Enhanced *in Vitro* Refolding of Insulin-like Growth Factor I Using a Solubilizing Fusion Partner†

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Received October 29, 1993; Revised Manuscript Received January 10, 1994®

ABSTRACT: We have previously shown that human insulin-like growth factor I (IGF-I), fused to ZZ (two domains derived from staphylococcal protein A), can be refolded at relatively high concentrations, without the use of solubilizing agents [Samuelsson, E., Wadensten, H., Hartmanis, M., Moks, T., & Uhlén, M. (1991) *Bio/Technology 9*, 363–366]. Here we have studied this phenomenon in detail by characterizing the *in vitro* refolding of IGF-I, fused to one or two solubilizing Z domains and without a solubilizing fusion partner. The characterization included solubility studies of the reduced proteins and an evaluation of the aggregation occurring during the refolding process. The results suggest that the applied fusion protein stategy can be used to obtain a cis-acting chaperone-like effect during refolding *in vitro*. Fusion to one or two Z domains resulted in more than a 100-fold increase in the solubility of reduced IGF-I. In addition, the Z or ZZ fusion partners decrease multimerization of the IGF-I moieties during the renaturation. The fusion protein strategy may be an option to overcome the obstacles of insolubility and aggregation, frequently encountered when designing *in vitro* refolding processes.

The expression of eukaryotic proteins in heterologous hosts often yields high production levels, but the product is often misfolded. This had led to an increasing interest in the development of efficient *in vitro* refolding processes. However, the folding of proteins, which are adapted to the environment of the eukaryotic cell, often results in low yields when performed *in vitro*. Aggregation of partially folded intermediates often drastically reduces the yield of the native product, unless the reactivation is performed in highly diluted solutions (Rudolph, 1990). Several approaches to solve this problem have been presented. Stepwise addition of the denatured protein to the refolding solution keeps the concentration of partially folded intermediates low, thereby limiting aggregation (Rudolph, 1990). Refolding yields may also be improved by including additives such as GdnHCl,[1] detergents (Tandon & Horowitz, 1986), or PEG (Cleland et al., 1992). An attempt to mimic the environment in the eukaryotic cell is to include molecular chaperones in the refolding reaction. Molecular chaperones bind to unfold or partially folded intermediates and thereby increase their solubility and prevent aggregation (Gething & Sambrook, 1992). The GroE complex has been shown to facilitate reconstitution of several proteins (Viitanen et al., 1990; Buchner et al., 1991; Mendoza et al., 1991), although the amount of chaperones needed was considerable; i.e., 2 mg/mL purified groEL and groES proteins were needed to refold 0.025 mg/mL rhodanese (Mendoza et al., 1991).

Recently, Samuelsson et al. (1991) described an alternative strategy to *in vitro* refolding in which a solubilizing fusion partner was used to improve the solubility during *in vitro* refolding of a recombinant protein. A fusion partner (ZZ), based on staphylococcal protein A, was used to solubilize unfolded and misfolded insulin-like growth factor I (IGF-I) during the purification and refolding process (Samuelsson et al., 1991). The solubilizing effect of protein A was also extended to the refolding of human IGF-II (Forsberg et al., 1992). In the paper, we have investigated the folding-improving effect of the Z domain further by comparing the solubility and refolding yields of human IGF-I fused to one or two Z domains with those of IGF-I without a fusion partner. IGF-I is a 70 amino acid peptide growth factor, containing 3 disulfide bonds, which are crucial for its three-dimensional structure (Hober et al., 1992). When produced as a secreted product in *Escherichia coli* or *Saccharomyces cerevisiae*, IGF-I is mainly found as precipitates of misfolded forms with intermolecular disulfides (Wong et al., 1988; Elliott et al., 1990). In addition, two monomeric forms with different disulfide patterns (mismatched and native) have been identified (Raschdorf et al., 1988), which, according to both disulfide equilibration refolding studies (Hober et al., 1992) and GdnHCl denaturation studies, show similar thermodynamic stabilities (Miller et al., 1993). This thermodynamic folding problem may be connected to the difficulties in obtaining high yields after *in vitro* refolding. However, using a fusion protein strategy may result in a facilitated refolding procedure. The results presented here show that the Z domain dramatically increases the solubility of unfolded and aggregated species of IGF-I and can be used to increase the yield of correctly folded IGF-I monomer during *in vitro* refolding.

