Differential Scanning Calorimetry Investigation of Formation of Poly(ethylene glycol) Hydrate with Controlled Freeze–Thawing of Aqueous Protein Solution

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ABSTRACT: The formation of poly(ethylene glycol) (PEG) hydrate during freeze–thawing of dilute lactate dehydrogenase solutions with the addition of 0.05–160 mg/mL PEG 6000 is investigated by differential scanning calorimetry and modulated temperature differential scanning calorimetry. The freeze–thawing process is performed with a controlled temperature history. A moderate cooling rate to a low freezing temperature in combination with a low heating rate seems to create the most stable PEG hydrate. The maximum amount and the most stable hydrate phase are obtained when the freezing temperature is at or below −60°C. The enthalpy of melting for the hydrate at −15°C is dependent on the heating rate but not on the cooling rate if the freezing temperature is −60°C. The effect of the addition of reduced form nicotinamide adenine dinucleotide to the PEG and protein solution indicates that competing interactions with the protein can increase the stability of the PEG hydrate. The amount of bound water in the PEG hydrate can be calculated directly from the melting enthalpy of the hydrate if an adequate temperature history is used. For solutions with >10 mg/mL PEG there are 1.7–2.7 water molecules bound per PEG unit. The PEG protection of the protein at freeze–thawing can be an effect of the amount of available PEG hydrate in relation to the amount of ice surface. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 91: 1626–1634, 2004

Key words: poly(ethylene glycol) hydrate; freeze–thawing; temperature history; differential scanning calorimetry; modulated temperature differential scanning calorimetry

INTRODUCTION

An increasing number of potential drugs are proteins or peptides, and they have limited stability in aqueous solutions. The most commonly used method for preparing protein pharmaceuticals is freeze-drying. Freezing is one important step in this process, but it induces several stresses capable of protein denaturation. Therefore, labile proteins require protection against freezing and dehydration stress. Protective additives are often incorporated to recover sufficient activity. There are different types of stabilizing additives like sugars, amino acids, certain salts, polymers, and nonionic surfactants.[1] Pharmaceutical formulations are thus quite complex, and it is important to understand the physical and chemical state of both protein and additives to predict the freeze-drying behavior and stability of the protein.

There are several possible mechanisms for the protection of proteins. One theory is that the additive is preferentially excluded from the surface of the protein.[2] In systems with high concentrations of poly(ethylene glycol) (PEG), the polymer is preferentially excluded from the protein surface at room temperature.[3] Stabilization by excluded volume is dependent on the PEG concentration and it becomes apparent only at relatively high concentrations (>0.3*M*).[4] The results of a previous study in our laboratory also showed that very low concentrations of PEG, which are far below the excluded volume range, give full protection of the model protein, lactate dehydrogenase (LDH).[5] This indicates a more complex protection mechanism. Mi et al. used circular dichroism studies to show that PEG at low concentrations can also protect the helix structure of LDH in solutions.[6]

Another theory for protection is that denaturation occurs at the ice–water interface and the protective effect depends on the fact that the additive can compete with the protein for these interfaces.[5,7,8] PEG itself is not a surface-active molecule, and the tendency to bind to the ice surface is minimal. However, the formation of a PEG hydrate might change the conditions for the ice surface interaction. The hydrated polymer chains can bind to the ice surface by hydrogen bonds between the ice and the PEG hydrate[9] or by water molecules in the ice being part of the PEG hydrate. An IR spectroscopy study showed that several types of

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hydrogen bonds between the PEG chain and water are present for low molecular weight PEGs.[10] The protein is thus hindered in reaching the destructive ice surface. A certain correlation between the ice crystal size and protection by PEG or PEG hydrate was previously found in our laboratory.[5]

Differential scanning calorimetry (DSC) is a well-established method of thermal analysis within the pharmaceutical sciences. It is commonly used to study freeze-dried formulations of proteins,[11,12] and it is also used to examine the freezing process of proteins in aqueous solutions.[5,8,13] Modulated (or oscillating) temperature DSC (MTDSC) is a type of DSC in which the usually linear or isothermal heating or cooling program is modulated by some form of perturbation. In this way the reversing and nonreversing components of a thermal event can be separated.[14] With an appropriate selection of variables the ratio between the enthalpy of the reversing component and the total enthalpy ($\Delta H_c/\Delta H$) can be an expression of the degree of crystallinity of the phases under investigation.[13,15]

The aim of the study was to investigate the effect of a controlled temperature history on the formation of PEG hydrate when protein solutions with PEG additions were freeze–thawed. We also intended to investigate the melting of the hydrate during different temperature histories and to determine the water content of the phase. LDH was used as the model protein, because this protein is inactivated during freeze–thawing in pure water. In some solutions its reduced nicotinamide adenine dinucleotide (NADH) cofactor was added and the protective effect was examined.

