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Disulfide Exchange Folding of Disulfide Mutants of Insulin-Like Growth Factor I *in Vitro*†

Sophia Hober,‡,§ Mathias Uhlén,§ and Björn Nilsson*,‡,§

Department of Biology, Preclinical Research, Pharmacia & Upjohn AB, S-112 87 Stockholm, Sweden, and Department of Biochemistry and Biotechnology, Royal Institute of Technology, S-100 44 Stockholm, Sweden

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ABSTRACT: We have previously concluded that insulin-like growth factor-I (IGF-I) is thermodynamically unable to quantitatively form its disulfide bonds under reversible redox conditions *in vitro*. From detailed analyses it was hypothesized that the 47−52 disulfide is energetically unfavorable in the native IGF-I structure [Hober *et al.* (1992) *Biochemistry 31,* 1749−1756]. In this paper, this hypothesis has been tested by refolding of IGF-I mutant proteins lacking either the 47−52 or 6−48 disulfide bond. The disulfide exchange folding equilibrium behavior of these mutated IGF-I variants were examined in a glutathione redox buffer. The mutant protein IGF-I(C47A,C52A) was demonstrated to form both remaining native disulfide bonds. In contrast, IGF-I(C6A,C48A) was unable to quantitatively form both of its disulfides and was shown to accumulate a one disulfide variant lacking the 47−52 disulfide bond. These folding data corroborate the hypothesis that the 47−52 disulfide bond of IGF-I is energetically unfavorable also in the absence of the 6−48 disulfide bond. The two IGF-I variants were purified in oxidized forms where both native disulfides are formed. Both variants were suggested to be structurally perturbed compared with the native molecule as determined by circular dichroism spectroscopy. Further, binding affinities to the IGF binding protein 1 and a soluble IGF type I receptor, respectively, were severely lowered in both disulfide mutant proteins compared to the native IGF-I molecule. Interestingly, the binding affinity toward the IGF type I receptor is higher for IGF-I(C6A,C48A) than for IGF-I(C47A,C52A) while the binding affinity to IGFBP-1 is higher for IGF-I(C47A,C52A) than for IGF-I(C6A,C48A). Thus, the structural changes due to removal of the 6−48 or 47−52 disulfide bonds, respectively, yield structural changes in different regions of the IGF-I molecule reflected in the different binding activities.

Protein folding is a spontaneous process that is thought to be thermodynamically driven to the lowest free energy of the system, representing the "native" folding state of the protein (Anfinsen, 1973). However, insulin-like growth factor I (IGF-I)[1] is thought not to follow this biophysical paradigm since it does not quantitatively reach its native state under reversible thiol exchange conditions similar to the redox state in the secretory vesicles (Hober *et al.*, 1992).

IGF-I is a basic single-chain peptide hormone containing 70 amino acid residues and originally isolated from serum. The growth factor is positively regulated by human growth hormone (GH) and is thought to mediate many of the growth promoting effects of GH. IGF-I is homologous to insulin, including the three disulfide bridges [for a review see Jones and Clemmons (1995)]. The three-dimensional structure of the growth factor has been solved by NMR spectroscopy (Cooke *et al.*, 1991; Sato *et al.*, 1993) (see Figure 1).

In an earlier study, we have demonstrated that IGF-I is unable to form its native disulfide bonds quantitatively *in vitro* in a redox buffer containing oxidized and reduced glutathione (Hober *et al.*, 1992). Instead of folding quantitatively into its native structure, an equilibrium of many different forms of IGF-I was established. The most predominant species were isolated and identified. In a buffer with 10 mM reduced and 1 mM oxidized glutathione, IGF-I with two native disulfides (6−48, 18−61) lacking the 47−52-disulfide is the most populated variant (Hober *et al.*, 1992). The described thermodynamic folding problem is present in a wide range of different redox conditions (Hober *et al.*, in preparation). From these studies it was hypothesized that the 47−52 disulfide is energetically unfavorable in the native three-dimensional structure of IGF-I. This inability of IGF-I to quantitatively reach its native three-dimensional structure in the glutathione buffer can be overcome by folding in the presence of equimolar amounts of IGF binding protein 1 (IGFBP-1) (Hober *et al.*, 1994).

In this paper, we report the disulfide exchange folding properties of mutants forms of IGF-I where two of the disulfide bonds (6−48 and 47−52, respectively) have been removed in two separate mutant proteins by substituting pairs of cysteine residues for alanines. The 47−52 disulfide bond of IGF-I has previously been proposed to be energetically

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* Corresponding author.
‡ Pharmacia & Upjohn AB.
§ Royal Institute of Technology.

