Mutations in the B-domain of insulin-like growth factor-I influence the oxidative folding to yield products with modified biological properties

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The oxidative folding of human insulin-like growth factor (IGF)-I yields two major disulphide folding isomers. In the present study, B-domain analogues of IGF-I were used to investigate the effect of mutations on the folding reaction and to investigate the functional implications of misfolding. The analogues used were substitutions of the native Glu³ by Gly or Arg, or the native Glu⁹ by Lys. IGF-I and these analogues were also prepared attached to a hydrophobic 13-amino-acid N-terminal extension, Met-Phe-Pro-Ala-Met-Pro-Leu-Ser-Ser-Leu-Phe-Val-Asn, referred to as 'Long-IGF-I' analogues. Each IGF was fully reduced and refolded to yield native and misfolded isomers, which were subsequently purified for biological characterization. Analysis of the folding reaction at equilibrium revealed a distribution of folding isomers characteristic for each peptide. The yield of the native disulphide folding isomer was increased for the Glu³ substitutions, but not for the Glu⁹ substitution. The main

alternative folding isomer was present in the IGF-I analogues in reduced proportions. Except for [Gly³]IGF-I the N-terminal extension increased the yield of the native isomer which was maximal for the analogue Long-[Arg³]IGF-I. A folding intermediate for the latter analogue was isolated and partially characterized. The biological assays showed that all the main alternative isomers bound poorly to IGF-binding proteins (IGFBPs) secreted by L6 myoblasts. Moreover, these isomers bound to the type 1 IGF receptor with 0.5–25% the affinity of the native isomer. In a rat L6 myoblast protein-synthesis assay, the observed biological activity of the native and main alternative isomers was explained by their modified IGFBP- and receptor-binding properties. We propose that the N-terminal extension imparts a steric constraint at a crucial point in folding, thus allowing native disulphide bonds to form efficiently.

INTRODUCTION

Human insulin-like growth factor (IGF)-I is a basic globular polypeptide of 70 amino acids, containing six cysteine residues that form three disulphide bonds. The three-dimensional structure of IGF-I has been modelled on the X-ray-crystallographic structure of insulin [1], and recently the tertiary conformation has been refined by NMR [2–4]. The native disulphide arrangement observed with IGF-I has the following disulphide linkages: Cys⁶–Cys⁴⁸, Cys¹⁸–Cys⁶¹ and Cys⁴⁷–Cys⁵² [5,6]. The oxidative folding of IGF-I *in vitro* appears to follow a pathway governed by thermodynamic rather than kinetic principles, with a late bifurcation resulting in two folding isomers of IGF-I with similar thermodynamic stabilities [7,8]. The two main products present at equilibrium are those of the native disulphide arrangement and an alternative disulphide arrangement of Cys⁶–Cys⁴⁷, Cys¹⁸–Cys⁶¹ and Cys⁴⁸–Cys⁵²; other minor products are stable intermediates of the folding pathway [7]. Thus it has been concluded that IGF-I contains insufficient information in the amino acid sequence to determine uniquely the native globular structure [8].

In the development of an efficient expression system for analogues of IGF-I with novel biological properties, we observed that a 13-amino-acid N-terminal fusion protein partner facilitated downstream refolding [9]. Thus, when a fusion protein analogue of IGF-I with a change in the B-domain (Met-Phe-Pro-Ala-Met-Pro-Leu-Ser-Ser-Leu-Phe-Val-Asn [Arg³]IGF-I) was refolded

from the fully reduced form, most of the protein was correctly folded [9]. This folding outcome was significantly different from that published for IGF-I refolding [10]. However, it was unclear whether the 13-amino-acid N-terminal fusion protein partner or the Arg substitution at Glu³, or both, were responsible for the favourable folding outcome. To examine this phenomenon further, we have investigated the effect of the presence of the 13-amino-acid N-terminal extension and three IGF-I analogues selected to evaluate the influence of charge at two different positions in the B-domain on the folding of IGF-I.