EXPERIMENTAL

Materials

The LDH in the study was from rabbit muscle as a crystalline suspension in 65% saturated $(NH_4)_2SO_4$ solution at pH 7.2 (ICN Pharmaceuticals Inc., Costa Mesa, CA). PEG with the formula $HO(C_2H_4O)_nH$ (PEG 6000, $n = 140$) had weight-average molecular weights of 5600–7000 (Janssen, Geel, Belgium) and 5000–7000 (Fluka Chemie AG, Buchs, Switzerland). The NADH in a preweighed vial (0.2 mg) and sodium pyruvate solution (22.7 m*M*, pH 7.5) were procured from Sigma (St. Louis). Potassium phosphate (0.1*M*, pH 7.5) and sodium citrate (10 m*M*, pH 6.4–6.5) buffers were used.

Preparation of solutions

Prior to some experiments the LDH suspension was dialyzed against 10 m*M* sodium citrate buffer (pH 6.4–6.5). The citrate buffer was selected because it has minimal pH change during freezing.[16] The dialyzed LDH was concentrated during centrifugation using Microsep Centrifugal Concentrators (Pall Filtron Company, Northborough, MA), and the concentration of the enzyme was determined spectrophotometrically with a Spectronic Genesys spectrophotometer (Milton Roy Company, New York) at 280 nm. The UV absorbance at 280 nm had a linear relation to the concentration range of 0.1–1.1 mg/mL. In the thermal analysis experiments the concentrations were 25 μg/mL LDH, 10–160 mg/mL PEG 6000, and 10 μg/mL NADH in deionized and filtered water (Minisart 0.2 μm sterile filter, Sartorius AG, Göttingen, Germany) or in 10 m*M* sodium citrate buffer (pH 6.4).

DSC and MTDSC analyses

The solutions were examined using a DSC 220C oscillating differential scanning calorimeter (Seiko Instruments Inc., Chiba, Japan) with or without oscillation. The samples were kept in aluminum pans in an atmosphere of nitrogen. The calorimeter was temperature and heat calibrated with indium, tin, gallium, and mercury as standards. The samples were analyzed using different combinations of variables. The cooling and heating rate was 1–10°C/min. The temperature history included freezing between −25 and −80°C, in some samples a ramp time at −40°C for 10 min, and then heating to 30°C. When oscillating mode was used, the frequency was 0.02 Hz and the amplitude was 2°C, giving cooling and heating in each cycle. The degree of oscillation for the MTDSC experiments was 0.96.[15] Thermograms were recorded with both cooling and heating. The results are presented as mean values with the standard deviations based on three determinations.

Assay of enzyme activity

The LDH activity was measured spectrophotometrically with a Spectronic Genesys spectrophotometer (Milton Roy). The 1.44-mL reaction mixture contained 55 μM NADH in 0.1*M* phosphate buffer (pH 7.5) and an aluminum pan with the 20 μL LDH sample (25 μg/mL). The enzymatic reaction was started by adding sodium pyruvate (1.9 m*M*), and it was monitored by measuring the decrease in absorbance at 340 nm. The recovered activity of a frozen LDH sample was calculated as the percentage of the activity of an identical unfrozen sample that had been stored in an aluminum pan.

RESULTS AND DISCUSSION

Phase transformations

The thermal transformations of aqueous solutions with different concentrations of PEG 6000 were studied by MTDSC measurements. At cooling the trans-

TABLE I
Peak Temperature (T_{peak}), Heat of Transformation (ΔH), and Relative Crystallinity ($\Delta H_c/\Delta H$) for Samples with LDH (25 μg/mL) and PEG 6000 Obtained by MTDSC