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; CD, circular dichroism; DTT, dithiothreitol; ER, endoplasmic reticulum; GH, growth hormone; GSH, reduced glutathione; GSSG, oxidized glutathione; IGFBP, insulin-like growth factor binding protein; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; PDMS, plasma desorption mass spectrometry; PFPA, pentafluoropropionic acid; RP-HPLC, reverse-phase high-performance liquid chromatography; VP, vinyl pyridine.

[2] In this paper, amino acid residues are numbered according to their position in the corresponding protein from its N-terminus. Similarly, disulfide bonds are designated as connected residue numbers, e.g., 47−52 is the disulfide connecting amino acid residues 47 and 52.

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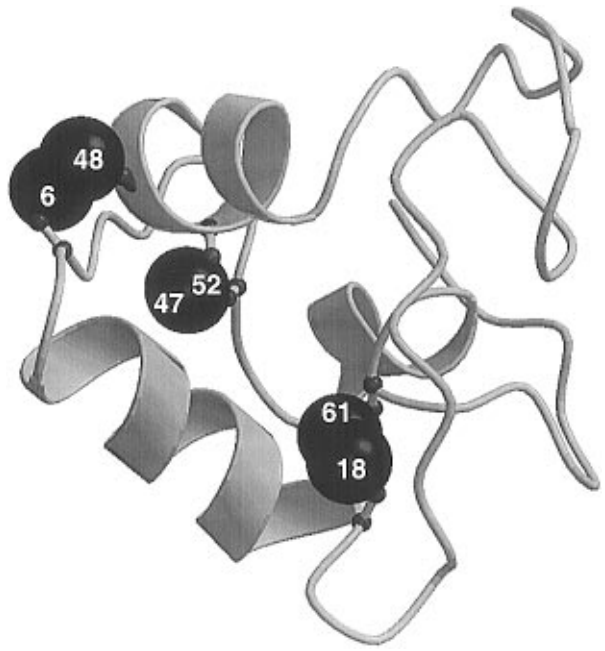

FIGURE 1: Ribbon computer graphics representation of the model of the structure of IGF-I using the coordinates from the NMR structure of Cooke *et al.* (1991) (Brookhaven Protein Structure Database). The three helices are shown as helical ribbons. The sulfurs are shown as black spheres with their respective residue number. The picture was generated by sequential use of the graphics software MOLSCRIPT (Kraulis, 1991) and RASTER 3D (Merritt & Murphy, 1994).

unfavorable in the native structure (Hober *et al.*, 1992). Accordingly, substitution of cysteines 47 and 52 for alanine residues should make the non-Anfinsen IGF-I molecule behave more like a normal protein and fold into a single form. These disulfide mutant IGF-I analogues have earlier been produced and structurally characterized by Nahri *et al.* (1993). Here, we extend the characterization by analysis of disulfide exchange folding of IGF-I(C6A,C48A) and IGF-I(C47A,C52A) under reversible conditions in a disulfide exchange buffer and by characterizing the IGF-I molecules with respect to binding of IGFBP-1 and a soluble form of the IGF type I receptor (sIGF-I^R). The results corroborate the model of the 47−52 disulfide as a high-energy bond in the native molecule.

MATERIALS AND METHODS

DNA Constructions and Mutagenesis. A synthetic IGF-I structural gene (Elmblad *et al.*, 1982) was cloned into the vector pRIT28 (Hultman *et al.*, 1988) using the restriction enzymes *Hin*dIII and *Eco*RI yielding the plasmid pRIT28i1. Site-specific mutagenesis was performed using PCR-mutagenesis as described by Landt *et al.* (1990). A one-step amplification was used to create the mutation encoding the C6A substitution using a mutagenesis primer spanning the *Eco*RI site. The mutagenesis primer used was (5′-TAGGGAATTCTAACGGTCCCGAAACCTTGGCCGGTGCTGAACTGG-3′) and the universal PCR primer used was (5′-TGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGG-3′). To create the other mutations yielding cysteine substitutions, two step PCR procedures were used. In the first step, the universal primer was used as well as two different mutagenesis primers; one to generate the mutation yielding the C48A amino acid substitution (5′-GTTGACGAATGCGCCTTTCGTTCTTGC-3′) and the other to generate the mutation resulting in the C47A,C52A substitutions (5′-GACGAAGCCTGCTTTCGTTCTGCCGACCTC-3′). In the second PCR step, generated PCR-fragments were used as one of the two PCR primers and a universal primer (5′-AATTGTGAGCGGATAACAATTTCACACAGG-3′) was used as the other. PCR fragments were cloned into a Z fusion production vector (Altman *et al.*, 1991), pKP594, using restriction enzymes *Eco*RI and *Hin*dIII. The C6A mutation was cloned into the C48A vector by using the restriction enzymes *Eco*RI and *Pst*I.

