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 during 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.

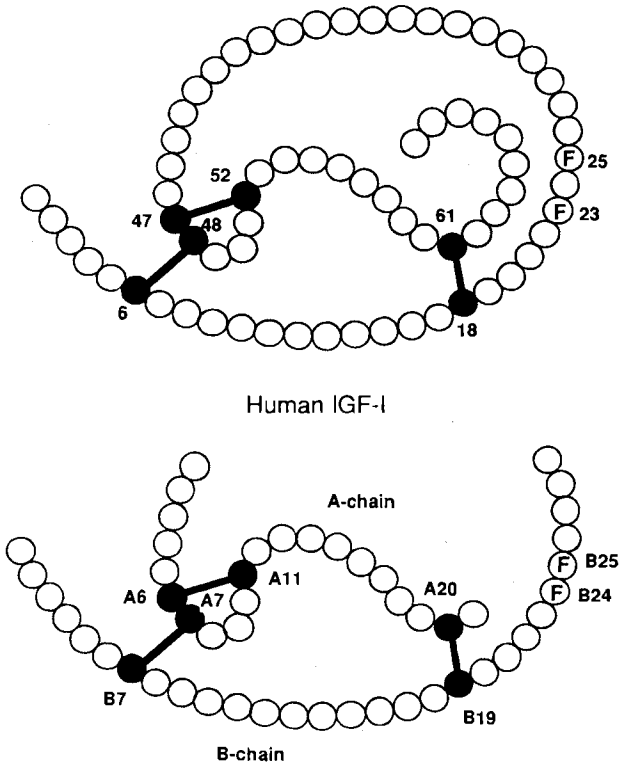

Fig. 1. Primary structures of human IGF-I and human insulin with disulfide bonds designated in black. The black residues designate the disulfide bonds. Human insulin consists of two polypeptide chains (A and B) linked by two disulfide bonds. Marked in the two proteins are the homologous phenylalanine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

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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.

Differential Scanning Calorimetry

Stability studies of different hIGF-I solutions were performed on a high sensitive MC-2 differential scanning calorimeter (DSC) (MicroCal, USA). The volume of the calorimetric vessel was 1.2 mL. All scans were performed with the respective formulation buffers under study as references. Baseline scans of each formulation buffer were performed in connection with each DSC scan of protein solution. The calorimetric data were analyzed with the Origin™ program. The scanning rate was 43°C/h, and scans were performed in the temperature range of 5–80°C.

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 $8.1 * 10^{-2}$.

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 ϕ_{app} was calculated by the relationship

$$\phi_{app_t} = \frac{1}{\rho_0}\left(1 - \frac{\Delta\rho}{C_2}\right)_t \quad (1)$$

Where ρ_0 is the density of the solvent, $\Delta\rho$ is the difference between the density of the protein solution and that of the solvent, C_2 is protein concentration in gram per milliliter and t is the temperature (20°C). Extrapolation of ϕ_{app} to infinite dilution gave the partial specific volume of the protein (ϕ_2^0) in the studied solution. The partial specific volumes at constant molality (ϕ_2^0) and at constant chemical potential ($\phi_2'^0$) were determined and the preferential interaction parameter $(\partial g_3/\partial g_2)_{t,\mu_1,\mu_3}$ in grams of excipient per gram of protein was obtained from the data by

$$\left(\frac{\partial g_3}{\partial g_2}\right)_{t,\mu_1,\mu_3} = \frac{(1 - \rho_0\phi_2'^0) - (1 - \rho_0\phi_2^0)}{1 - \rho_0\bar{\nu}_3} \quad (2)$$

where $\bar{\nu}_3$ is the partial specific volume of component 3 (20). The $\bar{\nu}_3$ was obtained by density measurements of solutions with different concentrations of the solute, correcting for concentration in a similar manner as for the ϕ_{app} for the protein and then extrapolating to infinite dilution. The interaction parameter $(\partial g_3/\partial g_2)_{t,\mu_1,\mu_3}$ 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.