PEG 6000 (mg/mL)	T_{peak} (°C)	SD	ΔH (J/g)	SD	$\Delta H_c/\Delta H$	SD	Cooling/heating rates (°C/min)
Crystallization of ice and PEG hydrate							
0.05	−9.7	1.0	−266	11			5/2
1	−11.4	0.8	−262	6			5/2
5	−10.1	0.2	−265	3			5/2
10	−15.5	2.4	−269	9			2.5/2.5
20	−15.4	1.6	−255	13			2.5/2.5
40	−15.0	0.2	−243	3			2.5/2.5
80	−18.8	2.1	−213	8			2.5/2.5
160	−12.7	1.3	−224	3			2.5/2.5
Transformation of ice and PEG hydrate							
10	−49.2	0.1	−0.2	0			2.5/2.5
20	−48.5	0.1	−0.5	0			2.5/2.5
40	−48.6	0.03	−1.2	0.1			2.5/2.5
80	−48.6	0.1	−2.4	0.2			2.5/2.5
160	−49.7	0.1	−5.5	0.5			2.5/2.5
Melting of PEG hydrate							
0.05	−15.2	0.2	0.029	0.011			5/2
1	−14.6	0.1	0.16	0.02			5/2
5	−14.5	0.2	1.2	0.02			5/2
10	−15.1	0.02	1.5	0.2	0.24	0.01	2.5/2.5
20	−13.9	0.3	4.2	0.3	0.31	0.04	2.5/2.5
40	−13.3	0.03	8.0	0.3	0.40	0.03	2.5/2.5
80	−12.7	0.1	15.9	0.4	0.19	0.01	2.5/2.5
160	−11.7	0.1	27.1	0.1	0.10	0.002	2.5/2.5
Melting of ice in PEG solution							
0.05	1.9	0.2	297	16			5/2
1	3.1	0.1	290	11			5/2
5	2.5	0.1	295	5			5/2
10	2.6	0.5	289	8	0.12	0.05	2.5/2.5
20	2.8	0.6	271	10	0.14	0.05	2.5/2.5
40	2.2	0.3	247	4	0.12	0.004	2.5/2.5
80	2.1	0.1	214	2	0.10	0.002	2.5/2.5
160	2.8	0.0	186	1	0.06	0.01	2.5/2.5

The sample with 160 mg/mL PEG was without LDH.

formation temperature of the system occurs at about −15°C (Table I). Most of the solid phase that is formed consists of hexagonal ice, but there is also metastable cubic ice.[17] In addition, some solid PEG hydrate may have started to form at that temperature.

The heating thermograms of the solutions are shown in Figure 1. When PEG was present, two specific transformations appeared that do not exist in solutions without PEG. One is an exothermic peak that appears in the heating process at about −48°C, and it is associated with changes in the ice structure.[18] This peak became larger at higher concentrations of PEG. The transformation was not observed in the reversing component (Fig. 1), and it is thus a nonreversing event. Different modifications of ice exist, of which hexagonal ice is the stable polymorph at the prevalent temperature and pressure and cubic ice is a metastable phase. The transition of cubic to hexagonal ice occurs at −47°C.[17] The transformation that is observed here indicates that with higher concentrations of PEG more metastable cubic ice might be formed during freezing. PEG is thus hindering the formation of thermodynamically stable hexagonal ice. It is also possible that additional formation and stabilization of PEG hydrate occurs. The ice structure might be crucial for the formation of a stable phase.

At −45°C there seemed to be an exothermal peak, which also appears in the heating thermogram of pure water. The size of this peak increased with the ramp time and became unreasonably large for long ramp periods. The only possible explanation of this is that the exotherm is an artifact, which is created when the

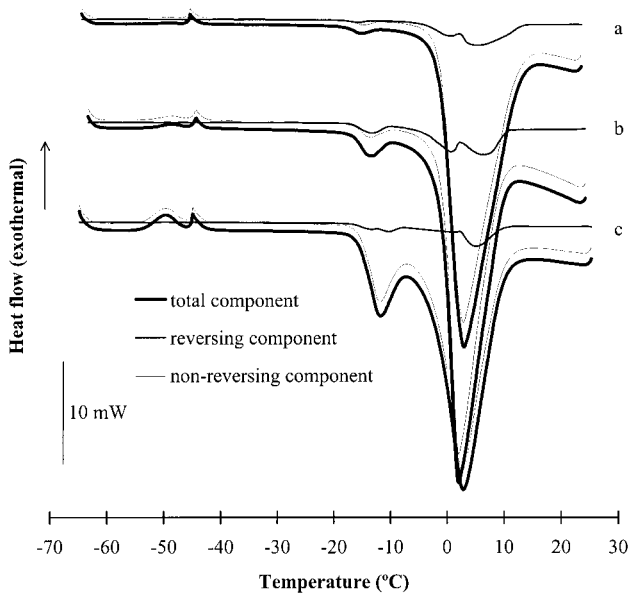

Figure 1 The heating thermogram obtained by modulated temperature DSC of samples with 25 μg/mL LDH and concentrations of PEG 6000 in water of (a) 10, (b) 40, and (c) 160 mg/mL. The cooling and heating rate was 2.5°C/min, the amplitude was 2°C, and the frequency was 0.02 Hz.

heat flow during a certain time at constant temperature (−40°C) is translated to one single point in the heat flow and temperature diagram.

The other specific transformation of the PEG solution was an endothermic peak in the heating process at about −15°C. With an increasing concentration of PEG, this peak, which reflects the melting of the PEG hydrate, became larger and appeared at higher temperature (Table I). The increased melting temperature indicates that a more stable compound is obtained when the PEG concentration is increased. The MTDSC revealed that the transformation has a reversible contribution to the enthalpy with a double melting peak at the highest concentration (160 mg/mL) of PEG 6000 (Fig. 1).

