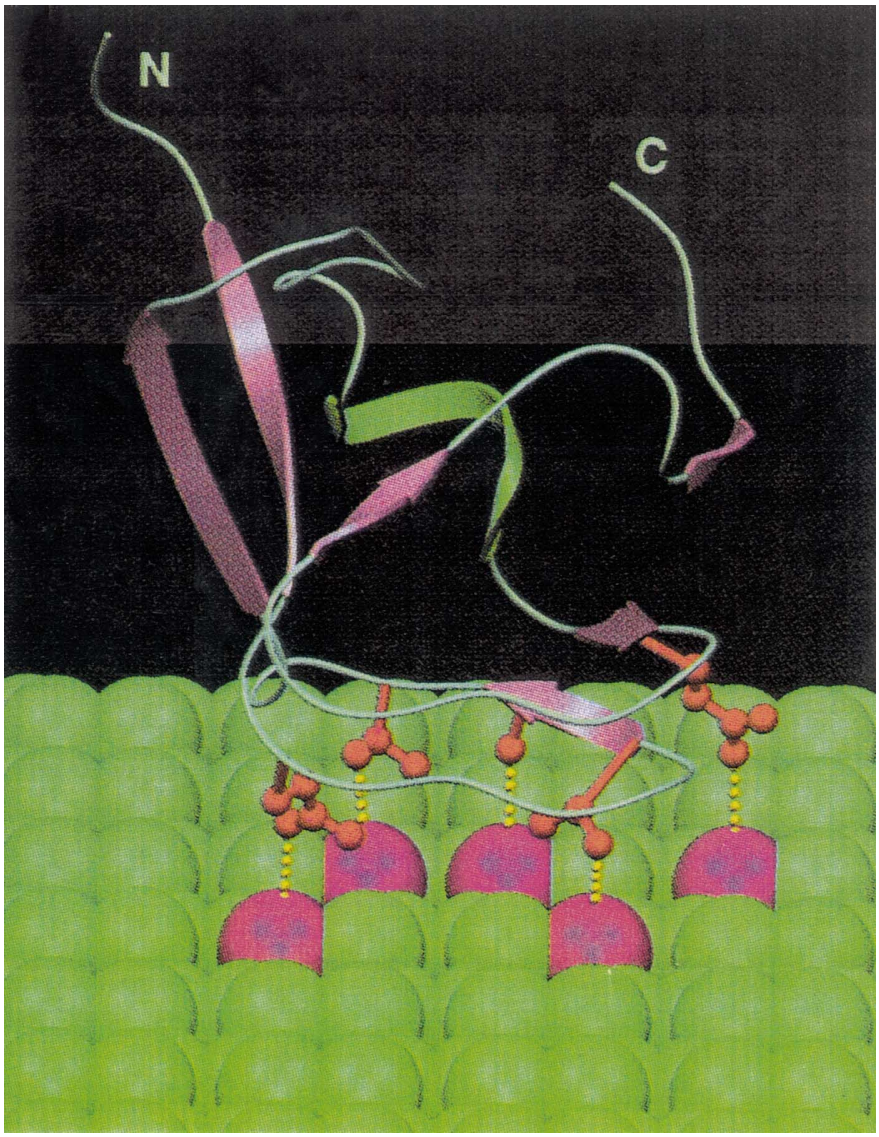

Figure 4. AFP-ice docking model of recombinant type III AFP. The plane of the ice lattice is presented, and the five prism-plane ice-binding residues are shown. The oxygen atoms of the ice lattice interacting with AFP through H bonds (yellow dashed lines) are coloured red. A 180° rotation of the planar amide group of Gln or Asn would fit the ice-binding model equally well. (Reprinted with the permission from the authors and Nature [100], © 1999, Macmillan Magazines Ltd, London).

The flatness and spatial geometry of the ice binding site are those structural requirements which exclude most globular proteins from binding to ice. Thus the 1.25-Å crystal structure of recombinant AFP type III, a globular antifreeze protein of eel pout origin, is of particular interest. The structural basis and mechanism for ice binding of this globular antifreeze has been revealed by Jia and co-workers [100] using high-resolution X-ray techniques together with site-specific mutations. The compact AFP III molecule contains imperfect β-strands, and one turn of α-helix together with many 'β-structure-like' interstrand main-chain hydrogen bonds that give the protein a rigid, globular fold. According to the X-ray structure, this small globular protein contains a flat amphipathic ice binding site, similar to type I AFPs. Consequently, there are also constraints to the side-chain flexibility of the ice-binding residues. The AFP-ice docking model shows that five atoms of the Asn, two Gln and two Thr residues can simultaneously form H bonds to the water 0 atoms of

the ice lattice, as demonstrated in figure 4. An automated algorithm was developed by Yang and co-workers [101] to analyze the surface planarity of the globular type III AFP from ocean pout, and it was used to identify the ice-binding surface (IBS) of this protein. This 'flatness function' algorithm is based on the flatness observed the IBS of the type I α-helical AFPs. The calculations indicated the same conclusion already found by the X-ray experiments of Jia and co-workers [100], namely that the specificity of interactions between an IBS and the plane of the ice crystal is based on surface complementarity.

Ice nucleation proteins (INPs). The aggregated INPs give the active forms, the ice nucleation activators (INAs) of various origins. INAs are present in a variety of organism: plant bacteria, fungi, other microorganisms, invertebrates and plants [90, 102, 103]. Many freeze-tolerant animals produce ice-nucleating proteins to ensure freezing at 'high' subzero temperatures, which involves then a slow freezing and in this way reduces the chances of intracellular freezing. The most extensively studied INPs are of bacterial origin [102]. Some protein sequences have been deduced from the elucidated complete DNA sequences of INA genes [104]. These large sequences of 1100–1300 amino acid residues consist of three segments, i.e. the N- and C-terminal nonrepeating parts and the longest middle part of repeating sequences [90]. The repeating section is composed of 8, 16 and 48 repeating amino acid residues. The nonrepeating N- and C-terminals do not determine the conformation of the large repetitive section of INP, according to the computations and model building. The large repetitive part is believed to play the main role in ice interactions. This in turn means, that a structural match should exist between this peptide section and the ice surface. Considering the relationship between the extent of INP aggregation of INA and ice nucleation activity, it has been shown that for a nucleus active at -2 °C, 132 INP units are required, whereas a monomeric INP would have a threshold temperature of -12 to -13 °C [90].

Thus protein-ice interactions play a significant role in the survival of many organisms. Moreover, antifreeze and ice-nucleation proteins have many unique properties of biotechnological importance.

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