Influence of Calcium Ions on the Structure and Stability of Recombinant Human Deoxyribonuclease I in the Aqueous and Lyophilized States

BEI CHEN,[†] HENRY R. COSTANTINO,[‡] JUN LIU,[†] CHUNG C. HSU,[†] AND STEVEN J. SHIRE*,[†]

Contribution from *Pharmaceutical Research and Development, Genentech, Inc., 1 DNA Way, South San Francisco, California 94080.*

Received July 7, 1998. Accepted for publication January 13, 1999.

Abstract □ The effect of calcium ions on the structure and stability of recombinant human DNase I (rhDNase) in the aqueous and solid (lyophilized) states was investigated. Fourier transform infrared (FTIR) spectroscopy was used to examine the overall secondary structure, while chemical stability was monitored in terms of deamidation and soluble aggregate formation at 40 °C. The exogenous calcium was removed by EGTA. This process can remove all but approximately one calcium ion per protein molecule. Analysis of the FTIR spectra in the amide III region in either the aqueous or lyophilized state demonstrated that removal of exogenous Ca^{2+} by EGTA-treatment had little effect on the secondary structure (and lyophilization-induced rearrangement thereof). For the aqueous solution, circular dichroism was used as an independent technique and confirmed that there was no large overall change in the secondary or tertiary structure upon the removal of calcium. The primary degradation route for the aqueous protein was deamidation. For the EGTA-treated protein, there was also severe covalent aggregation, e.g., formation of intermolecular disulfides facilitated by the cleavage of Cys173-Cys209. The aggregates exhibited a markedly different secondary structure compared to the native protein. For instance, the β-sheet band observed at ca. 1620 cm^{-1} wavenumber in the amide I second derivative spectra was increased. Enzymatic activity was completely lost upon aggregation, consistent with the cleavage of the aforementioned native disulfide. For the protein lyophilized in the presence of Ca^{2+}, there was no increase in deamidated species during solid-state storage; however, some aggregation was observed. For the lyophilized EGTA-treated protein, aggregation was even more pronounced, and there was some loss in enzymatic activity upon reconstitution. Thus, the removal of calcium ions by EGTA-treatment decreased the stability of rhDNase in both the aqueous and solid states even though no large overall calcium-induced structural changes could be observed by the techniques used in this study.

Introduction

It has been established that divalent metal cations, particularly Ca^{2+}, play an important role in the structure and function of deoxyribonuclease I (DNase).[1] For the bovine-derived molecule (bDNase), it was shown that the removal of Ca^{2+} from aqueous solution induced changes in the secondary structure, as evidenced in the far-UV circular dichroism spectrum, although no change was seen by sedimentation velocity analysis.[2] In addition, the presence of Ca^{2+} has been shown to increase bDNase's resistance to proteolysis (e.g., by trypsin),[2,3] play a role in refolding the reduced protein,[4] and impart stability toward pH-induced structural alterations.[5] The biological activity of bDNase is lost upon the removal of calcium ions.[1,6] According to Price,[7] bDNase has two strongly binding Ca^{2+} sites and several weakly binding ones. Similar results have demonstrated the importance of Ca^{2+} for the structure and function of the homologous recombinant human form of DNase (rhDNase).[8]

Suck and co-workers[9−11] have examined the structure of bDNase by X-ray crystallography. The protein has a mixed α/β composition, with a hydrophobic core of two, six-stranded β-pleated sheets flanked by α-helices and extensive loop regions. The electron density map also revealed two Ca^{2+} bound to the native protein, one located in a flexible loop formed by residues Gly100 to Gly105, and another in the loop formed by residues Asp201 to Cys209. Both sites are located in proximity to cystine bridges, one between Cys101 and Cys104 and the other between Cys173 and Cys209. The latter disulfide bridge, which is essential for enzymatic activity, is protected against reduction in the presence of Ca^{2+}.[4] It was hypothesized that the stabilizing effects of Ca^{2+} were due to configurational restraints imposed by binding of the cation in the flexible loop regions.[9,10] The putative catalytic Ca^{2+} binding site is located in the proximity of residues Glu78 and His134 at the active center.[11] The X-ray crystal structure of rhDNase has also been determined and is very similar to the bovine form.[8,12]

In the present investigation, we have focused on the effect of removal of exogenous Ca^{2+} on rhDNase structure and biochemical stability, in particular, aggregation and deamidation. The protein was studied both in aqueous solution and the freeze-dried form. The latter is often considered as a common dosage for biopharmaceuticals since many deleterious processes are retarded upon the removal of water (and thus shelf life can be extended).[13] The data reveal that the influence of Ca^{2+} on the rhDNase molecule observed for the aqueous solution also persists following lyophilization and storage in the solid state.

Materials and Methods

Materials—rhDNase (a glycosylated protein with MW ~ 32.7 kDa) was produced at Genentech, Inc. (South San Francisco). Deamidation is the major route of degradation of the protein in aqueous solution. The degradation happens during the manufacturing process, resulting in the starting material with ~60% deamidation. All other chemicals were of reagent grade and purchased from commercial suppliers.

