Insulin-like growth factors I and II are unable to form and maintain their native disulfides under in vivo redox conditions

Sophia Hober[a,*], Johanna Lundström Ljung[c], Mathias Uhlén[a], Björn Nilsson[a,b]

[a] *Department of Biotechnology, Royal Institute of Technology, S-100 44 Stockholm, Sweden*
[b] *Amersham Pharmacia Biotech, Box 605, S-751 25 Uppsala, Sweden*
[c] *The Medical Nobel Institute for Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institute, S-171 77 Stockholm, Sweden*

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Abstract Insulin-like growth factor (IGF) I does not quantitatively form its three native disulfide bonds in the presence of 10 mM reduced and 1 mM oxidized glutathione in vitro [Hober, S. et al. (1992) Biochemistry 31, 1749–1756]. In this paper, we show (i) that both IGF-I and IGF-II are unable to form and maintain their native disulfide bonds at redox conditions that are similar to the situation in the secretory vesicles in vivo and (ii) that the presence of protein disulfide isomerase does not overcome this problem. The results indicate that the previously described thermodynamic disulfide exchange folding problem of IGF-I in vitro is also present in vivo. Speculatively, we suggest that the thermodynamic disulfide exchange properties of IGF-I and II are biologically significant for inactivation of the unbound growth factors by disulfide exchange reactions to generate variants destined for rapid clearance.

Key words: Insulin-like growth factor I; Insulin-like growth factor II; Protein disulfide isomerase; Disulfide; Folding; Redox condition

1. Introduction

Human insulin-like growth factor I (IGF-I) is a single-chain peptide hormone of 70 amino acids which is homologous to proinsulin [1,2]. The single-chain polypeptide growth factor has six cysteine residues forming three disulfide bonds (Fig. 1). A three-dimensional model of IGF-I, based on the crystal structure of insulin, has been proposed by Blundell et al. [3] and the model has been corroborated by NMR spectroscopy [4,5].

IGF-I was originally isolated from serum [2]. The growth factor is synthesized by many different cell types and tissues, and it is thought to mediate most of the mitogenic and metabolic in vivo effects of growth hormone [6]. In serum and other extracellular fluids, most of the IGF-I is carried by at least seven different, but homologous, carrier proteins, designated IGFBP-1 to IGFBP-7 [7–9]. A number of papers report the different activities of these seven binding proteins including regulating half-life in circulation, transporting IGF-I to specific tissues, preventing hypoglycemia by inhibiting the binding of IGF-I to the insulin receptor [10,11] and mediating p53-induced cell cycle arrest [12,13].

We [14] and others [15] have previously reported a thermodynamic folding problem of IGF-I in vitro to form its native disulfide bonds. During refolding under redox conditions that normally favor the formation of protein disulfides (10 mM reduced glutathione (GSH) and 1 mM oxidized glutathione (GSSG), respectively) [16] several different forms of IGF-I are found, including two distinct three-disulfide species, differing only in their disulfide bond arrangements [14]. One of these forms of IGF-I has the native disulfide bonds expected from comparison with insulin and this molecular species stimulates proliferation through the IGF-I receptor (Fig. 1) [17,18]. The other form, designated 'mismatch', has two non-native disulfide bridges (6–47 and 48–52 instead of the native, 6–48 and 47–52) and lacks affinity for the IGF-I receptor [17,19]. This unusual folding behavior has been further confirmed by folding analysis of disulfide mutants of IGF-I [20]. We have also demonstrated that the presence of equimolar amounts of IGFBP-1 will efficiently overcome the folding problem of IGF-I in vitro, suggesting that IGFBPs may function to form and maintain correct disulfides of IGF-I in vivo [21].

