Folding of Insulin-like Growth Factor I Is Thermodynamically Controlled by Insulin-like Growth Factor Binding Protein†

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ABSTRACT: Insulin-like growth factor I (IGF-I) is thermodynamically unable to quantitatively form its native disulfides under reversible redox conditions *in vitro* [Hober et al. (1992) *Biochemistry 31*, 1749–1756]. These results prompted the question of how IGF-I may overcome this energetic problem in its folding *in vivo*. Here, we report that an IGF-I precursor, IGF-I-E_a, shows disulfide-exchange folding properties similar to those of mature IGF-I and, thus, is concluded not to overcome the identified folding problem of mature IGF-I. However, correct disulfide bonds are formed very efficiently when insulin-like growth factor binding protein 1 is added in equimolar amounts to IGF-I to the refolding mixture. On the basis of these results, we propose that one important function of at least one of the six homologous insulin-like growth factor binding proteins is to assist in the formation and maintenance of the native disulfides of IGF-I. To our knowledge, this is the first example where the folding of a mammalian protein or peptide in circulation has been demonstrated to be thermodynamically controlled by its binding protein. Speculatively, this could provide a mechanism to regulate the half-life of IGF-I *in vivo* by altering the interaction with insulin-like growth factor binding proteins.

Human insulin-like growth factor I (IGF-I)[1] is a three-disulfide 70 amino acid residue peptide growth factor (Humbel, 1990) with a defined three-dimensional structure in solution (Cooke et al., 1991). Recently, we (Hober et al., 1992) and others (Owers Narhi et al., 1993) reported that IGF-I is unable, as a result of its disulfide-exchange thermodynamic folding properties, to quantitatively form the native disulfides *in vitro*. The detailed analysis of the folding of IGF-I demonstrates the presence of two distinct three-disulfide-bonded forms (Hober et al., 1992): the native form (disulfides 6–48,[2] 18–61, and 47–52) and a non-native form designated "mismatched" (disulfides 6–47, 18–61, and 48–52). These two forms are equally represented in a disulfide-exchange equilibrium mixture (Hober et al., 1992), and they possess similar free energies of conformational stability (Owers Narhi et al., 1993). However, these two folding variants of IGF-I show significant differences in secondary structure contents (Hober et al., 1992), and only the native form is biologically active on the type 1 receptor (Raschdorf et al., 1988). This presence of two different, but thermodynamically equivalent, folding forms from one polypeptide chain is in conflict with the "thermodynamic hypothesis" of protein folding, as first proposed by Anfinsen (1973), and states that protein folding is expected to be energetically driven to a single native state. We have recently found that the thermodynamic folding problem of IGF-I is present not only at a redox potential comparable to that found in secretory vesicles (≈−200 mV) (Hober et al., 1992; Hwang et al., 1992) but also in more oxidizing environments (data not shown), similar to those found in plasma (≈0 mV) (Jellinek et al., 1992). Thus, the disulfides of IGF-I are thermodynamically unstable even in the oxidizing serum conditions. Since glutathione is abundant in serum (Shaheen & Hassan, 1991), the result would suggest that the folding behavior of IGF-I must be considered not only in the process of forming the disulfides but also in maintaining the native structure in the circulation.

It is unlikely that the formation of correct disulfides in IGF-I could be guided by classical protein folding chaperones, e.g., Hsp70, or folding enzymes, e.g., protein disulfide isomerase, since these have been shown only to affect the kinetic process of folding but not the folding thermodynamics (Nilsson & Anderson, 1991; Gething & Sambrook, 1992; Weissman & Kim, 1993). Instead, at least two different mechanisms can be proposed to act *in vivo* to quantitatively form and maintain correct disulfide bonds in IGF-I (Hober et al., 1992). First, the precursors of IGF-I, IGF-I-E_a and IGF-I-E_b (Rotwein, 1986), may show thermodynamic folding properties favoring the native disulfide bonds. Since these IGF-I precursors are not present in the circulation, the pro form of IGF-I could not function to maintain native disulfide configuration in the circulation, but it is still possible that folding would be promoted in this pro form in the secretory vesicles. This mechanism would be analogous to the enhanced folding properties of precursor forms of microbial proteases, e.g., subtilisin (Zhu et al., 1989), α-lytic protease (Baker et al., 1992), and carboxypeptidase Y (Winther & Sørensen, 1991). However, in all these cases, the propeptides seem to act at the kinetic level by accelerating the rate of folding, as was first demonstrated for α-lytic protease (Baker et al., 1992). A second hypothesis is that insulin-like growth factor binding proteins (IGFBPs) [for recent reviews, see Hintz (1990) and Shimasaki and Ling (1992)] assist in the formation of correct