Protein Lyophilization—rhDNase was provided in an aqueous solution consisting of ~18 mg/mL protein, 150 mM NaCl, and 1 mM $CaCl_2$ at pH ~ 6.5 ± 0.5. To remove calcium ions, this solution was exhaustively dialyzed against 1 mM EGTA (pH ~ 6.5 ± 0.5) at 5 °C. It should be noted that it is difficult to completely remove bound Ca^{2+}, and even after exhaustive dialysis against the chelating agent, there is approximately one calcium ion bound per rhDNase molecule.[8] Samples were filtered (0.22 μm, Millipore-

* To whom correspondence should be addressed.
[†] Genentech, Inc.
[‡] Current address: Alkermes, Inc., 64 Sidney Street, Cambridge, MA 02139.

10.1021/js980273g CCC: $18.00
Published on Web 03/12/1999

GV) and filled (2 mL) in 3-cc glass vials. The lyophilization cycle consisted of freezing for 4 h at −55 °C, followed by drying at a chamber pressure of 50 μm Hg and a shelf temperature which was ramped at a rate of 10 °C/h to a maximum of 25 °C and held for 50 h. A Leybold (Germany) model GT20 freeze-dryer was used. The moisture content of lyophilized samples was 6 ± 3%. There was no observed moisture transfer from the stoppers during storage (7 ± 2% after incubation for 24 months at 15 °C).

Determination of rhDNase Aggregation—Size exclusion chromatography (SEC) was used to monitor protein monomer, fragments, and soluble aggregates. A Hewlett-Packard 1090L system was used with a silica-based TSK Gel2000SWxl column (Tosohaas, Japan). Sample loadings were approximately 50 μg, and the flow rate for the mobile phase (phosphate-buffered saline; 5 mM sodium phosphate, pH 7.3, and 150 mM NaCl) was 0.5 mL/min. Molecular weight standards (BioRad) consisting of thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobulin (17 kDa), and cyanocobalamin (1.35 kDa) were used to calibrate the column. Silver-stained SDS−PAGE, under both reducing and nonreducing conditions, was employed to further examine protein fragments, monomer, and aggregates.

Determination of rhDNase Deamidation—The extent of deamidation of rhDNase was measured using tentacle ion-exchange chromatography.[14] Samples (~50 μg) were loaded on a LiChrosphere column (EM Separations) equilibrated with 1 mM $CaCl_2$, 0.1% acetate buffer, pH 4.7, and eluted with a linear gradient of 0−50% of 1 M sodium chloride in the same buffer. The flow rate was 0.8 mL/min.

Enzymatic Activity Assay—Measurement of rhDNase enzymatic activity was accomplished via a colorimetric method (using DNA complexed with methyl green as a substrate).[15] The activity of experimental samples was determined (in triplicate) relative to a rhDNase standard. Data are expressed as % specific activity, i.e., concentration of rhDNase determined by activity assay divided by that based on UV absorption of the sample.

Circular Dichroism (CD)—CD spectra were measured and analyzed as described previously.[8] All spectra were taken at a concentration of ~0.6 mg/mL rhDNase at room temperature using an Aviv 60DS spectropolarimeter. A 1-cm cylindrical cell was used for CD measurement above 250 nm with a 5-s averaging time for each data point taken at 0.5 nm interval and bandwidth of 0.2 nm. A 0.01-cm cell was used for CD measurement below 250 nm with a 5-s averaging time for each data point taken at 0.5 nm interval and bandwidth of 1.5 nm. Ellipticity values were converted to mean residue weight ellipticity using a mean weight of 112.8 for the protein.

Fourier transform Infrared (FTIR) Spectroscopy and Estimation of Secondary Structure—FTIR spectra were measured using an ATI-Mattson Galaxy 5022 IR spectrophotometer as described previously.[16] A total of 256 scans at 4 cm^{-1} resolution using Happ-Ganzel apodization were averaged to obtain each spectrum. Aqueous samples were measured using a 15-μm spacer between CaF_2 windows in a liquid cell (Spectra-Tech). The spectrum of pure water was subtracted out using Nicolet OMNIC 3.1 software in order to obtain the pure aqueous protein spectrum. Lyophilized samples were measured as KBr pellets (~1 mg of protein per 200 mg of KBr, pressure into a pellet at 5 kpsi compaction pressure); these conditions were shown not to induce any artifactual alterations in rhDNase secondary structure.[16] Spectra were analyzed by second derivatization (with smoothing equivalent to an approximate 10 cm^{-1} span) to determine the number of spectral bands and their approximate locations (OMNIC 3.1 software). Using this information as a starting point, the original spectra in the amide III region (1320−1220 cm^{-1}) were Gaussian curve-fitted using GRAMS/32 software (Galactic Industries), following the strategy detailed by Griebenow and Klibanov.[17] The secondary structure contents were calculated from the areas of the individual assigned bands and their fraction of the total area in the amide III region.[16,17] Data are presented as the average and deviation of at least three determinations. Secondary structure was also assessed and confirmed by qualitative examination of the second derivative amide I spectra (1700−1620 cm^{-1}).[18]

