The Effect of Benzyl Alcohol on Recombinant Human Interferon-γ

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Purpose. The goal of this study was to investigate the conformational change and aggregation of recombinant human interferon-gamma (rhIFN-γ) as a result of interaction between benzyl alcohol and the protein. The effects of buffer concentration, buffer species, ionic strength, rhIFN-γ and benzyl alcohol concentrations on the dynamics of the interaction in liquid formulations were also examined.

Methods. The effect of benzyl alcohol on the secondary and tertiary structure of rhIFN-γ in succinate and acetate buffers was studied using far-UV and near-UV circular dichroism spectrophotometry, respectively. Dynamic light scattering was employed to detect aggregate formation due to the interaction of benzyl alcohol with rhIFN-γ.

Results. The addition of benzyl alcohol at 0.9% (w/v) in various liquid rhIFN-γ formulations induced changes in circular dichroism (CD) spectra of the protein in the near-UV region, while the CD spectra in the far-UV region remained unaltered. There were gradual decreases in ellipticity with time throughout the near-UV CD spectra. The decreases in near-UV ellipticity induced by benzyl alcohol were accompanied by the formation of high molecular weight aggregates as measured by dynamic light scattering. Loss in near-UV ellipticity was accelerated at lower protein concentration and by increasing buffer or benzyl alcohol concentration. It was also faster in succinate than in acetate buffer. Formulation ionic strength did not affect the CD spectral changes in both the near- and far-UV regions.

Conclusions. Interaction between benzyl alcohol and rhIFN-γ is formulation dependent. Protein concentration, buffer species, buffer concentration, and preservative concentration play a significant role in determining the extent of the interaction and consequently the stability of the product.

KEY WORDS: recombinant human interferon-γ (rhIFN-γ); benzyl alcohol; circular dichroism; ellipticity; dynamic light scattering; aggregation.

INTRODUCTION

The addition of preservatives to parenteral product formulations is carried out to protect the product from microbial contamination during multiple use. Among the parenteral preservatives, benzyl alcohol is one of the most widely used and is regarded as one of the least toxic preservatives used in formulations for chronic administration of protein pharmaceuticals (e.g., Protropin®[6]). At a concentration of 0.9%, benzyl alcohol is effective against a wide variety of microorganisms and considered safe for healthy adult humans taking a single dose of 30 mL (1). Benzyl alcohol is also used as the bacteriostatic agent in bacteriostatic diluents such as water for injection and 0.9% sodium chloride. Although the safety, efficacy and toxicity of benzyl alcohol for parenteral administration have been studied and discussed (2,3), the interaction of benzyl alcohol with protein drug products has not been explored in detail. It has been reported that other preservatives such as phenol and phenolic compounds cause protein aggregation with human growth hormones after freeze-drying (4,5). The interaction of phenolic compounds with proteins has been investigated extensively in the purification of enzyme from plants (6,7). Phenols combine reversibly with proteins by hydrogen bonding of the phenolic hydrogen to the carbonyl oxygen group of the peptide bond. Formation of quinones as a result of oxidation of phenols can also enhance condensation reactions with proteins. Similarly, benzyl alcohol can also interact with protein by hydrogen bonding and its oxidized form, benzaldehyde, is a reactive agent.

In developing a multiple-dose liquid formulation for recombinant human interferon-gamma (rhIFN-γ) for the treatment of chronic granulomatous disease (8,9), it was noted that benzyl alcohol interacts with the protein resulting in the aggregation of rhIFN-γ with concurrent loss in bioactivity. The structural changes of rhIFN-γ due to denaturation of the protein under different conditions such as pH and temperature have been studied extensively. Human recombinant interferon-γ is unstable upon acid treatment, the protein loses its antiviral activity when exposed to low pH, even after return to neutral pH (10–13). Although circular dichroism analyses indicate that native rhIFN-γ unfolds at below pH 4.5 and refolds into a structure apparently identical to the native form during dialysis against neutral buffer, the refolded protein exists in both aggregated and non-aggregated forms (14). It has been suggested that aggregates are formed either after the monomer refolds into an unnatural conformation, or irreversibly at low pH with small conformational change (15).

Aggregation of rhIFN-γ can also occur via thermal denaturation. The reversible and irreversible thermal denaturation of the protein is pH dependent (16). At pH 6.0 and at 50°C, irreversible thermal denaturation and aggregation is associated with partial or complete unfolding of the protein. At pH 5.0, the protein is stable at the same temperature. It is believed that aggregation of rhIFN-γ during irreversible thermal denaturation follows a mechanism described by Lumry and Biltonen (17). The native folded rhIFN-γ undergoes an unfolded intermediate state which then forms inactive aggregates. The folded and unfolded states are in equilibrium and aggregates formed as a result of thermal denaturation are irreversible.