MATERIALS AND METHODS

Construction and Expression of the Recombinant Genes. The fusion protein ZZ–IGF-I was produced in *E. coli* secreted to the culture medium and recovered by IgG affinity purification, as described earlier (Moks et al., 1987). The fusion protein was further purified from degradation products using ion exchange chromatography (Samuelsson et al., 1991). On the basis of the construct used for the expression of ZZ–IGF-I, a new expression vector was assembled by removing a 174 base pair *Bgl*II fragment, leaving one Z domain intact.

† This project has been financially supported by the Swedish National Board for Industrial and Technical Development.

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® Abstract published in *Advance ACS Abstracts,* March 1, 1994.

[1] Abbreviations: Z and ZZ, IgG-binding domains derived from staphylococcal protein A; IGF-I, human insulin-like growth factor I; HPLC, high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GdnHCl, guanidine hydrochloride; CD, circular dichroism; DTT, dithiothreitol; T_m, melting temperature; PEG, polyethylene glycol.

0006-2960/94/0433-4207$04.50/0

The resulting construct encodes a Z–IGF-I fusion protein of 15.5 kDa. The fermentation and purification were performed according to the protocol used for ZZ–IGF-I. Pure IGF-I was produced as described earlier (Moks et al., 1987; Forsberg et al., 1991).

Ellman Assay for Thiols. Sulfhydryl groups in the IGF-I-containing proteins were determined by adding 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to the sample and by measuring the absorbance at 412 nm (Ellman, 1959).

Refolding. The reduction was performed at protein concentrations of 10, 20, 40, 80, and 160 μM in 100 mM ammonium acetate, pH 9.1, containing 0.5 mM EDTA, 0.05% Tween 20, and 1 M β-mercaptoethanol. The samples were incubated at 37 °C for 30 min. The reduced samples (100-μL portions) were desalted to the same buffer but without β-mercaptoethanol on a fast-desalting PC 3.2/10 column using the SMART System (Pharmacia LKB Biotechnology, Uppsala, Sweden). The flow rate was 100 μL/min. A 220-μL fraction containing the eluted protein was collected, diluting the sample to a volume approximately 2.2 times the volume of the injected sample. After reoxidation at 37 °C for 16 h, 20 μL of the protein was analyzed by gel filtration on a Superdex 75 PC 3.2/30 column (Pharmacia LKB) using the SMART System. The amounts of protein in different peaks were determined by integration using the software of the SMART System. The yields of monomers and multimers were calculated by comparing the different amounts before and after refolding.

Yields of Reduced Protein after Desalting. In order to determine eventual losses during reduction and desalting due to adsorption to column material or loss by precipitation, the following test was performed: Reduced samples of 10–160 μM IGF-I, Z–IGF-I, and ZZ–IGF-I were desalted on a fast-desalting PC 3.2/10 column, equilibrated with 100 mM acetic acid, pH 2.7, containing 0.5 mM EDTA and 0.05% Tween 20. The low pH prevented reoxidation of the cysteines in the proteins. Quantitative protein determination was performed by amino acid analysis on the samples before reduction and after desalting.

Protein Analysis. SDS–PAGE (Laemmli, 1970) was performed with and without β-mercaptoethanol in the sample buffer. Coomassie-blue (Pharmacia LKB) was used for staining according to the procedure described by the supplier. Amino acid analysis was performed on a Beckman 6300 amino acid analyzer, equipped with a System Gold data-handling system (Beckman). After hydrolysis with 6 M HCl at 155 °C for 45 min, the samples were analyzed on an ion exchange column with ninhydrin detection.

Reversed-Phase HPLC Analysis. The differently folded monomers of the IGF-I variants were separated by reversed-phase HPLC. The column used was a Vydac C_{18} (4.6 mm × 250 mm). The gradient used was 32–39% acetonitrile in 0.25% (v/v) pentafluoropropionic acid over 40 min at 40 °C and a flow of 1 mL/min.