Results and Discussion

Effect of Ca^{2+} on Secondary Structure of rhDNase by Fourier transform Infrared (FTIR) Spectroscopy—

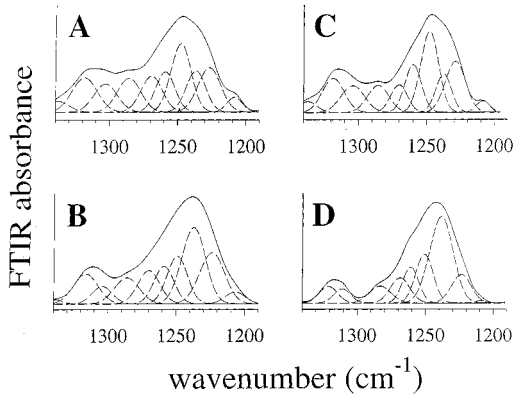

Figure 1—IR spectra for rhDNase I in the amide III region and their Gaussian curve-fitting: (A) in aqueous solution (EGTA-treated, no incubation); (B) in the lyophilized powder (EGTA-treated, no incubation); (C) deamidated form (aqueous protein incubated in 1 mM $CaCl_2$ for 120 days at 40 °C); (D) aggregated form (aqueous EGTA-treated protein incubated in the absence of exogenous calcium ions for 120 days at 40 °C). The solid and dotted curves represent the superimposed original spectra and curve-fit, and the dashed curves are individual Gaussian bands.

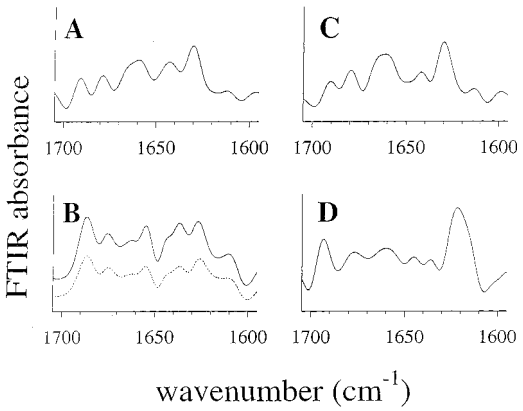

Figure 2—Inverted second derivative of the amide I IR spectra of rhDNase I: (A) in aqueous solution (EGTA-treated, no incubation); (B) lyophilized untreated power (solid line), and lyophilized EGTA-treated power (dotted line) (no incubation for either sample); (C) deamidated form (aqueous protein incubated in 1 mM $CaCl_2$ for 120 days at 40 °C); (D) aggregated form (aqueous EGTA-treated protein incubated in the absence of exogenous calcium ions for 120 days at 40 °C).

Recently, we examined the structure of rhDNase employing Fourier transform infrared (FTIR) spectroscopy, which allows for estimation of protein secondary structure in both aqueous and solid states.[16] It was seen that there was a dehydration-induced structural change, specifically a loss in α-helix and increase in β-sheet structures. Such alterations may be a common occurrence for proteins upon the removal of water.[17,18] In the present investigation, we have further extended FTIR spectroscopy to examine the effect of removing exogenous calcium ions on the protein structure in aqueous solution and also upon drying.

The FTIR spectrum, in the conformationally sensitive amide III region,[17] was measured for rhDNase in aqueous solution following EGTA-treatment to remove exogenous calcium ions (Figure 1A). The spectrum appears very similar to the protein in aqueous solution containing calcium ions, as presented earlier.[16] The amide I second derivative spectrum (Figure 2A) shows similar numbers of peaks and peak positions, but slightly different peak intensities, as for the spectrum for rhDNase with calcium reported previously.[16] To quantitate the structure in terms of the overall secondary structure, Gaussian curve-fitting of the original amide III spectra was employed.[17] A summary of the data is presented in Table 1 and shows that

Table 1—FTIR Analyses of Various rhDNase I Samples[a]

sample	secondary structure (%)		
	α-helix	β-sheet	other[b]
aqueous solution, with Ca^{2+}[c,d]	21 ± 2	23 ± 3	56 ± 6
aqueous solution, EGTA-treated	20 ± 2	26 ± 2	54 ± 3
lyophilized, with Ca^{2+}[d]	13 ± 2	41 ± 3	46 ± 2
lyophilized, EGTA-treated	14 ± 2	45 ± 3	41 ± 3
deamidated form[e]	21 ± 1	25 ± 2	54 ± 2
aggregated form[f]	10 ± 1	44 ± 4	46 ± 3

[a] The secondary structure of rhDNase was calculated by Gaussian curve-fitting the original amide III spectra. [b] Other secondary structure includes random coil and turns, and extended chains. [c] The aqueous solution contained 1 mM calcium chloride. [d] Data from ref 16. [e] Aqueous protein incubated in 1 mM $CaCl_2$ for 120 days at 40 °C. [f] Aqueous EGTA-treated protein incubated in the absence of exogenous calcium ions for 120 days at 40 °C.

removal of exogenous calcium had little effect on the overall secondary structure. The mixed α/β secondary structure estimated from the amide III FTIR spectra is in agreement with that predicted from consideration of the X-ray crystal structure of native rhDNase,[8,12] which is similar to bDNase.[9–11] As an independent method, we also employed circular dichroism (CD) to examine the structure of rhDNase in aqueous solution (data not shown). The secondary structure of rhDNase in the presence and absence of calcium as revealed by the far-UV CD were the same. Furthermore, the near-UV CD indicated that there was no change in the tertiary structure for the protein in the presence and absence of calcium ions. Therefore, data from two independent techniques, FTIR spectroscopy and CD, indicate that the removal of calcium does not affect the solution structure of rhDNase.