The goal of the present study was to investigate the effect of benzyl alcohol on the secondary and tertiary structure of rhIFN-γ using both far-UV and near-UV circular dichroism (CD). Dynamic light scattering (DLS) was also employed to detect aggregate formation as a result of interaction between benzyl alcohol and rhIFN-γ. The effect of buffer concentration, buffer species, ionic strength, rhIFN-γ and benzyl alcohol concentrations on the dynamics of the interaction were also examined.

MATERIALS AND METHODS

Recombinant DNA-Derived Human Interferon-γ

rhIFN-γ was expressed in *Escherichia coli* and purified by a series of chromatographic procedures described previously

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(18). The monomeric protein consists of 140 amino acids residues. Unlike natural human IFN-γ, recombinant human IFN-γ is not glycosylated and has an intact C-terminus (19,20).

Preparation of rhIFN-γ Solutions

To study the effect of benzyl alcohol on the degradation of rhIFN-γ in various solutions, rhIFN-γ at 1 mg/mL was dialyzed extensively against sodium succinate and sodium acetate buffers at pH 5.0 and 2–8°C with concentrations ranging from 1–60 mM and 5–250 mM, respectively. Benzyl alcohol was added to each solution to a final concentration of 0.9% (w/v) immediately prior to analyses by circular dichroism and dynamic light scattering. The effect of ionic strength on the interaction between rhIFN-γ and benzyl alcohol was investigated by preparing the protein at 1.0 mg/mL in 1 mM succinate buffer with ionic strength adjusted to 6.7 mM by adding sodium chloride directly to the solution. This ionic strength is equivalent to that of a 5 mM succinate buffer, pH 5.0. Benzyl alcohol was added to the formulation immediately before CD analysis.

The effect of protein concentration on the interaction between rhIFN-γ and benzyl alcohol was examined in 5 mM succinate, pH 5.0 at 0.5, 1.0, 1.5 and 2.0 mg/mL rhIFN-γ. Benzyl alcohol was added directly to each formulation containing various protein concentrations. Circular dichroism analysis was performed on these samples.

To study the effect of benzyl alcohol concentration on the structural change of rhIFN-γ (1.0 mg/mL), benzyl alcohol was used in a concentration range of 0.8–1.3% in 16 mM succinate buffer at pH 5.0 and 0.9–1.5% in 16 mM acetate buffer at pH 5.0. CD analysis was used to determine the structural change of the protein in these formulations as a function of benzyl alcohol concentration.

Protein Concentration Determination

The concentration of rhIFN-γ was determined spectrophotometrically using an extinction coefficient of 0.75 $(mg/mL)^{-1}cm^{-1}$ at 280 nm for rhIFN-γ before benzyl alcohol was added to each formulation. A corresponding formulation buffer was used for reference in each measurement.

Bioactivity Determination

The bioactivity of rhIFN-γ was determined based on its ability to protect A549 cells against infection by an encephalomyocarditis virus. The bioactivity expressed in units/mL is presented as the mean ± standard deviation of 12 replicates and normalized to a reference sample.

Circular Dichroism Measurement

An AVIV® spectropolarimeter Model 60 DS (AVIV Associates) equipped with water bath and data processor was used to measure circular dichroism. Measurements were made at 20°C for all the samples studied. Quartz cuvettes of 1.0 and 0.01 cm path length were used for measuring near-UV (325-275 nm) and far-UV (250-220 nm) CD, respectively. The near-UV CD spectra were taken at 0.5 nm intervals, with a 0.5 nm bandwidth, and 3.0 second averaging time. The far-UV CD spectra used a 0.5 nm interval, 1.5 nm bandwidth, and 3.0 second averaging time. Each CD measurement was taken continuously for 24 hours or more without removing the solution from the cuvette. The CD data were expressed as the mean residue ellipticity [θ], degree.cm^2/decimole, using the mean residue weight of 117 for rhIFN-γ.

Dynamic Light Scattering Measurement

A Brookhaven light scattering instrument (Brookhaven, Holtsville, NY) consisted of a B1200SM goniometer with a photomultiplier positioned at 90° to the incident ion laser (Model 95, Lexel, Fremont, CA) at 488 nm was employed to measure light scattering. Sample was placed in the sample holder in the goniometer with an index matching fluid, decalin, surrounding the sample. The photon data was collected using a BI2030 autocorrelator with 128 channels. Particle size distributions based on the method of Contin (21) was calculated using a personal computer. Each dynamic light scattering measurement was taken at zero, 2, 4, 6, 8 and 24 hours. At each time point, the sample was analyzed several times to assure the reproducibility of the particle size distribution. The results were expressed as the diameter of the particles in nanometer in the solution.