The redox state of the secretory pathway has been described to be more oxidative than our previous in vitro experiments [14,21] with a ratio of reduced to oxidized glutathione within the secretory pathway from 1/1 to 3/1 [22]. In addition, the redox potential in serum, though variable, is normally even more oxidizing [23]. Since there are several different equilibrium constants to consider, one for each disulfide in a distinct conformation, and only some of these are known [14], the final in vivo distribution of IGF-I variants cannot be predicted. The knowledge of individual equilibrium and rate constants is not enough to interpret the global folding behavior of the intact molecule. Also, this has led to disputes in the literature [24,25]. Therefore we have extended our analysis to redox conditions similar to the situation in the endoplasmic reticulum (ER). Furthermore, the disulfide exchange folding properties of IGF-I have been analyzed in the presence of bovine protein disulfide isomerase (PDI). PDI is abundant in the secretory vesicles [26] and has been demonstrated to act as a true catalyst of disulfide bond formation and isomerization in vitro to form native protein disulfides in many different proteins, for example insulin [27]. If PDI possessed

*Corresponding author. Fax: (46) (8) 25 54 52.
E-mail: sophia@biochem.kth.se

Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; DTT, dithiothreitol; ER, endoplasmic reticulum; GH, growth hormone; GSH, reduced glutathione; GSSG, oxidized glutathione; IAA, iodoacetic acid; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; PDI, protein disulfide isomerase; PDMS, plasma desorption mass spectrometry; PFPA, pentafluoropropionic acid; RP-HPLC, reverse phase high-performance liquid chromatography; VP, vinyl pyridine

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 as the disulfide connecting amino acid residues 47 and 52.

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different accessibility to the various disulfide bonds of IGF-I as previously proposed [28], the distribution of different IGF-I variants could be affected in high (mM levels) PDI concentrations in the ER. The disulfide exchange folding properties of IGF-II have also been analyzed. This growth factor shows different molecular endocrinological properties from IGF-I, including a separate receptor [2]. However, IGF-II is carried by the same set of IGFBPs as IGF-I. Therefore, this growth factor is also a candidate for dependence on IGFBP to form and maintain correct disulfides.

The results presented in this paper suggest that IGF-I possesses a thermodynamic folding problem in vivo and is dependent on IGFBP for correct folding. Interestingly, IGF-II shares these unusual folding properties. These results are utilized to propose a mechanism of inactivation of IGF-I and IGF-II involving disulfide exchange reshuffling of the native disulfides.

2. Materials and methods

2.1. Materials

Native and mismatch forms of IGF-I were produced and purified as fusion proteins in *Escherichia coli* and the different IGF-I forms were separated on reverse-phase HPLC [19,29,30]. Recombinant IGF-II was produced in *E. coli* as described previously [31]. BPTI was kindly provided by Bayer AG, Germany. Bovine PDI was purified as described by Lundström et al. [32].

Reduced protein (IGF-I, IGF-II or BPTI) was prepared according to Hober et al. [14].

2.2. Protein analysis

Protein concentrations for native and mismatch IGF-I were calculated from quantitative amino acid analysis as described by Hober et al. [14].

The concentration of reduced IGF-I and PDI was determined by measuring the absorbance at 280 nm using a spectrophotometer (Kontron, Switzerland) and calculating the protein content by using the specific absorption constant A_{280} (1 cm, 1 mg/ml) = 2.1 for IGF-I [14] and 0.8 for PDI [32].

Molecular masses were determined using a ^{252}Cf plasma desorption mass spectrometer, Bio-Ion 20 (Applied Biosystems, USA) [14], or a Jeol SX102 mass spectrometer equipped with an electrospray unit (Jeol, Japan).

2.3. Disulfide exchange reactions

Disulfide exchange reactions were performed at 37°C for 1 h, 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 GSSG (1, 2, 5, 10, or 10 mM) and GSH (10, 10, 5, 2, or 1 mM), respectively. Where applicable, bovine PDI was added to the reaction mixture to a final concentration of 1, 10, or 30 μM. Disulfide exchange reactions were quenched by pyridylethylation of free thiols with 160 mM vinyl pyridine (VP) [33]. The alkylation reaction was allowed to proceed for 15 min in the dark [14]. Thereafter, the buffer was immediately exchanged to 10 mM HCl by ultrafiltration (Centricon 3, microconcentrator, Amicon) or by gel filtration using Sephadex G-25 (Pharmacia, Sweden).