It is intriguing that a previous study on bDNase concluded that there was some change in the protein conformation upon addition of calcium based on circular dichroism (CD) data.[2] It was found that the addition of calcium (10^{-4} to 10^{-2} M) to bDNase resulted in an intensification of the CD bands with major peaks centered at 275 and 284 nm. It was also found that as the pH was decreased (from pH 8 to pH 3), the change in the CD spectrum (change in ellipticity at 215 nm) induced by 10^{-3} M $CaCl_2$ was eliminated. It was hypothesized that the affinity of bDNase for binding calcium was greatly decreased due to protonation of the amino acid side chains involved in binding calcium.[2] The explanation for the difference in the effect of calcium observed for rhDNase herein compared to the earlier bDNase study is uncertain. The two protein forms have a high structural homology, including the ability to bind calcium ions.[1,8–12] In our current study, we started with rhDNase in the presence of 1 mM $CaCl_2$. Even after exhaustive dialysis in the presence of the chelating agent EGTA, there is still approximately one calcium ion bound per rhDNase molecule.[8] In the bDNase study, no information was provided regarding the calcium level of the starting material. It is possible that the binding of calcium is less strong for bDNase, leading toward a more complete removal. A direct comparison of rhDNase and bDNase starting from the same, known level of calcium on the protein is necessary to shed light on this issue.

Chan et al.[20] have studied the thermal denaturation of rhDNase as a function of calcium ion concentration by solution differential scanning calorimetry (DSC). It was found that there was a significant increase in T_m and H_m (putative "melting" temperature and enthalpy corresponding to an endothermic event associated with protein denaturation) as the exogenous calcium ion concentration was increased. Furthermore, this event was associated with unfolding and irreversible aggregation of rhDNase. It was hypothesized that the effect was due to stabilization of rhDNase structure in its native state and protection of disulfides near calcium binding sites (which may be involved in aggregation via intermolecular disulfide formation). The latter effect has been well-established for bDNase and is also likely to play a role in the thermal denaturation of rhDNase. The former effect, if true, may be minor since there was quantitatively a similar percentage of ordered secondary structures found for the protein in the presence of calcium and following EGTA-treatment. It should be noted that such solution DSC studies, while valuable in obtaining information regarding denaturation at elevated temperatures, cannot necessarily be used as an indication of stability in aqueous solution at lower temperatures, or upon freeze-drying. The binding of calcium may stabilize rhDNase via mediation of the free energy of denaturation. For the classic, native-to-denatured state transition, the native state would be favored (lowered free energy) if it binds calcium more strongly than the denatured state. This view is consistent with an increase in T_m, without calcium-induced changes in protein conformation at lower (e.g., ambient) temperatures.

Next, we examined the secondary structure of solid (lyophilized) rhDNase. The amide III spectra (Figure 1B depicts data for the protein that was EGTA-treated prior to freeze-drying) clearly indicate that some structural perturbations occurred upon drying. These alterations were quantified by curve-fitting of the original spectra, as summarized in Table 1. It can be seen that there was a significant decrease in α-helical structures and increase in β-sheets for the EGTA-treated protein. To further confirm this change, we also examined the second-derivative amide I spectra for the aqueous (Figure 2A) and lyophilized (dashed line in Figure 2B) EGTA-treated protein. These data also reveal alterations upon lyophilization, in particular, augmentation of the band centered at approximately 1691 cm^{-1}, which likely indicates increased β-sheets in the solid state as a result of increased protein−protein interactions.[18] The nature and extent of the drying-induced structural alterations for the lyophilized EGTA-treated protein were similar to that for rhDNase freeze-dried from aqueous solution containing 1 mM calcium chloride (Figure 2).[16]

Therefore, although calcium ions increase rhDNase structural stability with respect to thermal unfolding, there was little effect on the stability toward dehydration-induced structural perturbation. This result is similar to that presented in a previous investigation of the enzyme lysozyme.[21] In that study, the protein lyophilized from a series of pHs from 1.9 to 5.1, corresponding to a wide range in T_m values from 45 °C to 75 °C (determined by solution DSC), exhibited very similar structural rearrangements upon lyophilization (estimated from FTIR spectra in the amide III region).[21]