An endothermic peak appeared at about 2°C in the heating thermograms, representing the melting of a two-phase mixture of ice in a PEG solution.[20] The reversible component showed a double melting peak (Fig. 1), as was also demonstrated for pure water in previous studies.[13,19] This can indicate the complex melting of two ice phases with different stability and/or crystallinity. However, it cannot be excluded that it is an effect of the temperature modulation, which induces a phase lag at the melting.[19] The shape of this double peak varies with the concentration of PEG 6000, being less distinct for the higher concentrations where the most amorphous phase is created.

Total enthalpy changes at transformations

The values for the heat of transformations and peak temperatures for some of the investigated solutions are shown in Table I. Some samples have large standard deviations in the ΔH values, reflecting the difficulties of obtaining reproducible conditions in DSC and MTDSC measurements of dilute aqueous solutions, where the processes are relatively fast.[19]

The absolute value of the heat of crystallization at about −15°C decreased when the concentration of PEG was increased to 80 mg/mL. In addition, the heat of melting of ice and the PEG solution at about 2°C followed that pattern. This indicates that, for this range of concentrations, a less stable structure or a decreasing phase amount is transformed when the PEG concentration increases.

In solutions with low concentrations of PEG a larger value for the heat of fusion is obtained than for the heat of crystallization. The difference between the absolute values of ΔH with melting at 2°C and that with crystallization at −15°C decreases with the increasing concentration of PEG. The amount of ice that is formed is probably reduced and reflected in the ΔH value of melting. It is a well-known fact that the growth of nuclei formed at first crystallization occurs during further cooling if the diffusion is high enough and the mass of ice formed in a solution during freezing decreases with increasing solute concentration.[21] PEG is believed to reduce the ice nucleation temperature and stabilize the structures that exist in undercooled water, probably because of its effect on the diffusional motion of water.[22] In addition, the formation of a PEG hydrate can contribute to delaying the crystallization of ice and hindering the growth of ice crystals. For the highest concentration of PEG (160 mg/mL) the difference between the ΔH values is negative, which is partly dependent on the reduced amount of ice that is melting compared to PEG hydrate at higher concentrations of PEG. Moreover, at this high concentration of PEG, the PEG chains start to interact with each other and therefore its effect on the nucleation and ice growth is changed.

The transformation enthalpy of ice and PEG hydrate at −48°C was significantly increased with the PEG concentration. The peak became larger at higher concentrations of PEG, giving a linear relationship between the ΔH value and PEG concentration with a correlation coefficient of 0.997.

The melting peak of PEG hydrate at −15°C increased as the concentration of PEG 6000 increased. The relationship between the ΔH and the concentration of PEG was linear with a correlation coefficient of 0.990. Increasing concentrations of PEG thus yield a larger amount of the PEG hydrate, assuming constant crystallinity.

The MTDSC experiments were used to tentatively evaluate the degree of crystallinity of the samples. The relative degree of crystallinity ($\Delta H_c/\Delta H$) showed minor differences in the melting process of ice and the PEG solution for samples with low concentrations of

TABLE II
Peak Temperature (T_{peak}) and Heat of Transformation (ΔH) for Samples with LDH (25 μg/mL) and PEG 6000 (80 mg/mL) Obtained by DSC

	Cooling				Heating				Cooling and heating	
Cooling rate (°C/min)	T_{peak} (°C)	SD	ΔH (J/g)	SD	T_{peak} (°C)	SD	ΔH (J/g)	SD	ΔH (J/g)	SD
Transformation of ice and PEG hydrate										
1	−49.0	0.1	−2.0	0.3	−51.3	0.1	−1.1	0.1	−3.0	0.2
2.5	−51.2	0.2	−0.7	0.1	−51.7	0.1	−3.5	0.2	−4.1	0.2
5	—		—		−51.7	0.1	−4.5	0.2	−4.5	0.2
10	—		—		−51.9	0.1	−4.6	0.1	−4.6	0.1
Melting of PEG hydrate										
1					−13.3	0.0	14.9	0.4		
2.5					−13.3	0.0	15.1	0.2		
5					−13.3	0.0	14.9	0.4		
10					−13.2	0.1	14.8	0.6		

All samples were frozen to −60°C and the heating rate was 1°C/min.

PEG, but it decreased significantly when the PEG concentration was above 80 mg/mL (Table I). This shows that a more amorphous structure is formed at higher concentrations of polymer. The relative degree of crystallinity of the PEG hydrate showed an increasing value to 40 mg/mL PEG and a distinct decrease at higher concentrations of PEG (Table I). However, the enthalpy values are very small and thus the ratio describing the crystallinity is very uncertain.