RESULTS

Expression of the Various IGF-I Derivatives. Three IGF-I derivatives were analyzed in this study, and all were expressed in *E. coli*. The fusion partner was the Z domain, based on staphylococcal protein A and originally designed for affinity purification of recombinant products, due to its IgG-binding properties (Nilsson et al., 1987). A model of the structure of ZZ–IGF-I is displayed in Figure 1. The production of ZZ–IGF-I, including expression of the plasmid vector pEZZ–IGF-I in *E. coli*, followed by affinity purification of the product,

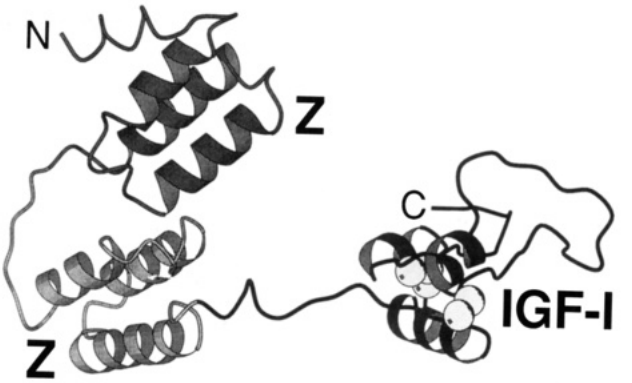

FIGURE 1: A computer graphic ribbon representation of the ZZ–IGF-I fusion protein. The Z moieties are fused to the N-terminal of IGF-I. Helices are shown as coils. Filled spheres on IGF-I show the six sulfur atoms involved in the three disulfide bonds. The N-terminus is shown by "N" and the C-terminus is shown by "C". The display is based on the structure of IGF-I (Blundell et al., 1978) and staphylococcal protein A (Torigoe et al., 1990) and was generated using the program MOLSCRIPT (Kraulis, 1991).

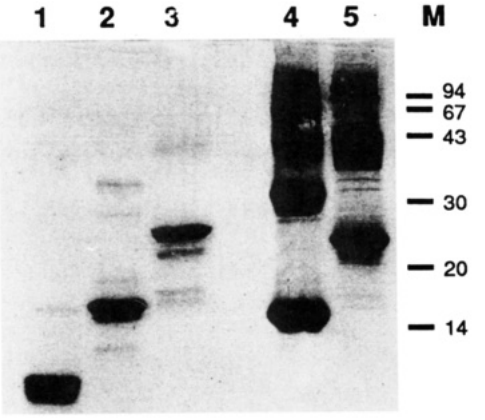

FIGURE 2: SDS–PAGE analysis of IGF-I, Z–IGF-I, and ZZ–IGF-I: reduced samples of (1) IGF-I, (2) Z–IGF-I, and (3) ZZ–IGF-I and nonreduced samples of (4) Z–IGF-I and (5) ZZ–IGF-I analyzed on 16% polyacrylamide gel electrophoresis (Laemmli, 1920). The fusion proteins were purified by IgG affinity chromatography and ion exchange chromatography (as described in the Materials and Methods) prior to the electrophoresis.

recovered from the culture medium, has been described previously (Moks et al., 1987). The native IGF-I was produced by site-specific cleavage, followed by purification of the resulting IGF-I moiety (Forsberg et al., 1990). A new expression vector was assembled to allow the production of a monomeric protein A domain (Z), fused to IGF-I (see Materials and Methods for details). The fusion proteins were further purified by ion exchange chromatography to separate degraded material from the full-length product (Samuelsson et al., 1991). Both Z–IGF-I and ZZ–IGF-I were analyzed by reducing and nonreducing SDS–PAGE (Figure 2) to determine the fraction of fusion protein in multimeric forms. A high fraction of disulfide-bonded multimers were observed in the materials obtained from the fermentation.