Effect of Ca^{2+} on Protein Stability in the Aqueous and Lyophilized States—As described by Shire,[8] the dominant degradation pathway for rhDNase in current liquid formulation is deamidation of Asn74. The pseudo-first-order rate constants are not affected by the starting levels of deamidation of the protein (data not shown). Furthermore, deamidation, which results in partial loss of the enzymatic activity, does not significantly alter the protein's structure, as evidenced in the far- and near-UV CD.[8]

Figure 3A depicts data for the deamidation of rhDNase in aqueous solution at the accelerated stability condition of 40 °C. This condition is well below the T_m for rhDNase, even in the absence of calcium ions.[20] As expected, based on previous data,[8] the molecule readily deamidated. The starting material was already largely deamidated (about 60%), and complete deamidation was observed after 120

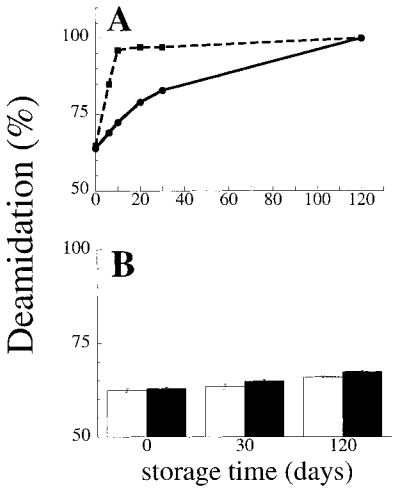

Figure 3—Deamidation of rhDNase at 40 °C: (A) in aqueous solution: solid line depicts data for untreated protein in the presence of 1 mM $CaCl_2$ and dashed line represents EGTA-treated protein in the absence of exogenous calcium ions (note that all error bars are too small to be seen); and (B) in the lyophilized untreated powder (open symbols) and lyophilized EGTA-treated powder (filled symbols).

days at 40 °C for the protein in the presence of 1 mM $CaCl_2$ (dashed line in Figure 3A). For this latter sample, which was fully deamidated (and did not exhibit any significant aggregate formation as discussed below), we measured the amide III FTIR spectra (Figure 1C) and estimated the secondary structure (Table 1), in addition to qualitative inspection of the amide I second derivative spectra (Figure 2C). Despite the conversion of asparagine to isoaspartate (i.e., the placement of an extra methylene group in the peptide backbone), there was no difference in the secondary structure, consistent with previous data obtained by CD.[8]

Deamidation was then monitored for the EGTA-treated protein which was incubated in aqueous solution without calcium ions (solid line in Figure 3A). In the absence of calcium, the degradation occurred even more rapidly compared to the protein in the presence of calcium ions. There was nearly complete deamidation seen after only a 10-day incubation at 40 °C. This result is different from that observed previously under a milder condition.[8] In that investigation, rhDNase was treated with EDTA to remove calcium and reformulated in isotonic 10 mM phosphate buffer. Deamidation of rhDNase under this condition did not increase significantly compared to untreated samples over ~0.5 year stored at 25 °C.[8]

We also examined the protein by size-exclusion chromatography (SEC), which was useful in detecting soluble aggregate formation. For the protein in the aqueous solution of 1 mM $CaCl_2$, there was virtually no aggregation observed by SEC, even after 120 days at 40 °C (filled symbols in Figure 4A). However, for the case of the protein in aqueous solution in the absence of calcium, there was rapid deterioration, with nearly complete aggregation following only 30 days of incubation (open symbols in Figure 4A).

Therefore, the data show that the removal of calcium ions results in increased rates of both deamidation and soluble aggregate formation in aqueous solution. These findings are consistent with results of bDNase, demonstrating that the removal of calcium was associated with increased rates of proteolysis (e.g., by trypsin),[2,3] susceptibility toward pH-induced structural alterations,[5] and reduction of the Cys173-Cys209 disulfide.[4] The latter may play a role in covalent aggregation via intermolecular disulfide formation. Majority of aggregates as observed by SDS−PAGE were dissociable in the presence of free thiol

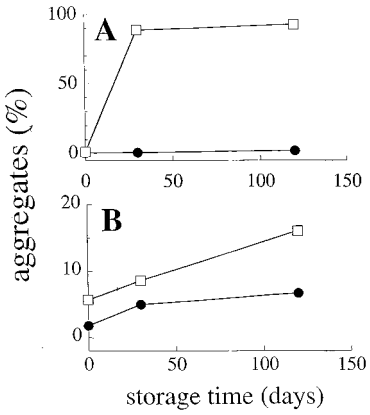

Figure 4—Aggregation of rhDNase at 40 °C: (A) in aqueous solution; (B) in the lyophilized powder. Filled symbols depict data for untreated protein in the presence of 1 mM $CaCl_2$, and open symbols represent EGTA-treated protein in the absence of exogenous calcium ions (note that all error bars are too small to be seen).

