Effects of Additives on Heat Denaturation of rhDNase in Solutions[1]

Hak-Kim Chan,[2,3,6] Kwok-Leung Au-Yeung,[2,4] and Igor Gonda[2,5]

Received November 22, 1995; accepted February 1, 1996

Purpose. To study the thermal stability of recombinant human deoxyribonuclease I (rhDNase) in aqueous solutions.

Methods. Differential scanning calorimetry (DSC) was used to measure the denaturation or melting temperature (T_m) and enthalpy (H_m) of rhDNase. The effects of denaturants (guanidine HCl and urea) and additives (mainly divalent cations and disaccharides) were investigated at pH 6–7.

Results. The T_m and H_m of rhDNase in pure water were measured as 67.4 °C and 18.0 J/g respectively, values typical of globular proteins. The melting peak disappeared on re-running the sample after cooling to room temperature, indicating that the thermal denaturation was irreversible. The latter was due to the occurrence of aggregation accompanying the unfolding process of rhDNase. Size exclusion chromatography indicated that during heat denaturation, rhDNase formed soluble high molecular weight aggregates with a molecular size >300kD estimated by the void volume. Of particular interest are the divalent cations: Ca^{2+} stabilizes rhDNase against thermal denaturation and elevates T_m and H_m while Mg^{2+}, Mn^{2+} and Zn^{2+} destabilize it. Sugars also stabilize rhDNase. As expected, denaturants destabilize the protein and lower the T_m and H_m. All destabilization of rhDNase can be prevented by adding Ca^{2+} to the solutions.

Conclusions. $CaCl_2$ and sugars were found to stabilize rhDNase against thermal denaturation while divalent cations, urea and guanidine HCl destabilize the protein. The effects could be explained by a mixture of mechanisms. For Ca^{2+} the protective effect is believed to be due to an ordering of the rhDNase structure in its native state, and by prevention of breaking of a disulfide bridge, thus making it less susceptible to unfold under thermal stress.

KEY WORDS: rhDNase; heat denaturation; protein; additives; calorimetry.

INTRODUCTION

The technique of scanning calorimetry has been applied extensively to study reversible thermal transitions in proteins (1). Equilibrium thermodynamic data (heat capacity, enthalpy and entropy) of native and unfolded proteins have been obtained to investigate, e.g., 2-state denaturation as well as specific protein interactions such as solute or ligand complexation. Such studies are not possible with proteins which undergo irreversible aggregation during heating. However, the denaturation temperature and enthalpy can be used as a screening tool for the effects of additives on protein stability (2). Recombinant human deoxyribonuclease I (rhDNase I, dornase alfa, Pulmozyme®) has been found to reduce the viscosity of cystic fibrosis sputum *in vitro* (3), and to be clinically effective for treatment of cystic fibrosis (4). The protein is in a liquid formulation used for nebulization. The biochemical properties of rhDNase have been described previously (5). In the present study, differential scanning calorimetry was carried out to screen the effects of additives on thermal denaturation of rhDNase in solutions.

MATERIALS AND METHODS

Materials

rhDNase at a concentration of 20.0 mg/ml in deionized water was used as the stock solution. The solutes (additives) which were studied for their effects on thermal stability of rhDNase were: α-lactose monohydrate (Sigma, lot 72H0563), mannitol (Sigma, lot 31H0181), trehalose (Sigma, lot 112H3903), sucrose (AR, Mallinckrodt, lot 8360 KBTA), calcium chloride dihydrate (Baker Analyzed), magnesium chloride.$6H_2O$ (Baker Analyzed), manganese chloride.$4H_2O$ (AR, Mallinckrodt, lot KTHG), sodium chloride (AR, Mallinckrodt, lot 7581 KDME), PVP-40 (Sigma, lot 57F-0008), guanidine hydrochloride (Sigma, lot 102H5601), urea (crystallized, ultrapure grade, Boehringer Mannheim, lot EGA159). Sodium hydroxide (AR, Mallinckrodt) and hydrochloric acid (AR, Mallinckrodt) were used for pH adjustment.