The preferential interaction parameter is related to the preferential hydration parameter $(\partial g_1/\partial g_2)_{t,\mu_1,\mu_3}$ by

$$\left(\frac{\partial g_1}{\partial g_2}\right)_{t,\mu_1,\mu_3} = -\frac{1}{g_3}\left(\frac{\partial g_3}{\partial g_2}\right)_{t,\mu_1,\mu_3} \quad (3)$$

where g_3 is the concentration of component 3 (the solute) in grams per gram of water (19).

RESULTS

Solubility Study

The solubility of hIGF-I in different solvents as a function of temperature are shown in Figure 2. In the figures the OD at 276 nm (the protein), and at 320 nm (light scattering particles) are presented. For hIGF-I in 290 mM mannitol with or without 140 mM benzyl alcohol the protein solubility remained unchanged in the range of 50 to 10°C. See Figure 2A for a typical temperature scan. For hIGF-I in 145 mM sodium chloride the protein solubility remained also unchanged in the same temperature range. The hIGF-I solution with 145 mM sodium chloride and 140 mM benzyl alcohol exhibited a clear temperature dependent solubility (Figure 2B). The OD at 276 nm was unchanged from 65°C down to 30°C where the OD increased substantially. This increase in OD was paralleled by the appearance of light scattering particles which is seen by the large increase in OD at 320 nm. The light-scattering causes a shift in the baseline at the lower wavelengths. By correcting the OD_{276} for the base-line shift it is obvious that the OD_{276} decreases substantially, which suggests a decreased solubility of hIGF-I in this medium at low temperatures, as seen in Figure 2B.

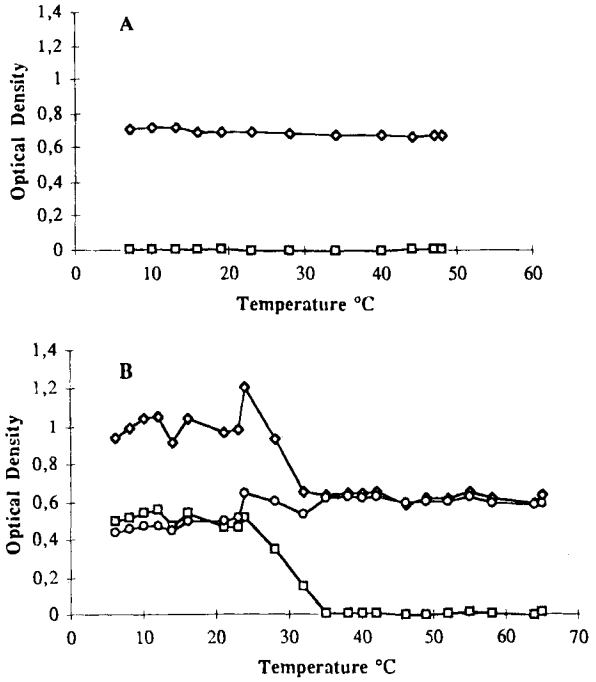

Fig. 2. Optical densities of aqueous solutions of hIGF-I with (A) 290 mM mannitol and 140 mM benzyl alcohol and (B) 145 mM sodium chloride and 140 mM benzyl alcohol at different temperatures and pH 6 at different temperatures. The OD was determined at 276 nm (◇) and at 320 nm (□). For B the OD at 276 nm was also corrected for light scattering (○) as described in the methods.

Precipitation Study

A light blockage technique was used to study the effect of temperature on the solubility of hIGF-I in different aqueous media. The quantification of particles larger than 1.25 or 8.9 μm was chosen as a probe for solubility. The contents of light blocking particles larger than 8.9 μm in concentrated hIGF-I solutions with different compositions at 75, 25 and 7°C are shown in Figure 3. In a sodium chloride/benzyl alcohol medium a drastic increase in light blocking particles with decreasing temperature was found. The particles in this solution were formed instantly when the temperature was lowered to 20–25°C. This phenomenon indicated that at this temperature the solution was saturated with a protein complex with low aqueous solubility. The precipitate dissolved when the temperature was raised above 30°C, which suggested a reversible formation of a complex with low aqueous solubility. The mannitol formulation with benzyl alcohol exhibited the lowest particle content of all formulations investigated and also showed no significant change in particle content with the temperature.

