Reversibility of Heat-Induced Denaturation of the Recombinant Human Megakaryocyte Growth and Development Factor

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Purpose. The present study was performed to examine the effect of solution conditions on the reversibility of the thermal denaturation of megakaryocyte growth and development factor (rHuMGDF).

Methods. Changes in the far UV CD spectra of rHuMGDF with temperature were used to monitor the thermal denaturation of the protein, and the recovery of folded protein following a return to room temperature. The effect of protein concentration, scan rate, and buffer composition on thermal denaturation and on the reversibility were determined. Surface tension measurements were used to determine the effect of this unfolding reaction on the surface adsorption of the protein. Sedimentation velocity was used to assess recovery of native monomer and the size of soluble aggregates. In addition, monomeric protein remaining in solution after incubation at 37°C for 2 weeks in either 10 mM imidazole of 10 mM phosphate was determined.

Results. In phosphate buffer the rHuMGDF irreversibly precipitates upon unfolding under all the conditions examined. In imidazole the unfolding is at least partially reversible, with no visible precipitate seen; the degree of reversibility increased by lowering both protein and salt concentrations, and the amount of time spent at elevated temperature. In order to compare thermal unfolding occuring with different degrees of reversibility, the melting temperature was defined as the temperature at which melting begins. The melting temperature itself is relatively independent of the buffer composition, or experimental conditions. At low protein concentrations the protein stabilizer sucrose had a marginal effect on the thermal transition of rHuMGDF, while at protein concentrations of about 2 mg/ml the inclusion of sucrose increased the apparent melting temperature by about 4°C, to that seen at low protein concentrations, but had little effect on the reversibility of denaturation. Inclusion of 1 or 2 M urea did not affect the reaction. Surface tension measurements of rHuMGDF solutions showed little difference before and after melting, and in the presence or absence of sucrose. When unfolding is irreversible, the MGDF appears to form soluble aggregates of tetramers to 14-mers, while under reversible conditions native monomer is recovered. More monomeric MGDF remained in solution following storage for 2 weeks at 37°C in imidazole than in phosphate, in both the presence and absence of sucrose.

Conclusions. These results can be explained by assuming that thermal denaturation proceeds as a two-step reaction, the first step being the equilibrium between folded and unfolded states, while the second step is a slow irreversible aggregation. The different buffer systems affect the rate of the aggregation step, but not the intrinsic thermal stability nor the rate of the unfolding step.

KEY WORDS: heat-induced denaturation; MGDF; reversibility; sucrose; circular dichroism.

INTRODUCTION

Mpl ligand, or thrombopoietin, is a member of the four-helical bundle cytokine family (1,2,3), and is responsible for the growth and differentiation of megakaryocytes and platelets. rHuMGDF is a truncated form of the Mpl ligand which contains the receptor-binding domain, and was expressed in *E. coli* and refolded. This protein is currently undergoing clinical trials as a therapeutic for use in bone marrow transplantation and as an adjuvant to chemotherapy (4,5,6).

The long-term shelf life of protein therapeutics can often be improved by finding the appropriate buffer, pH, and excipients for the formulation of each particular protein. Changes in melting temperature induced by alterations in these conditions can be used to aid in developing the optimum formulation, since the thermal stability of proteins is often a reliable indicator of their long term stability during storage, with an increase in the melting temperature in general resulting in an enhanced shelf life (7,8,9). The melting temperature can be determined by various calorimetric and spectroscopic techniques. However, a parameter that often is ignored in these measurements is the reversibility of the unfolding reaction itself. When the unfolding reaction is at least partially irreversible, as is true for many cytokines and growth factors, minimizing this irreversibility would also be expected to increase the storage stability of the protein.

We analyzed the reversibility of thermal denaturation, using changes in the CD spectrum, to screen for the optimum solvent conditions which would improve the long-term stability of rHuMGDF. Interestingly, we observed a drastic difference in the reversibility of the thermally-induced unfolding of rHuMGDF in phosphate versus imidazole. We therefore undertook a detailed examination of the thermally-induced unfolding of rHuMGDF, and the effects of solvent and experimental parameters on this reaction. The results of these studies are reported here.

MATERIALS AND METHODS

The protein analyzed in this study was rHuMGDF, the N-terminal receptor binding domain of the Mpl ligand thrombopoietin. This protein was obtained from Amgen Clinical. It consists of the 163 N-terminal amino acids of thrombopoietin with a Met added as the amino terminus, and was cloned, expressed in *E. coli*, refolded and purified using several chromatographic steps. The protein was dialyzed overnight into the desired buffer and centrifuged immediately prior to analysis to remove any soluble aggregates that might be present. As a control protein for the surface tension experiment recombinant IL-1ra, interleukin-1 receptor antagonist, a protein with a molecular weight of 17,258, was obtained from Amgen Clinical.

CD Analysis

The characteristics of the thermally-induced unfolding of rHuMGDF were determined by following changes in the far

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ABBREVIATIONS: rHuMGDF, megakaryocyte growth and development factor; CD, circular dichroism; PBS, phosphate-buffered saline; IL-1RA, interleukin-1 receptor antagonist.

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UV CD spectra at 225 nm with increasing temperature from 24 to 86°C, using different heating rates, protein concentrations and buffers. A Jasco J-720 spectropolarimeter and a JTC-345 Peltier thermal control unit were used for these experiments, with a 0.1 cm pathlength rectangular thermal cuvette. Thermal stability was analyzed by comparing the temperature, T_i, at which melting began. Thus, the T_i is defined as an initial temperature of unfolding throughout the paper. This was determined using custom software which fits the baseline before and after melting and the transition region, independently. The T_i is the temperature at which 5% of the signal has been lost. The degree of reversibility was determined by cooling the solution to 24°C over 10 min as soon as the temperature reached 90°C, incubating the protein at 24°C for 30 min, and then repeating the heating cycle except otherwise indicated. The amount of signal regained during the 24°C incubation relative to the initial signal was measured, and used to determine the percent reversibility. Spectra in the near UV CD region (340–240 nm, 1 cm cuvette) and the far UV CD region (250–190 nm, 0.02 cm cuvette) were obtained before and after heating with a Jasco J-715 spectropolarimeter.