MATERIALS AND METHODS

Materials

Receptor-grade recombinant IGF-I, [Arg³]IGF-I and [Met¹]pGH(1–11)-Val-Asn-[Arg³]IGF-I (Long-[Arg³]IGF-I) were supplied by GroPep Pty., Adelaide, S.A., Australia. [Gly³]IGF-I, [Met¹]pGH(1–11)-Val-Asn-IGF-I (Long-IGF-I) and [Met¹]pGH(1–11)-Val-Asn-[Gly³]IGF-I (Long-[Gly³]IGF-I) were prepared as described previously [9,11]. Inclusion bodies containing the fusion protein [Met¹]pGH(1–11)-Val-Asn-[Lys⁹]IGF-I (Long-[Lys⁹]IGF-I) were produced essentially as described for similar fusion proteins [9]. Carrier-free Na¹²⁵I and L-[4,5-³H]Leucine were obtained from Amersham Australia Pty., Sydney, N.S.W., Australia. IGF-I was iodinated to specific radioactivities of 30–40 Ci/g with Chloramine-T [12]. Foetal-

Abbreviations used: IGF, insulin-like growth factor; IGFBP, IGF-binding protein; pGH, porcine growth hormone; Long-IGF-I, [Met¹]pGH(1–11)-Val-Asn-IGF-I; Long-[Gly³]IGF-I, [Met¹]pGH(1–11)-Val-Asn-[Gly³]IGF-I; Long-[Arg³]IGF-I, [Met¹]pGH(1–11)-Val-Asn-[Arg³]IGF-I; Long-[Lys⁹]IGF-I, [Met¹]pGH(1–11)-Val-Asn-[Lys⁹]IGF-I.

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bovine serum was obtained from Cytosystems Pty., Castle Hill, N.S.W., Australia. All other reagents were of analytical grade.

Preparation of Long-[Lys⁹]IGF-I and [Lys⁹]IGF-I

The Long-[Lys⁹]IGF-I inclusion bodies were solubilized and refolded to the biologically active form as described previously [9]. This process yielded 35 mg of biologically active Long-[Lys⁹]IGF-I in the final reverse-phase HPLC purification step from 3 g of inclusion bodies. [Lys⁹]IGF-I was prepared by hydroxylamine cleavage of the Asn-Gly bond of Long-[Lys⁹]IGF-I by a procedure employed to minimize hydroxyamination of susceptible Asn and Gln residues [13]. Briefly, 20 mg of Long-[Lys⁹]IGF-I was dissolved in 10 ml of 10 mM HCl, diluted to 0.25 mg/ml with a cleavage buffer containing 1 M hydroxylamine, 0.1 M Tris and 1 mM EDTA, pH 8.65, and incubated at 37 °C for 24 h under nitrogen. The cleavage reaction was stopped by lowering the pH to 6.0 using acetic acid, with cooling. The cleavage products were subsequently purified to homogeneity by two preparative reverse-phase HPLC steps, essentially as described previously [11]. The reaction yielded 5 mg of purified [Lys⁹]IGF-I in the final reverse-phase HPLC step.

Purity of IGF-I analogues

Each IGF-I analogue was analysed on a microbore C₄ reverse-phase HPLC column (2.1 mm diameter × 10 cm long; Brownlee Laboratories, Santa Clara, CA, U.S.A.) using a gradient of 25–40% (v/v) acetonitrile over 150 min in the presence of 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.5 ml/min. Purities were confirmed by N-terminal-sequence analysis and electrospray MS [14].

Oxidative folding of IGF-I analogues

Each purified IGF-I analogue was reduced for 60 min at 22 °C, at a protein concentration of 4 mg/ml, in a 8 M urea buffer containing 40 mM glycine, 0.1 M Tris, 1 mM EDTA and 16 mM dithiothreitol, pH 8.7. The solution of reduced peptide was then diluted to 0.1 mg of protein/ml, 2 M urea, 10 mM glycine, 0.1 M Tris, 1 mM EDTA and 0.4 mM dithiothreitol at 22 °C prior to the addition of 2-hydroxyethyl disulphide (1 mM), to initiate the oxidative folding reaction. The progress of the reaction was monitored, and the folding intermediates stabilized by removing samples at various times and acidifying them to pH 2.1 with trifluoroacetic acid [15]. Analysis was carried out by microbore C₄ reverse-phase HPLC employing a gradient of 20–45% (v/v) acetonitrile over 25 min in the presence of 0.1% (v/v) trifluoroacetic acid, pH 2.1, at a flow rate of 0.5 ml/min. The reverse-phase HPLC elution profiles were used to calculate the percentage abundance of folding products at each time. In all the refolding reactions quantitative yields were recorded, and there was no evidence of aggregation of peptide. For preparation of large quantities of folding isomers, the folding reaction was stopped after 180 min by acidification to pH 2.1 with concentrated HCl. The refolding isomers were then purified by one or two preparative reverse-phase HPLC steps, employing a C₄ PrepPak column (25 mm diameter × 10 cm long; Millipore-Waters, Milford, MA, U.S.A.) and a linear 0.1% (v/v) acetonitrile/min gradient in the presence of 0.1% (v/v) trifluoroacetic acid.