Circular Dichroism Studies

Typical near-UV scans of aqueous solutions of hIGF-I in different concentrations in 10 mM sodium phosphate buffer, pH 6.0 at 22°C are shown in Figure 4. The spectra for the formulations with 290 mM mannitol or 145 mM sodium chloride both without benzyl alcohol are identical and do not deviate from the spectrum for hIGF-I in 10 mM sodium phosphate buffer (Figure 5A). These spectra basically demonstrate a large negative band at 240 nm, three weaker positive bands at 250–270 nm and a broad positive shoulder starting from 275 nm and going towards higher wavelengths. The addition of 140 mM benzyl alcohol to these solutions changed the band shape at 250–270 nm. In the mannitol/benzyl alcohol solution the spectra was significantly perturbed in comparison to the solutions without benzyl alcohol. For the sodium chloride solution the bandshapes were significantly more sharpened than for

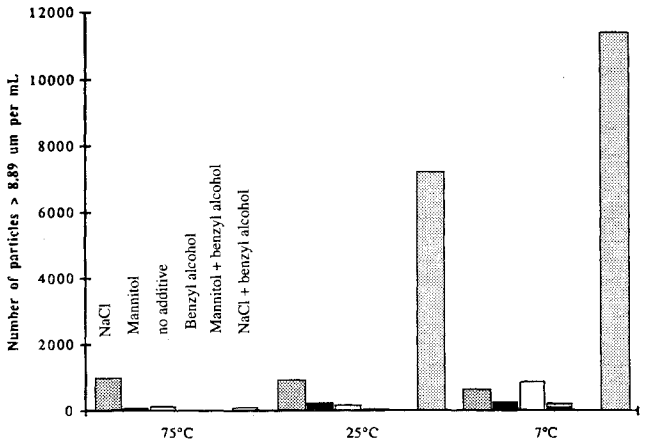

Fig. 3. The content per mL of particles larger than 8.89 μm in different hIGF-I solutions determined at 75, 25 and 7°C with light blockage technique. The solutions contained hIGF-I 8–10 mg/mL and a 10 mM sodium phosphate buffer of pH 6.0.

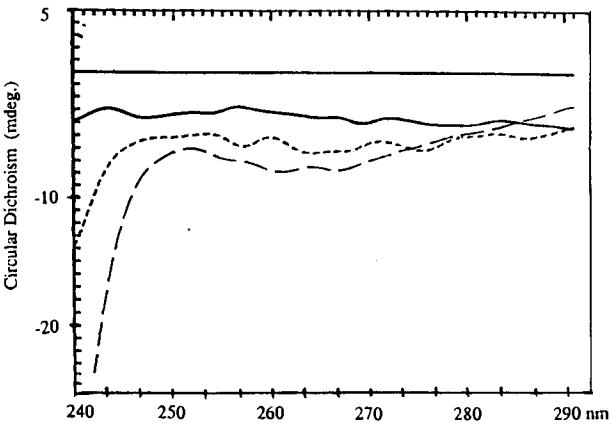

Fig. 4. Near-UV CD spectra at 22°C of hIGF-I 0.1 mg/mL (——), 2 mg/mL (- - - -) and 7 mg/mL (— —) in a 10 mM sodium phosphate buffer of pH 6.0.