Sedimentation Velocity

Sedimentation velocity experiments were carried out at 20°C using dual-channel charcoal EPON centerpieces and sapphire windows in a Beckman Optima XL-I analytical ultracentrifuge. For g(s*) analysis rapid bursts of 40–60 Rayleigh interference scans were acquired and analyzed using the DOS version of the program DCDT from the National Analytical Ultracentrifugation Facility at the University of Connecticut. Absorbance scans at 280 or 229 nm were also acquired at various times during the runs, and in some cases these data were analyzed using the program SVEDBERG (10) to more accurately determine the sedimentation coefficient and amount of the monomer fraction in the sample. The total absorbance due to monomer returned by this analysis was then compared to the total absorbance seen in scans early in the run to calculate the fraction of the monomer.

Surface Tension

The surface tensions of various protein solutions were determined with the Wilhelmy plate method using a Krüss K12 dynamic tensiometer. The temperature scan parameters were controlled by a Neslab RT-110 programmable recirculator. Protein concentration was 0.05 mg/ml. Surface pressure represents the difference in surface tension between the protein solution and its buffer; i.e., surface tension (buffer)-surface tension (protein solution).

Storage Stability

Solutions of 1 mg/ml of rHuMGDF were stored for 2 weeks at 37°C, and the amount of monomeric protein remaining in solution was determined from the area of the peak eluting from the gel filtration column.

RESULTS

Effect of Buffer Conditions on Thermal Denaturation

When the thermal stability of the secondary structure of rHuMGDF was analyzed in either PBS or citrate buffers (anionic buffers) from pH 4 to pH 7 the thermally-induced denaturation was accompanied by precipitation. Thus when the sample was cooled to 24°C no soluble protein remained, and none of the signal was recovered. This is shown in Fig. 1, and tabulated in Table 1. The precipitation occurred after the onset of melting, and interfered with the accurate determination of the thermal transition curve by causing a slow loss of protein (and therefore ellipticity) as well as by increasing the light scattering (Fig. 1A vs. 1C-E). Therefore, the thermal stability was assessed by comparing the onset of melting T_i, rather than the midpoint of the transition, as determined by fitting the melting curve as described in Methods since this temperature is less influenced by precipitation. However, when the buffer was 10 mM imidazole, 10 mM histidine or 10 mM Tris (cationic buffers) from pH 6 to pH 8, no precipitation was ever observed, and about 50% of the signal was recovered after cooling under these conditions. The absorbance of the individual buffers varies, and therefore the baseline ellipticity is slightly different from sample to sample. The effect of heating on the near UV CD spectra in imidazole is similar, with a complete loss of signal occurring at the same temperature as that of the secondary structure, while the protein remained soluble (data not shown) and some reversibility was observed. This indicates that at elevated temperature the rHuMGDF is unfolded, with little or no secondary or tertiary structure retained, and that the unfolding of both secondary and tertiary structure occurs simultaneously. However, due to the weak signal in the near UV CD region, along with the larger amounts of protein used for this experiment, the analysis of thermally-induced unfolding of tertiary structure was not routinely performed.

Following heating in the cationic buffers rHuMGDF may remain as a monomer, or may form soluble aggregates; either way the final unfolded state is very different from that of the precipitated unfolded state obtained in the anionic buffers. This suggests that there is a specific interaction between the buffer and the protein for one of these systems. Either the cationic buffers bind to the unfolded protein and prevent aggregation, or the phosphate and citrate bind to specific sites and enhance aggregation or precipitation. In unbuffered solutions the melting is fully reversible, suggesting that the latter explanation is the most likely. Interestingly, the type of buffer affected the degree of reversibility without affecting the temperature at which melting began. This suggests that only the unfolded protein aggregates, and that the aggregation occurs at a rate such that it does not affect the apparent melting temperature.

Effect of Ionic Strength

The effect of the imidazole concentration on the melting temperature and degree of reversibility was compared next, using a protein concentration of 0.2 mg/ml and a heating rate of 50°C/hr. The temperature at which melting began and the percentage of the signal recovered after 60 min at 24°C are indicated in Table 2. A clear trend of greater reversibility at lower ionic strength is observed, while the melting temperature remains constant.

The effect of NaCl concentration on the reversibility in 10 mM imidazole pH 7 was also analyzed, and is included in Table 2. Again, higher ionic strength results in decreased reversibility, or increased aggregation. In this case, it also results in a lower melting temperature.