Differential Scanning Calorimetry (DSC)

Measurements were carried out on a Seiko DSC 120 differential scanning calorimeter which has a detectability <2 μW. The solutions were sealed in a silver sample cell (60 μl) with water used as the reference. Preliminary runs were done on a series of pure rhDNase solutions using scanning rates ranging 0.1–2.0 °C/min. Generally, a fast heating rate will result in a broader peak with a shift in the baseline, while slow heating will give a low signal to noise ratio. On the other hand, while low protein concentrations will not give a sufficient DSC signal, high concentrations will facilitate aggregation. For rhDNase, the optimal protein concentration and heating rate were found to be around 10 mg/ml and 1.2 °C/min respectively which were subsequently employed throughout this study.

Preparation of rhDNase Solutions Containing Additives

Aqueous solutions of different additives were prepared by dissolving a known amount of the solute in deionized water. The solution pH was adjusted using dilute (0.005–0.01 N) NaOH or HCl to a value between 6 and 7 (this will give rise to a difference within 1.0 °C in the T_m and 0.5 J/g in H_m, see Results and Discussion). An aliquot of 70–100 μl of the 20 mg/ml rhDNase stock solution was then mixed with an equal volume of additive solutions (which was substituted by pure water for the control rhDNase samples). The final pH of the

[1] Partly presented at the 9th annual meeting of AAPS, San Diego, California.

[2] Department of Pharmaceutical Research and Development, Genentech, Inc. 460 Point San Bruno Boulevard, South San Francisco, California 94080.

[3] Present affiliation: Department of Pharmacy, University of Sydeny, NSW 2006, Australia.

[4] Present affiliation: Department of Chemical Engineering, University of California, Berkeley, California.

[5] Present affiliation: Aradigm Corp., Hayward, California 94545.

[6] To whom correspondence should be addressed.

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solutions was found in the range of 6.6–7.2, except for the $CaCl_2$ samples at 100 and 1000 mM (pH 6.3) and samples in the pH dependent study (pH 5–9). No buffer was added in order to avoid possible intereactions of the buffer species with rhDNase and/or additives.

Size Exclusion HPLC

Size exclusion chromatography (SEC), as previously described (6), was employed to determine the amount of monomer and aggregates in heated rhDNase solutions. In the present study, the urea samples were measured to determine if aggregation of rhDNase still occurred in the presence of the denaturant. Heated solutions of rhDNase in 2 and 4 M urea were collected from the DSC sample cell and diluted with the SEC mobile phase to approximately 1 mg/ml rhDNase for the measurement. The mobile phase was: 4 M urea, 5mM HEPES, 150mM NaCl, 1mM $CaCl_2.2H_2O$, adjusted to pH 7.0 with NaOH. The running conditions were: absorbance measured at 280 nm, injection volume 100 μl (1mg/ml), flow rate 1.0 ml/min, running time 20 min. The % amount of aggregates was expressed as a fraction of the peak area eluted at 5.6 min to the total area at 5.6 min and 7.6 min.