Preparation of Protein

Production and Purification of IGF-I(wt), IGF-I(C6A,C48A), and IGF-I(C47A,C52A). Mature IGF-I was produced and purified as secreted fusion protein in *Escherichia coli*, as described by Moks *et al.* (1987a,b). The different IGF-I folding forms were separated using reverse-phase HPLC (Forsberg *et al.*, 1990).

IGF-I-mutant forms were produced intracellularly in *E. coli* and purified as a fusion protein with an analogue (Z) of an IgG-binding domain of staphylococcal protein A based on the expression system described by Altman *et al.* (1991). After production, cells were disrupted by incubation for two hours at 20 °C in 6 M GuHCl, 50 mM phosphate buffer, pH 6.5, 150 mM NaCl, and 0.5 mM EDTA. The dissolved cell solutions were diluted 6-fold in 10 mM Tris, pH 8, 0.05% Tween 20, 200 mM NaCl, 1.25 mM EDTA, and subsequently purified by IgG affinity chromatography. Purified fusion proteins were chemically cleaved with hydroxyl amine as described (Moks *et al.*, 1987b). During cleavage at the high pH, protein disulfides are readily formed by air oxidation. Released and oxidized IGF-I analogues were separated from uncleaved material (Z-IGF-I), as well as released fusion partner (Z), by a second passage through an IgG affinity column. A final purification step was performed using reversed-phase HPLC using an HP1090 system (Hewlett Packard, Palo Alto, CA). The column used was a Kromasil C_8 with 7 μm particles having a pore diameter of 18 nm (Eka Nobel, Surte, Sweden). The gradient used was 30−45% acetonitrile in 0.25% pentafluoropropionic acid (PFPA) over 30 min for IGF-I(C47A,C52A) and 35−40% in 20 min for IGF-I(C6A,C48A). The flow rate used was 1 mL/min, and column oven temperature was 40 °C. Proteins were detected by a diode array detector and a fluorescence detector in series.

Production and Preparation of IGFBP-1. Recombinant IGFBP-1 was produced in DON cells transfected with a bovine papilloma viral vector containing an expression cassette with the cloned human IGFBP-1 gene (Luthman *et al.*, 1989). IGFBP-1 was purified to homogeneity by IGF-I affinity purification followed by cation-exchange chromatography.

Production and Preparation of IGF Type I Receptor. The extracellular part of the IGF type-I receptor was produced as a fusion protein with Z as an affinity handle. A kidney cell line, 293, was transfected with a vector containing the receptor gene under control of a hCMV promoter. The receptor was purified with IgG affinity purification. Details of this production system will be published elsewhere.

Reduction of IGF-I Mutant Proteins and Native IGF-I. Reduced proteins were prepared by incubating purified

proteins at a concentration of 1 mg/mL, in a buffer containing 0.1 M Tris, pH 8.7, 0.2 M KCl, 1 mM EDTA, 10 mM reduced DTT, and 8 M urea. The proteins were incubated at 37 °C for 1 h. After reduction, the buffer was changed by gel filtration using Sephadex G-25 medium (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 10 mM HCl (Hober *et al.*, 1992). To confirm the reduced state of the protein, free thiols were alkylated with vinyl pyridine and alkylated protein was analyzed by RP-HPLC to confirm a single peak. Isolated material was analyzed by both mass spectrometry and absorbance amplitude at 254 nm to confirm that the mass and absorbance increase correspond to six pyridylethyl moieties. Reduced proteins can be stored for months at −80 °C without any detectable decrease in alkylation competence.

Protein Analysis

To verify the disulfide pattern of isolated IGF-I analogues, purified proteins were digested with porcine pepsin (Sigma) in 10 mM HCl, with a protease to IGF-I ratio of 1:10 (w:w). The digestion was allowed to proceed for 3 h at 20 °C. Generated polypeptide fragments were separated on reverse-phase HPLC. Gradient used was 0−45% acetonitrile in 0.1% trifluoroacetic acid over 40 min at a temperature of 30 °C and a flow rate of 1 mL/min. Column used was a Kromasil C_8 (Eka Nobel, Surte, Sweden) with a particle size of 7 μm and a pore diameter of 10 nm (Hober *et al.*, 1992).