The purified folding isomers were quantified by calculating the area under the peak of analytical microbore C₄ reverse-phase HPLC absorbance (A_{214}) profiles and comparing it with that of a reference Long-[Arg³]IGF-I preparation. The homogeneity of the folding isomer preparations was established by analysis on

the microbore C₄ reverse-phase HPLC system but this time employing a linear gradient of 25–40% (v/v) acetonitrile over 150 min in the presence of 0.1% (v/v) trifluoroacetic acid.

Purification and alkylation of Long-[Arg³]IGF-I intermediate

Long-[Arg³]IGF-I was reduced and refolded as described in the preceding section. However, after 1.5 min of refolding, the reaction was stopped by acidification and the resultant mixture purified by reverse-phase HPLC using a 25 mm diameter × 10 cm long C₄ PrepPak column and a linear 0.1% (v/v) acetonitrile/min gradient in the presence of 0.1% (v/v) trifluoroacetic acid. The fractions containing the major intermediate were dried under vacuum. The intermediate was subsequently alkylated [15]. Briefly, 500 µl of a solution containing 500 mM sodium iodoacetate, pH 8.7, was added to 50 µg of the Long-[Arg³]IGF-I intermediate and mixed for 30 s. The mixture was immediately desalted by injection on to a C₄ reverse-phase HPLC column previously equilibrated with 0.1% (v/v) trifluoroacetic acid. The bound peptide was eluted using a linear 0.1% (v/v) acetonitrile/min gradient in the presence of 0.1% (v/v) trifluoroacetic acid. The fractions containing the single peak were dried and analysed by electrospray MS [14].

Measurement of binding to IGF-BPs

L6 myoblast IGF-BPs were prepared from serum-free Dulbecco's Minimal Essential Medium conditioned by confluent monolayers of cells for 24 h. The medium was filtered through a 0.22 µm filter and stored at –20 °C until used. The binding of various IGF analogues and isomers (100 pg–2000 ng) to IGF-BPs was measured by their competition for the binding of 0.2 ng of ¹²⁵I-labelled IGF-I. The peptides were incubated for 18 h at 4 °C in phosphate buffer, pH 6.5, containing 0.1% (w/v) BSA. Binding was expressed as the percentage of ¹²⁵I-labelled-IGF-I bound in the absence of added unlabelled IGF peptide [16]. The concentration of added peptide giving 20% displacement of labelled ligand is defined as the ED₂₀ value.

Binding of IGF peptides to cell receptors

The relative binding of various IGF analogues and folding isomers (50 pg–1000 ng) to confluent monolayers of rat L6 myoblasts was measured by their competition for the binding of 0.2 ng of ¹²⁵I-labelled IGF-I [17]. The monolayers were incubated for 18 h at 4 °C in a total volume of 0.5 ml in 0.1 M Hepes buffer, pH 7.6, containing 0.5% (w/v) BSA. Binding was expressed as the percentage of ¹²⁵I-labelled-IGF-I bound in the absence of added unlabelled IGF peptide. The ED₅₀ values were calculated as the concentration of added peptide giving 50% displacement of labelled ligand.

Effect of IGF peptides on protein synthesis

Stimulation of protein synthesis in rat L6 myoblasts by IGF peptides was measured as described previously [18]. Briefly, stimulation of protein synthesis was measured as the increased incorporation of L-[4,5-³H]leucine into total cell protein over an 18 h incubation period, at pH 7.4 and at 37 °C, compared with a buffer control. The biological potencies were calculated as the amount of added peptide required to give 50% of the maximum stimulation achieved by IGF-I.