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[1] Abbreviations: DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; IGFBP, insulin-like growth factor binding protein; IGF-I, insulin-like growth factor I; IGF-I-E_a, insulin-like growth factor I precursor containing the C-terminal E_a peptide extension of 35 amino acid residues; PDMS, plasma desorption mass spectrometry; PFPA, pentafluoropropionic acid; RP-HPLC, reverse-phase high-performance liquid chromatography.

[2] In this paper, amino acid residues are numbered according to their position in the corresponding protein from its N-terminus, and disulfide bonds are designated as connected residue numbers, e.g., 18–61 as the disulfide connecting cysteine residues 18 and 61.

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disulfide bonds by their binding to IGF-I. Both of these hypotheses have now been tested and are reported in this paper.

MATERIALS AND METHODS

Preparation of Native, Mismatched, and Reduced Forms of IGF-I. Native and mismatched forms of IGF-I were produced as a fusion protein in *Escherichia coli* and purified essentially as described [see Moks et al. (1987) and Forsberg et al. (1990)]. Reduced IGF-I was prepared by incubating the native protein in a buffer containing 10 mM reduced DTT and 8 M urea. After reduction, the buffer was changed to 10 mM HCl using gel filtration chromatography (Hober et al., 1992).

Production and Preparation of IGF-I-E_a. IGF-I-E_a was 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 efficient expression system described by Altman et al. (1991). After production, cells were disrupted by incubation for 2 h at 20 °C in 6 M GuHCl, 50 mM phosphate buffer, pH 6.5, 150 mM NaCl, and 0.5 mM EDTA. The protein solution was diluted six times in 10 mM Tris, pH 8, 0.05% Tween 20, 200 mM NaCl, and 1.25 mM EDTA and subsequently purified by IgG affinity chromatography. The eluted fusion protein was immediately subjected to Mono S cation-exchange chromatography (Pharmacia Biotech, Uppsala, Sweden). After the protein was washed with 40 mM ammonium acetate, pH 5.5, containing 15% acetonitrile, Z-IGF-I-E_a was eluted with 2 M ammonium acetate, pH 5.2, containing 15% acetonitrile. The fusion protein was chemically cleaved with hydroxylamine as described in Moks et al. (1987). Released IGF-I-E_a was separated from uncleaved fusion protein (Z-IGF-I-E_a) as well as the fusion partner (Z) by a second passage through the IgG affinity column. The final purification of IGF-I-E_a was performed using Mono S cation-exchange chromatography. The column was preequilibrated in starting buffer (40 mM ammonium acetate, pH 7.0, 6 M urea, and 10 mM reduced DTT). Before application onto the column, the protein was reduced by incubation (1 h) in starting buffer at 37 °C. The reduced IGF-I-E_a was eluted with 1 M ammonium acetate, pH 7.0, 6 M urea, and 10 mM reduced DTT. The buffer was subsequently changed to 10 mM HCl by gel filtration chromatography using a Sephadex G25 column (Pharmacia Biotech, Uppsala, Sweden).

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 by IGF-I affinity purification followed by cation-exchange chromatography (details will be published elsewhere).