Circular dichroism (CD) spectra were collected in a J-720 spectropolarimeter (JASCO, Japan) at room temperature. The buffer used was 10 mM potassium phosphate, pH 7.0. The scanning speed was 20 nm/min, and each spectrum was averaged from five scans. Cell path length was 1 mm, and protein concentration was 0.1 mg/mL. Secondary structure predictions were performed by using a variable selection method (Manavalan & Johnson, 1987). Protein concentrations were determined by quantitative amino acid analysis.

Quantitative amino acid analysis was performed by hydrolyzing each protein or peptide in 6 M HCl at 155 °C for 45 min. The samples were analyzed on an ion-exchanger column, and the amino acids were detected with ninhydrin. The analyses were done on a Beckman 6300 amino acid analyzer and evaluated with the System Gold data-handling system (Hober *et al.*, 1992).

For determination of the molecular masses, ^{252}Cf plasma desorption mass spectrometry (PDMS) using Bio-Ion 20 (Applied Biosystems, Foster City, CA) (Hober *et al.*, 1992) and electrospray mass spectrometry using a VG Platform mass spectrometer (Micromass, Manchester, U.K.) were used.

Disulfide exchange reactions were performed at 37 °C for 1 h and at a protein concentration of 30 μM. The buffer used contained 0.1 M Tris, pH 8.7, 0.2 M KCl, 1 mM EDTA, and different concentrations of reduced and oxidized glutathione. Disulfide exchange reactions were terminated by pyridylethylation of free thiols with 160 mM vinyl pyridine. The alkylation reaction was allowed to proceed for 15 min in the dark (Hober *et al.*, 1992). Thereafter the buffer was exchanged to 10 mM HCl by gel filtration on G25 column (Pharmacia Biotech, Uppsala, Sweden).

Binding Studies

Competitive Binding Assay. A competitive binding assay was used to study the different IGF-I variants ability to compete with ^{125}I-labeled IGF-I in the binding to a soluble IGF type I receptor. First, a microtiter plate was coated with 100 μL of human IgG, 5 μg/mL in a carbonate/bicarbonate buffer, pH 9.6. The plate was incubated for 1 h in 37 °C and washed six times with PBST (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 140 mM NaCl, 2.7 mM KCl, pH 7.3, and 0.05% Tween 20). Soluble IGF-I receptor fused to Z (100 μL of 0.1 μg/mL) was added to the wells in order to bind to the coated IgG molecules. The microtiter plate was incubated at 37 °C for 2 h and washed six times with PBST. A fixed amount of ^{125}I-labeled IGF-I was mixed with a serial dilution of unlabeled mutant proteins or native IGF-I, respectively. All proteins were diluted in assay buffer (50 mM phosphate pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.05% Tween 20), and 100 μL was added to each well. The samples were allowed to equilibrate at 20 °C for 3 h and washed three times with assay buffer. Radioactivity (cpm) for each sample was determined by using a γ counter (Wallac Oy, Finland).

BIAcore Analysis. Sensorchip CM5, Surfactant P20, and amine coupling reagents [*N*′-ethyl-*N*′-(dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), ethanolamine hydrochloride] were obtained from Pharmacia Biosensor (Uppsala, Sweden). Kinetic measurements were performed with IGFBP-1 as the immobilized acceptor molecule. For immobilization, a solution of 50 mM NaAc, pH 4.7, with IGFBP-1 at a concentration of 50 μg/mL was used (Löfås & Johnsson, 1990). The immobilization, was performed at 5 μL/min with 1 × HBS (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.05% P20) as driving buffer and was allowed to continue to a final resonance between 1000 and 2000 RU. Kinetic binding experiments were performed at a flow rate of 8 μL/min, using 1 × HBS as driving buffer. Each protein sample was analyzed at five different concentrations, and samples were injected twice in random order over the same surface. The concentrations used were 0.019−0.303 nM for native IGF-I, 4.848−0.303 μM for IGF-I(C47A,C52A), and 23.700−1.481 μM for IGF-I(C6A,C48A). Kinetic measurements were performed by injection of each protein for 300 s followed by buffer flow for 400 s. The immobilized IGFBP-1 was regenerated by injecting 12 μL of 100 mM HCl. The BIAcore data were evaluated by using software (BiaEvaluate) supplied by the BIAcore hardware producer.

RESULTS

Two separate mutant IGF-I proteins were constructed by site-directed mutagenesis, produced in *E. coli*, and purified. Both mutant proteins were shown to contain two disulfides by pyridylethylation, peptide mapping, and mass spectrometry. Each IGF-I variant had one of the native disulfide bonds removed by replacing pairs of cysteine residues by alanine residues. In one of the mutant forms, cysteines 6 and 48 were replaced by alanine residues [IGF-I(C6A,C48A)] and the other variant had cysteine residues 47 and 52 replaced by alanines [IGF-I(C47A,C52A] (Figure 1).