Stability of Long-[Arg³]IGF-I in the receptor-binding assay

A 5 µg portion of the main alternative folding isomer of Long-[Arg³]IGF-I was added, in 0.1 M Hepes buffer, pH 7.6, and in

the presence of 0.5% (w/v) BSA, to confluent monolayers of rat L6 myoblasts under the assay conditions described for binding of IGF peptides to cell receptors. After incubation for 18 h at 4 °C, the medium was removed and centrifuged at 10000 *g* for 3 min; 400 μ l was removed, acidified with 100 μ l of 5% (v/v) trifluoroacetic acid and centrifuged again. Portions of this sample were either mixed with an equivalent amount of receptor-binding activity of the correctly folded isomer of Long-[Arg³]IGF-I in 10 mM HCl, or diluted with 10 mM HCl. The treated samples were analysed by C₄ analytical reverse-phase HPLC employing a linear gradient of 28–40% (v/v) acetonitrile over 60 min in the presence of 0.1% (v/v) trifluoroacetic acid. Fractions were collected for the analysis of receptor-binding activity. The elution times of the alternative and native folding isomers of Long-[Arg³]IGF-I from the C₄ column were confirmed by mixing control incubation samples that contained no IGFs with the appropriate folding isomers of Long-[Arg³]IGF-I.

Analysis of data from biological assays

Curves were drawn for the biological assays using a computer-fitted logistic dose-response function employing TableCurve version 3.1 software (Jandel Scientific, Corte Madera, CA, U.S.A.). This logistic dose-response function was used to calculate biological-potency data in each assay.

RESULTS

To investigate the role of the 13-amino-acid N-terminal extension and the charge substitution at Glu³ on the refolding of Long-[Arg³]IGF-I, we compared the folding properties of Long-IGF-I and [Arg³]IGF-I with those of Long-[Arg³]IGF-I and the native sequence IGF-I (Figure 1). To examine further the contribution of charge at position 3 of IGF-I, we studied the folding properties of the neutral-amino-acid substitution, [Gly³]IGF-I, and its 'Long' counterpart, Long-[Gly³]IGF-I. Another analogue of IGF-I was constructed containing both the N-terminal extension and a charge reversal, but at a position in the B-domain other

Figure 1 Analogues of IGF-I

The analogues of IGF-I are substitutions of Glu (E) at position 3 by Gly (G) or Arg (R) and at position 9 by Lys (K) in the B-domain, either separated from, or still attached to, the 13-amino-acid N-terminal extension. The primary structure of IGF-I is shown using standard single-letter codes with the native disulphide assignment (—) and with a mismatched disulphide arrangement involving Cys⁶, Cys⁴⁷, Cys⁴⁸ and Cys⁵² that corresponds to the main alternative folding isomer (•••).

Figure 2 Purity of folding isomers

The purified alternative (A) and native (N) folding isomers of IGF-I and Long-[Arg³]IGF-I were assessed by microbore C₄ reverse-phase HPLC. Proteins were eluted with a gradient of 25–40% (v/v) acetonitrile over 150 min in the presence of 0.1% (v/v) trifluoroacetic acid and were monitored as A₂₁₄. The chromatograms of the separate injections are overlapped to indicate the relative retention times of the alternative and native isomers for each analogue.

than that of Glu³. A charge substitution of Lys for Glu⁹, near the start of helix I (IGF-I residues 8–18), was chosen. Lys was used instead of Arg because of the similar packing volume to Glu {0.171 nm³ (171 Å³) versus 0.155 nm³ (155 Å³); [19]} compared with Arg [0.202 nm³ (202 Å³)]. Since such a charge reversal in the helix is potentially disruptive and may have a detrimental effect on refolding, we therefore investigated the possibility that the N-terminal extension could also modulate the refolding of this analogue.

The IGF-I and IGF-I analogues (Figure 1) used in the refolding experiments and the folding isomers used in the biological experiments were characterized by reverse-phase HPLC, as demonstrated in Figure 2 for IGF-I and Long-[Arg³]IGF-I. The identity of each peptide was confirmed by N-terminal-sequence analysis and electrospray MS (results not shown).