Protein Analysis. Purified proteins were analyzed by SDS–PAGE (Laemmli, 1970) using the Phast system (Pharmacia Biotech, Uppsala, Sweden). The concentrations of IGF-I, IGF-I-E_a, and IGFBP-1, respectively, were determined by quantitative amino acid analysis or by measuring the absorbance at 280 nm using the specific absorption constant A_{280} (1%, 1 cm) = 2.1 for IGF-I (Hober et al., 1992) and 2.4 for IGF-I-E_a.

Molecular masses of the different forms of IGF-I were determined using ^{252}Cf plasma desorption mass spectrometry (Bio-Ion 20, Applied Biosystems, Foster City, CA) (Hober et al., 1992). Molecular masses of IGF-I-E_a, and derivatives thereof, were determined using a JEOL SX102 (Tokyo, Japan) mass spectrometer equipped with an electrospray unit.

Disulfide-Exchange Reactions of IGF-I and IGF-I-E_a. The disulfide-exchange reactions were performed as described

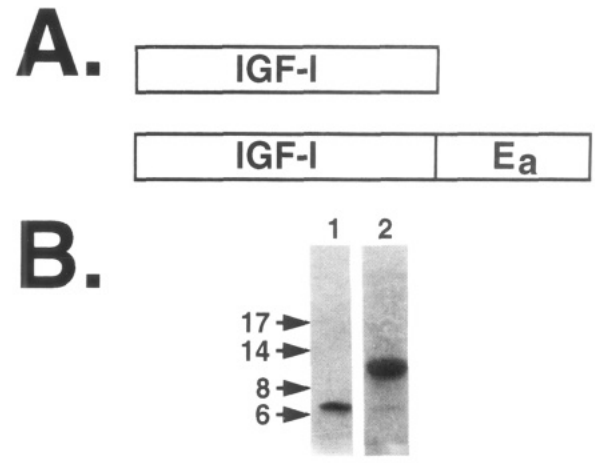

FIGURE 1: (A) Schematic representation of IGF-I and IGF-I-E_a. IGF-I is a protein of 70 amino acid residues, and IGF-I-E_a represents a pro form of IGF-I, having a C-terminal extension of 35 amino acid residues (Rotwein, 1986). (B) SDS–polyacrylamide gel electrophoresis analysis of purified recombinant IGF-I and IGF-I-E_a: lane 1, IGF-I; lane 2, IGF-I-E_a. Molecular mass, as measured by mass spectrometry, of purified recombinant IGF-I was 7650 ± 5 Da (calculated 7650 Da) and of purified recombinant IGF-I-E_a was 11682 ± 3 Da (calculated 11 684 Da).

(Hober et al., 1992) at an IGF-I concentration of 30 μM and at an IGF-I-E_a concentration of 5.5 μM at 37 °C for 1 h, which is at least 30 times that needed to reach equilibrium of reduced mature IGF-I under the conditions used (data not shown). The concentrations of oxidized (GSSG) and reduced (GSH) glutathione were 1 and 10 mM, respectively. The reactions were terminated by alkylating free thiols using 160 mM vinylpyridine as described (Hober et al., 1992). The method will efficiently pyridylethylate free thiols but not yield any detectable alkylation of non-thiol groups in IGF-I under the conditions used (Hober et al., 1992). After alkylation, the different forms of IGF-I and IGF-I-E_a, respectively, were separated on reverse-phase HPLC (Hober et al., 1992). IGFBP-1 is apparently unaffected in the alkylation reactions, and it elutes as a single peak at a retention time of ≈30 min in the HPLC system used (data not shown).