Different IGF-I variants were separated by reverse-phase HPLC according to Hober et al. [14]. Molecular masses of material isolated from each peak revealed possible numbers of covalently bound pyridylethyl or glutathione groups. The presence of pyridylethyl groups was also detected by its chromophore, showing a strong absorbance at 254 nm [14] (data not shown). The relative quantities of IGF-I in the different peaks were determined by integrating their fluorescence spectra (excitation at 280 nm and emission at 305 nm) (Fig. 3), which has been demonstrated to be similar for the different forms of IGF-I [14]. Pyridylethyl groups do not add to the fluorescence of IGF-I and the relative numbers of pyridylethyl groups could be determined by dividing the integrated absorbance at 254 nm and the integrated fluorescence [14].

Separation of different IGF-II variants were performed as described for IGF-I but with a gradient of 33–48% in 50 min. Similarly, for the separation of BPTI variants, the gradient used was 25–40% in 30 min.

2.4. Calculations

Relative free energies in the presence of reduced and oxidized glutathione were calculated relative to native IGF-I from determined equilibrium constants (K) using the equation $\Delta G = -RT \ln (K)$.

3. Results

3.1. Refolding of IGF-I under different redox conditions

Native, mismatch and reduced IGF-I were equilibrated in glutathione redox buffers differing in their respective concentrations of GSSG and GSH. Ratios of GSH/GSSG [mM/mM] used were 10/1, 10/2, 5/5, 2/10, or 1/10, respectively (Table 1). As a control, reduced IGF-I was incubated for 1 h in the absence of glutathione, resulting in only minor oxidation of IGF-I. Thus, we conclude that the reduced and oxidized glutathione is responsible for essentially all of the observed disulfide exchange reactions under the conditions used. Since the results using different starting materials (reduced, mismatch and native, respectively) were indistinguishable, it could be concluded that equilibrium had been established for all conditions used (data not shown). In fact, the disulfide exchange equilibrium of IGF-I under these conditions is formed within 5–10 min (data not shown). In Fig. 2, results from an experiment starting from reduced IGF-I are shown. The different forms of IGF-I isolated from the equilibrium mixtures were analyzed. The number of pyridylethylated thiols of each IGF-I form was determined by dividing the integrated absorbance at 254 nm and the integrated fluorescence [14] (Table 2). The identities of the different isolated major IGF-I components in the equilibrium mixtures were further analyzed by peptide mapping techniques and by retention time comparison in RP-HPLC [14] (Table 2). Relative amounts of IGF-I were determined by the integrated fluorescence (Table 1, Fig. 3). This is a previously validated method for quantitative analyses of relative amounts of IGF-I [14].

A separate experiment was performed as a control for possible artifacts due to the alkylation reaction. The disulfide exchange folding reactions in the 10 mM GSH/1 mM GSSG equilibrium mixture were slowed down by lowering the pH to 2 with HCl. The different folding variants were without delay

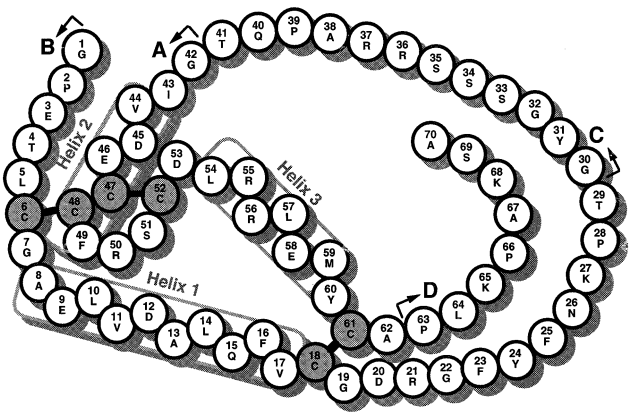

Fig. 1. A two-dimensional representation of IGF-I. The amino acids are shown as circles and written in one-letter code. The disulfides are connected as in the native structure of IGF-I. Helices are indicated by boxes. The fragments A, B, C, D and E are named according to the nomenclature of insulin.