RESULTS AND DISCUSSION

Proteins unfold or 'melt' at elevated temperatures and this usually involves endothermic heat changes. The apparent denaturation temperature (T_m) and enthalpy (H_m) of 10 mg/ml rhDNase in pure water at pH 6.8 were found to be 67.4 ± 0.3 °C and 18.0 ± 0.2 J/g (n = 4) respectively, values typical of globular proteins (7). The melting peak disappeared on re-running the sample after cooling to room temperature, indicating that the thermal denaturation was irreversible. The latter is attributed to aggregation of rhDNase accompanying the unfolding. The unfolding is supported by two-dimensional proton NMR spectroscopy of rhDNase (Zhiwen Zheng and O. Jardetsky, Stanford Magnetic Resonance Laboratory, unpublished results). Overnight NMR runs in D_2O showed that while the amide protons at 7–8 ppm were still clearly visible in the native rhDNase spectrum, there was an almost complete disappearance of these protons in the spectrum for aggregated rhDNase. The more rapid deuterium exchange in the aggregates indicates a more open or unfolded structure of the protein molecules. Unfolding is further supported by a significant decrease in the NMR peak dispersion in the spectrum. Size exclusion chromatography indicated that during denaturation, rhDNase formed aggregates with a molecular size >300kD estimated by the void volume (c.f. 33 kD for the monomer). Therefore, the peak temperature and enthalpy measured may not be the 'true' values for melting of rhDNase alone as there would be heat changes (usually exothermic) contributed from the aggregation event. The aggregates were found to dissociate to monomers in sodium dodecyl sulfate (SDS) gel without dithiothreitol as the reducing agent, suggesting that they were largely non-covalent nor linked by disulfide bonds. In addition, some higher molecular weight aggregates were not dissociable. These non-dissociable aggregates ran as monomer in the presence of dithiothreitol, indicating that they are linked by disulfide bonds.

Effects of pH

Figure 1 shows that the T_m decreases with increase in pH which coincides with increased departure from the isoelectric point, pI ≈ 3 − 4 (8), suggesting the involvement of electrostatic charge in the protein's stability. As will be described below, this may also be related to the pH-dependence of calcium binding. The T_m decrease is accompanied by a slight H_m increase (Figure 2). Outside the pH range of 6–8, the thermal transition becomes significantly broader (denaturation onset temperature becomes lower, Figure 1) which may suggest involvement of multiple denaturation pathways.

Effects of Denaturants

Urea and guanidine HCl are well known protein denaturants. Although both urea and guanidine are capable of hydrogen bonding with proteins, their denaturation mechanisms may not be the same, as guanidine HCl is also capable of electrostatic interactions with proteins. As expected, rhDNase was destabilized in both denaturants. In urea, both T_m and H_m decrease monotonically with the denaturant concentration (Figure 3a). In guanidine HCl (plot not included), T_m decreases to 65° and 60.1 °C at 0.15 and 0.5 M of the denaturant, respectively. Guanidine HCl is also known to be the more potent denaturant. For rhDNase, 0.5 M guanidine HCl lowers T_m to the similar extent (i.e., 61 °C) as 2 M of urea. As shown in Figure 3b for urea, the destabilization can be protected by Ca^{2+}. Similarly, when 10 mM Ca^{2+} was added to rhDNase containing 0.15 M guanidine HCl, the T_m increases to 70 °C. The Ca^{2+} stabilization will be further discussed under 'Effects of Cations'.

One possible way to obtain the 'true' melting data is to measure the T_m and H_m in the presence of a denaturant such as urea. It is anticipated that a protein might remain as monomer due to denaturant binding. However, sizing HPLC showed that the thermally denatured rhDNase was still highly aggregated at urea concentrations of 2 and 4 M (88 and 98% aggregates, respectively). The values of T_m and H_m measured at zero urea concentration almost coincide with values extrapolated from measurement at higher urea concentrations (Figure 3a). Higher urea concentrations were not attempted as it becomes difficult to locate the T_m accurately at a urea concentration ≥4M.

One concern of using urea at high temperature is the possible decomposition of urea to cyanate which could lead to modification of the protein by carbamylation reaction. However, in view of the close agreement between the extrapolated value of T_m and H_m and the measured values at zero urea concentration, this reaction did not appear to have happened.

Effects of Sugars

Figure 4 shows the protective effects of different sugars (lactose, sucrose, and trehalose as disaccharides; mannitol as monosaccharide) on rhDNase against thermal denaturation. In general, both the T_m and H_m appear to increase monotonically with the sugar concentration. The disaccharides are very similar in their stabilizing effect based on their close T_m and H_m values. The effect of mannitol also appears to be comparable to the dissacharides.

