Solvent Effects on the Solubility and Physical Stability of Human Insulin-Like Growth Factor I

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Purpose. The solubility and physical stability of human Insulin-like Growth Factor I (hIGF-I) were studied in aqueous solutions with different excipients.

Methods. The solubility of hIGF-I was determined by UV-absorption and quantification of light blocking particles. The physical stability of hIGF-I was studied with differential scanning calorimetry (DSC) and circular dichroism (CD) spectroscopy.

Results. Human IGF-I precipitated at low temperature in the presence of 140 mM benzyl alcohol and 145 mM sodium chloride. CD data showed that the tertiary structure of hIGF-I in these conditions was perturbed compared to that in 5 mM phosphate buffer. In the presence of benzyl alcohol 290 mM mannitol stabilized hIGF-I. Sodium chloride or mannitol by themselves had no effect on either the solubility or the tertiary structure. Benzyl alcohol was attracted to hIGF-I, whereas sodium chloride was preferentially excluded. The attraction of benzyl alcohol was reinforced by sodium chloride leading to salting-out of hIGF-I. The CD-data indicated interactions of benzyl alcohol with phenylalanine in hIGF-I. Thermal denaturation of hIGF-I occurred in all solutions with sodium chloride, whereas mannitol or benzyl alcohol had no effect on the thermal stability. The thermal stability of hIGF-I was thus decreased in 145 mM sodium chloride although it was excluded from hIGF-I.

Conclusions. The self-association and thermal aggregation of hIGF-I is driven by hydrophobic interactions. Benzyl alcohol is attracted to hIGF-I and induces changes in the tertiary structure causing hydrophobic attraction of the protein at low temperatures.

KEY WORDS: hIGF-I; benzyl alcohol; preferential interaction; stability; preservative.

INTRODUCTION

Human insulin-like growth factor I (hIGF-I) is a single chain globular protein consisting of 70 amino acids (1), see Figure 1. Human IGF-I shares structural similarities with the homologous proteins insulin, proinsulin, relaxin and hIGF-II (2). These proteins share a sensitivity to solution conditions and form associated forms or precipitates (3,4). The secondary and tertiary structure of hIGF-I is pH-dependent and the protein has been found to aggregate covalently at pHs around its isoelectric point at pH 8.2 (5,6). It has been demonstrated that solvent polarity has a major effect on the folding selectivity of hIGF-I (7).

The solubility and physical stability of proteins are dependent on different solvent conditions, e.g., pH, ionic strength and additional solutes. The stability of proteins in aqueous solutions is often discussed in terms of preferential interactions between the protein and solutes (8–10). Solvent components, solutes, can be either attracted to or excluded from the protein surface. It has recently been suggested that solutes are excluded from the peptide-backbone and attracted to the amino acid residue side chains (11). Both these phenomena can stabilize the protein structure. Stabilization is obtained when the ΔG of denaturation is increased for the protein-solute complex or the protein with excluded solute compared to the protein in a solution without the solute.

Benzyl alcohol is a common preservative for injectables. Reports on enhanced aggregation of proteins and decreased thermal stability in the presence of benzyl alcohol (12,13) suggest that benzyl alcohol interacts with protein structures. In this paper, we present an investigation on the solubility and physical stability of hIGF-I in the presence of some common formulation components; benzyl alcohol, mannitol and sodium chloride.

The concentrations of the excipients were chosen to be relevant for a parenteral formulation for subcutaneous administration.

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Fig. 1. Primary structures of human IGF-I and human insulin with disulfide bonds designated in black. The black residues designate the disulfide bonds. Human insulin consists of two polypeptide chains (A and B) linked by two disulfide bonds. Marked in the two proteins are the homologous phenylalanine residues expected to be affected by interactions with phenol compounds.
MATERIALS AND METHODS

Materials

In-house yeast-derived recombinant hIGF-I (14) and ultra pure MilliQ water (Millipore Corp., USA) were used. Analytical grade disodium phosphate dodecahydrate, monosodium phosphate monohydrate, sodium chloride, mannitol and benzyl alcohol were purchased from Merck (USA).

Solubility Study

The optical densities (OD) at 276 and 320 nm in protein solutions with hIGF-I 10 mg/mL and different excipients were determined by a UV/VIS spectrophotometer Perkin Elmer Lambda 2 (Perkin-Elmer, Sweden). The respective vehicles without protein were used as reference solutions.

