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 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 occurring 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 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
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, Tᵣ, at which melting began. Thus, the Tᵣ 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 Tᵣ 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 carboxyl 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 DCIT 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 Tᵣ, 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 (caticonic 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 caticonic 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 caticonic 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.