RESULTS AND DISCUSSION

Interaction of Benzyl Alcohol and rhIFN-γ in Solution

The effect of benzyl alcohol in various formulations was studied using circular dichroism and dynamic light scattering techniques. Figure 1 shows the effect of 0.9% benzyl alcohol on the near-UV CD spectrum of 1.0 mg/mL rhIFN-γ in 5 mM succinate at pH 5.0. The spectrum of the control sample containing no preservative is characterized by two positive peaks, at 287 and 280 nm. The addition of benzyl alcohol (0.9%) in this rhIFN-γ formulation resulted in a gradual decrease in CD signal with time. After 21 hours at 20°C, the two positive peaks of the near-UV CD spectrum (325–275 nm) completely disappeared (Figure 1). In contrast, the far-UV CD spectrum (250–220 nm) of the same sample did not show any change

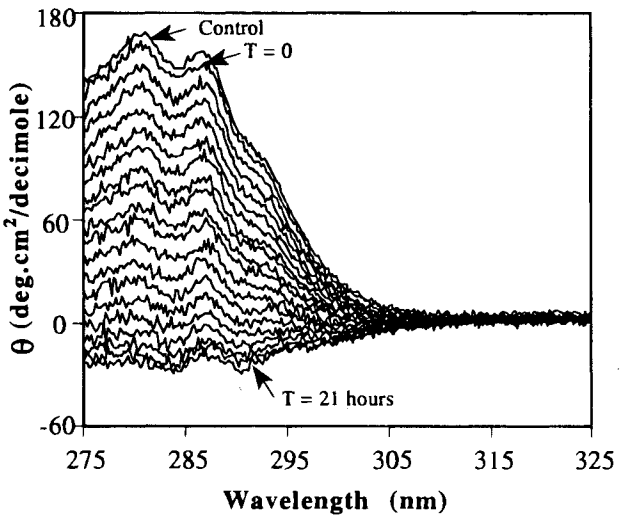

Fig. 1. Time course of change in near-UV circular dichroism spectrum of 1.0 mg/mL rhIFN-γ in a solution consisting of 5 mM succinate, pH 5.0 and 0.9% benzyl alcohol.

in signal after 24 hours (data not shown). This indicates that the loss in near-UV CD signal is not due to light scattering from protein aggregates. One would expect greater scattering and thus greater losses in CD signal at the lower wavelengths. Under the same conditions, the control sample without benzyl alcohol has been shown to be stable for several months. These observations indicate that the addition of benzyl alcohol (0.9%) in a rhIFN-γ formulation consisting of 5 mM succinate at pH 5.0 induces changes in the tertiary structure of rhIFN-γ without affecting the secondary structure of the protein. No change in far-UV CD was observed down to 220 nm, indicating no substantial loss of α-helical structure occurred. Native rhIFN-γ formulated in 5 mM succinate at pH 5.0 has been reported to be 55% α-helix and 1% β-sheet (22). Due to the high absorption by benzyl alcohol, lower wavelengths (below 220 nm) where β-sheet contributes could not be monitored. Upon removal of benzyl alcohol from the rhIFN-γ sample after 24 hours of incubation with the preservative, almost full retention of near-UV CD spectrum was observed. However, no recovery of near-UV CD spectrum was obtained in the rhIFN-γ sample that had been incubated with the preservative for 1 month. This demonstrates that the changes observed within 24 hours are reversible but become irreversible upon further incubation. Additionally, the protein lost <10% of its bioactivity after 24 hours of incubation with benzyl alcohol (0.9% w/v) as compared to >95% loss after 1 month at 25°C.