Protection of proteins against heat denaturation by sugars is a well documented phenomenon (9–11) and has been explained thermodynamically by preferential exclusion of the sugar from

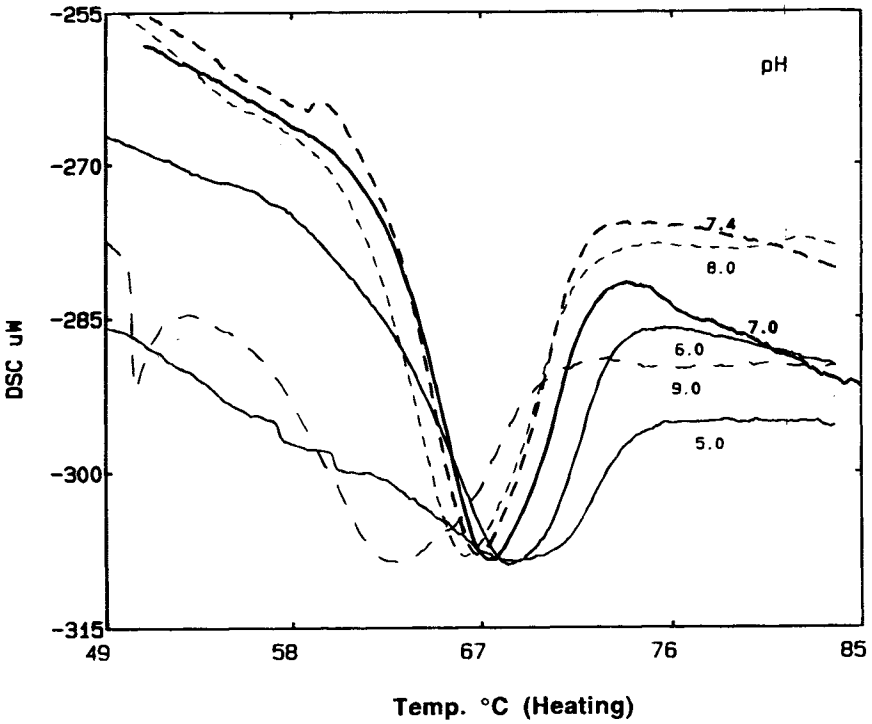

Fig. 1. DSC thermograms of rhDNase at different pH.

the surface of the protein molecules (11). The basic assumption is that the chemical nature of the interactions between the protein and its environment does not change when the protein unfolds. This will not be valid if there are specific protein-solute bindings.

Effects of Cations of Inorganic Salts

Ca^{2+} was found to stabilize rhDNase against thermal denaturation. Initially rhDNase was prepared in deionized water without free $CaCl_2$. The ratio of total Ca^{2+} to rhDNase was determined to be 3 by atomic emission spectroscopy. These Ca^{2+} are most likely bound to the protein molecules rather than freely existing in the solution (see below). Figure 5 shows the concentration dependent effect of Ca^{2+} on stabilization of rhDNase. When the $CaCl_2$ concentration changes from zero to 10 mM, the increases in T_m and H_m are most rapid. The effect reaches a maximum at around 100 mM; further increase in concentration to 1000 mM does not raise the T_m or H_m. In the presence of another stabilizer, lactose, a combined effect was observed (T_m = 76.4 °C, H_m = 21.9 J/g at $CaCl_2$ 50 mM, lactose 100 mg/ml). The effects of divalent cations on the enzymatic activity of bovine pancreatic deoxyribonuclease were reported (12, 13). It was shown that Ca^{2+} was able to protect bovine DNase from reduction of the two disulfide bonds by reducing agents (12). The molecular details of the effects of divalent ions on the structure of rhDNase are not known yet. However, in view of the extensive (~80%) homology and general similarity in biochemical properties, it is expected that rhDNase behaves in many respects similar to bovine DNase. For example, the four cysteine residues are in the same relative sequence positions as in the bovine DNase (3), and the same disulfide pairing occuring in both proteins has been confirmed (14). In addition, a histidine residue at position 131 in bovine DNase has been shown to be essential for enzymatic activity (15, 18), and is conserved in rhDNase (3, 14). Spectroscopic studies showed that circular dichroism and optical rotatory dispersion of bovine DNase undergo significant changes as a result of calcium ion binding. Mn^{2+} causes smaller changes while magnesium ions cause no such changes at all (16). These structural changes detected by optical means are paralleled by the level of protection these cations provide against inactivation of DNase by trypsin, with calcium giving complete protection, suggesting an important role of calcium in the preservation of the native conformation. Bovine DNase unfolds at high pH as observed by changes in the hydrodynamic behavior (17). When calcium ions are bound to at least two sites on the protein, the pH-induced change is