The IGFs were fully reduced and then refolded to investigate the effect of charge in the B-domain and the presence of the N-terminal extension on the refolding outcome. For each IGF the most abundant product of folding, which also exhibits the greatest biological potency, is referred to as the 'native' isomer; the most abundant non-native folding product is referred to as the 'alternative' isomer. Within 1.25 min of initiating refolding, reduced IGF-I formed numerous folding intermediates (Figure 3a). These intermediates rearranged with time and, after 10 min, the native and alternative isomers were present in similar proportions to those observed at 120 min. Similar analysis with Long-[Arg³]IGF-I revealed a pathway different from that for IGF-I, in that the early products of folding were dominated by an intermediate that constituted up to 45% of the total products (Figure 3b). The rapid appearance of this intermediate was followed by a decline and the emergence of the native and alternative folding isomers of Long-[Arg³]IGF-I (see the inset to Figure 3b). The final equilibrium distribution of Long-[Arg³]IGF-I was reached after approx. 100 min. Analysis of the refolding of other analogues of IGF-I revealed folding characteristics intermediate between those of IGF-I and Long-[Arg³]IGF-I (results not shown).

The most abundant intermediate of Long-[Arg³]IGF-I (Figure 3b) was acid-trapped, purified by reverse-phase HPLC and alkylated with sodium iodoacetate for 30 s at pH 8.7.

Figure 3 Analysis of the refolding reactions of IGF-I and Long-[Arg³]IGF-I

The oxidative refolding reactions of reduced IGF-I (a) and Long-[Arg³]IGF-I (b) were monitored by acid-quenching samples with time and analysis on microbore C₄ reverse-phase HPLC. Proteins were eluted with a gradient of 20–45% (v/v) acetonitrile over 25 min in the presence of 0.1% (v/v) trifluoroacetic acid and were monitored as A₂₁₄. The elution profiles were analysed to calculate the percentage abundance of folding isomers and intermediates with time (insets). Abbreviations: R, reduced protein; N, native folding isomer; A, alternative folding isomer; and I, intermediate.

Table 1 Folding patterns at equilibrium

The percentage abundance of the folding isomers at equilibrium for each of the IGF-I analogues was calculated from the reverse-phase HPLC profiles for two separate folding experiments.

Analogue	Abundance of folding isomers at equilibrium (%)		
	Alternative	Native	Others
IGF-I	24, 24	45, 45	31, 31
[Gly ³]IGF-I	22, 22	64, 67	14, 11
[Arg ³]IGF-I	10, 12	75, 74	15, 14
[Lys ⁹]IGF-I	13, 14	35, 38	52, 48
Long-IGF-I	23, 27	62, 65	15, 8
Long-[Gly ³]IGF-I	23, 26	58, 57	19, 17
Long-[Arg ³]IGF-I	8, 10	80, 79	12, 11
Long-[Lys ⁹]IGF-I	15, 17	73, 72	12, 11

Table 2 Binding properties of IGF isomers in rat L6 myoblasts

The binding of IGF isomers to rat L6 myoblast IGF-BPs in conditioned medium and to the type 1 IGF receptor were expressed respectively as ED₂₀ and ED₅₀ values (ng/ml).

	Binding to IGF-BPs (ED ₂₀)		Binding to the type 1 IGF receptor (ED ₅₀)	
	Native	Alternative	Native	Alternative
IGF-I	0.3	79	6.8	1200
[Gly ³]IGF-I	1.0	430	5.3	610
[Arg ³]IGF-I	5.1	1050	5.4	610
[Lys ⁹]IGF-I	0.5	340	39	> 2000
Long-IGF-I	4.2	93	9.8	300
Long-[Gly ³]IGF-I	21	> 2000	10	890
Long-[Arg ³]IGF-I	190	460	17	65
Long-[Lys ⁹]IGF-I	6.8	56	31	350

Electrospray MS of the resultant modified peptide showed the major component to have a mass of 9231.2 ± 0.9 . Native Long-[Arg³]IGF-I has a mass of 9111.7, indicating that the modified peptide has a mass increase consistent with the presence of two S-carboxymethylcysteine residues (theoretical mass 9229.8).

The folding outcome for each of the IGF-I analogues was compared by analysis of the equilibrium distribution of the isomers after 120 min (Table 1). Substitution of the negatively charged Glu³ by a neutral Gly or a positively charged Arg in the 70-amino-acid protein increased the yield of the native folding isomer from 45 to 66 and 75 % of the folding products respectively (Table 1), reducing the abundance of the alternative folding isomer and the more hydrophobic minor products. A charge reversal at Glu⁹ resulted in an equilibrium distribution where the yield of the native isomer was decreased from 45 to 37 % of the total folding products (Table 1). Analysis of the refolding of the Long-IGF-I analogues showed that the 13-amino-acid N-terminal extension increased the abundance of the native isomer for Long-IGF-I, Long-[Arg³]IGF-I and Long-[Lys⁹]IGF-I to 64, 80 and 73 % of the total folding products respectively. The folding pattern for Long-[Gly³]IGF-I was only slightly altered by the addition of the N-terminal extension peptide (Table 1).