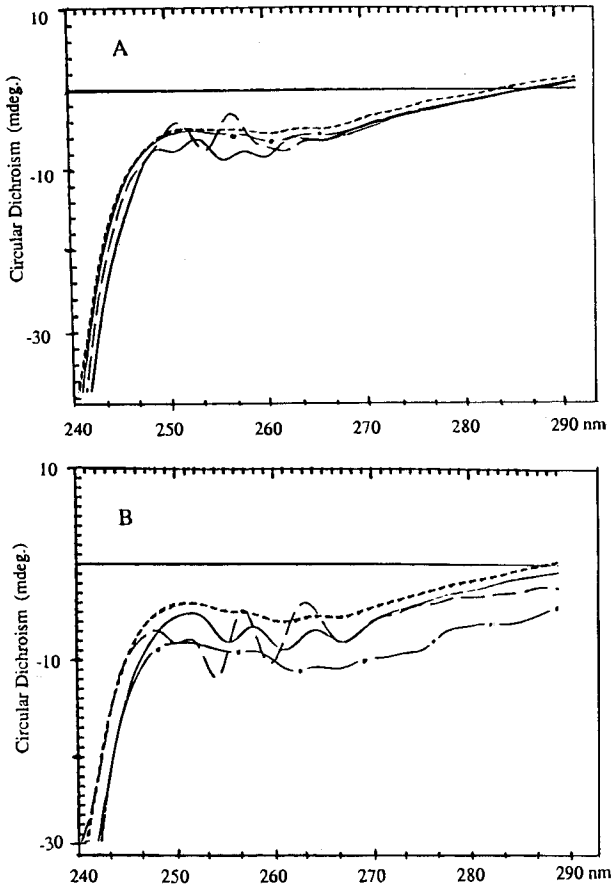

Fig. 5. Near-UV CD spectra of hIGF-I 7 mg/mL in different solutions with pH 6 at (A) 22°C and (B) 7°C. The solutions contained mannitol 290 mM and benzyl alcohol 140 mM (——), mannitol 290 mM (- - -), sodium chloride 145 mM and benzyl alcohol 140 mM (— —) and sodium chloride 145 mM (— • —).

mannitol and also shifted towards lower wavelengths when benzyl alcohol was added. A small additional positive band at 252 nm also appeared.

Increasing the temperature from 22 to 55°C at constant protein and benzyl alcohol concentrations did not affect the overall shape of the absorption bands, with the exception that at 55 °C the spectral perturbations in the benzyl alcohol solutions were less pronounced than at 22°C compared to the other solutions. Lowering the temperature to 7°C (Figure 5B) at the same protein and benzyl alcohol concentrations resulted in a reinforcement of the spectral changes in the region 250–270 nm. These changes appeared both for mannitol and sodium chloride solutions but they were much more pronounced for the latter case. At lower hIGF-I concentrations, i.e. 0.1 and 2 mg/mL, the impact of benzyl alcohol on the CD spectra was even greater (not shown). Spectral changes in near-UV CD reflect alterations in the tertiary structure of the protein, particulary the the aromatic residues and disulfides. Aggregation of proteins can also alter the shape and intensity of near-UV CD (21) and the large changes in near-UV CD in this study can thus indicate that benzyl alcohol induces aggregation of hIGF-I. All the changes in the tertiary structure of hIGF-I were reversible as all the spectra after exchange of solvent by exhaustive dialysis for 48 hours were identical to the original spectrum in phosphate buffer solution.

Far-UV CD reflect the secondary structure of proteins and can be used for estimations of the relative amounts of alpha-helices, beta-sheets and turn structures in a particular protein. Far-UV scans of hIGF-I exhibited only a minor temperature dependence at pH 6 (not shown). Addition of mannitol, sodium chloride or benzyl alcohol did not provoke any significant changes of the spectra. The secondary structure appears therefore not to have been affected by the solution conditions in this study.

Preferential Interaction Parameters

The results from the determinations of partial specific volumes and the interaction parameters are summarized in Table 1. Mannitol 290 mM exhibited a small positive interaction term indicating minor attraction of solute to hIGF-I. For 145 mM sodium chloride the interaction parameter was of negative sign but of the same amount as for mannitol. Sodium chloride would therefore be preferentially excluded from the protein surface. Benzyl alcohol, 140 mM had a larger positive interaction parameter and would be more markedly attracted to hIGF-I. The phenomenon became more pronounced when the preferential hydration parameters were compared. Mannitol exhibited a small negative value, sodium chloride a large positive value and benzyl alcohol a large negative value reflecting a massive exclusion of water from the protein surface in the latter case. For the four-component systems (hIGF-I with benzyl alcohol and either sodium chloride or mannitol) the interaction parameters could be calculated only under the assumption that the two non-protein solutes did not interact. The value for preferential hydration of hIGF-I in the mannitol/benzyl alcohol solution was in the same range as for mannitol alone, and hIGF-I was significantly better hydrated in this solution than in the solution with benzyl alcohol only. This showed that mannitol by being preferently excluded balances the decreased hydration of hIGF-I caused by the attraction of benzyl alcohol from hIGF-I during