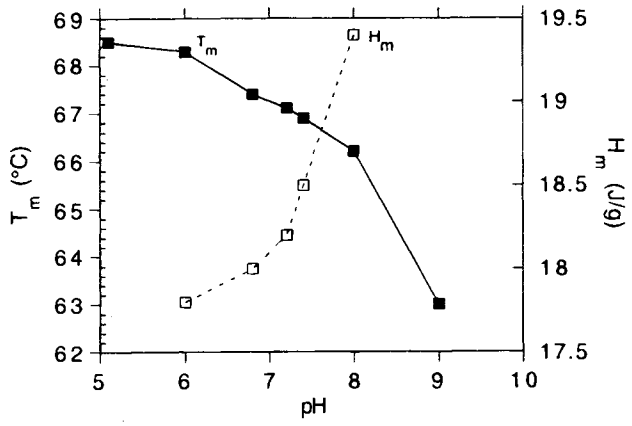

Fig. 2. The T_m and H_m of rhDNase at different pH (H_m at pH 5 and 9 are not included because the broad transition peaks lead to a large uncertainty).

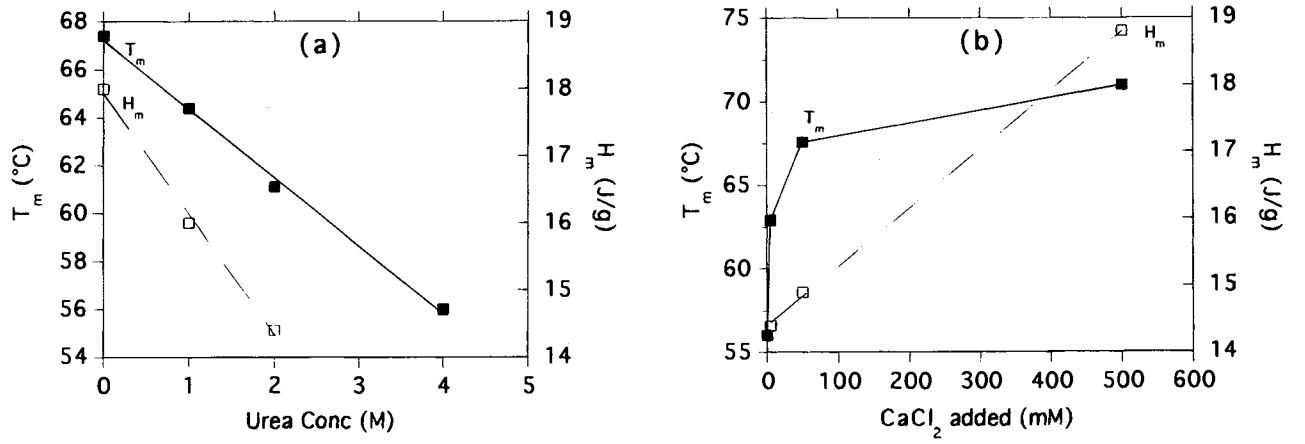

Fig. 3. (a) the effect of urea at different concentrations on the T_m & H_m of rhDNase, (b) the protection against 4M urea denaturation of rhDNase by adding $CaCl_2$ at different concentrations.