Folding Analyses

In order to evaluate whether this substitutions could affect the thermodynamic folding behavior of IGF-I, we equilibrated each IGF-I analogue in a redox buffer containing reduced and oxidized glutathione (Hober *et al.*, 1992).

Mature IGF-I, reduced or oxidized, was allowed to equilibrate in a redox buffer composed of 10 mM reduced

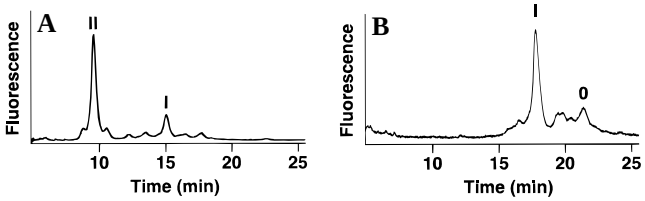

FIGURE 2: RP-HPLC separation of different disulfide variants of mutant proteins of IGF-I. The chromatograms show samples from vinylpyridine-trapped folding mixtures after equilibration in the redox buffer. The fluorescence (excitation at 280 nm and emission at 305 nm) of the different peaks was measured. The peaks are numbered according to the number of disulfides. A. RP-HPLC separation of different IGF-I(C47A,C52A) variants. The gradient was linear, 30−45% acetonitrile in 0.25% PFPA over 30 min. B. RP-HPLC separation of different IGF-I(C6A,C48A) variants. The gradient used was 35−40% acetonitrile in 0.25% pentafluoropropionic acid (PFPA) over 20 min.

Table 1: Mass Spectrometric Analysis of Different Isolated Forms of IGF-I(C47A,C52A) after Disulfide Exchange Equilibrium in Oxidized (1 mM) and Reduced (10 mM) Glutathione

peak	designation	number of VP	calculated mass (Da)	measured mass (Da)
1	II	0	7586.6	7584.2
2	I	2	7796.9	7798.5
3	0	4	8007.2	8007.9

and 1 mM oxidized glutathione, in two separate experiments. After 30 min of incubation, the components in the two different equilibrium mixtures were trapped by thiol alkylation using VP. We have earlier shown by comparison with acid disulfide exchange quenching that the IGF-I equilibrium is not significantly shifted during the pyridylethylation under the conditions used (data not shown).

Separation of the different IGF-I forms in the mixtures was performed using RP-HPLC. Peak material was collected and further analyzed by PDMS and peptic mapping. Masses reveal possible number of covalently bounded pyridylethyl or glutathione groups. The presence of pyridylethyl groups was also detected by its chromophore, showing a strong absorbance at 254 nm (Hober *et al.*, 1992). The relative numbers of pyridylethyl groups could be determined by dividing the integrated absorbance at 254 nm and the integrated fluorescence (excitation at 280 nm and emission at 305 nm). Addition of up to six pyridylethyl groups per IGF-I molecules do not add significantly to the IGF-I fluorescence (Hober *et al.*, 1992).

IGF-I(C47A,C52A). Starting from either reduced or oxidized form, two major molecular species accumulated. Figure 2A shows a chromatogram from a sample starting from reduced protein, but the corresponding experiment starting from the oxidized two-disulfide IGF-I molecule appears indistinguishable (data not shown). Mass spectroscopy and absorbance at 254 nm revealed that the main peak (corresponding to approximately 75%) in the chromatogram is a two-disulfide form (II) and that the smaller peak (25%) is a one-disulfide variant (I) of the molecule (Table 1). The disulfides were analyzed with peptic mapping (data not shown) (Hober *et al.*, 1992). In both molecular species, the disulfide bond between cysteine residues 18 and 61 was formed. Thus, the major proportion of this IGF-I variant is able to keep both disulfides under the redox conditions used.

IGF-I(C6A,C48A). Folding equilibrium of the IGF-I(C6A,C48A) analogue, starting from reduced or oxidized material, revealed only one main peak (Figure 2B). The major molecular form was isolated and analyzed by the peptide mapping methods described above. The results suggest that the main peak contains IGF-I(C6A,C48A) with only one disulfide bridge (Table 2). In this HPLC system, the two-disulfide form of the mutant has a retention time only about 1 min longer than the one-disulfide variant. However, mass analysis confirmed that the peak did not contain any detectable two-disulfide specie. Peptic mapping revealed that the disulfide bond (18−61) was also formed in this mutant (data not shown). On the basis of the absorbance at 254 nm, the latest eluting peak in the chromatogram is suggested to contain reduced material (Figure 2B).