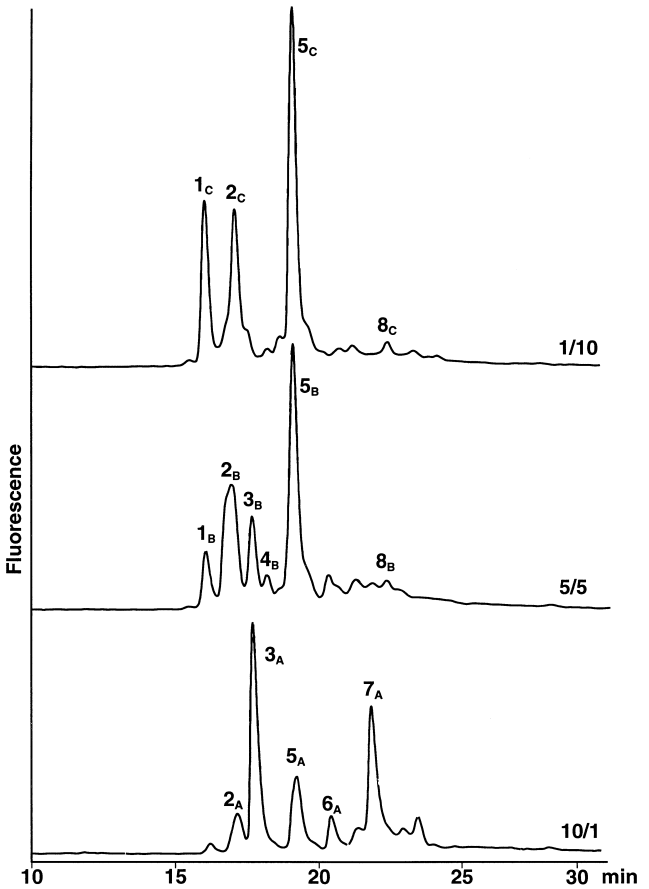

Fig. 2. RP-HPLC separation of different IGF-I variants from VP-trapped folding mixtures. The gradient was linear, 30–45% acetonitrile in 0.25% PFPA, over 30 min. The fluorescence (excitation at 280 nm and emission at 305 nm) of the different peaks was monitored. The chromatograms show the analyses of IGF-I samples after incubation in different redox buffers. The major peaks are numbered according to the order of migration and are further described in the text. Ratios (1/10, 5/5, 10/1) denote the GSH/GSSG concentrations in mM/mM.

separated by RP-HPLC and collected. All isolated IGF-I species were alkylated with VP at alkaline pH and reanalyzed by RP-HPLC. Each isolated molecular species was maintained as a single peak after alkylation demonstrating that no detectable disulfide rearrangement occurred during alkylation. In addition, retention times and absorbance at 254 nm revealed that the distribution of intermediates was indistinguishable from the original VP-trapping experiment [14]. These results suggest that the IGF-I equilibrium is unaltered during the pyridyl ethylation under the conditions used.

Table 1
Amounts and relative free energies of native (III) and mismatch (III′) IGF-I in different redox potentials

GSSG/GSH	III (%)	III′ (%)	Ratio III/III′	$\Delta\Delta G$ (kcal/mol)
1/10	22	7	3.1	0.7
1/5	29	13	2.2	0.5
1/1	42	23	1.8	0.4
5/1	51	27	1.9	0.4
10/1	51	27	1.9	0.4

The differences in relative energy were calculated relative to native IGF-I.

3.2. Analysis of the different equilibrium mixtures

3.2.1. 10 mM GSH/1 mM GSSG. The 1/10 redox conditions are identical to what has been utilized previously to define the thermodynamic folding problem of IGF-I [14,21]. It is also identical to what is commonly used to study disulfide exchange folding of proteins [16,34,35]. Five different forms of IGF-I, isolated from the equilibrium mixture (2_A–7_A), were analyzed. The obtained results are similar to what has been described by us earlier [14] (Tables 1 and 2, Figs. 2 and 3).

3.2.2. 10 mM GSH/2 mM GSSG. Materials from five different peaks (data not shown) were isolated and analyzed (Table 1, Fig. 3).

3.2.3. 5 mM GSH/5 mM GSSG. Material from six separate peaks (1_B–8_B) was isolated and further analyzed (Tables 1 and 2, Figs. 2 and 3).

3.2.4. 2 mM GSH/10 mM GSSG. Three different peaks (data not shown) were isolated and analyzed (Table 1, Fig. 3).