Solubility of Reduced IGF-I, Z–IGF-I, and ZZ–IGF-I. The solubility of IGF-I, Z–IGF-I, and ZZ–IGF-I was determined by reducing the proteins at different concentrations and measuring any precipitate spectrophotometrically. Increasing amounts of protein were dissolved at pH 8.0 and reduced with 100 mM DTT for 30 min at 37 °C at protein concentrations spanning from 20 to 8000 μM. The results presented in Figure 3 show that precipitates of IGF-I can be observed at concentrations as low as 65 μM (0.5 mg/mL), whereas both fusion proteins remain soluble at concentrations

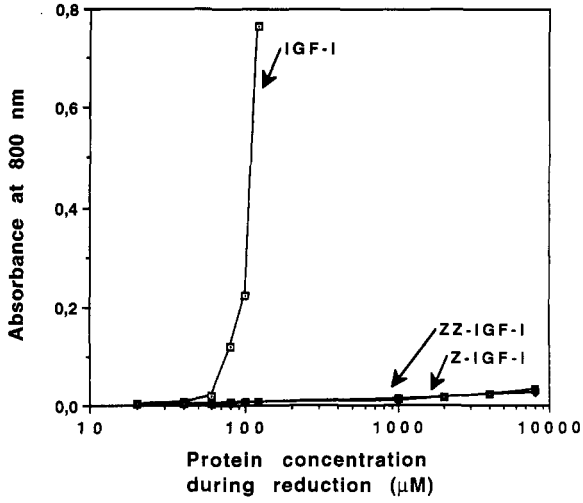

FIGURE 3: Solubility of the reduced proteins IGF-I, Z–IGF-I, and ZZ–IGF-I. Increasing millimolar concentrations of protein were reduced in a 100 mM Tris, pH 8.0, buffer containing 100 mM DTT at 37 °C for 30 min. The absorbance at 800 nm was measured as an indication of precipitation.

of 8000 μM (corresponding to 60 mg of Z–IGF-I/mL and 90 mg of ZZ–IGF-I/mL). This demonstrates that the small and soluble fusion partner Z can increase the solubility of the equally small but poorly soluble reduced IGF-I by a factor of at least 120. At 8000 μM, the viscosity of the fusion proteins is high and a further increase in concentration is of little practical interest.

Refolding of IGF-I, Z–IGF-I, and ZZ–IGF-I. In order to investigate the influence of the fusion partner Z on aggregation during refolding of IGF-I, a scheme was designed for the refolding and analysis of IGF-I, Z–IGF-I, and ZZ–IGF-I. The following steps were included: (i) reduction of the different proteins, (ii) removal of the reducing agent using a desalting column, (iii) reoxidation by air, and (iv) analysis of the refolded material using size exclusion chromatography to determine the yield of monomers and multimers (see Materials and Methods for details). To ensure that no losses of protein occurred during the early steps, the yield before and after the reduction and desalting was determined using amino acid analysis. A yield of approximately 95% was obtained for all three IGF-I-containing proteins (data not shown).

IGF-I, Z–IGF-I, and ZZ–IGF-I were reduced at protein concentrations ranging from 10 to 160 μM, corresponding to 0.075–1.2 mg/mL IGF-I. An ammonium acetate buffer at pH 9.1 containing 1 M β-mercaptoethanol completely reduced both IGF-I and the IGF-I fusion proteins after 30 min at 37 °C according to analysis using Ellmans' reagent (data not shown). To avoid losses of the recombinant proteins, due to unspecific adhesion or precipitation during the reduction step, a low amount (0.05%) of a nonionic detergent (Tween 20) was included in the buffer. After reduction all samples were transferred to the reoxidation buffer, using a desalting column.

The desalted samples were reoxidized by air for 16 h at 37 °C. The resulting ratios of monomeric and multimeric forms of the refolding protein were determined using size exclusion chromatography. Integration of the size exclusion chromatograms allowed calculations of the yield of monomer and multimers as a percentage of the total amount of protein before refolding. The results of the refolding are shown in Figure 4. As expected, the yield of monomeric protein decreases with increased concentrations for all three derivatives. However, as seen in Figure 4B the relative proportion of monomer at a fixed protein concentration can be enhanced by the fusion