The solutions were filled in sealed vials, heated to 65°C and then carefully transferred to 1 mm quartz cuvettes (UVIR, Sweden). The heating of the solutions were made to dissolve protein particles in the solutions.

Precipitation Study

Determinations of particle content per mL hIGF-I solution at the temperatures 75, 25 and 7°C were performed by a light blockage technique (15). A liquid sampler HIAC/ROYCO 3004A (Ninolab, Sweden) equipped with a liquid sensor HR-LD 150 and a 9064 sizing counter was used. Sample volumes of 2–5 mL were used. Three separate determinations were made for each formulation. Vehicle solutions without protein were used for blank determinations.

Circular Dichroism Study

Circular dichroism spectroscopy was performed on a JASCO J-720 (Japan). Circular dichroism spectra were recorded for solutions with 0.1, 2 and 7 mg/mL hIGF-I in the different formulations. The temperature was controlled by a Neslab RTE-110 water bath (Chemical Instruments, Sweden) monitored by a temperature probe at the cuvette holder. Quartz cuvettes with path lengths of 1 and 10 mm were used (UVIR, Sweden). Scans were obtained with a 1 nm slit and 50 mdeg sensitivity. Triplicate scans were obtained for each formulation and correction for background dichroism was made by subtracting the CD-spectra recorded for the respective vehicle solution. Noise reduction was performed on all CD-spectra by the Fourier transformation algorithm in the instrument’s J-700 software.

Densitometry

The densities of the solutions were determined with a high precision Mettler Toledo KEM DE-310 (Switzerland) densitometer at 20.00 ± 0.05°C. Solutions were prepared with protein concentrations ranging from 2–15 mg/mL and with either constant molality (m) or constant chemical potential (μ) of the different excipients. Solutions with constant molality of excipients were prepared by mixing aliquots of the hIGF-I stock solution with weighed amounts of excipients and adding water to make solutions with hIGF-I of the desired concentrations. Solutions with constant chemical potential of excipients were prepared by dialyzing volumes of 3–5 mL of hIGF-I in different concentrations against 2000 mL of the respective solvent for 48 h at room temperature with one change of solvent. The solutions were withdrawn from the dialysis bags immediately prior to the densitometric determinations.

Immediately after densitometry the protein concentrations in all solutions were determined from the optical density (OD) at 276 nm on a UV/VIS spectrophotometer Perkin Elmer Lambda 2 (Perkin-Elmer, Sweden) assuming a molar absorptivity of 8.1 × 10⁻³.

Calculation of the Preferential Interactions of the Solutes with hIGF-I

The calculations of the preferential interactions were made as described by Arakawa and Timasheff (16,17). The investigated protein formulations contained water (component 1), protein (component 2) and excipients (component 3) according to the notation of Scatchard (18) and Stockmayer (19). The apparent specific volume φₐₚ was calculated by the relationship

\[
φₐₚ = \frac{1}{ρ₀} \left( 1 - \frac{Δρ}{C_z} \right)
\]

(1)

Where ρ₀ is the density of the solvent, Δρ is the difference between the density of the protein solution and that of the solvent, C₂ is protein concentration in gram per milliliter and t is the temperature (20°C). Extrapolation of φₐₚ to infinite dilution gave the partial specific volume of the protein (φ₂₀) in the studied solution. The partial specific volumes at constant molality (φ₂₀) and at constant chemical potential (φ₂₀) were determined and the preferential interaction parameter \(\langle δg_j/δg_k \rangle_{j,k=1,3}\) was determined by the data by

\[
\left( \frac{δg_j}{δg_k} \right)_{j,k=1,3} = \frac{(1 - ρ₀φ₂₀) - (1 - ρ₀φ₂₀)}{1 - ρ₀\bar{v}_3}
\]

where \(\bar{v}_3\) is the partial specific volume of component 3 (20). The \(\bar{v}_3\) was obtained by density measurements of solutions with different concentrations of the solute, correcting for concentration in a similar manner as for the φₐₚ for the protein and then extrapolating to infinite dilution. The interaction parameter \(\langle δg_j/δg_k \rangle_{j,k=1,3}\) is a measure of the excess of component 3 in the immediate proximity of the protein compared to its bulk concentration. A positive value indicates a higher concentration of component 3 around the protein than in the bulk solution, i.e. preferential attraction to the protein. A negative value indicates lower concentration of component 3 near the protein, i.e. preferential exclusion.