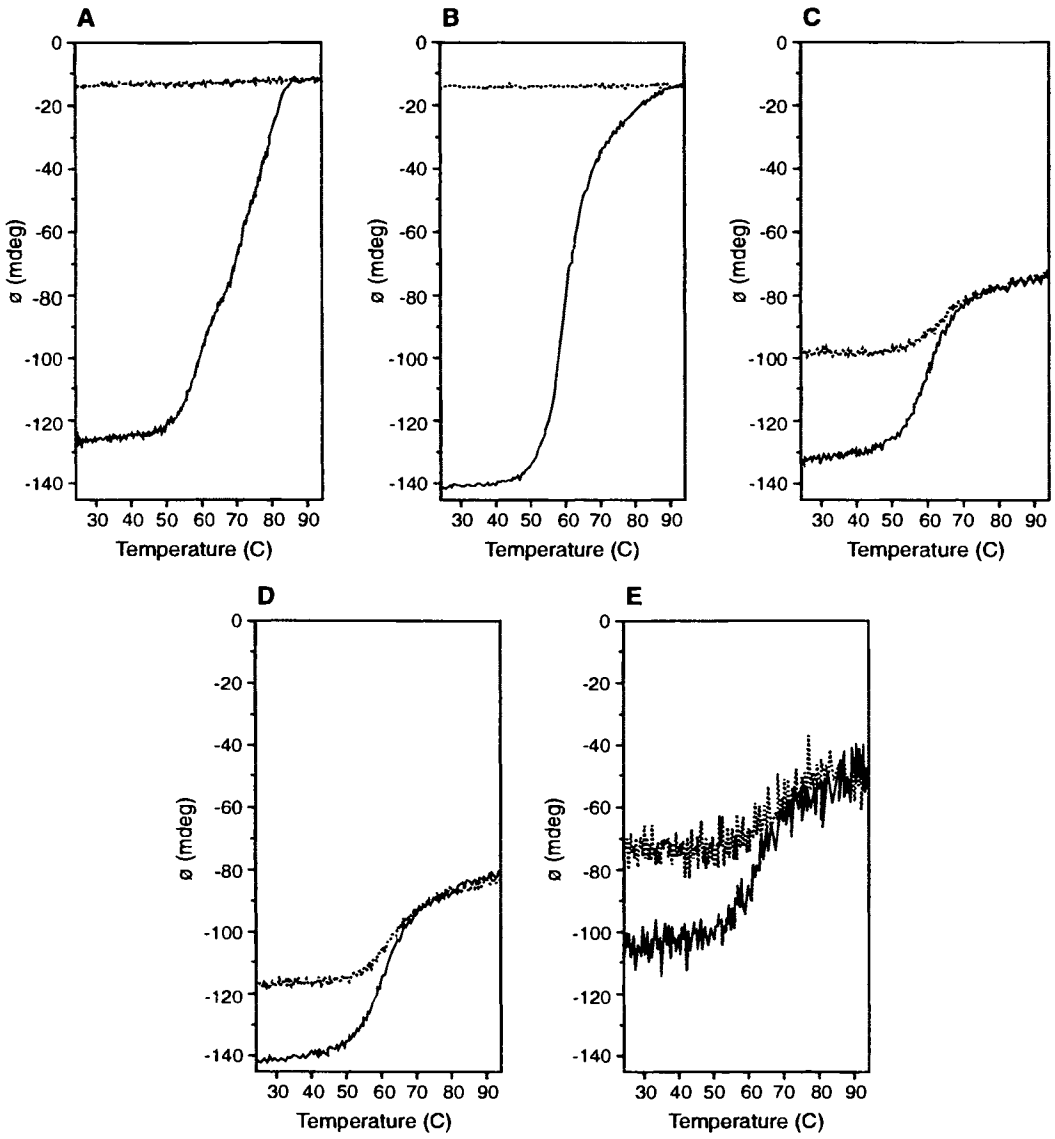

Fig. 1. The reversibility of thermally-induced denaturation of rHuMGDF. Protein solutions of 1 mg/ml were heated at 50°C/hr, and changes at 225 nm were monitored with temperature. As soon as the protein reached 90°C, the solution was cooled to 24°C and incubated for 30 min at 24°C before a second thermal scan was recorded. The solid line represents the first scan, the dotted line represents the second scan. (A) Using 10 mM Na phosphate pH 7.0; (B) Using 10 mM Na citrate pH 7.0; (C) Using 10 mM imidazole pH 7.0; (D) Using 10 mM histidine pH 7.0; (E) Using 10 mM Tris pH 7.0.

Effect of Protein Concentration

The effect of protein concentration on the melting temperature and the degree of reversibility of the thermally-induced denaturation was studied next. The protein was heated at 50°C/hr in 10 mM imidazole, pH 6.5. The results are shown in Table 3. Higher protein concentrations result in lower reversibility. Interestingly, increasing the protein concentration from 0.1 to 1 mg/ml results in decreased reversibility, but does not affect the temperature at which the unfolding reaction begins to occur. However, when the protein concentration is increased to between 2 and 10 mg/ml, both reversibility and melting temperature are decreased (5° and 7°C lower at 2 and 10 mg/ml), similar to the results obtained at high NaCl concentration.

Effect of Heating Rate

The final variable that was analyzed was the effect of heating rate on the melting reaction, using protein solutions of 0.2 mg/ml in 10 mM imidazole pH 6.5. After heating from 24 to 90°C at a rate of 20°C/hr 70% of the original signal was recovered, after heating at 50°C/hr 89% was recovered, and after heating at a rate of 100°C/hr 90% of the original signal was recovered. Heating to 64°C at 20°C/hr and cooling immediately resulted in the recovery of 99% of the original signal, while heating to 84°C and incubating at that temperature for 1 hour resulted in only 40% recovery. Thus, slower heating rates result in more protein lost to aggregation. Also, stopping the heating before the thermal transition is completed decreases aggregation

Table 1. Effect of Buffer Composition on Melting Monitored by CD

Buffer	Temperature at which melting began (T_i, °C)	Precipitation	Reversibility (% of signal recovered after incubation at 24°C)
10 mM imidazole pH 6	51	no	~46%
10 mM His pH 7	52–51	no	~50%
10 mM Na phosphate pH 7.1	~52	yes	0
10 mM Na citrate, pH 7.1	~52	yes	0
10 mM Tris pH 8.6	~52	no	~65%

Note: The experiments were done with an rHuMGDF concentration of 1 mg/ml in buffer, at a rate of 50°C/hr, following changes at 225 nm while heating to 85°C. Reversibility was checked by performing a second scan following a 30 min incubation at room temperature (see Fig. 1).