3.2.5. 1 mM GSH/10 mM GSSG. Materials from four different peaks (1_C–8_C) were isolated and analyzed. The mass, the absorbance at 254 nm, comparison of retention times in the HPLC system and peptide mapping gave the identity of the peaks (Tables 1 and 2, Figs. 2 and 3).

Relative amounts of native and mismatch IGF-I in the different redox buffers were used to calculate free energies relative to native IGF-I from equilibrium constants (Table 1).

Under all redox conditions tried we found at least four different IGF-I variants (Fig. 2). Their relative amounts var-

Table 2
PDMS analysis of material in the peaks from RP-HPLC (Fig. 2)

Peak	Designation	Number of VP	Number of GS	Calculated mass	Measured mass
1_B[a],1_C[b,c]	mixed	0	2	8262.2	8261.8
2_A[b],2_B[b],2_C[b]	III′	0	0	7649.6	7649.3
3_A[a],3_B[b]	II	2	0	7859.9	7860.9
4_B	II-mix	1	1	8061.0	8061.0
5_A[b],5_B[b],5_C[b]	III	0	0	7649.6	7648.8
6_A[a]	II″	2	0	7859.9	7861.0
7_A[a]	I	4	0	8070.2	8069.5
8_B[b],8_C[b]	III?	0	0	7649.6	7648.9

The numbers of disulfide bonds in the intermediates are indicated in Roman numerals. A prime after the Roman numeral indicates a molecule where all formed disulfides are as in the mismatch form of IGF-I. Two primes after the Roman numeral indicates at least one disulfide bridge which is found neither in the native nor in the mismatch form of IGF-I.

[a]Identity determined by peptide mapping techniques.

[b]Identity determined by retention time comparision in RP-HPLC.

[c]Peptide mapping of the material in this peak revealed that the 18–61 disulfide was formed.

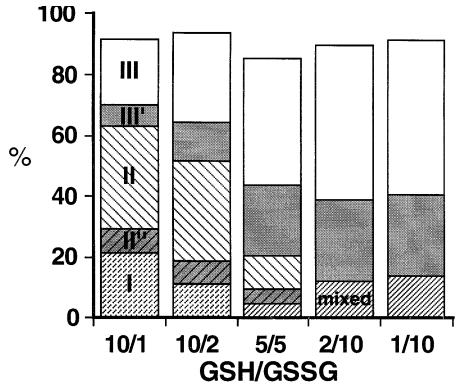

Fig. 3. Relative amounts of different IGF-I species vary with the redox potential. The percentages are calculated according to the total peak area from the RP-HPLC chromatograms (Fig. 2). Different molecular species are marked as the designation in Table 2. Ratios (1/10, 2/10, 5/5, 10/2, 10/1) denote the GSH/GSSG concentrations in mM/mM.

ied with the redox conditions (Fig. 3). Even in the most oxidative environment tested, more oxidative than what has been proposed to exist in the secretory pathway [22], we found only about 65% of IGF-I as three disulfide species. The ratio between native and mismatch IGF-I is about the same in the different glutathione concentrations tried. As expected, the relative amount of three disulfide-bonded species of IGF-I increases with increasing redox potential.

3.3. Disulfide exchange equilibrium of IGF-I in a glutathione buffer in the presence of PDI

The disulfide exchange equilibrium folding properties of IGF-I were analyzed in a buffer containing 10 mM GSH, 1 mM GSSG and 1, 10, or 30 μM of PDI. The disulfide exchange reaction was trapped using VP and different species were analyzed using RP-HPLC. PDI elutes later in the chromatogram than all previously analyzed IGF-I equilibrium variants (data not shown). Fig. 4 shows a chromatogram from a refolding reaction which started from reduced IGF-I in the presence of 10 μM PDI. The results appear indistinguishable from refolding reactions starting from native hormone and/or with other concentrations of PDI. The results demonstrate that catalytic or equimolar amounts of PDI per se do not overcome the thermodynamic folding problem of IGF-I.