RESULTS AND DISCUSSION

Refolding of IGF-I-E_a. A recombinant IGF-I precursor, IGF-I-E_a (Figure 1A), having a C-terminal extension of 35 non-cysteine amino acid residues, was produced in *E. coli* and purified (Figure 1B). The identity of the purified IGF-I-E_a polypeptide chain was confirmed by mass spectroscopy. The thermodynamic properties of its disulfide-exchange folding was studied in a redox buffer containing reduced and oxidized glutathione. The redox potential in the experiment is comparable to that described for secretory vesicles of mammalian cells (Hwang et al., 1992), and these conditions were previously used to study the disulfides of IGF-I (Hober et al., 1992). In addition, these conditions are expected to favor the formation of protein disulfides and are typically used *in vitro* to generate reversible thiol-exchange conditions to study kinetics and thermodynamics of protein disulfide bond formation (Saxena & Wetlaufer, 1970; Creighton, 1984). The components in the refolding mixture were trapped by thiol alkylation using vinylpyridine. Separation of the different IGF-I-E_a forms was performed by RP-HPLC (Figure 2). The materials in the different peaks were collected and analyzed by mass spectrometry. These determined masses, and the absorbances at the pyridylethyl chromophore at 254 nm, were utilized to calculate the number of covalently attached pyridylethyl or glutathione groups. The relative amounts of

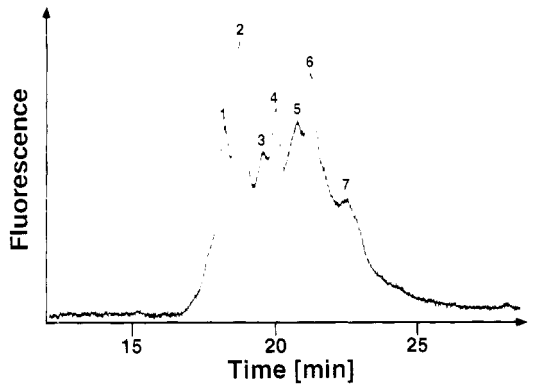

FIGURE 2: RP-HPLC separation of different IGF-I-E_a components after incubation in a redox buffer followed by pyridyl ethylation of free thiolate groups (Hober et al., 1992). The elution was monitored by a fluorescence detector and a diode array detector (data not shown) in series.

IGF-I-E_a polypeptide in the different peaks were determined by integrating their respective fluorescence (data not shown). The analysis of fractionated materials from the different peaks indicated that the major peak (peak 2 in Figure 2) is a two-disulfide-bonded form with two attached pyridylethyl groups. In the two peaks eluting before (peak 1) and after (peak 4) the major peak are three disulfide-bonded forms (data not shown). If the order in which the different forms of IGF-I and IGF-I-E_a elute from the reverse-phase HPLC system is maintained between IGF-I and IGF-I-E_a, the two-disulfide major peak (peak 2) of the chromatogram would correspond to IGF-I-E_a lacking the disulfide bond corresponding to 47–52 in native IGF-I, preceded by the peak containing mismatched IGF-I-E_a (peak 1) and followed by IGF-I-E_a with native disulfides (peak 4). A one-disulfide IGF-I-E_a species elutes late in the chromatogram (peak 6), which is also consistent with the elution profile of vinylpyridine-trapped forms of the mature IGF-I under these conditions (Hober et al., 1992). Even though the detailed analysis of the different IGF-I-E_a forms in the mixture remains to be performed by peptide mapping, the experiment implies that, under these redox conditions, IGF-I-E_a possesses a similar thermodynamic folding problem as previously described for IGF-I (Hober et al., 1992). Thus, we conclude that the analyzed precursor of IGF-I, IGF-I-E_a, fails to guide quantitative formation of native disulfides under the conditions used. However, it should be mentioned that only a single refolding time point (1 h) was used. Even though this refolding time exceeds the time needed for IGF-I to reach equilibrium by at least 30 times under the conditions used (data not shown), it is still possible that the IGF-I precursor indeed favors native disulfides but that the disulfide exchange rates become extremely slow by the precursor peptide extension. However, we find this possibility less likely in light of the results below and in light of the disulfide-exchange rates found *in vitro* for other disulfide-containing proteins (Creighton, 1984).