Table 2. The Effect of Buffer Concentration on Thermal-Induced Denaturation of rHuMGDF

Buffer	Temperature at which melting began (T_i, °C)	Reversibility (determined as above)
1 mM imidazole	52	90%
10 mM imidazole	52	89%
25 mM imidazole	52	60%
50 mM imidazole	52	54%
10 mM imidazole 50 mM NaCl	52	70%
10 mM imidazole 100 mM NaCl	43	50%
10 mM imidazole 200 mM NaCl	40	0%

Note: These experiments were performed with an rHuMGDF concentration of 0.2 mg/ml; otherwise the procedure was the same as for Table (1). The buffers were all pH 7. The rHuMGDF all precipitated in phosphate buffer regardless of the molarity of the buffer (1 mM to 50 mM).

and increases reversibility, while prolonged incubation above the melting transition results in decreased reversibility. It appears that as more time is spent at elevated temperatures, more aggregation occurs, and therefore the melting transition

Table 3. Effect of Protein Concentration on Thermal Denaturation of rHuMGDF

Sample concentration	% reversible	Temperature when melting began (T_i, °C)
0.1	95	52
0.2	89	51
1	46	51
2	25	46
10	0	44

Note: Samples were heated at 50°C/hr in 10 mM imidazole pH 6.5 from 24 to 86°C, incubated at ambient temperature for 1 hr, and then remelted.

becomes less reversible. However, in all cases, the melting temperatures appear to be unaffected by the heating rate used, with T_i occurring at 51°C for all of these samples.

Conformational State of Renatured Protein

Under optimum conditions the thermal unfolding of rHuMGDF can be 90–100% reversible as measured spectroscopically. In order to determine whether the renatured protein has the same hydrodynamic properties as the native monomer, and in order to assess the size of the irreversibly-denatured soluble aggregates, we have carried out sedimentation velocity experiments on rHuMGDF samples which were heated at various concentrations and at various rates in the CD cell and then allowed to refold. When rHuMGDF in 10 mM imidazole pH 6.5 is thermally denatured at a concentration of 2.0 mg/ml (where the reaction is only about 25% reversible by CD) and then cooled to room temperature, the majority of the sample sediments very rapidly with very broad boundaries, indicating a wide range of aggregate sizes. The sedimentation coefficient distribution, g(s*), for such a sample, as obtained using the time-derivative method developed by Stafford (11), is shown as the dashed line in Fig. 2 and indicates a broad distribution of soluble aggregates with sedimentation coefficients from ~5 to 13 S, plus some smaller material below 4 S (which has not cleared the meniscus and thus will be poorly resolved at this time in the run). If these soluble aggregates are assumed to be globular rather than rod-like, then a sedimentation coefficient range of 5–13 S corresponds roughly to a size range from tetramers to 14-mers of rHuMGDF. The exact size distribution of the soluble aggregates varies somewhat from sample to sample and is dependent on details such as heating and cooling rates, and aggregates greater than 40 S are seen if the sample is held longer at high temperature (not shown).

In contrast, for samples which have been unfolded at a concentration of 0.2 mg/ml (about 95% reversible by CD), the

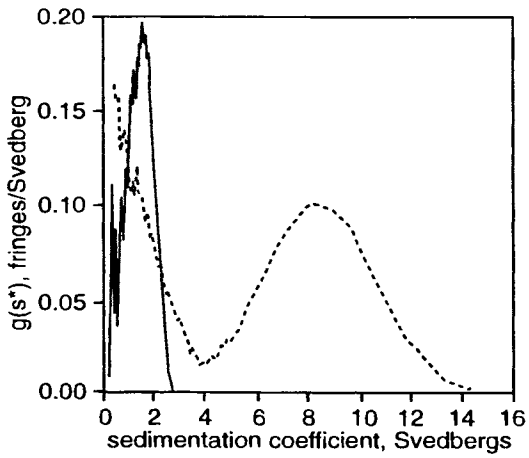

Fig. 2. Sedimentation coefficient distribution function g(s*), of rHuMGDF samples after heating. The dashed line gives results for a sample heated at a concentration of 2.0 mg/ml (run at ~0.4 mg/ml in the centrifuge). The solid line gives results for a sample that was heated at 0.2 mg/ml (~0.1 mg/ml in the centrifuge). The distributions have not been normalized for the sample concentration to make them easier to see on the same vertical scale, and the sedimentation coefficients are raw values (not corrected to standard conditions).

bulk of the sample sediments slowly. No material sedimenting in the 5–15 S range can be detected by g(s*) analysis, however, it is likely that small amounts of aggregates would not be detectable at the lower concentration (about 4-fold) of this velocity sample. The solid line in Fig. 2 shows a g(s*) distribution from data taken much later in the run, which shows a single narrow peak centered at around 1.6 S, matching control data for native rHuMGDF. Since the g(s*) analysis is not the optimum method for proteins as small as the rHuMGDF monomer, we also used direct boundary fitting methods (10) to more accurately assess whether the renatured monomer truly adopts a native conformation and to more accurately determine the amount of monomer recovered. This analysis gives a sedimentation coefficient of 1.60 S for the renatured material, which is not significantly different than the 1.62 S value for a side-by-side native control, and indicates that this 1.60 S species represents 95% ± 2% of the material initially loaded into the cell, in agreement with the percentage recovery of the native CD signal.

The conformational state of the soluble protein following unfolding and subsequent renaturation was also determined by CD spectroscopy. The sample was filtered through a 100,000 molecular weight membrane. Based on the sedimentation data this should remove a majority of soluble aggregates, leaving monomer in solution. The filtered samples were then analyzed by near UV CD (Fig. 3A), far UV CD (Fig. 3B), and fluorescence spectroscopy (Fig. 3C). The soluble protein has tertiary and secondary structure resembling those of the starting material, indicating that the renatured rHuMGDF is the native monomer. This is in contrast to the CD spectra at 86°C, which indicated complete unfolding of the protein, with loss of both tertiary and secondary structure (data not shown).