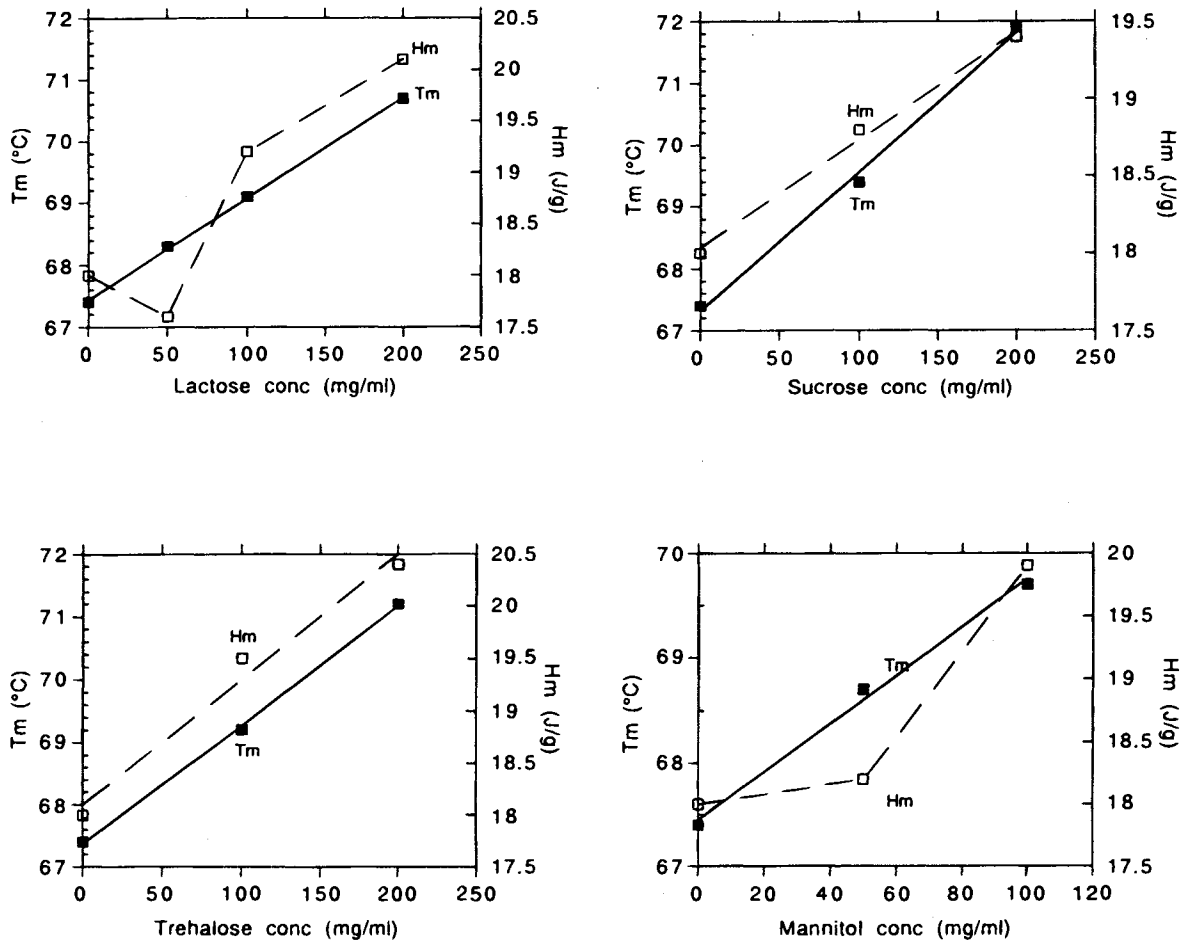

Fig. 4. The effect of sugars at different concentrations on the T_m & H_m of rhDNase.