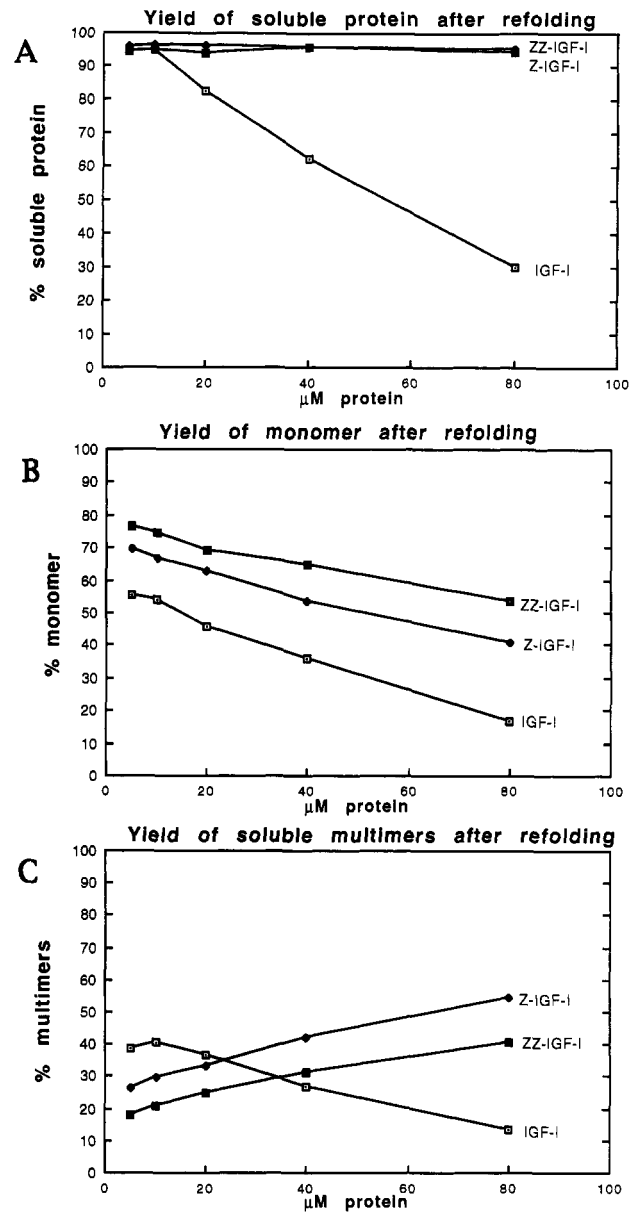

FIGURE 4: Recovery of soluble-IGF-I-containing protein (A), monomeric-IGF-I-containing protein (B), and multimeric-IGF-I-containing protein (C) as a function of protein concentration during refolding. The yield after refolding is expressed as a percentage of total protein before refolding. The calculations were performed as described in the Materials and Methods.

partner Z. Even at protein concentrations below the solubility limit of native IGF-I (5 μM), one domain Z increases the yield of monomer by 33% and two domains increase the yield by 45% as compared to IGF-I without a fusion partner (Figure 4B). At higher protein concentrations, the effect of the Z domain becomes even more pronounced. The yield of monomer is more than doubled using the Z fusion and tripled using the ZZ fusion when the refolding is performed at 70 μM.

A dramatic difference in the yield of multimers can also be observed between the native IGF-I and the two derivatives containing Z or ZZ as solubilizing domains (Figure 4C). In fact, the overall yield of IGF-I containing a fusion protein is approximately 95% after the refolding, while most IGF-I is lost during refolding without a fusion partner, when performed at higher concentrations (Figure 4A). Approximately 70% of the nonfused IGF-I is not recovered at 70 μM, most likely due to precipitation of multimers formed during reoxidation.