The amount of secondary and tertiary structure recovered after heating is equivalent to the amount of native monomer that is recovered. This is consistent with the previous results, and the hypothesis that the thermal denaturation is a two-step process, with the transition between native and unfolded protein being a reversible reaction, followed by an irreversible transition between monomer and aggregate.

Effect of Sucrose and Urea

rHuMGDF was dialyzed into 10 mM imidazole pH 6.5, and then mixed with 2 M sucrose to give stock solutions in the same buffer. The melting curves obtained with a final solution of 1 M sucrose, and either 0.2 or 2 mg/ml protein are shown in Fig. 4. At 0.2 mg/ml protein, 1 M sucrose had a marginal effect on the melting temperature with the reversibility of rHuMGDF unfolding unchanged. This is also true in 10 mM sodium phosphate at pH 7, where the thermal unfolding was completely irreversible in the presence and absence of 1 M sucrose. However, the results are quite different at 2 mg/ml. Although 1 M sucrose resulted in little effect on reversibility, it increased the melting temperature by about 4°C, restoring it to a value similar to that seen at low protein concentrations (see Table 3). As described, the melting temperature decreases without sucrose at 2 mg/ml by 5°C. Similar results for high protein concentrations were obtained in 10 mM sodium phosphate, with the melting temperature increasing a few degrees but no change in reversibility occurring. This suggests that sucrose is affecting the rate of aggregation, but not the unfolding of rHuMGDF (see Discussion, below).

rHuMGDF was also melted following the procedure used for the surface tension measurements (below). The protein was diluted to 0.05 mg/ml in either H_2O or 1M sucrose and changes at 222 nm followed as the temperature was quickly raised (over 30 min). Following an incubation for 30 min at 24°C the solution was again heated. Both the H_20 and 1M sucrose samples began melting at 50°C, with 99% reversibility. When rHuMGDF was heated in imidazole in either 1 or 2M urea the thermal transition temperature was again unchanged. The lack of effect of these typical protein stabilizers and destabilizers at low protein concentration suggests that there is something very unusual in the surface properties of unfolded rHuMGDF.

Surface Tension

Usually protein unfolding is accompanied by an increase in surface area. This increase in surface area results in increased preferential interactions of excipients such as sucrose (negative) or urea (positive) with the protein, and this is why these excipients affect protein stability. Therefore, the lack of an effect of these additives on the thermally-induced unfolding of rHuMGDF suggests that unfolding occurs without any significant change in the surface area of the protein. Although CD tells us that the protein is unfolded with little secondary or tertiary structure remaining, it does not give any information on the compactness of the unfolded protein. Hydrodynamic measurements are difficult to perform at high temperatures. Therefore, we examined the surface tension of rHuMGDF as a function of temperature in the presence and absence of sucrose. As a control protein, IL-1ra, which exhibits increased thermal stability in the presence of sucrose (as determined by differential scanning calorimetry DSC; B. Chang, unpublished data), was also examined. The surface tensions of water at 25°C and 68°C were 72.3 and 65.3 (mN/M), respectively. Addition of 1 M sucrose to water resulted in an increase of surface tension to 73.7 and 66.1 (mN/M), respectively. When proteins were added to water, the surface tension decreased to 71–72 mN/M at 25°C. The surface tensions of solutions containing protein and 1M sucrose were 68–70 mN/M. This large decrease suggests that sucrose is facilitating the adsorption of proteins to the air-water interface. The temperature dependence of the surface pressure (defined in the Methods section) of IL-1ra and rHuMGDF in solution is also shown in Fig. 4. The surface pressure of the IL-1ra solution gradually decreases with the temperature even before thermally-induced unfolding of the protein occurs, reflecting the decreased hydrogen bonding between water molecules. Around 60°C, the surface pressure sharply decreases due to unfolding of the protein, which increases its surface area and hydrophobicity, and hence its surface activity. Sucrose increases the temperature of this sharp surface pressure change, reflecting its stabilizing effect on the thermal stability of the protein, in agreement with the DSC results. In contrast, rHuMGDF showed no sharp decrease in surface pressure during melting, suggesting at most a small increase in surface activity, and hence a much smaller change in the surface area or volume of the protein upon unfolding. There seems to be no stabilizing effect of 1M sucrose using surface pressure measurements (done at 0.05 mg/ml) to follow thermally-induced denaturation, consistent with the CD data obtained at low protein concentrations (0.05 and 0.2 mg/ml).