Influence of temperature history on transformations

Samples with 80 mg/mL PEG 6000 were freeze–thawed with different temperature histories. The temperature history had very little influence on the crystallization of ice and PEG hydrate at −15°C in the cooling process and the melting of ice and the PEG solution at 2°C in the heating process. The values presented in Table I for this concentration are thus representative for all temperature histories. However, the transformations of the phases at −15°C in the heating process and at −48°C in the cooling and heating processes were greatly affected by the temperature (Tables II, III).

Melting of PEG hydrate at −15°C

The melting endotherm of PEG hydrate observed at −15°C was strongly affected by the freezing temper-

TABLE III
Peak Temperature (T_{peak}) and Heat of Transformation (ΔH) for Samples with LDH (25 μg/mL) and PEG 6000 (80 mg/mL) Obtained by DSC

	Cooling				Heating				Cooling and heating	
Heating rate (°C/min)	T_{peak} (°C)	SD	ΔH (J/g)	SD	T_{peak} (°C)	SD	ΔH (J/g)	SD	ΔH (J/g)	SD
Transformation of ice and PEG hydrate										
1	−49.0	0.1	−2.0	0.3	−51.3	0.1	−1.1	0.1	−3.0	0.2
2.5	−48.9	0.0	−2.2	0.2	−47.8	0.1	−1.2	0.1	−3.4	0.2
5	−48.8	0.1	−2.1	0.3	−44.6	0.2	−1.3	0.3	−3.5	0.2
10	−49.1	0.4	−2.5	0.3	−41.0	0.2	−0.8	0.3	−3.3	0.1
Melting of PEG hydrate										
1					−13.3	0.0	14.9	0.4		
2.5					−13.2	0.1	14.0	0.4		
5					−12.7	0.2	9.8	0.4		
10					−12.0	0.1	6.1	0.5		

All samples were frozen to −60°C and the cooling rate was 1°C/min.

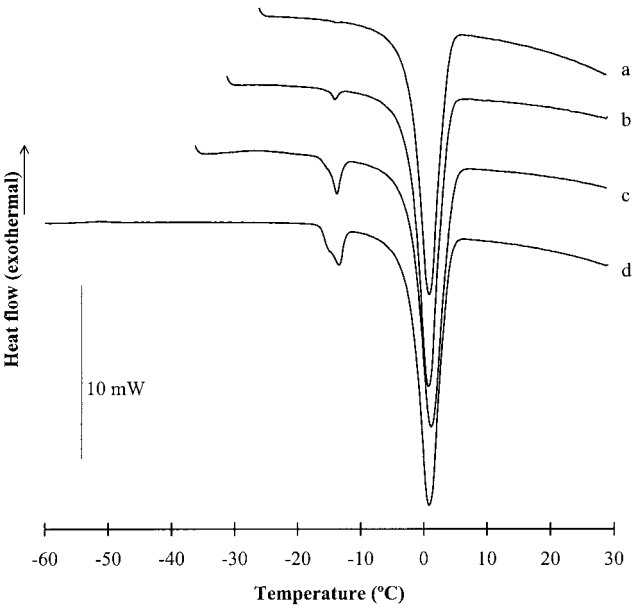

Figure 2 The heating thermogram obtained by DSC of samples with 25 μg/mL LDH and 80 mg/mL PEG 6000 in water at freezing temperatures of (a) −25, (b) −30, (c) −35, and (d) −60°C. The cooling and heating rate was 1°C/min.

ature (Fig. 2). A distinct endotherm was obtained when the minimum temperature was less than or equal to −30°C. When the minimum temperature was lowered, the enthalpy of the hydrate transformation increased from 1 J/g at a freezing temperature of −30°C to 15 J/g when the sample was frozen to −60°C. A still lower freezing temperature (−80°C) did not increase the enthalpy of melting further.

If the freezing temperature was −60°C at a constant heating rate, the variation of the cooling rate did not influence the temperature or enthalpy of the transformation (Table II). Freezing to −35°C at a high cooling rate decreased the enthalpy of the PEG hydrate transformation somewhat from 9.1 to 7.6 J/g. An increased heating rate at a constant cooling rate induced a less pronounced transformation with a lower enthalpy (Table III). The stability of the PEG hydrate thus seems to be affected by fast heating. However, we cannot exclude that the melting process of ice and the melting of PEG hydrate overlap when the heating is too fast, giving reduced ΔH values for the PEG hydrate. A previous study found that the amount of PEG hydrate did not increase with a ramp period of 60 min at −60°C.[9] However, if the sample was kept at −30°C for 60 min, the amount of PEG hydrate increased compared to a cycle without the ramp period.[9] The polymer hydrate is thus stabilized by freezing to a very low temperature or by a ramp time at a higher temperature.