Table 2: Mass Spectrometric Analysis of Different Isolated Forms of IGF-I(C6A,C48A) after Disulfide Exchange Equilibrium in Oxidized (1 mM) and Reduced (10 mM) Glutathione

peak	designation	number of VP	calculated mass (Da)	measured mass (Da)
1	I	2	7796.9	7799.5
2	II	0	7586.6	7586.3
3	0	4	8007.2	8011.1

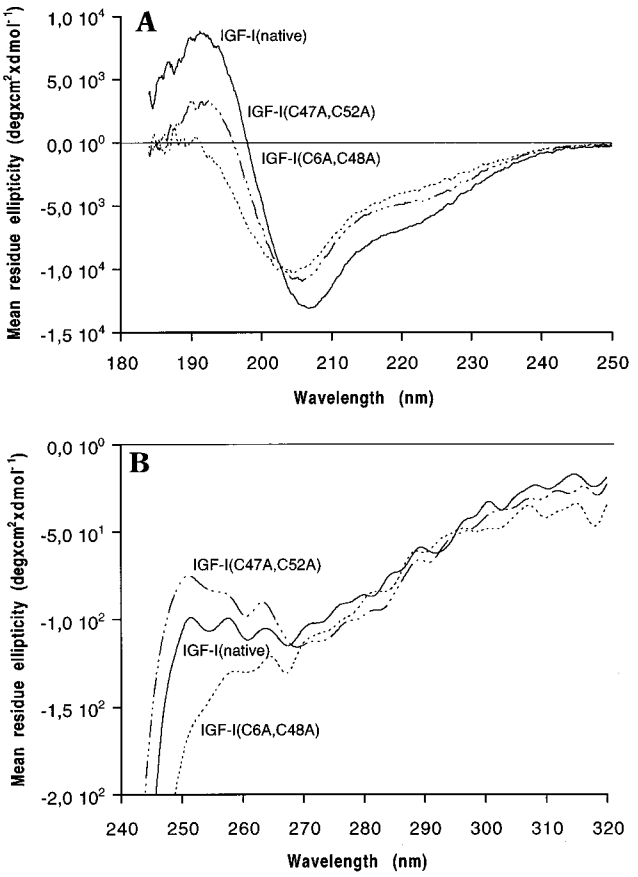

FIGURE 3: Far-UV (A) and near-UV (B) circular dichroism spectra of native IGF-I (—), IGF-I(C6A,C48A) (···) and IGF-I(C47A,C52A) (—···—).

CD Spectroscopy Analyses

Far- and near-UV CD spectra of the disulfide mutant variants of IGF-I and native IGF-I were collected and evaluated (Figure 3A and 3B). In native IGF-I, the minima at 222 and 208 nm in a combination with a maximum between 185 and 190 nm suggest that there is α-helicity in the molecule (Johnson, 1990), in accordance with the

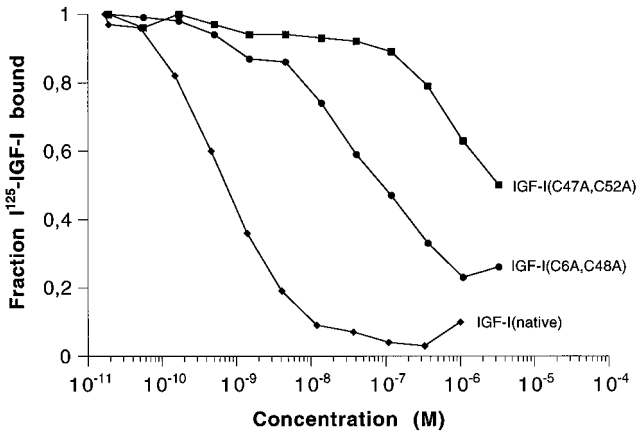

FIGURE 4: Competitive protein binding assay between [^{125}I]IGF-I and unlabeled IGF-I(native), IGF-I(C6A,C48A), and IGF-I-(C47A,C52A) for binding to the IGF type-I receptor.

published NMR structure of native IGF-I under acidic conditions (Cooke *et al.*, 1991; Sato *et al.*, 1993). Secondary structure prediction using a variable selection procedure (Manavalan & Johnson, 1987) suggests that native IGF-I has an α-helical content of 20%. The CD spectra of the IGF-I analogues show significantly lower α-helicity than the native molecule. Predictions of the secondary structure from the spectra suggest that IGF-I(C6A,C48A) has an α-helicical content of only about 8% and that IGF-I(C47A,C52A) has 13%. Thus, IGF-I is dependent on its disulfides to fold into a conformation with maintained secondary structure content. Calculated secondary structure contents from CD spectroscopy data for native IGF-I differ by less than 2% from earlier published results (Hober *et al.*, 1992). Comparision of the near-UV CD spectra is also interesting. The spectra of the two-disulfide mutant proteins are distinct from each other dispite having the same number of disulfides. This shows that the changes in spectra presumably are due to structural differences in the molecules and cannot simply be an effect of differences in the number of disulfides.