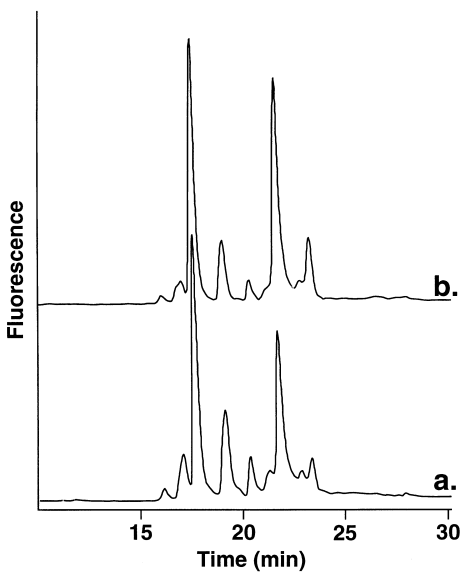

Fig. 4. RP-HPLC separation of different IGF-I variants from VP-trapped folding mixtures. The lower chromatogram (a) shows a sample from incubation in a buffer with PDI. The upper chromatogram (b) shows a sample from an identical redox buffer but without PDI. The concentration of glutathione in the buffer was 10 mM GSH and 1 mM GSSG. The gradient was linear, 30–45% acetonitrile in 0.25% PFPA, over 30 min. The fluorescence (excitation at 280 nm and emission at 305 nm) of the different peaks was measured.

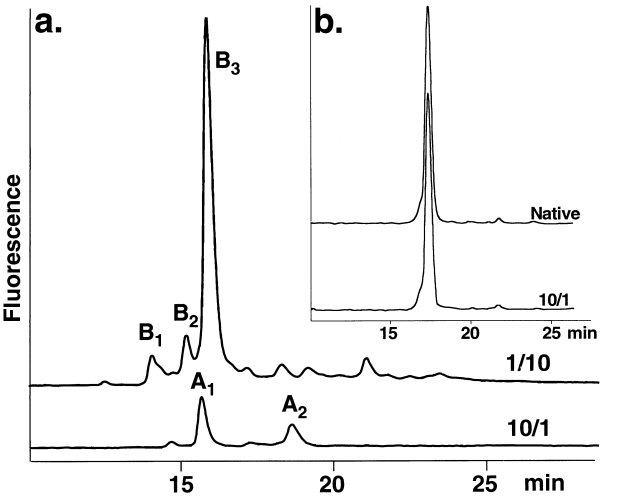

Fig. 5. A: RP-HPLC separation of different IGF-II variants from VP-trapped equilibrium mixtures. The gradient was linear, 33–48% acetonitrile in 0.25% PFPA, over 50 min. The fluorescence (excitation at 280 nm and emission at 305 nm) of the different peaks was measured. The chromatograms show samples from incubations in buffers at different redox conditions. The major peaks are numbered according to the order of migration. B: BPTI variants from VP-trapped equilibrium folding mixtures. The folding buffer has a concentration of 10 mM GSH and 1 mM GSSG. The upper chromatogram shows a reference with oxidized BPTI. The gradient was linear, 30–50% acetonitrile in 0.25% PFPA, over 30 min. The fluorescence (excitation at 280 nm and emission at 305 nm) of the different peaks was measured. Ratios (1/10, 10/1) denote the GSH/GSSG concentrations in mM/mM.

3.4. Disulfide exchange folding of IGF-II

Oxidized IGF-II was incubated for 1 h in glutathione buffers at two different redox conditions, 10 mM or 1 mM GSH and 1 mM or 10 mM GSSG, respectively. Thus, this redox range will span what was used for IGF-I, but with only two different conditions (GSH/GSSG 10/1 and 1/10, respectively). The disulfide exchange reactions were trapped with VP similar to the conditions used for IGF-I [14]. The different disulfide species were separated by RP-HPLC (Fig. 5). The materials in different peaks were collected and analyzed with mass spectrometry, UV absorbance spectroscopy and fluorescence (excitation at 280 nm and emission at 305 nm). The masses and the ratio of the integrated absorbance at 254 nm and the integrated fluorescence revealed the possible number of covalently bound pyridylethyl groups. Based on these two results, the two predominant peaks in 10 mM GSH and 1 mM GSSG are a three-disulfide species (A_1) and a one-disulfide species (A_2). When refolded at 1 mM GSH and 10 mM GSSG, we found one IGF-II variant with two disulfides and two cysteines with glutathione covalently bound (B_1), and two

three-disulfide species (B_2 and B_3). Even though detailed analysis of the different IGF-II forms in the mixtures should be corroborated with detailed disulfide mapping, these result implies that IGF-II possesses a similar thermodynamic folding problem as was previously reported for IGF-I [14].