Dynamic light scattering (DLS) was used to monitor the formation of large molecular weight species resulting from interaction of benzyl alcohol and rhIFN-γ. DLS has been employed to determine the actual average size change of macromolecules in solution as a result of thermal denaturation (23) or refolding (24) of globular proteins. Figure 2 illustrates the results obtained for a 1.0 mg/mL rhIFN-γ in 5 mM succinate in the presence of 0.9% benzyl alcohol. The increase in average size of the protein molecule expressed as the diameter (D in nm) and the loss in ellipticity at 280 nm were plotted as a function of time. The ellipticity appears to follow a zero order kinetic decay whereas D increases with time following a sigmoidal relationship. It is apparent that the spectral changes in the near-UV CD region correlates with the formation of high molecular weight species. rhIFN-γ exists in the native state as a non-covalently bound homodimer with D ≈ 5 nm. When the ellipticity of the sample dropped to 50% of the initial value at the 7.5 hour timepoint, D was approximately 60 nm. The lag time in the D vs. time curve is probably due to the insensitivity of the detector to small increases in average size. The quantitative ratio of aggregate/monomer is difficult to determine as the weight averaged intensity of the scattered light is proportional to the third power of D. Thus, as the size of the aggregate grows, the observed intensity of scattered light would be primarily generated by larger species even though on a weight average basis, these aggregates may account for only a small fraction of the total protein. The correlation between loss in near-UV CD signal and increase in average molecular diameter may be explained as follows: benzyl alcohol may induce a loosening of rhIFN-γ tertiary structure to form molten globules. Alternatively, benzyl alcohol may selectively bind to the protein, trapping the protein into specific isomeric structures which might result in a loss of near- UV CD signal. These structural changes may predispose rhIFN-γ to aggregation. Specific binding of benzyl alcohol to rhIFN-γ should not contribute to the observed ellipticity in the 280–300 nm region due to its low extinction coefficient in this spectral range.

Dependence on Protein and Benzyl Alcohol Concentrations

The effect of protein concentration on the change in near-UV CD of rhIFN-γ in a fixed concentration of benzyl alcohol (0.9% w/v) was studied within a range of 0.5 to 2.0 mg/mL protein in 5 mM succinate at pH 5.0. The time course of change in circular dichroism was followed for up to 24 hours after the addition of benzyl alcohol to each protein solution. These results are depicted in Figure 3. At a fixed benzyl alcohol concentration, the degradation (expressed as loss in ellipticity at 280 nm with time) is faster at lower protein concentrations such as 0.5 and 1.0 mg/mL than at higher protein concentrations such as 1.5 and 2.0 mg/mL.

Benzyl alcohol concentration was also studied for it effect on change in near-UV CD of rhIFN-γ. At a fixed rhIFN-γ concentration, increasing benzyl alcohol concentration resulted in an increase in the rate of loss in ellipticity for the protein formulated at 1 mg/mL in either 16 mM succinate or acetate

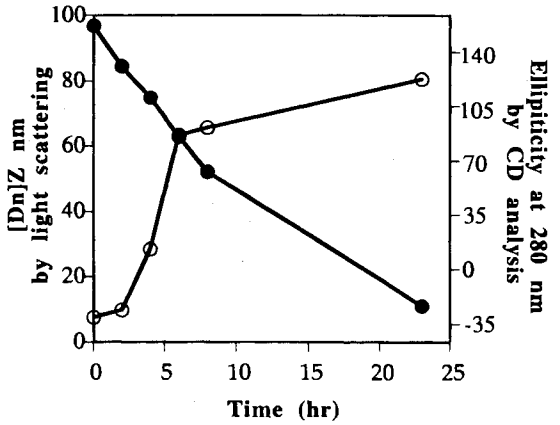

Fig. 2. Time course of aggregate formation of 1.0 mg/mL rhIFN-γ in 5 mM succinate, pH 5.0 in the presence of 0.9% benzyl alcohol as determined by dynamic light scattering (○) and by circular dichroism analysis (●).

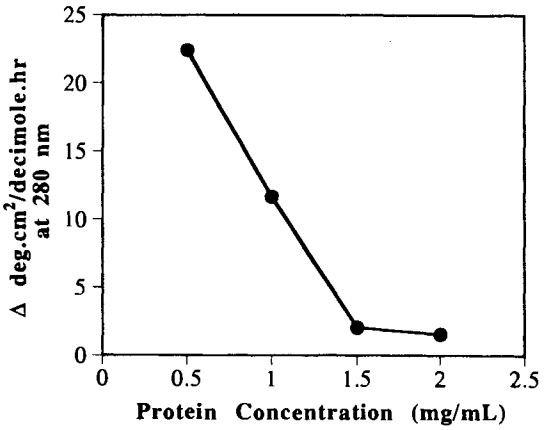

Fig. 3. Effect of protein concentration on degradation of rhIFN-γ formulated in 5 mM succinate, pH 5.0, and 0.9% benzyl alcohol.

at pH 5.0 (Figure 4). The plot of rate of loss in ellipticity versus benzyl alcohol shown in Figure 4 is bi-phasic. In 16 mM succinate, the degradation increases slowly with increasing benzyl alcohol up to a concentration of 1.1% where a second phase begins in which the rate of loss in ellipticity increases 7 fold. At the same buffer concentration, the rate of loss in ellipticity for rhIFN-γ in the acetate formulation is approximately 8 to 10 fold slower than in succinate and the second phase begins at a much higher preservative concentration (1.3%). Thus, benzyl alcohol-induced unfolding of rhIFN-γ is also buffer dependent.