Table 1. Preferential Interaction and Hydration Parameters of hIGF-I with Different Solutes in Aqueous Solution at pH 6.0 and +20°C

Solute	Conc. mol/kg	g_3 g/g	ρ_0 g/mL	$\bar{\nu}_3$ mL/g	ϕ_2^0 mL/g	$\phi_2'^0$ mL/g	$\phi_2^0 - \phi_2'^0$ mL/g	$\left(\frac{\partial g_3}{\partial g_2}\right)_{t,\mu_1,\mu_3}$ g/g	$\left(\frac{\partial g_1}{\partial g_2}\right)_{t,\mu_1,\mu_3}$ g/g
Reference[a]	—	—	0.9989	—	0.701	—	—	—	—
Mannitol	290	0.046	1.0143	0.650	0.773	0.729	0.043	0.13	−2.8
NaCl	145	0.0085	1.0042	0.281	0.605	0.703	−0.097	−0.14	16
Benzylalcohol	140	0.015	0.9992	0.935	0.702	0.677	0.026	0.26	−18
Mannitol + Benzylalcohol	290 / 140	0.046 / 0.015	1.0152	1.585[b]	0.493	0.668	−0.175	0.29	−4.8
NaCl + Benzylalcohol	145 / 140	0.0085 / 0.015	1.0052	1.216[b]	0.513	0.773	−0.260	1.18	−50

[a] 5 mM sodium phosphate buffer, pH 6.
[b] Obtained by adding the respective $\bar{\nu}_3$ of the two non-protein solutes. It was assumed that the two solutes did not interact.

these conditions. In the solution with benzyl alcohol/sodium chloride the hydration of hIGF-I was lowest in all the solution variants and was significantly lower than in the solution with benzyl alcohol alone.

Differential Scanning Calorimetry Studies

Typical DSC scans on hIGF-I in different formulations are shown in Figure 6. There is no simple way to obtain quantitative information on aggregation from heat capacity measurements. However, thermograms from DSC scans of protein solutions showing drifts or irregularities indicate changes in the systems which preferably are interpreted as aggregation. For the 290 mM mannitol formulation the heat capacity increased almost linearly with temperature indicating aggregation. The 145 mM sodium chloride formulations with or without 140 mM benzyl alcohol showed a large increase in heat capacity starting at 20–30°C. The latter behavior indicated aggregation and the protein was also precipitated in the sample cell. As seen in the figures, the scans on hIGF-I mannitol formulations with or without benzyl alcohol were not identical. The formulation with mannitol and benzyl alcohol showed a broad "shoulder" in the range 30–70°C. This behaviour could either indicate formation of small aggregates, e.g. dimers, or unfolding of the protein. We could, however, not fit DSC data to any simple unfolding process. Neither of the hIGF-I 290 mM mannitol formulation nor the mannitol and benzyl formulation resulted in any precipitation when performing the DSC scans.