Binding Studies

To understand the conformational changes observed in the disulfide variants in relation to biochemical activities, binding studies were made. Native IGF-I and the two variants thereof were studied with respect to their affinity to IGFBP-1 and soluble IGF type-I receptor.

Receptor Affinity. To analyze the affinity to the receptor, a competitive displacement assay was used. Binding curves of different IGF-I analogues and native IGF-I are shown in Figure 4. IGF-I(C47A,C52A) shows the lowest affinity to the IGF type-I receptor. The binding IC_{50} value to the receptor affinity is lower by approximately 5×10^3 than that for the native protein [IC_{50} for IGF-I(C47A,C52A) is $<3 \times 10^{-6}$ M]. The receptor affinity of IGF-I(C6A,C48A) is 100-fold lower than for native IGF-I [IC_{50} for IGF-I-(C6A,C48A) is approximately 8×10^{-8} M].

Affinity to IGFBP-1. The affinity toward IGFBP-1 was determined using a BIAcore instrument with the IGFBP-1 molecule immobilized to the dextran surface. Table 3 shows the binding constants of IGF-I and the disulfide-mutated variants. The affinity constant of native IGF-I to the binding protein is fairly high. Binding affinity of IGF-I(C47A,C52A) is lowered by 3 orders of magnitude compared to native. Within the limits of the binding assay, no measurable IGFBP-1 binding was exhibited by the other IGF-I analogue, IGF-I(C6A,C48A) (Table 3).

Table 3: BIAcore Analysis of the Binding of IGF-I and Disulfide Mutants thereof in Their Binding to IGFBP-1[a]

	calculated binding parameters		
protein	k_{on} (1/M s)	k_{off} (1/s)	K_a (1/M)
IGF-I(native)	6.4×10^5	4.1×10^{-4}	1.6×10^9
IGF-I(C47A,C52A)	5.3×10^3	1.5×10^{-3}	3.4×10^6
IGF-I(C6A,C48A)	no measurable binding		

[a] On-rate (k_{on}), off-rate (k_{off}), and binding affinity (K_a) constants were calculated.

DISCUSSION

We (Hober *et al.*, 1992) and others (Miller *et al.*, 1993) have demonstrated that IGF-I is a protein with a "non-Anfinsen" folding behavior in a redox buffer system. Thus, the molecule possesses a thermodynamic folding problem involving two distinct three-disulfide forms, native and "mismatched", in similar quantities and in disulfide exchange equilibrium. This thermodynamically based folding inability to quantitatively reach its native conformation is present not only in a redox potential corresponding to the secretory vesicle but also in more oxidizing environments (unpublished results). In this paper, the structural importances of the 6−48 and 47−52 disulfide bonds were determined by replacing pairs of cysteine residues by alanines. Further, the mutant IGF-I molecules were used to examine whether it would be possible to turn this molecule into a protein which would fit more into the folding dogma by Anfinsen and possess a thermodynamically driven folding into one native form (Anfinsen, 1973). The two IGF-I analogues were constructed, produced in *E. coli*, and purified. Thereafter, the IGF-I variants were allowed to equilibrate in a buffer composed of reduced and oxidized glutathione. When the IGF-I(C47A,C52A) equilibrium sample was analyzed, it was found that the predominant form had the two remaining native disulfides, formed between cysteines 18−61 and 6−48 (Figure 2A). Thus, IGF-I(C47A,C52A) is able to readily form both possible, native disulfides under the conditions used. In contrast, the IGF-I form that lacks the 6−48 disulfide, IGF-I(C6A,C48A), was not able to keep the two remaining disulfides oxidized in this environment. The most populated form in this case had only the 18−61 disulfide formed (Figure 2B). Thus, IGF-I(C6A,C48A) possesses a thermodynamic folding problem similar to the one of native IGF-I (Hober *et al.*, 1992). Most interestingly, the native 18−61 disulfide bond is readily formed in both mutant IGF-I proteins as well as in parent IGF-I under the conditions used. This specificity of the 18−61 disulfide bond suggests that all these IGF-I variants follow a common characteristic of a native-like folding behavior.