Table 2—Enzymatic Activities of Various rhDNase I Samples[a]

sample	enzymatic activity after incubation at 40 °C for[b] 0 days	30 days	120 days
aqueous solution, with Ca^{2+},[c]	100 ± 12	67 ± 5	58 ± 2
aqueous solution, EGTA-treated	90 ± 9	<9	<6
lyophilized, with Ca^{2+}	100 ± 13	89 ± 4	89 ± 2
lyophilized, EGTA-treated	92 ± 6	75 ± 6	53 ± 5

[a] Enzymatic activity was determined by the methyl green assay. [b] Data are expressed as % specific activity, or the amount of protein determined by the activity assay divided by that based on UV absorption of the sample. [c] The aqueous solution contained 1 mM calcium chloride.

agent (data not shown). Therefore, these aggregates were linked by disulfides.

Since reduction of the Cys173-Cys209 disulfide is known to affect the enzymatic activity, we used the methyl green assay[15] to probe the integrity of the active site of the molecule. For the protein in aqueous solution containing 1 mM calcium chloride, there was a substantial drop in activity, up to about 40% loss after 120 days at 40 °C (Table 2), even though there was no significant aggregation observed (Figure 4A). In this case, the drop in activity is a result of increased deamidation (Figure 3A), which is known to result in decreased activity.[8]

If the disulfide bridge Cys173-Cys209 had been cleaved for aqueous rhDNase in the absence of calcium ions, then it follows that the enzymatic activity would be lost, based on observations for the bovine-derived protein.[4] Thus, we measured the enzymatic activity of the EGTA-treated protein in aqueous solution without calcium ions. As predicted, there was over 90% loss of enzymatic activity after only 30 days of incubation (Table 2), concomitant with the aggregation of the molecule (Figure 4A). These data, along with the confirmation of the presence of disulfides in the rhDNase aggregates, strongly support the view that removal of calcium from binding sites near native disulfides made these areas more susceptible to cleavage and ultimately intermolecular bond formation. The increased susceptibility toward deamidation in the absence of calcium may be linked to increased configurational freedom as a result of the disulfide cleavage and/or exchange.

It was interesting to also examine the secondary structure of the rhDNase aggregates (generated by incubation of EGTA-treated protein for 120 days in aqueous solution). To this end, we measured the amide III FTIR spectra for the rhDNase aggregates (Figure 1D) which showed sig-

nificant perturbation compared to the protein prior to incubation, particularly an increase in the β-sheet band centered at approximately 1238 cm^{-1} (Figure 1D). Estimation of the secondary structure (Table 2) confirmed that there was an increase in the overall β-sheet content in the aggregates. Protein aggregates tend to have increased β-sheet contents, largely intermolecular in nature.[18] This was also confirmed in the amide I second derivative spectra (Figure 2D) which exhibited a band at $\sim$1620 cm^{-1}. It has been reported that this band indicates the presence of intermolecular aggregates, and has been correlated with protein aggregation.[18]

Having established the influence of calcium ions on the solution structure/stability of rhDNase, we then next examined the lyophilized form of the protein. Generally, proteins exhibit superior stability in the dehydrated state due to several factors, such as the increase in molecular rigidity upon dehydration, which should retard deleterious reactions.[22,23] The level of deamidation (Figure 3B) shows that there was no significant increase in the extent of deamidation, regardless of the absence of calcium. This result may stem from the fact that deamidation is a hydrolytic process, i.e., requires water;[24] it is logical then, that removal of water would coincide with reduction of deamidation.

Figure 4B depicts the formation of aggregates for rhDNase in the solid state. It can be seen that for the protein lyophilized from the aqueous solution containing Ca^{2+}, there was slight aggregation ($\sim$7% after 120 days at 40 °C). Note that under this condition in aqueous solution, there was essentially no aggregation observed. Therefore, from the standpoint of intermolecular processes, i.e., aggregation, the protein exhibited inferior stability in the solid compared to the aqueous state; the reverse was true for the intramolecular process of deamidation. This finding is consistent with the view that the proximity of protein molecules in the dried state facilitates intermolecular pathways over intramolecular ones.[25]

For the EGTA-treated protein, the extent of solid-state aggregation was more substantial (Figure 4B), although not as severe as observed for the aqueous solution (Figure 4A). Thus, in this case (EGTA-treated), superior stability was observed for the lyophilized protein. As expected, there was also significant loss in rhDNase enzymatic activity for the EGTA-treated protein (Table 2), whereas there was little change for the protein lyophilized from 1 mM $CaCl_2$. From the data it is clear that the activity loss does not directly correspond to the loss of monomer, suggesting that some monomers have become inactivated (e.g., cleavage of Cys173-Cys209). As was the case for the protein aggregates in aqueous solution, the aggregates generated in the solid-state had over 50% disulfide-bonded character, as revealed by reducing and nonreducing SDS−PAGE.