Refolding of IGF-I in the Presence and Absence of IGFBP-1. The majority of IGF-I and IGFBP-1 in circulation is coexpressed in the liver (Humbel, 1990), while in the plasma IGF-I is carried mainly by a high molecular weight form of IGFBP-3 (Hintz, 1990). Thus, one function of IGFBP-1 is thought to be the transport of newly synthesized IGF-I from the liver out into the circulation. We decided to study if IGFBP-1 additionally assists in folding by directing the formation of correct disulfides in IGF-I. Native, mismatched, and reduced IGF-I, respectively, were incubated in a glutathione redox buffer in the presence or absence of IGFBP-1. Separation of the different IGF-I components was performed

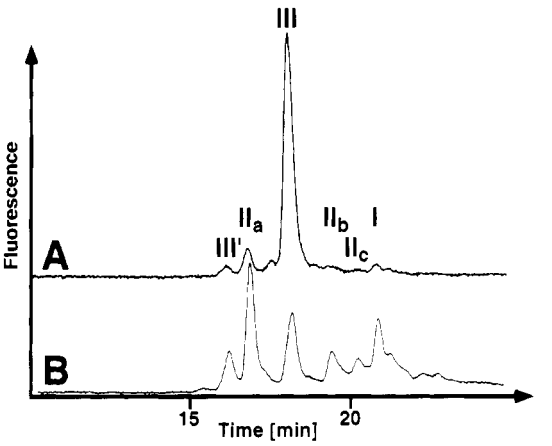

FIGURE 3: RP-HPLC separation of different IGF-I forms after incubation in the presence (A) or in the absence (B) of IGFBP-1 in a glutathione redox buffer. Here, the refolding was performed by starting from reduced IGF-I, but the results were indistinguishable by starting from either mismatched or native IGF-I (data not shown). IGFBP-1 elutes at ≈30 min (data not shown).

Table 1: Relative Amounts of the Different Forms of IGF-I in the Presence and Absence of Variable Amounts of IGFBP-1[a]

reaction conditions			forms of IGF-I					
IGF-I	IGFBP-1 (μM)	BSA (μM)	III (%)	III′ (%)	IIa (%)	IIb (%)	I (%)	O (%)
O	30	0	89	<4	11	<4	<4	<4
O	0	0	22	11	30	12	25	<4
O	0	30	23	10	30	11	26	<4
III′	30	0	84	5	11	<4	<4	<4
III′	0	0	22	10	31	12	25	<4
III	30	0	86	4	10	<4	<4	<4
III	3	0	29	9	30	8	24	<4
III	0.3	0	26	11	32	8	23	<4
III	0	0	22	10	32	9	27	<4

[a] The IGF-I concentration was 30 μM in all experiments. The amount of IGF-I in each peak was determined by integrating the fluorescence signal from the HPLC chromatogram, which has been shown to be consistent (within 5%) with quantitative amino acid analysis of isolated materials for all these peaks of IGF-I (Hober et al., 1992). Peak designations for **III**, **III′**, $\mathbf{II_a}$, $\mathbf{II_b}$, and **I** are as described in the text. **O** is the form with six pyridylethyl groups. As a control experiment, disulfide-exchange reactions were performed in equimolar amounts of bovine serum albumin. The IGF-I column shows the form of IGF-I (**O**, **III**, or **III′**) that was used as starting material in the different redox reactions.

on RP-HPLC (Figure 3). The identity of each peak was confirmed by its retention time in the HPLC system as well as by mass spectrometry analysis of isolated peak materials. Most of these peaks have previously been analyzed in detail by peptide mapping techniques (Hober et al., 1992). Roman numbers in the chromatogram represent the different trapped forms of IGF-I (Hober et al., 1992). **I** is a form with only the native disulfide between amino acid residue 18 and 61, $\mathbf{II_a}$ is a form with two of the three native disulfides present between amino acid residues 18–61 and 6–48, $\mathbf{II_b}$ corresponds to a variant of IGF-I with two disulfide bonds of which one is native (18–61) and the other (6–52) is not present in native IGF-I, and peak $\mathbf{II_c}$ contains an IGF-I variant with two disulfide bonds that remain to be analyzed. **III′** is the mismatched IGF-I which is a form with three disulfides of which one is native (18–61) and the other two (6–47 and 48–52) are not present in the native and biologically active molecule, and **III** is the native molecule with disulfides 6–48, 18–61, and 47–52. It was found that only in the presence of IGFBP-1 does IGF-I quantitatively attain its native three-disulfide conformation (Figure 3, Table 1). Refolding experiments with an excess of IGF-I over IGFBP-1 (Table 1) suggest that equimolar amounts of IGFBP-1 and IGF-I are