reversed and DNase is maintained in the more active structure. Such and Oefner (18) identified three calcium-binding sites in the structure of bovine pancreatic DNase: one is associated with the active site of the enzyme, whereas the other two structurally important calcium-binding sites are in vicinity of the two disulfide bridges in the molecule. Calcium ion binding was shown to prevent breaking of the disulfide bridge between Cys_{98}-Cys_{101} on DNase; loss of this S-S bond inactivate the enzyme (12). Human DNase extracted from serum and pancreas similarly retains activity after treatment with disulfide reducing agents in the presence of calcium ions (19). At a pH of 7.5, bovine DNase binds 2 calcium ions strongly. At pH 5.5, bovine DNase has

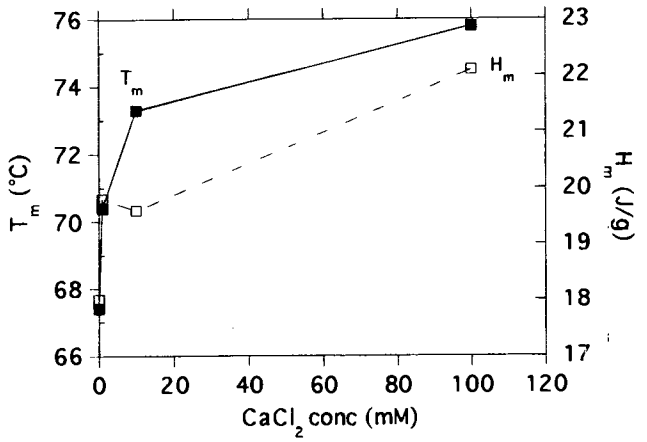

Fig. 5. The effect of $CaCl_2$ at different concentrations on the T_m & H_m of rhDNase.

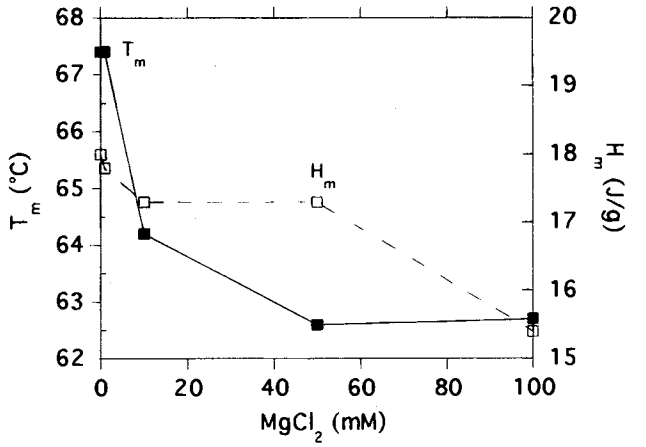

Fig. 6. The effect of $MgCl_2$ at different concentrations on the T_m & H_m of rhDNase.

only 1 strong calcium binding site. Recent work with rhDNase suggests similar properties to the bovine DNase: at pH 5–6 one calcium ion binds strongly, probably in the vicinity of the active site. Several other sites with weaker binding are present (14). Calcium ions stabilise rhDNase against trypsin digestion which also suggests the role of calcium in stabilizing the native conformation of this enzyme (14). This prior work does not address directly the role of calcium in protecting rhDNase against heat denaturation. However, taking together with the current observations, we surmise that calcium ions stabilize the native conformation of rhDNase against heat denaturation by an ordering of the structure in its native state, as suggested for bovine DNase (17) and by prevention of breaking of a disulfide bridge that would lead to unfolding, denaturation and loss of activity under the thermal stress. Since it is expected that negatively charged residues would be involved in the coordination of the Ca^{2+}, pH is likely to play a role. The results of the effect of pH on stability of rhDNase described earlier may also be partly due to the change of calcium binding.