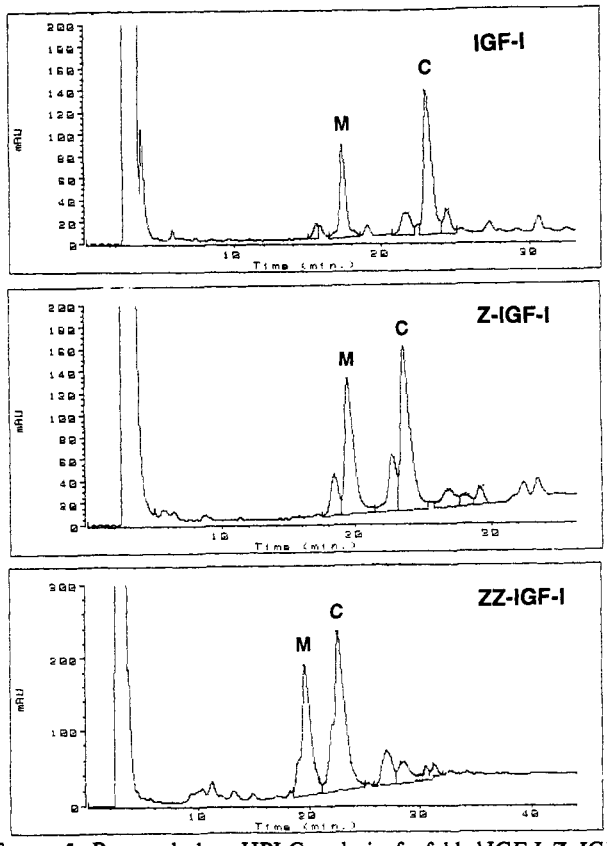

FIGURE 5: Reversed-phase HPLC analysis of refolded IGF-I, Z–IGF-I, and ZZ–IGF-I. The following peaks are identified: M, misfolded monomer; N; nativelike monomer. The gradients used were 31–36% (for IGF-I), 32–37% (for Z–IGF-I), and 34–39% (for ZZ–IGF-I) acetonitrile in 0.025% (v/v) pentafluoropropionic acid (PFPA) over 40 min at 40 °C and a flow rate of 1 mL/min. The column was a Vydac C_{18} (4.6 mm × 250 mm).

Analysis of the Monomeric Products. Although aggregation, due to intermolecular disulfide bonds formed during reoxidation, constitutes the major obstacle to successful refolding of IGF-I at higher concentrations, the yield of a native product is also limited by the formation of a misfolded monomer (Raschdorf et al., 1988). Therefore, the refolding products from the various protocols were analyzed using reversed-phase HPLC (see Materials and Methods for details) to determine the ratio of correctly folded and misfolded monomer. The peaks corresponding to misfolded and native fusion protein were collected and site-specifically cleaved using hydroxylamine (Moks et al., 1987). An HPLC analysis (Forsberg et al., 1990) allowed identification, in all three cases, of the peak corresponding to misfolded and correctly folded IGF-I-containing monomer. Interestingly, a comparison of the products obtained after refolding of the various IGF-I constructs (Figure 5) shows that approximately the same ratio between native (N) and mismatched (M) forms is obtained. The monomers of all three IGF-I variants after refolding consisted of at least 50% correctly folded product (Figure 5).

DISCUSSION

In this study we have investigated how the fusion partner Z can improve the *in vitro* refolding behavior of IGF-I. Several reports concerning precipitation, aggregation, and misfolding of IGF-I, when using various expression systems and refolding schemes, have been presented (Samuelsson et al., 1991; Elliott et al., 1990; Iwai et al., 1989; Forsberg et al., 1990). It has been suggested that the amino acid sequence of mature IGF-I does not contain all information for its folding into a native form. This statement is based on an analysis of disulfide exchange folding of IGF-I under reversible redox conditions (Hober et al., 1992) and has also been supported by a study on the oxidative refolding of IGF-I (Miller et al., 1993). The two monomeric three-disulfide forms of IGF-I (mismatched and native) obtained after completed refolding were found to have similar thermodynamic stabilities, with free energies differing by less than 0.25 kcal/mol (Miller et al., 1993). These properties in addition to IGF-I's tendency to form aggregates clearly illustrate the problems of folding eukaryotic proteins in prokaryotic hosts. We believe that these "problems" make IGF-I suitable as a model system to develop methods to improve *in vitro* refolding.

