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The cold-induced denaturation of lactate dehydrogenase at sub-zero temperatures in the absence of perturbants

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The cold-induced denaturation of lactate dehydrogenase has been determined in an unfrozen, cryoprotectant-free solution at sub-zero temperatures. The cold-induced denaturation temperature (T_L) has been found to be −28°C. The results for the first time clearly establish that temperature alone can induce denaturation in a cooled protein solution. The validity of earlier data, obtained in the presence of perturbants (particularly pH or guanidinium chloride), is discussed.

Lactate dehydrogenase; Enzyme denaturation, cold; pH; Temperature; Cryosolvent

1. INTRODUCTION

The cold inactivation of enzymes has been studied for some time; see, for instance, phosphofructokinase [1], ATPase [2], and glucose-6-phosphate dehydrogenase [3]. The phenomenon has recently begun to be subjected to thermodynamic and mechanistic analyses, following the first direct measurements in 1985 [4] and 1986 [5], which showed that the process resembled the well-known thermal denaturation. However, extrapolations of protein free energy profiles indicate that in the majority of cases cold denaturation would occur below 0°C [6]. Freezing has to be avoided, since freeze denaturation (e.g. [7]) is an entirely different process, due mainly to concentration effects. Thus, the practical difficulties involved in studying such sub-zero temperature phenomena have required that in all cases it has been necessary to use some destabilising influence (usually pH, guanidinium chloride or cryosolvent) [8,9], to bring about denaturation at a temperature above the onset of freezing. Consequently, the possibility that cold denaturation is caused mainly by these perturbations and might not be induced by temperature alone, cannot be excluded.

Recently Hatley and Franks [10] have extrapolated the data obtained from a series of experiments in which lactate dehydrogenase was cold-denatured in mixed methanol/aqueous phosphate buffer solutions and estimated the expected cold-induced denaturation temperature (T_L) of this enzyme in a cryosolvent-free solution. From the extrapolation, −30°C was the value predicted for T_L of lactate dehydrogenase (LDH) in such an environment. Although a precedent is set by the accuracy of such extrapolations in predicting heat denaturation (T_H) data [11,12] the only way to confirm its validity is to directly observe cold denaturation under perturbant-free conditions.

In order to achieve the necessary sub-zero temperature conditions, ice nucleation must be inhibited. This can conveniently be done by dispersing the aqueous phase in the form of microdroplets in an inert carrier fluid. The theory and practice of this procedure have been described in detail [13,14]. It has also been applied to the study of enzyme-catalysed reactions [15–17], where it has been established that the solution contained in the emulsion droplets behaves in all respects as a bulk aqueous environment. Comparisons of the kinetics and pathways of several such reactions have shown up significant differences between aqueous media (emulsified or bulk) and mixed organic/aqueous cryosolvents. It has been demonstrated that undercooled water as reaction medium is best suited for studies of the effects of low temperatures and that cryosolvents can, and do alter kinetic and mechanistic details, even though they may not qualitatively affect the overall nature of the reaction.

We have therefore employed the undercooling technique to investigate the cold-induced transition of LDH. It has already been clearly demonstrated that the storage of LDH, for extended periods (years), in a

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Abbreviations: T_L, temperature at which cold-induced denaturation occurs; T_H, temperature at which thermal denaturation occurs; LDH, lactate dehydrogenase (EC 1.1.1.27); pH(T), temperature dependence of pH

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dilute undercooled solution at −20°C has no detrimental effect on its activity, following its recovery [18]. This is in contrast to frozen solutions where rapid deterioration is observed at sub-zero temperatures. Hence, the undercooled aqueous droplets can be considered to be a series of micro-environments in which the conditions closely resemble those in a bulk solution.

The technique is therefore suitable for the study of the low temperature behaviour of LDH in a cryosolvent-free solution and we report here the first observation of cold-induced denaturation in the absence of chemical perturbants.

2. EXPERIMENTAL

Protein denaturation was followed by monitoring the UV absorbance at 240 nm where the change in absorbance is greater during denaturation than at the more commonly used wavelength of 286 nm [19,20]. The temperature was monitored with a 170 μm foil thermocouple inserted directly into the sample solution. LDH (rabbit muscle type II, Sigma Chemical Co.) was diluted in 0.01 M phosphate buffer (pH 7 at 25°C) to a concentration of 2.5 mg/ml. 1 ml of this solution was then emulsified into 9 ml of mineral oil, using a low shear mixer. The emulsion was stabilized by placing 2 ml in a precooled cuvette. The cuvette was placed into the cell holder in a Perkin-Elmer 557 spectrophotometer. A blank was made in the same way, except that 1 ml of buffer was used in place of the enzyme solution and this was placed in the reference beam. Cooled methanol was circulated around the cells while a stream of dried nitrogen was used to prevent condensation on the cuvette faces. The emulsions were then cooled while the absorbance at 240 nm was monitored.

3. RESULTS

The observed transition in an undercooled solution at −28°C (fig.1) is in agreement with the value earlier predicted from an extrapolation of the T_L data obtained with cryosolvent mixtures [10].