Hart *et al.* (1994) have recently challenged our previous conclusion that the disulfide bonds in native IGF-I are important to direct IGF-I folding and that their energetics can be the cause of the unusual folding behavior of IGF-I. Their conlusion is incorrectly based on the assumption that the different isolated forms are kinetic "intermediates", neglecting the fact that these trapped forms evidently are in chemical equilibrium. The results published in this paper, as well as in a previous report by Nahri *et al.* (1993) demonstrating that the disulfide replacement variants of IGF-I

are structurally perturbed, further corroborate our initial conclusion that the native IGF-I fold is dependent on, and directed by, at least two of its three native disulfide bonds. The CD spectra of the disulfide analogues further support that the three-dimensional structure of IGF-I is dependent on the disulfides, consistent with earlier results (Hober *et al.*, 1992; Hua *et al.*, 1996; Nahri *et al.*, 1993). Predictions of the secondary structure from the far-UV CD spectra (Figure 3A) suggest that IGF-I(C6A,C48A) has only about 8% α-helix compared to native IGF-I which has about 20%. The second IGF-I disulfide variant, IGF-I(C47A,C52A), is concluded from the CD spectra to be more native like in terms of secondary structure content (13% α-helix). This results is also in accordance with the near-UV CD spectra (Figure 3B) where the two-disulfide mutants have distinct spectra despite same number of disulfides. Thus, the native conformation of IGF-I is dependent on at least the 47−52 and 6−48 disulfides. In comparison, any of the three disulfides of BPTI (5−55, 30−51, and 14−38) can be mutated and the molecule still behaves native-like in terms of three-dimensional structure (Eigenbrot *et al.*, 1992; Staley & Kim, 1992; van Mierlo *et al.*, 1991a). At least two of the three BPTI disulfides must be removed in order to obtain non-native structural and biochemical behavior of the molecule (van Mierlo *et al.*, 1991b, 1993).

There are drastic changes in binding constants after deleting either the 6−48 or the 47−52 disulfide IGF-I in the affinities both to sIGF-I^R and to IGFBP-1. To analyze the affinity to the receptor, a competitive binding assay was used. IGF-I(C47A,C52A) shows the weakest affinity to the IGF-I-receptor. It is more than 5000 times lower than that of the native protein. The IGF-I(C6A,C48A) protein is 100 times less active than native IGF-I. This is in accordance with earlier results published by Narhi *et al.* (1993). In serum and in other extra cellular fluids, IGF-I and IGF-II are carried by binding proteins. There are several reports concerning the activity of these proteins, including protection from degradation, transportation to specific tissue, and protection from hypoglycaemia by inhibition binding to the insulin receptor (Jones & Clemmons, 1995). Recently we showed that IGFBP-1 could assist in folding by directing native disulfide formation of IGF-I. Refolding experiments in the prescence of an excess of IGF-I over IGFBP-1 suggest that equimolar amounts of IGF-I and IGFBP-1 are needed to form and maintain the native structure of IGF-I (Hober *et al.*, 1994). The mutant forms of IGF-I were characterized with respect to binding to IGFBP-1. The BIAcore studies showed that the affinity constant of native IGF-I to IGFBP-1 is fairly high. Binding of IGF-I(C47A,C52A) was rather affected, compared to native IGF-I; it was 500-fold less. Interestingly, the lowered affinity seems to mainly result from a slower on rate of binding (Table 3). It may be explained by the requirement of a conformational change upon binding and by all complexes formed *per se* possessing native-like interactions between the IGF-I molecules and IGFBP-1. Within the limits of the binding assay, no detectable IGFBP-1 binding was exhibited by the other mutant form, IGF-I-(C6A,C48A). Thus, the IGF-I variant that lacks the 6−48 disulfide is the most disordered, and it displays no detectable binding to IGFBP-1 (Table 3).

Interestingly, the mutant that lacks detectable affinity for IGFBP-1 and also has a very disordered structure, compared to native, seems to bind the IGF type-I receptor quite well. On the other hand, IGF-I(C47A,C52A) has nearly no detectable affinity to the receptor but exhibits affinity to IGFBP-1. An explanation to this phenomenon could be that the binding protein and the receptor bind on opposite sides of the IGF-I molecule. This theory is also supported by binding studies of other IGF-I mutant forms Bayne *et al.*, 1989, 1990; Cascieri & Bayne, 1994, Jansson *et al.*, 1996; Zhang *et al.*, 1994). Structural studies of these mutant proteins could guide the understanding of which portions of the IGF-I molecule bind to the IGF-I receptor and IGFBP-1.

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