3.5. Disulfide exchange of BPTI

Native and reduced BPTI were incubated in GSH/GSSG redox buffers and subsequently alkylated with VP. In both redox conditions studied (10 mM or 1 mM GSH and 1 mM or 10 mM GSSG, respectively) starting from either oxidized or reduced BPTI, after pyridylethylation using VP, we found only one major peak in the HPLC analysis (Fig. 5). The retention time, mass and the absorbance at 254 nm were identical to what was found for the native three-disulfide form of BPTI. Thus, as predicted from other reports [36], BPTI is able to refold and maintain its native disulfide bonds in the redox range tested and under the conditions used.

4. Discussion

In this paper we have analyzed disulfide formation of IGF-I in different glutathione redox conditions (Table 1). A broad range of different glutathione redox conditions has been tried, including conditions similar to those reported for the secretory pathway of eukaryotic cells [22]. Since IGF-I theoretically has 75 possible disulfide conformations, there are several unknown equilibrium constants to consider. Therefore it is hard to predict the final distribution of different IGF-I variants, based on the limited number of previously identified equilibrium constants for the formation of individual disulfides. Similar components and amounts were found at equilibrium under each redox condition starting from either reduced, mismatch or native IGF-I demonstrating that an equilibrium had been established. From the experiments we conclude that IGF-I is unable to quantitatively maintain its native three-disulfide conformation under all conditions tried. The predominant variants of IGF-I under the conditions tested were isolated and most of these were identified (Table 2). In the redox range tested, native IGF-I increased from 22% to 51% and mismatch from 7% to 27% (Table 1), going to more oxidizing conditions. Interestingly, the relative proportions of native to mismatch IGF-I are essentially constant through the redox range; the relative free energies between these two forms are kept in a rather narrow range (0.4–0.7 kcal/mol) (Table 1). Comparison of the amounts of different disulfide variants of IGF-I shows that the similar thermodynamic folding energies probably are due to a similar structural folding core. IGF-I is a rather flexible molecule and the hydrophobic core that stabilizes the three-dimensional structure is small [4,37]. By making disulfide mutant proteins and studying their structure it has been possible to conclude that IGF-I is dependent on, at least, the 6–48 and 47–52 disulfide bonds to keep the structure of the native molecule [20,37,38]. The structural content of the different disulfide mutants and those lacking disulfides has been compared by circular dichroism. From these studies one can conclude that all disulfide variants have at least partly ordered structure [14,20,37,38]. Also, analyses of the affinity constants of the mutated molecules towards the IGF-I receptor and the IGF-BP-1 suggest partly native-like structures [20,38]. Through NMR studies on an alanine model of IGF-I lacking the 47–52 disulfide, Hua et al. [37] have demonstrated that the removal of one disulfide is associated with a local unfolding of the second α-helix. In the native structure this helix is connected to the hydrophobic core by the 47–52 disulfide. These results indicate that the small folding core, similar for the different folding species, is located around the 18–61 disulfide.

Redox equilibria were established for IGF-II in two different redox conditions (Fig. 5). The results demonstrate that the three native disulfide bonds are not quantitatively formed in IGF-II under the conditions used, suggesting that also IGF-II is unable to quantitatively form and maintain its native disulfides. This result suggests that IGF-II may utilize IGFBP to form its native disulfides in vivo, as has been demonstrated for IGF-I in vitro [21]. In contrast, as expected from the known equilibrium constants of the individual disulfides of BPTI [36], the BPTI molecule maintains its native disulfides in both tested redox potentials (Fig. 5).