The formation and stability of the PEG hydrate is thus greatly dependent on the temperature history at freeze–thawing. A moderate cooling rate with a low freezing temperature (−60°C) in combination with a low heating rate seems to create the most stable PEG hydrate.

Transformation at −48°C

The exotherm at −48°C, which reflects the transformation from cubic to hexagonal ice with or without the PEG hydrate involved, was influenced by the cooling and heating rates (Tables II, III, Fig. 3). If the heating rate was low and constant, a higher cooling rate induced an increased heat of transformation. At a low cooling rate of below 2.5°C/min, a transformation was also observed in the cooling range but the sum of the enthalpy in the cooling and heating process was almost constant (Table II). This shows that the transformation starts in the cooling process if the cooling rate is low enough. If the cooling rate is low and constant, a varying heating rate induced a transformation at both cooling and heating. The sum of the transformation enthalpies was constant (Table III). A lower freezing temperature (−80°C) increased the sum of enthalpies to −5.3 J/g.

The ramp period also affects the transformation as shown in Figure 3. In all cases the transformation was irreversible, and no reversing component was indicated. When the lack of a ramp period was combined with a moderate cooling rate, the transformation occurred only in the heating range with an enthalpy change of −3.8 J/g. With a ramp period at −40°C in both the cooling and heating processes, the transfor-

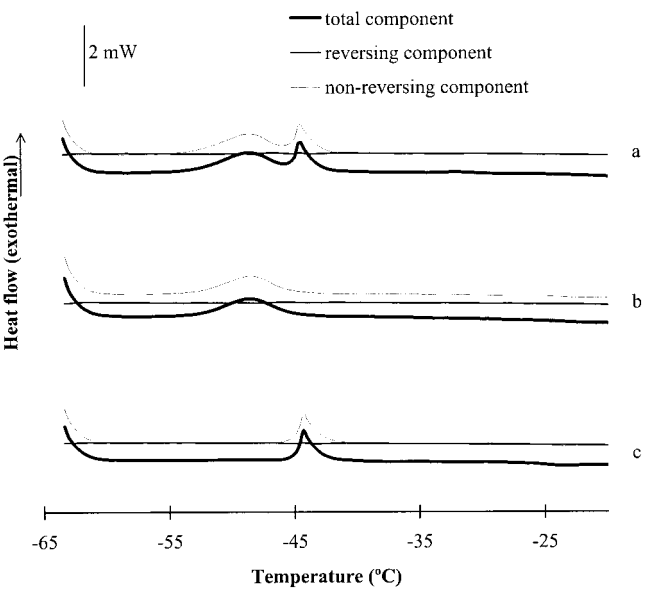

Figure 3 The heating thermogram obtained by modulated temperature DSC of samples with 25 μg/mL LDH and 80 mg/mL PEG 6000 in water (a) with a ramp period at −40°C in the heating process, (b) without a ramp period, and (c) with a ramp period at −40°C in both the cooling and heating processes. The cooling and heating rate was 2.5°C/min, the amplitude was 2°C, and the frequency was 0.02 Hz.

TABLE IV
Calculated Amount of Water Molecules/PEG Segment (repeat unit) for Samples with LDH (25 μg/mL) and PEG 6000

PEG 6000 (mg/mL)	Water molecules/ PEG segment	SD
0.05	11.6	5.32
1	1.43	0.48
5	3.46	0.10
10	1.19	0.48
20	2.64	0.36
40	2.40	0.18
80	2.37	0.12
160	1.65	0.02

mation peak in the heating range disappeared. It instead appeared during the ramp period in the cooling process ($\Delta H = -4.5$ J/g). This shows that the transformation of cubic ice to a stable hexagonal phase, including occasional formation and stabilization of PEG hydrate, occurs during the cooling phase if there is enough time for the process to proceed; otherwise, the transformation occurs in the heating process.

Determination of bound water in PEG hydrate

The methods described earlier to determine the amount of bound water in PEG hydrate are in all cases built on the determination of the fraction of water that freezes into ice in the sample. The amount of bound or nonfreezing water has been obtained by the difference. In all cases, very concentrated solutions have been examined by their cooling or melting peaks and the hydrate that has been observed includes two or three water molecules per ethylene oxide unit.[9,18,20]

Relatively dilute solutions have been investigated here and the amount of bound water has been determined by another method. By evaluating the enthalpy values of the PEG hydrate that is melting at −15°C in the heating process, the number of H_2O molecules per C_2H_4O unit (x) was calculated by using the following equation:

$$w_{\mathrm{PEG}} + \frac{w_{\mathrm{PEG}}}{M_{\mathrm{PEGunit}}} \cdot x \cdot M_{\mathrm{water}} = \frac{\Delta H_{\mathrm{hydrate,measured}}}{\Delta H_{\mathrm{hydrate.theoretical}}}$$

where w_{PEG} is the weight fraction of PEG, $M_{\mathrm{PEG\ unit}}$ is the molecular weight of the PEG repeat unit, and M_{water} is the molecular weight of water. In the calculation the theoretical enthalpy of melting for the PEG hydrate was assumed to be 101 J/g and all available PEG was assumed to transform into the hydrate. The results of the calculations are presented in Table IV.