When reduced, IGF-I loses most of its native tertiary and secondary structure. In fact, the CD spectrum of the reduced IGF-I molecule, lacking all three disulfide bonds, resembles a CD spectrum close to that of a random coil (Hober et al., 1992). The Z and ZZ moieties on the other hand contain no cysteines, and their structures are most likely independent of disulfide reagents. The Z domain has a very temperature stable three-dimensional structure, with a T_m exceeding 90 °C at neutral pH (data not shown) and a midpoint of transition at 3.9 M concentration of GdnHCl during denaturation (Cedergren et al., 1993). We have previously shown that unfolded, misfolded, and multimeric ZZ–IGF-I are soluble, which simplifies the refolding and purification process. Here we have made a comparison of the solubility of reduced IGF-I, Z–IGF-I, and ZZ–IGF-I. The results revealed that reduced IGF-I, which precipitates at low concentrations (70 μM under the conditions used), is at least 120 times more soluble when fused to either Z and ZZ (Figure 3). Both reduced Z–IGF-I and ZZ–IGF-I were soluble at protein concentrations of at least 8000 μM, correponding to about 60 mg of Z–IGF-I/mL or 90 mg of ZZ–IGF-I/mL. This remarkable increase in solubility was accomplished in an aqueous buffer, without any addition of solubilizing agents such as denaturants, detergents, or organic solvents.

In addition to the improved solubility of reduced IGF-I, it was found that the yield of soluble protein after renaturation was increased when using the fusion proteins. The results after reoxidation of the three proteins revealed that the recovery of total protein (including both monomers and multimers) was dependent on the protein concentration only in the case of IGF-I (Figure 4A). The yield of IGF-I was high (about 95%) at concentrations below 10 μM but decreased to 35% at 70 μM. The yield decreased due to precipitation, most likely due to poorly soluble multimers formed during reoxidation. In contrast, both the monovalent and divalent Z fusions of IGF-I gave a high total recovery (about 95%) even at 70 μM protein concentration (Figure 4B,C). These results clearly illustrate the solubilizing effect of the Z domain during refolding.

The Z moiety not only prevented precipitation but also decreased multimerization of IGF-I. The yield of monomer was considerably enhanced by fusion of IGF-I to the domain Z, although the highest yield was achieved using the divalent (ZZ) fusion (Figure 4B). Even at low concentrations (below 10 μM) where no precipitation of IGF-I was observed after reoxidation, the yield of monomer was higher for the Z fusions (Figure 4B). At higher concentrations (80 μM), the yield of monomer was 3 times higher for the ZZ fusion as compared with "native" IGF-I. The mechanism behind this decrease in multimerization is not clear, but may be a sterically shielding effect of the Z moieties which decreases contact between IGF-I molecules and subsequent multimerization. An alterntive

explanation is an electrostatic effect resulting from the highly charged Z moieties. Although the mechanism remains unclear, a comparison can be made with the known function of molecular chaperones (Nilsson & Anderson, 1991; Gething & Sambrook, (1992). By definition, chaperones act in folding by inhibiting protein aggregation by binding to the polypeptide chain during the folding process. A common feature of many unfolded proteins is their low solubility, and one role of molecular chaperones may be to solubilize and prevent precipitation of nascent polypeptides emerging from the ribosome (Nilsson & Anderson, 1991). Similarly, Z or ZZ acts as a solubilizer and also diminishes aggregation.

When a fusion partner has been introduced to facilitate the folding or purification of a recombinant protein, a suitable cleavage method has to be chosen to obtain the native product. Here, hydroxylamine cleavage of an asparagine–glycine linker could be used since IGF-I does not contain this site (Moks et al., 1987). However, for fusions to other proteins, other cleavage methods may be more appropriate. A variety of chemical and enzymatic methods have been used for site-specific cleavage of fusion proteins [for a review, see Carter (1990)].

In conclusion, this study shows that the Z and ZZ fusion handles are useful for refolding of human recombinant IGF-I, due to their solubilizing effect on reduced and multimeric IGF-I, and also their capability of limiting multimerization during refolding. These properties are especially valuable when designing refolding processes on a commercial scale, since higher protein concentrations are permitted and the use of denaturing agents can be avoided. The solubilizing-fusion strategy may be a useful tool to solve solubility and aggregation problems for other recombinant proteins.

ACKNOWLEDGMENT

We are grateful to Bert Larsson and Christina Kalderén for the fermentations and Kristina Zachrisson for the amino acid analysis.

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