Thus, the disulfide equilibrium studies under the different redox conditions utilized in this paper suggest that IGF-I and IGF-II cannot by themselves *form* or *maintain* their disulfides in vivo. The obvious question is how IGF-I and IGF-II overcome this problem. We have previously shown that the IGF-I precursor, IGF-I-E_a, has similar disulfide exchange folding properties as mature IGF-I and is therefore unlikely to play any role in shifting the disulfide exchange equilibrium properties of the growth factor during folding [21]. As demonstrated in this paper, the protein PDI, which is responsible for the disulfide bond formation in the lumen of the ER in eukaryotic cells, seems to act as a true catalyst with very little effect on the disulfide exchange equilibrium of IGF-I under the conditions used (Fig. 4). Also when equimolar amounts of the disulfide isomerase are used the distribution of different IGF-I variants is rather unchanged. Accordingly, Joly and Swartz showed [39] that DsbA, a disulfide isomerase from *E. coli*, is able to catalyze disulfide shuffling of IGF-I, but the ratio of native to mismatch seemed to be unaffected. It has previously been shown in vitro that IGFBP could aid in the formation of native disulfides of IGF-I through binding only the native IGF-I molecule [21]. Thus, the previously presented IGFBP model for how native disulfides might be formed and maintained in vivo is consistent with data presented in this paper. In this mechanism, IGF-I folds into a native state assisted by binding to the IGFBP, at the precursor level (IGF-I-E) or in the form of the mature IGF-I polypeptide. In fact, IGFBP has been demonstrated to assist in the folding of both IGF-I and IGF-I-E in vitro [21].

Our model for a folding switch for inactivation of IGF-I/IGF-II has not been demonstrated in vivo. While the redox conditions used are similar to the in vivo situation, one might suspect that the alkaline pH (8.7) could create in vitro artifacts. We have repeated the equilibrium disulfide exchange reactions at neutral pH in the presence and absence of PDI. The only difference is the rate at which equilibrium is reached, up to 24 h. Using such long incubations would require strict anaerobic conditions, in practice impossible to obtain. We chose to continue the experiments at pH 8.7 and conclude that the properties now established for IGF-I most likely are significant for its folding in vivo.

The similar three-dimensional structure of insulin and the insulin-like molecules (IGF-I and IGF-II) raises the question why the folding information is specified in the insulin case but not in the cases of IGF-I and IGF-II. Even though the native

fold for insulin and the insulin-like molecules is very similar and also the amino acid sequence, there are significant differences in their disulfide exchange folding behavior. This is, however, not very surprising because small changes in primary structure might cause large changes in the overall fold, since the relative importance of different amino acids varies. Hua et al. claim that the molecules' relative stabilities are due to variabilities in the side chains of the A and B domains of the molecules [40]. Also, Dalal et al. have shown that changing less than 50% of the sequence identity of a former β-sheet protein gives a four-helix bundle conformation [41]. We propose that the difference in these molecule foldabilities are of crucial evolutionary importance. Hence, the rationale behind this IGFBP folding mechanism is probably to construct a very reliable switch so that IGF-I and -II lose their three-dimensional structures as well as biological activities in the absence of IGFBP and thereafter are rapidly cleared. This hypothesis is corroborated by the demonstrated inability of IGF-I and IGF-II to maintain their native three-disulfide conformation in the broad redox range analyzed in this report, covering redox ranges found both in the secretory machinery [22] and in the circulation [23]. Why would IGF-I and IGF-II evolve such molecular switches? IGF-I and IGF-II bind two orders of magnitudes more weakly than insulin to the insulin receptor, but the concentrations of IGF-I and IGF-II are rather high in serum. In adult man, normal concentrations of IGF-I are about 200 ng/ml and of IGF-II 700 ng/ml, about 1000 times that of insulin [2]. In the absence of IGFBP, IGF-I and IGF-II would cause a hypoglycemic response [8,10]. Therefore, a dependence on IGFBP to form *and* maintain the disulfides of IGF-I and IGF-II could guarantee a rapid clearance in the absence of IGFBP. Thus, a hypoglycemic reaction would be minimized if the normally tightly regulated IGFBP levels in circulation or locally would decrease under the IGF levels. The disulfide exchange folding switch proposed in this paper could be general, and could be found also in other molecular systems. Carrier proteins are found for many growth factors, including growth hormone and epidermal growth factor [42]. It is possible that a folding dependence on the carrier protein is relevant also for some of these other growth factors in addition to the IGF-I/IGF-II system.

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