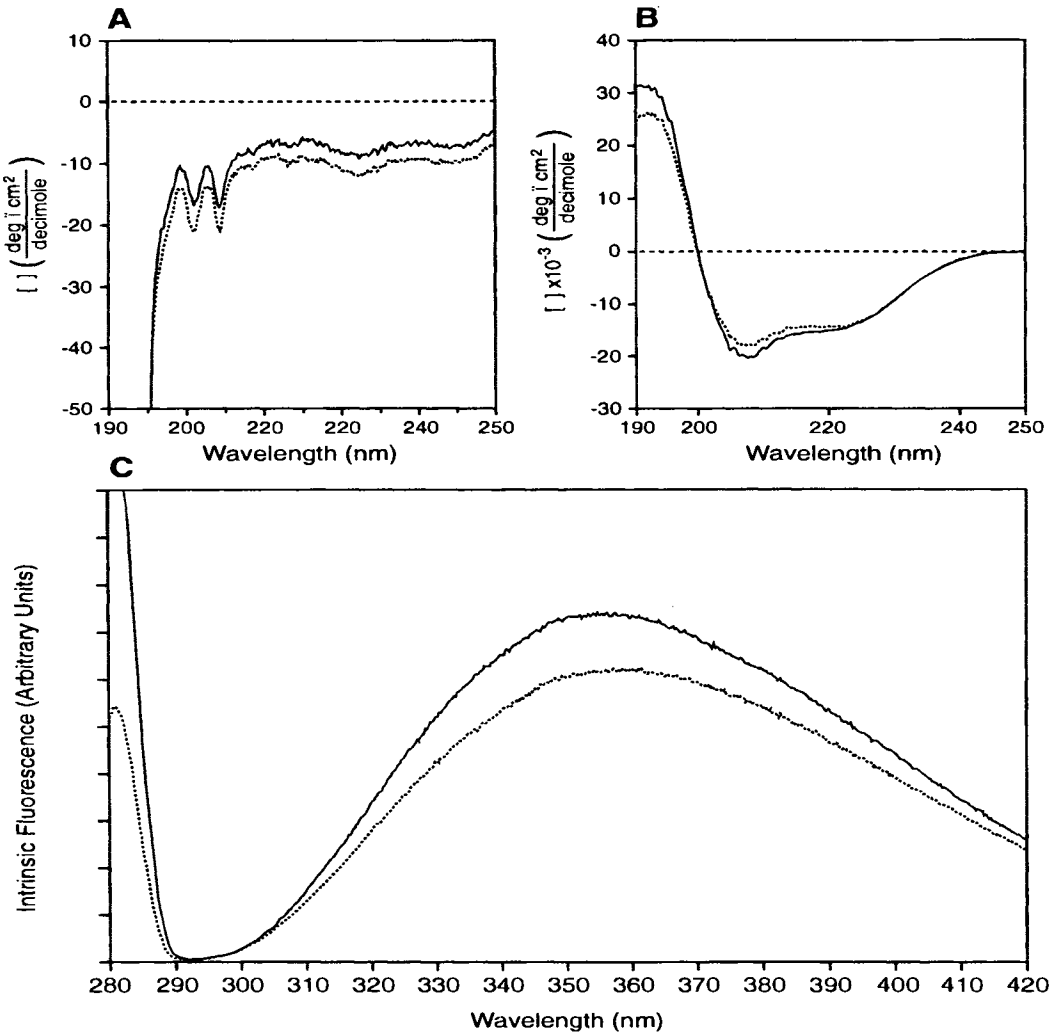

Fig. 3. A comparison of the solution structure of soluble rHuMGDF before and after heating. rHuMGDF was heated at 50°C/hr in 10 mM imidazole, pH 6.5, at 0.2 mg/ml. After reaching 86°C the protein was cooled to 24°C, incubated overnight, and soluble protein separated from aggregates by filtering through a 100,000 molecular weight cutoff filter. The spectra of the soluble protein (——) were compared to that of the standard protein obtained at the same concentration (.1 mg/ml) (-----). Panel A shows the near UV CD spectra, panel B shows the far UV CD spectra, and Panel C shows the fluorescence spectra.

Storage Stability

Thus far, we have discussed the effect of these buffers on the thermal unfolding of rHuMGDF. Does the difference in reversibility of unfolding affect the kinetics during storage as well? To determine this, we compared the amount of monomeric protein remaining in solution following a 2 week incubation at 37°C. Vials of rHuMGDF that were stored in 10 mM phosphate had 65% monomer remaining, vials stored in 10 mM imidazole had 76% remaining, vials of rHuMGDF stored in phosphate plus 1 M sucrose had 75% remaining, and vials stored in 10 mM imidazole plus 1 M sucrose had 87% remaining. These results do show a correlation between the reversibility of thermal unfolding and the amount of monomeric protein remaining after storage, and suggest that optimizing the one can improve the other.

DISCUSSION

rHuMGDF appears to exhibit two unique characteristics during thermally-induced denaturation. First, the reversibility depends on the type of buffer used. Second, sucrose and urea showed little stabilizing or destabilizing effects at low protein concentrations under conditions where the unfolding is highly reversible. Unfolding was completely irreversible in phosphate (anionic buffers), while it was at least partially reversible in imidazole (cationic buffers). However, the melting temperature was not affected by the reversibility and was identical in the anionic and cationic buffers. Melting temperatures usually decrease when reversibility is decreased because the aggregation reaction shifts the thermal unfolding equilibrium toward the denatured state. We can explain the behavior of rHuMGDF by assuming a slow aggregation rate in both phosphate and