necessary to quantitatively form and maintain the native disulfides of IGF-I. This would indicate that the molecular mechanism by which IGFBP-1 is assisting in the folding of IGF-I is a mass-action effect by recognition of only the native IGF-I molecule in forming the heterodimeric complex. Thus, complex formation with IGFBP-1 can overcome the previously described thermodynamic problem in quantitatively forming correct disulfides in IGF-I.

In serum and in other extracellular fluids, IGF-I is carried by at least six distinct IGFBPs, and there are a large number of reported different activities of these homologous molecules (Hintz, 1990; Shimasaki & Ling, 1992), and the most important of these are (i) to protect IGF-I from clearance and proteolytic degradation, (ii) to transport IGF-I to specific tissues, (iii) to play a role in hormone regulation, (iv) to prevent hypoglycemia by inhibiting the binding of IGF-I to the insulin receptor, (v) to increase the potency of IGF-I by interacting with the cell surface, (vi) to remove IGFs from tissue and circulation, and (vii) to inhibit the biological activity of IGFs. On the basis of our results, we propose that an additional and important function of the IGFBPs is to form and maintain native disulfides in IGF-I. Since the majority of IGF-I in circulation is carried by IGFBP-3, the question of whether this, and other IGFBPs, could also act in forming and maintaining the native IGF-I disulfides arises. Because of the significant homologies between the different IGFBPs and the identified problem to maintain IGF-I disulfides in the circulation, we find this likely.

IGFBP-1 contains 18 cysteine residues furnishing 9 intramolecular disulfide bonds (Hintz, 1990). Do these disulfides participate in the refolding of IGF-I? In fact, we have recently addressed this issue and found that IGFBP-1 significantly accelerates the refolding rate of reduced IGF-I (S. Hober and B. Nilsson, data not shown). However, the mechanism of this acceleration could be found in other explanations than IGFBP-1 acting as a disulfide isomerase. Even though the folding problem of IGF-I that has been addressed in this paper is of thermodynamic and not of kinetic nature, we find this effect on the IGF-I refolding rate interesting.

Can IGFBP-1 act also at the precursor level? When the pro form IGF-I-E_a (Figure 1) was allowed to fold *in vitro* in the presence of IGFBP-1, IGF-I-E_a with disulfide bonds corresponding to those in native IGF-I was quantitatively formed (data not shown). This result demonstrates that IGFBP could act to promote correct folding of IGF-I not only at the level of the mature polypeptide chain but also at the precursor level.

The results presented in this paper suggest that IGFBP assists in the folding of IGF-I *in vivo*, which would solve both the forming and the maintenance problem of the energetically unfavorable native disulfides present in the noncomplexed IGF-I molecule. Thus, thermodynamically, IGF-I could be considered as the receptor binding subunit in a heterodimeric complex with an insulin-like growth factor binding protein. Speculatively, the binding of IGF-I to IGFBP provides a mechanism to regulate the half-life of IGF-I in the circulation, since the stability of noncomplexed IGF-I molecules can be predicted to be low due to the instability of its disulfide bonds under serum redox conditions. A truncated form of IGF-I, lacking the three N-terminal amino acid residues, is present in brain (Sara et al., 1986) and in other tissue (Ogasawara et al., 1989). This form of IGF-I, des(1–3)-IGF-I, binds poorly to IGFBP-1 but is apparently more potent in receptor binding than IGF-I (Ballard et al., 1987). From our results we suggest that the significance of this posttranslationally modified form of IGF-I is to exclusively act locally and to become rapidly inactivated in the circulation.

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