For the molecular weight of PEG studied here the amounts of bound water ranged from 2.4 to 2.6 H_2O molecules per PEG unit for solutions with 10–80 mg/mL PEG 6000. When the concentration of PEG was increased to 160 mg/mL, the amount of bound water decreased, probably because the melting of the PEG hydrate and the ice in the PEG solution greatly overlap, inducing an uncertain enthalpy value for the calculation. It is also possible that in the concentrated solution the effective PEG amount for hydrate formation is reduced because of PEG chain interaction. Such a condition lowers the calculated number of water molecules per PEG unit. At very low concentrations the amount of bound water was largely varied, which is probably an effect of the very small and therefore uncertain enthalpies that were obtained.

The amount of bound water was calculated for a special temperature history with a cooling and heating rate of 2.5°C/min and a freezing temperature of −60°C, yielding a maximum amount of PEG hydrate. The effects of different temperature histories on the amount of bound water or the formation of hydrate were examined for the 80 mg/mL PEG solution. Independent of the cooling rate, 2.1 H_2O molecules per PEG unit was obtained if the heating rate was 1°C/min. If the cooling rate was constant at 1°C/min, the value of bound water was dependent on the heating rate. If the heating rate was higher than 2.5°C/min, the value of bound water was much lower than 2.1, which is again the effect of overlapping endotherms giving uncertain ΔH values for the melting of the hydrate.

The results presented here confirm the fact that two or three water molecules per PEG unit are included in the PEG hydrate that is formed in an aqueous solution. The amount of bound water can be directly calculated from the melting enthalpy of the PEG hydrate in a certain concentration range and with an adequate temperature history.

Effect of cofactor NADH on PEG hydrate

In a previous study we showed that the addition of LDH made the PEG hydrate less stable.[13] When NADH was present with LDH, the peak temperature for the melting of PEG hydrate increased, indicating that the hydrate structure was somewhat stabilized (Table V). If the cofactor binds to the enzyme, the interaction of LDH with PEG, which was observed by fluorescence spectroscopy,[5] might be reduced. Therefore, the PEG molecules are free to interact with the water molecules and the PEG hydrate may be stabilized. The relative crystallinity of the solutions was not influenced by the addition of NADH (Table V).

Activity of LDH in relation to PEG hydrate

The recovered activity of LDH was high (>79%) and independent of the PEG 6000 concentration in a range of 10–80 mg/mL (1–8%, w/v). The activity of LDH after freeze–thawing with 80 mg/mL PEG and differ-

TABLE V
Peak Temperature (T_{peak}), Heat of Transformation (ΔH), and Relative Crystallinity ($\Delta H_c/\Delta H$) for Samples with LDH (25 μg/mL), NADH (10 μg/mL), and PEG 6000 (10 mg/mL)

Sample	T_{peak} (°C)	SD	ΔH (J/g)	SD	$\Delta H_c/\Delta H$	SD
Melting of PEG hydrate						
PEG + NADH + LDH	−14.1	0.1	1.8	0.1	0.22	0.01
PEG + LDH[a]	−15.1	0.02	1.6	0.1	0.25	0.02
PEG[a]	−13.5	0.03	2.0	0.1	0.49	0.08

[a] According to Aldén and Magnusson.[13]

ent temperature histories was >86%, indicating that at this high concentration the PEG itself protects LDH very well and the temperature history is of minor importance for these samples. Because PEG alone protects LDH, the addition of NADH to the solution did not influence the recovered activity of LDH.

It was previously demonstrated that solutions of 1–10% (w/v) PEG 8000 (10–100 mg/mL) fully protected LDH during freezing and thawing.[11] A recent study at this laboratory found that very low concentrations of PEG 6000 (0.5 μg/mL or 0.00005%, w/v) were needed for complete protection of LDH at freezing with a low cooling rate. With enhanced freezing rates, higher concentrations of PEG were needed for full protection.[5] It was also found that the protection ability of PEG increased with the molecular weight and concentration up to about 1% (w/v) when LDH was frozen in liquid nitrogen.[6]

The formation of a PEG hydrate can be crucial for the protective ability of PEG. Hydrogen bonding and the structural matching between the PE oxide chain and the water molecules are important in the stabilization of the PEG chain in water.[10] Several types of hydrogen-bonded bridges are suggested. Water molecules can form hydrogen-bonded bridges between the adjacent ether oxygen atoms or between the ether oxygens separated by two PE units.[10]

Because PEG is able to form a hydrate at low temperature when all water outside the complex is transferred to ice, as found by IR spectroscopy,[8] it is possible that hydrogen bonds are formed between the ice surface molecules and the water molecules in the PEG hydrate. Another possibility is that the water molecules on the ice surface can form part of a PEG hydrate complex. An illustration of such interactions is presented in Figure 4. The bonding between the PEG hydrate and the ice can prevent the protein from reaching the destructive ice surface, thereby avoiding denaturation.