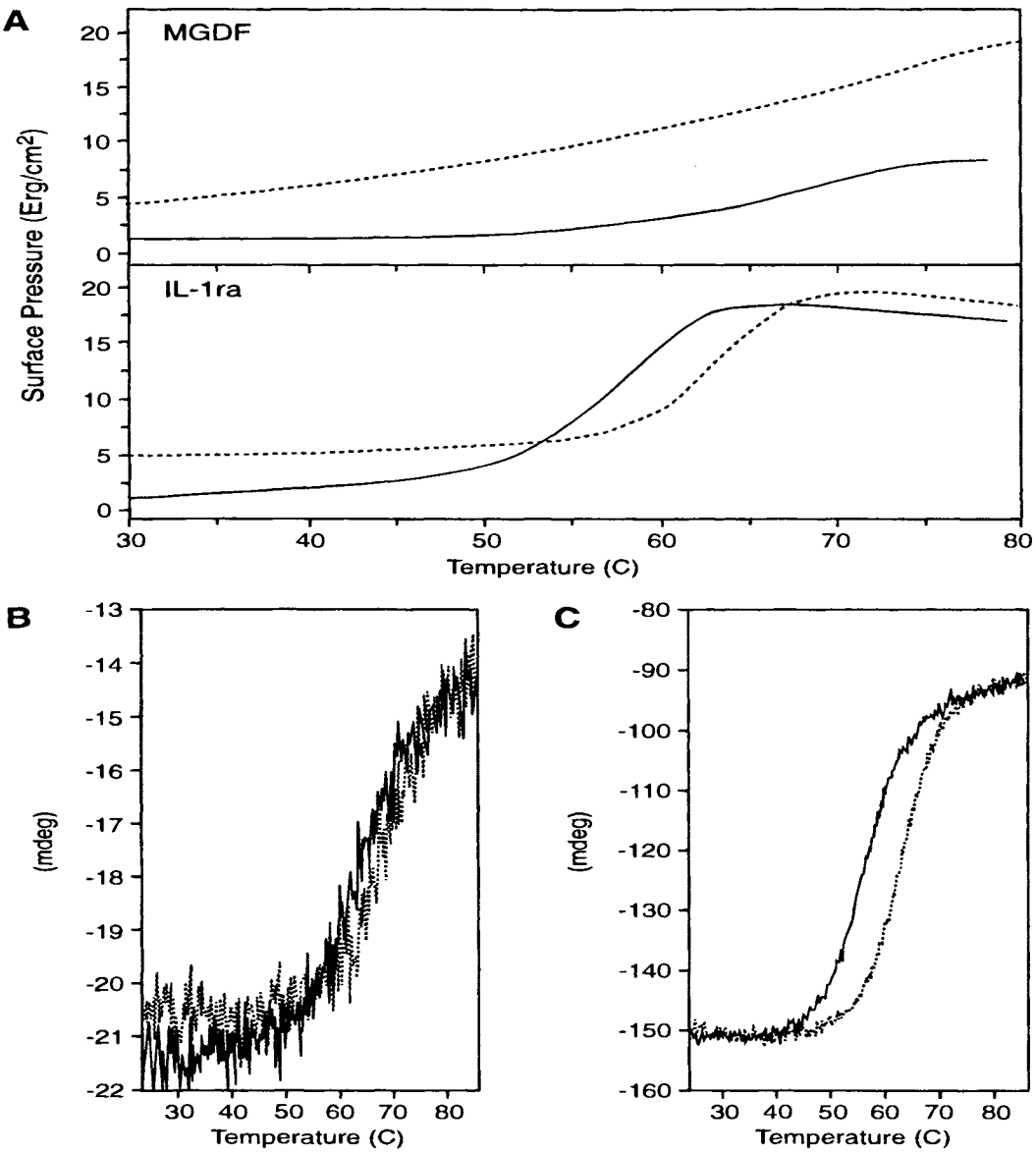

Fig. 4. Effect of sucrose on melting, reversibility and surface tension of rHuMGDF in imidazole. Panel A. Effect of thermal denaturation on the surface tension of protein solutions of 0.05 mg/ml protein in the presence (dotted lines) and absence (solid line) of 1 M sucrose. The surface tension was determined as the temperature was increased from 30°C to 80°C over 30 min. Panel B: rHuMGDF at protein concentrations of 0.2 mg/ml was heated to 86°C, recording the ellipticity at 222 nm. The protein was in either 2 mM imidazole, alone (solid line) or in 1 M sucrose, 2 mM imidazole (dotted line). Panel C: the same experiment, only with protein solutions of 1.6 mg/ml.

imidazole according to Scheme 1, essentially following the Lumry and Eyring model (12),

$$N \underset{K}{\overset{k1}{\leftrightarrow}} D \xrightarrow{k2} Agg$$

Schematic 1

where K is the equilibrium constant of thermal unfolding, k_1 is the rate constant of unfolding, and k_2 is the rate constant of aggregation. To simplify, we assume that k_1 and K are not affected by the solvents, i.e., they are identical in cationic and anionic buffers. Then, the observed difference in reversibility in these two buffers can be explained by differences in k_2, this value being much smaller in imidazole. Although the unfolding reaction itself could be reversible in phosphate, it is the value of k_2 that makes this reaction apparently irreversible.

With this reaction scheme, the apparent melting temperature is affected by the value of k_2 relative to the scan rate. Let us assume that the scan rate is slow enough, relative to k_1, so that the unfolding equilibrium is achieved at each temperature. The lack of any scan rate dependence of Ti (Table 4) is consistent with this assumption. When the unfolding reaction is reversible $k_2 = 0$, and it does not matter how slowly you scan the temperature. However, when the unfolding is irreversible, as is the case here, the scan rate becomes critical. When k_2 increases relative

to the scan rate, the unfolding equilibrium is shifted to the denatured state during the thermal scan and hence the apparent melting temperature decreases. This was not observed for rHuMGDF in phosphate, or at higher imidazole concentrations where reversibility is decreased, indicating that k_2 is still small enough, relative to the scan rate, to have no effect on the apparent melting temperature. However, at high protein or NaCl concentrations, both reversibility and melting temperature are affected, indicating that k_2 is now large enough to affect the melting temperature. Davoodi *et al.* (13) have shown that based on the scan rate dependence of calorimetric unfolding temperature a disulfide mutant of xylanase also exhibits this behavior.

However, all the reversibility and melting temperature results cannot be explained simply by the effects of solvents on k_2, since based on this hypothesis some correlation should exist between reversibility (or aggregation) and melting temperature. For example, phosphate does not change the melting temperature but does reduce the reversibility to 0%, while in 10 mM imidazole the addition of 0.1M NaCl decreases the melting temperature by 9°C, yet does not change the reversibility. The physical characteristics of the aggregate formed also differs between these two conditions, with heating in phosphate producing a true precipitate while heating in imidazole results in soluble aggregates. This suggests that different types of aggregation are occurring under these two different conditions. Although the k_2 value may be more affected in the latter solvent, imidazole appears to result in formation of an aggregate that can be at least partially reversed upon cooling and hence imidazole increases the reversibility.