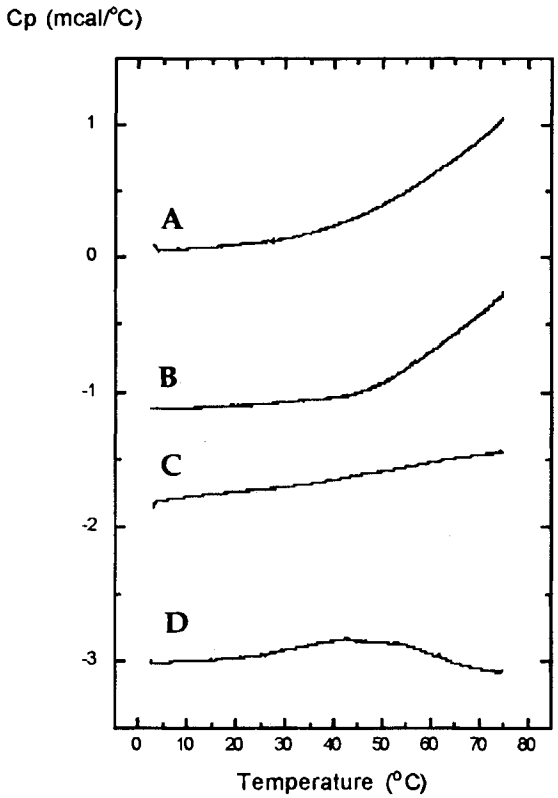

Fig. 6. DSC scans of 10 mg/ml hIGF-I in different formulations. The plot shows baseline subtracted rawscans of the protein solutions. The baseline scan of the respective formulation buffer was performed as the scan on the hIGF-I solution, except that in the sample vessel was filled with the formulation buffer, 10 mM sodium phosphate, pH 6. The thermograms of each scan are all adjusted on the Cp-scale to give a good overview over the results. The different hIGF-I formulations shown on the graph are: (A) 145 mM NaCl, (B) 145 mM NaCl and 140 mM benzyl alcohol, (C) 290 mM mannitol, and (D) 140 mM benzyl alcohol and 85 mM mannitol.

DISCUSSION

Effects on the Structure of hIGF-I

The effect on the tertiary structure was temperature-dependent, being significant only at low temperatures and parallel to the decrease in hIGF-I solubility. The near-UV CD detected no changes in tertiary structure of hIGF-I 10 mg/mL in either 290 mM mannitol or 145 mM sodium chloride when compared to that in 5 mM phosphate buffer. Thus the tertiary structure of hIGF-I is not dependent on the ionic strength for up to 145

mM sodium chloride. Although it is difficult to fully interpret the distinct sharpening of the CD spectrum for hIGF-I in the sodium chloride solution when benzyl alcohol was added without further structural studies, some conclusions can be drawn. The significant distortions of the near-UV CD of hIGF-I were in the range 250–270 nm as seen in Figure 5. In this interval, it is mainly phenylalanine residues and disulfides that determine the ellipticity (22). The change in near-UV CD was more pronounced for the lower protein concentrations when the benzyl alcohol concentration was kept constant.

Near-UV CD studies on insulin in aqueous solutions with phenol have detected ellipticity changes in the range 250–270 nm (23). A number of alcohol ligands, have been found to interact with insulin (24), and it can be expected that benzyl alcohol would interact with hIGF-I in a similar manner. The primary structure of human insulin is illustrated in Figure 1. The interaction with insulin appears to be primarily with the A-chain cysteine 6 (24) but it affects also the phenylalanine 25 in the insulin B-chain adjacent to the disulfide B19-A19 (25). Both the B25 phenylalanine and the A6 cysteine in insulin are located at the surface and therefore easily accessible to solution components. This non-covalent interaction between cysteine and the alcohol ligand results primarily in a fixation of the amino acid residue and a structural change. The main effect of the presence of the alcohol ligand and the structural change is the formation of an insulin dimer and further precipitation of an insulin hexamer. This supports our hypothesis for hIGF-I that benzyl alcohol actually induces structural changes in hIGF-I which results in association of monomers. However, the association of hIGF-I is weaker than that of insulin as the precipitated hIGF-I readily dissolves at higher temperatures.

From the CD data it is evident that phenylalanines or disulfides in hIGF-I interact with benzyl alcohol. In the single-chain hIGF-I, phenylalanines are located at positions 23, 25 and 49 while disulfides are located at 6-48, 18-61 and 47-52. From the structural similarities of insulin and hIGF-I, the phenylalanine 23 or 25 in hIGF-I correspond to the B25 phenylalanine in insulin and cysteine 47 in hIGF-I with A6 cysteine in insulin (see Figure 1).