The data clearly demonstrate that the removal of calcium destabilizes rhDNase in the solid state. This result is intriguing considering the conjecture that protein biochemical stability is linked to the glass transition temperature (T_g) of the formulation.[24] Although it is difficult to measure the T_g for pure proteins,[26] it is expected that the T_g for rhDNase is much higher than that of calcium. Therefore, removal of calcium should raise the T_g, yet the data show that rhDNase stability was decreased. The other component present in our system, namely water, also has a very low T_g and is known to destabilize proteins.[22,23] Another possibility to explain the effect of calcium is that its removal results in increase in residual moisture level and/or increase in moisture over storage (e.g., moisture transfer from the lyophilization stopper). To check this, we measured the moisture levels of lyophilized rhDNase in the presence and absence of calcium after a storage period of 24 months at 15 °C. We found there was no significant difference in moisture levels between the samples in the presence and absence of calcium. In addition, the average moisture level ($n = 10$) was found the same as previous data,[16] suggesting that no moisture transferred from the stopper over time. These data strongly suggest that for the case of rhDNase the stabilizing influence of calcium is due to some specific effect (e.g., binding) rather than mediated by influence on the T_g of the system.

In addition to the formulation, the reconstitution medium may also affect protein stability. For instance, Zhang et al.[27] have studied the effect of additives in the reconstitution medium on aggregation and activity of lyophilized recombinant interleukin-2 and ribonuclease A. It was found that additives such as 0.05% sodium polyphosphate or 0.5% sulfated β-cyclodextrin increased recovery of activity of RNase (from 62% to 97% and 77%, respectively) after storage of the lyophilized proteins for three weeks at 45 °C. In the current investigation, we tested whether the addition of calcium in the reconstitution medium (but not the dried formulation) could influence rhDNase stability. EGTA-treated samples were stored for 2 years at 40 °C and then reconstituted in the absence and presence of calcium (1 mM $CaCl_2$). The data revealed the same activity loss, 60 $\pm$ 2% vs 55 $\pm$ 5%, and increase in aggregates, 17 $\pm$ 1% vs 15 $\pm$ 2% for the protein reconstituted in the absence and presence of calcium, respectively. This excludes the possibility that rhDNase is stabilized solely by calcium binding during the reconstitution step.

Both EGTA-treated and nontreated rhDNase exhibited similar alterations in their structure upon lyophilization as determined by FTIR spectroscopy, yet the two had different stability. This suggests that the predominant mechanism of solid-state instability, aggregation, did not depend on the overall secondary structure. If similar mechanisms hold for the aqueous and solid states, then it is likely that the local structure near a labile site, for instance, Cys173-Cys209, is influenced by calcium ions (such an effect is not detectable by examination of overall secondary structure by FTIR spectroscopy).

In summary, this investigation reports the effect of calcium ions on the structure and stability of rhDNase in the aqueous and solid (lyophilized) states. The data reveal that the removal of calcium ions by EGTA-treatment had little effect on the secondary structure (and lyophilization-induced rearrangement thereof) as determined by FTIR spectroscopy. The CD spectra also confirmed that the removal of calcium did not alter the secondary or tertiary structure for the aqueous protein. The primary degradation route for the aqueous protein at 40 °C was deamidation. There was also rapid aggregation observed for the aqueous EGTA-treated protein, presumably via a mechanism not related to the overall structure, but rather a labile site, e.g., reduction of Cys173-Cys209. For the protein lyophilized in the presence of calcium, there was some aggregation observed at 40 °C; thus the solid-state stability was actually worse than that in aqueous solution. For the EGTA-treated protein lyophilized in the absence of calcium, solid-state aggregation was much more pronounced compared to protein in the presence of calcium. Thus, the removal of calcium ions by EGTA-treatment has a similar effect on the structure and stability of rhDNase in both the aqueous and solid states.

References and Notes

1. Moore, S. Pancreatic DNase. In *The Enzymes Vol. XIV*; Academic Press: San Diego, CA, 1981; pp 281−295.
2. Poulos, T. L.; Price, P. A. Some effects of calcium ions on the structure of bovine pancreatic deoxyribonuclease A. *J. Biol. Chem.* **1972**, *247*, 2900−2904.