4. DISCUSSION

This study indicates that the cold-induced denaturation of LDH in a cryosolvent-free buffer does take place, as previously predicted at ~ −30°C. If the absorbance of the heat-denatured protein is extrapolated back to T_L, then the magnitude of the absorbance change at T_L corresponds to that measured at T_H. It is uncertain whether at either T_L or T_H the protein is completely unfolded [21,22]; our results do indicate that the degree of residue exposure is approximately the same at the two temperatures.

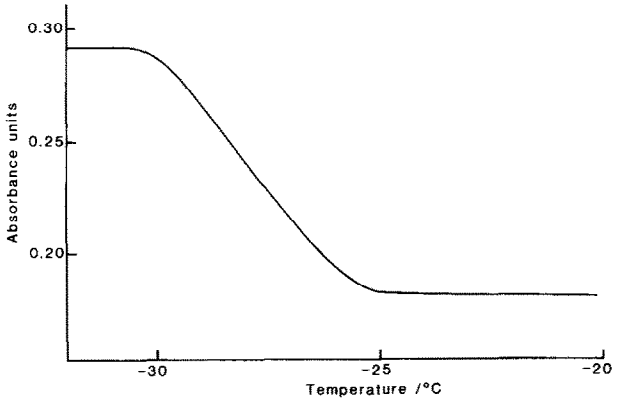

Fig.1. The change in absorbance corresponding to the cold-denaturation of lactate dehydrogenase in an unfrozen, cryoprotectant-free, pH 7.2 (at 25°C), phosphate buffer.

Many reliable data exist on the thermal denaturation processes of proteins in aqueous solutions at physiological pH values [23]. A comparison with similar data obtained from low temperature studies would be helpful in leading to a clearer understanding of protein conformational stability. However, to date, all attempts to measure T_L have relied on the partial destabilisation of the protein by some external chemical perturbant to bring the transition into a measurable temperature range. Such perturbants include pH, urea, guanidinium chloride or organic solvents. The influences of these destabilising materials on thermal denaturation have been extensively documented, and it has been demonstrated that they can have markedly different effects on both the thermodynamics of denaturation and even the conformation of the denatured protein [21,22]. When two or more influences (e.g. pH and temperature) are combined, the overall interpretation of the data has to rely on complex empirical models [24].

Although such models are able to represent the interrelationship of the mixed perturbants with respect to their effects on T_H, there is no obvious reason why they should equally well describe T_L, since cold-induced denaturation is likely to be dependent on entirely different factors [25]. Where the influence of mixed solvents on both T_H and T_L has been directly compared, the observed effects have been different. Consequently, the empirically derived model that explains the solvent effect on T_H has been found to be totally inadequate in attempts to describe the effects on T_L (Hatley and Franks, in preparation). Therefore, the common device of raising T_L by a change in pH is only of limited value, because the derived thermodynamic quantities are only applicable to the particular system under study and cannot reliably be used to validate either the general case or the separate effects of temperature or pH.

In the work here reported, the only variation in the environment of the protein, apart from the measured temperature change, was the change in pH with temperature pH(T). It is impossible in a simple scanning experiment to separate these two factors but their relationship should not be overlooked.

In thermal denaturation studies, the pH(T) relationship has usually been ignored; the quoted pH refers usually to the measured value at some near ambient temperature [8]. If denaturation measurements are made on solutions with a pH near the protein stability

optimum, then the results will be accurate, because the ΔpH – depending on the buffer – will be at most 0.5 units [14] during heating to T_H. The relatively broad pH/stability profile [23] of most enzymes will ensure that the enzyme structure is not markedly affected by this change.

However, if pH is deliberately used as a perturbant to reduce the stability of an enzyme – as has been the case in some allegedly 'cold-induced' denaturation experiments [8] – then the pH(T) dependence can become of great significance. The pH selected for such studies is usually near the protein stability limit, where the protein becomes extremely pH sensitive. Hence the small change in pH, induced by a reduction in the temperature, can result in a significant destabilisation of the protein. A difficulty then arises in clearly defining which effects are due to pH and which due to temperature. As mentioned earlier, this can lead to significant errors in the interpretation of experimental data.

In the case of cold denaturation, it was particularly important that major pH shifts, induced by temperature, should be avoided to enable the establishment of cold-induced denaturation as a real phenomenon to be achieved. Undercooling allows the protein to be prepared in a buffer at the pH stability maximum. Consequently, on cooling, any effect of pH changes on the stability of the enzyme is negligible and measured values during cooling can be shown to be directly and unambiguously related to a temperature-induced transition.

Results from undercooled systems could provide more direct thermodynamic information and so lead to a better insight into the balance of the forces which determine the conformational stability of proteins and to the part played by low temperature transitions in the cold adaptation and cold resistance of living organisms [14].

Undercooling has permitted the observation of a cold denaturation at sub-zero temperatures in a cryosolvent-free solution at physiological pH. Therefore, it has been established for the first time that cold-denaturation is a real phenomenon induced by a temperature change and not merely a side-effect of the temperature dependence of some perturbant influence.

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