The formation of PEG hydrate is a slow process, and the hydrate cannot be formed with very high cooling rates. At the same time, with fast cooling a very large ice area is created that claims a large amount of PEG hydrate. The protection of the protein will thus be insufficient, and the denaturation increases at the ice surface.

PEGs with low molecular weights cannot form as many hydrogen bonds as those with high molecular weights. The observation by Mi et al. that the protection increased with the molecular weight[6] supports the theory of the protective PEG hydrate. In our laboratory we found that additions of maltodextrin to PEG solutions decreased the amount of PEG hydrate that was formed and decreased the protection of LDH.[13] Izutsu et al. also reported that sugars and polymers inhibit the formation of PEG hydrate.[23]

The results from this study show that the amount of ice surface in relation to the amount of PEG hydrate can be crucial for the protection of LDH, if it is assumed that the hydrate protects the protein mainly by

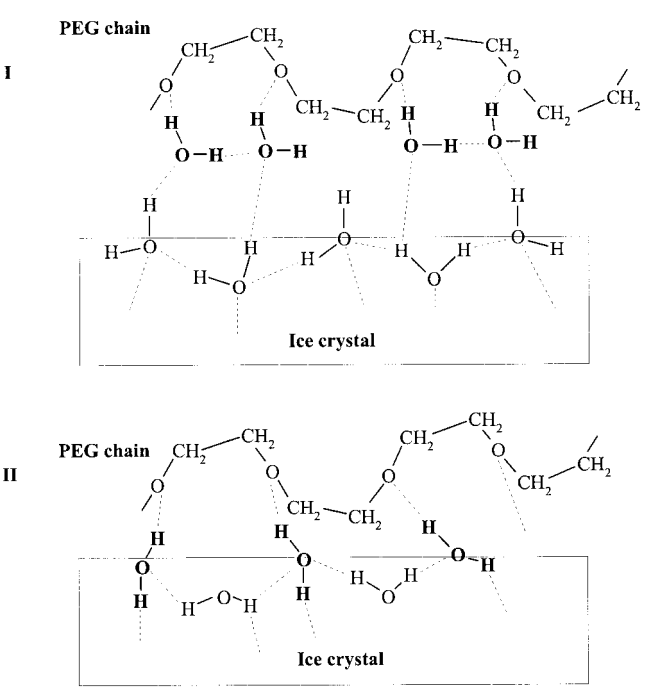

Figure 4 A schematic illustration of two possible interactions (I and II) between PEG 6000 and ice.

hindering its destructive interaction with the ice crystals.

CONCLUSIONS

Increasing concentrations of PEG yield a larger amount of PEG hydrate. The formation and stability of the PEG hydrate at low temperatures is greatly dependent on the temperature history in the freeze–thawing process. The maximum amount of hydrate and the most stable phase is obtained when the freezing temperature is low (≤ -60°C) or by giving the process time to proceed at a higher temperature using a very low cooling rate or a ramp period. The melting enthalpy of the hydrate at -15°C is dependent on the heating rate at thawing but not on the cooling rate of the freezing process if the freezing temperature is -60°C. A moderate cooling rate and a low freezing temperature in combination with a low heating rate thus seems to create the most stable PEG hydrate. The transformation at -15°C is a partly reversible process.

The amount of bound water can be directly calculated from the melting enthalpy of the PEG hydrate at -15°C in a certain concentration range and with an adequate temperature history. The number of water molecules per PEG unit in 20–80 mg/mL PEG 6000 solutions was 2.4–2.7. The values are independent of cooling rate if a low heating rate is used, but a higher heating rate affects the stability of the hydrate.

The effects of the addition of NADH to the PEG–protein solution indicate that competing interactions between the protein and the cofactor can influence the stability of the PEG hydrate.

The results from this study indicate that the amount of ice surface in relation to the amount of available PEG hydrate can be crucial for the protection of LDH if it is assumed that the hydrate protects the protein mainly by hindering its destructive interaction with the ice crystals. The formation of PEG hydrate is a slow process, and the hydrate cannot be formed with very high cooling rates. By contrast, with fast cooling a very large ice area is created that claims a large amount of PEG hydrate. Therefore, the protection of the protein will be insufficient and the denaturation increases at the ice surface.

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