Stability of hIGF-I

It is often found that destabilization of tertiary and secondary structure and subsequent attraction of hydrophobic residues are prerequisites for covalent aggregation of proteins (27,28). The thermal stability is also often increased by solutes which stabilize the protein structure such as low-molecular sugars, polyols and salts (29). These stabilizers are unspecific and stabilize simply by being excluded from the protein vicinity and thereby increasing the surface tension of the solvent surrounding the protein (9,16). In 145 mM sodium chloride hIGF-I was irreversibly aggregated at high temperatures, although sodium chloride was significantly excluded from hIGF-I. This is contrary to the general belief that excluded solutes stabilize proteins. Instead, ionic strength induces hydrophobic attraction of hIGF-I. Interestingly, the attraction appears to be independent of the presence of 140 mM benzyl alcohol. Benzyl alcohol 100 mM has previously been found to significantly decrease the denaturation temperature of e.g. hGH (13) and could be expected to have a similar effect on hIGF-I. However, the effects on the tertiary structure of hIGF-I which was induced by benzyl alcohol were significant only at temperatures around 25°C and below. This is consistent with the lack of effect of benzyl alcohol on the thermal stability of hIGF-I at temperatures above 25°C. The high thermal stability of hIGF-I in solutions with low ionic strength, irrespective of the presence of 140 mM benzyl alcohol, indicated that hydrophobic attraction is the main factor that determines the thermal aggregation of hIGF-I. Temperature scans of hIGF-I both with and without mannitol exhibited a similar increase in heat capacity, which suggested that 290 mM mannitol is a far too low concentration to stabilize hIGF-I significantly.

Solubility of hIGF-I

The solubility of hIGF-I is temperature-dependent which is illustrated by the precipitation of human IGF-I at low temperatures in the presence of 140 mM benzyl alcohol and 145 mM sodium chloride. This is indicated by an increase in light-scattering particles (Figure 2B) and decrease in the concentration of dissolved protein. Since the hydration of hIGF-I as seen in table 1 is significantly decreased in this solvent the precipitation appears to be a "salting-out" of the protein (12). A salting-out mechanism for the precipitation is also supported by the perturbed tertiary structure of hIGF-I in this medium (12). Aromatic solutes such as benzyl alcohol interact with the phenylalanine in hIGF-I which apparently causes structural changes similar to those of insulin and subsequently a decrease in the surface charge of the protein and increased hydrophobic attraction. Even in a solvent with a moderate ionic strength, e.g. in 145 mM sodium chloride, the hydrophobic attraction becomes sufficiently strong for self-association of hIGF-I. Mannitol, on the other hand, causes a better hydration of hIGF-I and balances the hydrophobic effect caused by benzyl alcohol.

Analogous studies on zinc-free insulin have shown that inonic strength and low temperatures favor association in aqueous solution (30,31) which supports our hypothesis of a similar behaviour of insulin and hIGF-I.

CONCLUSIONS

The present study shows that the self-association of hIGF-I in aqueous solution is mainly driven by hydrophobic interactions. The influence that different solutes will have on the association of hIGF-I is consequently determined by their effect on this mechanism. It can be concluded that pharmaceutical preparations of hIGF-I with benzyl alcohol are possible only in solutions with low ionic strength.

Benzyl alcohol interacts with phenylalanine in the hIGF-I chain, in a similar manner that phenol and other alcohols interact with insulin. Basically, the interaction leads to changes in the tertiary structure of hIGF-I and a decreased hydration of the protein and subsequently a decreased solubility. The interaction is increased in an aqueous ionic solvent demonstrating a hydrophobic mechanism. Mannitol stabilizes the tertiary structure and balances the perturbing effect of benzyl alcohol which results in a better hydration of the protein.

hIGF-I aggregates at higher temperatures. The thermal aggregation appears to be driven by the hydrophobic effect since it is reinforced in an aqueous ionic solvent. The effect of 140 mM benzyl alcohol decreases with temperature.

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