Dependence on Buffer and Ionic Strength

The effect of buffer concentration and buffer species on the interaction between rhIFN-γ and 0.9% benzyl alcohol was studied at a rhIFN-γ concentration of 1.0 mg/mL in succinate and acetate buffer concentrations ranging from 1 to 60 mM and 5 to 100 mM at pH 5.0, respectively. Benzyl alcohol was added directly to each formulation prior to CD measurements. The rate of loss in ellipticity in the near-UV region is related to the concentration of buffer in both acetate and succinate as shown in Figure 5. However, the rates observed in acetate buffered solutions are much slower. The rate of decrease in ellipticity at 280 nm was 126 times less for the sample in 5 mM acetate than in 5 mM succinate at the same pH. The role of ionic strength was discounted based on an experiment in which a rhIFN-γ solution formulated in 1 mM succinate which is stable in the presence of 0.9% benzyl alcohol, was adjusted to an ionic strength equivalent to that of a 5 mM succinate solution (6.7 mM) using NaCl. This solution was not sensitive to the overall ionic strength of the solution and that the faster reaction rate in succinate is buffer specific, namely the divalence nature of the succinate salt. An attempt was made to study the interaction in citrate buffer, a trivalent anion failed. rhIFN-γ precipitates in citrate-buffered solution even in the absence of benzyl alcohol. Again, no spectral change in the far-UV region was recorded in rhIFN-γ formulation consisting of 60 mM acetate at pH 5.0 and 0.9% benzyl alcohol, indicating that the secondary structure of rhIFN-γ is not affected by the presence of 0.9% benzyl alcohol in the acetate formulation.

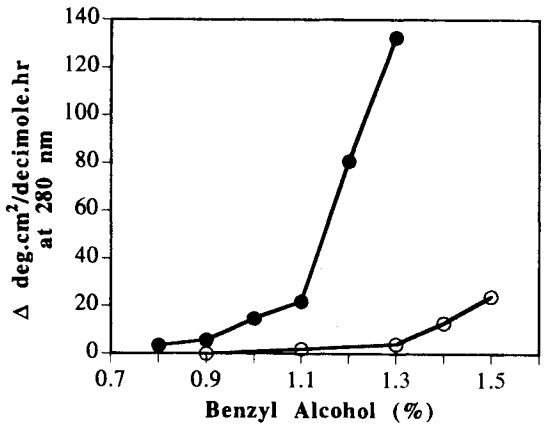

Fig. 4. Loss in ellipticity at 280 nm of rhIFN-γ as a function of benzyl alcohol concentration in 16 mM acetate buffer at pH 5.0 (○) and 16 mM succinate buffer at pH 5.0 (●).

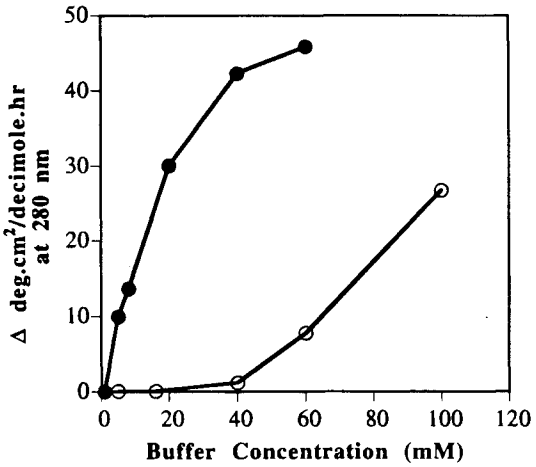

Fig. 5. Loss in ellipticity at 280 nm of rhIFN-γ in the presence of 0.9% benzyl alcohol as a function of buffer concentration in the formulations. Acetate buffer at pH 5.0 (○); succinate buffer at pH 5.0 (●).

CONCLUSIONS

The interaction between benzyl alcohol and rhIFN-γ is a complex phenomenon involving the preservative, the protein and the formulation buffer. This interaction may result in the loosening of the rhIFN-γ tertiary structure which in turn predisposes the protein to aggregation. In the presence of benzyl alcohol, rhIFN-γ may also be trapped into specific isomeric structures which result in a loss of near-UV CD signal. The mechanism of interaction is not clear at this time, but minimization of preservative concentration and the selection of acetate as the buffer led to a stable preserved multiple-dose liquid formulation of rhIFN-γ.

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