The specific effect of imidazole versus phosphate suggests there is a specific interaction with one of these buffers and the surface of rHuMGDF. The imidazole (and other cationic buffers) could bind to unfolded rHuMGDF, making the aggregation reversible. Or alternatively, the phosphate (and other anionic buffers) could bind to the folded protein, making the aggregation irreversible. The second mechanism is the more likely for several reasons. First, in water the melting reaction was completely reversible, indicating that something about the protein has been changed in order to produce an irreversible reaction. Secondly, rHuMGDF has a very high (10.7) isoelectric point, such that at pH 7 the protein should theoretically have 5.7 positive charges, making charge-charge repulsion a possible inhibitor of aggregate formation. Interactions with the negatively charged phosphate and citrate would be more favorable than interactions with the positively charged Tris or His. It is also possible that binding of these anionic groups could result in neutralization of the charge, decreasing the inhibition of aggregation due to charge-charge repulsion. At higher concentrations of imidazole or NaCl the chloride present (HCl is used to titrate imidazole) could have the same effect on aggregation. Finally, there are many reports of phosphate binding at the amino terminal end of α-helices, near a loop or β-turn, which contains Gly (14,15). MGDF is a four-helix bundle cytokine, and this contains several potential phosphate binding sites. More than one ligand can interact with phosphate (14), so perhaps the phosphate itself acts as a nucleation site for aggregation.

The observation that 1 M sucrose or 1 to 2 M urea have very little effect on the thermal unfolding of rHuMGDF are at first glance quite puzzling. rHuMGDF is, to our knowledge, the first protein where addition of 1 M sucrose to solutions of 0.05 to 0.2 mg/ml protein fails to increase the melting temperature of the protein. In general, the melting temperatures of proteins are increased by several degrees by the addition of 1 M sucrose and other sugars, independent of the protein concentrations (16–19). It is well known that sucrose is preferentially excluded from protein surfaces and its stabilizing action arises from the increased surface area of the protein which occurs during temperature-induced unfolding (16–19). Therefore, the lack of effect of sucrose implies that for rHuMGDF there is no change in preferential exclusion of sucrose and hence no change in the surface area upon unfolding. This is indeed a very unusual property for a protein, but appears to be consistent with the observation that no change in the surface tension of rHuMGDF occurred upon unfolding.

The addition of 1 and 2 M urea also showed no effect on the melting temperature of rHuMGDF at 0.2 mg/ml. This is also surprising given the general destabilizing effects of urea on the thermal unfolding of native proteins (20–24), but is consistent with the results obtained with rHuMGDF in 1 M sucrose. The destabilizing effect of urea arises from preferential binding of urea to proteins, which is greater for the unfolded state (23). If the thermal unfolding of rHuMGDF results in no change in the surface area, as demonstrated by the lack of effect of 1 M sucrose, then urea should be unable to destabilize it, as well. However, 1 M sucrose does increase the apparent melting temperature of rHuMGDF at 2 mg/ml protein. Does this mean that at 2 mg/ml the thermal unfolding of rHuMGDF in fact increases its surface area and hence the thermal-unfolded state of rHuMGDF depends on protein concentration? We believe there is a better alternative explanation. We hypothesize that the decreased melting temperature of rHuMGDF with protein concentrations above 2 mg/ml or at high salt concentrations is due to an increased rate of aggregation. The stabilization of rHuMGDF at 2 mg/ml by 1 M sucrose may then in fact be due to the effect of sucrose on the rate of aggregation, rather than its usual effect on the thermodynamic equilibrium of thermal unfolding. (Indeed, the melting temperature is only increased to that seen at low protein concentrations in the absence of sucrose.) The effect of 1 M sucrose may be to slow down the rate of aggregation through its interaction with the thermally-unfolded protein or its effect on solvent properties. The viscosity of a 1 M sucrose solution is increased relative to that of buffer alone, and could slow down the rate of intermolecular collision and hence aggregation. Either way, at low protein concentrations the rate of aggregation of rHuMGDF is slow relative to unfolding, hence the apparent melting temperature reflects unfolding itself, and sucrose fails to change this. However, at high protein concentrations the rate of aggregation is increased relative to the scan rate, lowering the apparent T_i. Sucrose slows the aggregation and thus raises the apparent T_i without affecting the unfolding step itself. However, it is also possible that sucrose and urea affect both the equilibrium constant, K, and the rate of aggregation, k_2, such that the net effect is apparently zero at low protein concentrations.

Storage stability, assessed by the remaining monomer in solution incubated at 37°C for 2 weeks, correlates with the reversibility, not the unfolding temperature, of the thermal unfolding reaction. More monomeric protein remains in solution in imidazole than in phosphate. Sucrose also increases the amount of monomer, consistent with the proposed hypothesis that it slows down the rate of aggregation of the unfolded

protein during the thermal scan. This correlation demonstrates the importance of comparing the reversibility of the thermal unfolding as well as the temperature it occurs at, when screening solvent conditions.

The IL-1ra tested here clearly exhibited a drastic decrease in the surface tension upon thermal unfolding. This is probably due to large scale unfolding of the protein and hence due to the denatured protein being more effective in decreasing the surface tension of the solution. Conversely, the rHuMGDF undergoes little change in surface area upon thermal unfolding. However, the far UV CD spectra revealed a large change in the α-helical secondary structure. Therefore, the thermally denatured state of rHuMGDF may be characterized as a compact, but disordered structure. It is then interesting that this structure reversibly converts to the native conformation upon cooling, as observed by both CD and fluorescence spectroscopy, and the sedimentation coefficient of the refolded protein.

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