Monovalent ions such as sodium chloride are expected to display a rather nonspecific interaction with proteins via a general ionic strength effect or charge neutralization (19, 20). The net result depends on the relative effect of the ions on the native and denatured protein. The T_m was found to first decrease to 65.3 °C at 75 mg/ml of NaCl and then increase monotonically with the sodium chloride concentration to 70.1 °C at 200 mg/ml of NaCl. H_m shows a general decrease with increase in NaCl concentration (H_m = 14.5 J/g at 200 mg/ml NaCl). The higher T_m and yet lower H_m suggest stronger intramolecular interactions in the presence of the electrolyte (thus higher T_m), but in a less cooperative fashion such that not as much energy is required to disrupt the protein structure (hence the lower H_m). Another explanation for the lower H_m is due to an increased positive heat contribution from aggregation. The presence of NaCl will tend to neutralize the surface charge of the unfolded protein molecules. Consequently, the molecules will be more favorably brought together for aggregation. Aggregation event is expected to be exothermic since heat is produced from formation of new bonds intermolecularly. This would make the overall heat apparently less endothermic.

Figure 6 shows the concentration dependent effect of Mg^{2+} on destabilization of rhDNase. A further increase of the $MgCl_2$ concentration to 1000 mM slightly elevates T_m by 0.4°C which is likely due to a general ionic strength effect. In bovine DNase, Mg^{2+} was found to inhibit the enzymatic activity by competing for Ca^{2+} binding to one of the two Ca^{2+} binding sites (12, 13). Such competition between Ca^{2+} and Mg^{2+} may also occur in the human recombinant DNase. rhDNase has one strong calcium binding site and several weaker ones (14), some or all of them are presumably required for structural stability. The protection of rhDNase by Ca^{2+} against Mg^{2+}-induced destabilization supports this competition hypothesis (Figure 7). Similar protection by Ca^{2+} against thermal denaturation was also observed in the case of Mn^{2+} (plot not included). In the presence of 100 mM $MnCl_2$ the T_m was lowered to 65.3 °C, which was elevated to 69.5°C when 10 mM $CaCl_2$ was added. $ZnCl_2$ at 1 mg/ml was found to precipitate rhDNase, and was not studied further.

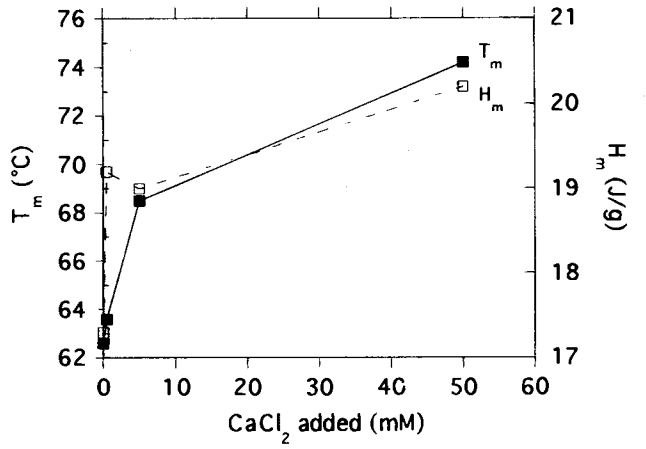

Fig. 7. The protection of rhDNase against 50 mM $MgCl_2$-induced destabilization by adding $CaCl_2$ at different concentrations.

CONCLUSIONS

Thermal denaturation of rhDNase was studied in the presence of additives by DSC. $CaCl_2$ and sugars were found to be stabilizers (protectants). Destabilizers included divalent cations, urea and guanidine HCl. The effects could be explained by a mixture of mechanisms: specific solute binding (dominant for

Ca^{2+}), electrostatic charge (probably most important for pH and NaCl), Timasheff's preferential hydration or solute exclusion (for sugars). Compared to the sugars, even a relatively low Ca^{2+} concentration (say, 10 mM, i.e., 1.5 mg/ml) imparts good stability to the protein.

ACKNOWLEDGMENTS

We thank Dr. Linda DeYoung for general advice on protein unfolding and escpecially for the suggestion to use urea to prevent aggregation, Dr. Steve Shire for general discussions and for a wealth of information on DNase, and Ms. Henrietta Ong for running the HPLC sizing of the urea samples.

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