3. Price, P. A.; Liu, T.-Y.; Stein, W. H.; Moore, S. Properties of chromatographically purified bovine pancreatic deoxyribonuclease. *J. Biol. Chem*. **1969**, *244*, 917−923.
4. Price, P. A.; Stein, W. H.; Moore, S. Effect of divalent cations on the reduction and re-formation of the disulfide bonds of deoxyribonuclease. *J. Biol. Chem*. **1969**, *244*, 929−932.
5. Lizarraga, B.; Sanchez-Romero, D.; Gil, A.; Melgar, E. The role of Ca^{2+} on pH-induced hydrodynamic changes of bovine pancreatic deoxyribonuclease A. *J. Biol. Chem*. **1978**, *253*, 3191−3195.
6. Price, P. A. The essential role of Ca^{2+} in the activity of bovine pancreatic deoxyribonuclease. *J. Biol. Chem*. **1975**, *250*, 1981−1986.
7. Price, P. A. Characterization of Ca^{2+} and Mg^{2+} binding to bovine pancreatic deoxyribonuclease A. *J. Biol. Chem*. **1972**, *247*, 2895−2899.
8. Shire, S. J. Stability characterization and formulation development of recombinant human deoxyribonuclease I [Pulmozyme®, (Dornase alpha)]. In *Formulation, Characterization and Stability of Protein Drugs*; Pearlman, R., Wang, Y. J., Eds.; Plenum Press: New York, 1996; pp 393−426.
9. Suck, D.; Oefner, C.; Kabsch, W. Three-dimensional structure of bovine pancreatic DNase I at 2.5 Å resolution. *EMBO J*. **1984**, *3*, 2423−2430.
10. Oefner, C.; Suck, D. Crystallographic refinement and structure of DNase I at 2 Å resolution. *J. Mol. Biol*. **1986**, *192*, 605−632.
11. Suck, D.; Oefner, C. Structure of DNase I at 2.0 Å resolutions suggests a mechanism for binding to and cutting DNA. *Nature* **1986**, *321*, 620−625.
12. Wolf, E.; Frenz, J.; Suck, D. Structure of human pancreatic DNase at 1.8 Å resolution. *Protein Sci*. **1995**, *4*, 115.
13. Nail, S. J. Formulation of Proteins and Peptides. In *Controlled Drug Delivery. Challenges and Strategies*; Park, K., Ed.; American Chemical Society: Washington, D. C., 1997; pp 185−203.
14. Cacia, J.; Quan, C. P.; Vassar, M.; Sliwkowski, M. B.; Frenz, J. Protein sorting by high-performance liquid chromatography. I. Biomimetic interaction chromatography of recombinant human deoxyribonuclease I on polyionic stationary phases. *J. Chromatogr*. **1993**, *634*, 229−239.
15. Sinicropi, D.; Baker, D. L.; Prine, W. S.; Shiffer, K.; Shak, S. Colorimetric determination of DNase I activity with a DNA-methyl green substrate. *Anal. Biochem*. **1994**, *222*, 351−358.
16. Costantino, H. R.; Chen, B.; Liu, J.; Hsu, C. C.; Shire, S. J. Fourier transform infrared (FTIR) spectroscopic investigation of the secondary structure of recombinant human DNase I in the aqueous and dried states. *Pharm. Pharmacol. Commun*. **1998**, *4*, 391−395.
17. Griebenow, K.; Klibanov, A. M. Lyophilization-induced changes in the secondary structure of proteins. *Proc. Natl. Acad. Sci U.S.A*. **1995**, *92*, 10969−10976.
18. Dong, A.; Prestrelski, S. J.; Allison, S. D.; Carpenter, J. F. Infrared spectroscopic studies of lyophilization and temperature-induced protein aggregation. *J. Pharm. Sci*. **1995**, *84*, 415−424.
19. Bloemendal, M.; Johnson, W. C. Structural information on proteins from circular dichroism spectroscopy: Possibilities and limitations. In *Physical Methods to Characterize Pharmaceutical Proteins*; Herron, J. N., Crommelin, D. J. A., Eds.; Plenum Press: New York, 1995; pp 65−93.
20. Chan, H.-K.; Au−Yeung, K.-L.; Gonda, I. Effects of additives on heat denaturation of rhDNase in solution. *Pharm. Res*. **1996**, *13*, 756−761.
21. Costantino, H. R.; Griebenow, K.; Mishra, P.; Klibanov, A. M. Fourier transform infrared (FTIR) spectroscopic investigation of protein stability in the lyophilized form. *Biochim. Biophys. Acta* **1995**, *1253*, 69−74.
22. Hageman, M. J. Water sorption and solid-state stability of proteins. In *Stability of Protein Pharmaceuticals. Part A. Chemical and Physical Pathways of Protein Degradation*; Ahern, T. J., Manning, M. C., Eds.; Plenum Press: New York, 1992; pp 273−309.
23. Costantino, H. R.; Langer, R.; Klibanov, A. M. Solid-phase aggregation of proteins under pharmaceutically relevant conditions. *J. Pharm. Sci*. **1994**, *83*, 1662−1669.
24. Cleland J. L.; Powell, M. F.; Shire, S. J. The development of stable protein formulations. A close look at protein aggregation, deamidation, and oxidation. *Crit. Rev. Ther. Drug Carrier Syst*. **1993**, *10*, 307−377.
25. Hageman, M. J.; Bauer, J. M.; Possert, P. L.; Darrington, R. T. Preformulation studies oriented towards sustained delivery of recombinant somatotropins. *J. Agric. Food Chem*. **1992**, *40*, 348−355.
26. Bell, L. N.; Hageman, M. J.; Muraoka, L. M. Thermally induced denaturation of lyophilized bovine somatrotropin and lysozyme as impacted by moisture and excipients. *J. Pharm. Sci*. **1995**, *84*, 707−712.
27. Zhang, M. Z.; Pikal, K.; Nguyen, T.; Arakawa, T.; Prestrelski, S. J. The effect of the reconstitution medium on aggregation of lyophilized recombinant interleukin-2 and ribonuclease A. *Pharm. Res*. **1996**, *13*, 643−646.

Acknowledgments

The authors thank Sylvia Wu for assistance in lyophilization, Dee Ann Henderson for technical support, Dennis Brooks for helping with moisture content determination, Tom Bewley and Tom Patapoff for critical reading and valuable suggestion about modification of the manuscript, and Tue Nguyen for project support.

JS980273G