We then studied the effect of changes in tertiary structure (resulting from disulphide-bond isomerization) on biological function in rat L6 myoblasts. The binding of folding isomers of IGF-I and analogues to IGF-BPs secreted by rat L6 myoblasts was measured in a competition binding assay (Table 2). For the

Figure 4 Stimulation of protein synthesis in rat L6 myoblasts by folding isomers of IGF-I analogues

Stimulation of protein synthesis by native (closed symbols) and alternative (open symbols) folding isomers of (a) IGF-I (●, ○), [Gly³]IGF-I (▲, △), [Arg³]IGF-I (▼, ▽) and [Lys⁹]IGF-I (■, □) and (b) Long-IGF-I (●, ○), Long-[Gly³]IGF-I (▲, △), Long-[Arg³]IGF-I (▼, ▽) and Long-[Lys⁹]IGF-I (■, □). Each folding isomer was tested in triplicate at each peptide concentration. The S.E.M. values are indicated by descending bars where they are larger than the symbols.

native folding isomers of the 70-amino-acid peptides the order of potency was: IGF-I > [Lys⁹]IGF-I > [Gly³]IGF-I > [Arg³]IGF-I. The alternative folding isomers of these analogues showed a similar order of potency with a reduction in binding of at least 200-fold (Table 2). The presence of the 13-amino-acid N-terminal extension had the effect of reducing binding to IGF-BPs for the native folding isomers of the Long-IGF-I analogues. The alternative folding isomer of Long-[Arg³]IGF-I had an apparent affinity for L6-conditioned-medium IGF-BPs similar to that of the native folding form. However, the alternative folding isomers of the other 83-amino-acid analogues exhibited much lower binding potencies compared with their native isomer forms (Table 2).

Binding of the folding isomers of the IGF-I analogues to the type 1 IGF receptor on L6 myoblasts was measured in a competition binding assay. Each of the alternative folding isomers competed with ¹²⁵I-IGF-I for binding to the type 1 IGF receptor less well than the corresponding native folding isomer (Table 2). For the native 70-amino-acid IGF-I analogues the order of potency was: [Gly³]IGF-I ≥ [Arg³]IGF-I > IGF-I > [Lys⁹]IGF-I. This order of potency was similar for the alternative folding isomers, but with a reduction in binding of at least 100-fold (Table 2). Except for Long-[Lys⁹]IGF-I, the presence of the N-terminal extension reduced the binding of the native folding isomers to the type 1 IGF receptor. The alternative folding

Figure 5 Stability of the alternative isomer of Long-[Arg³]IGF-I in the type 1 receptor-binding assay

The alternative folding isomer of Long-[Arg³]IGF-I was incubated for 18 h in the presence of rat L6 myoblasts under the conditions used for the receptor-binding assay. The mixture was then separated by C₄ reverse-phase HPLC using a 28–40% (v/v) acetonitrile gradient over 60 min in the presence of 0.1% (v/v) trifluoroacetic acid. Fractions were analysed for receptor-binding activity (○). A second gradient separation was carried out with the native isomer included as an internal standard (●).

isomer of Long-[Arg³]IGF-I had one quarter the potency of the native folding isomer, whereas the alternative folding isomers of Long-IGF-I, Long-[Gly³]IGF-I and Long-[Lys⁹]IGF-I had only 3, 1 and 9% of the binding potency compared with the respective native folding isomers.

We have determined the effect of altered IGFBP and type 1 IGF receptor binding on the growth-factor activity of the various native and alternative folding isomers (Figure 4). In L6 myoblasts, all folding isomers stimulated protein synthesis substantially, and, with the exception of the alternative folding isomers of IGF-I and [Lys⁹]IGF-I, achieved responses nearly equivalent to those observed with 10% (v/v) foetal-bovine serum. Higher concentrations of all alternative folding isomers were required to stimulate protein synthesis compared with the respective native folding isomer (Figure 4). For the 70-amino-acid IGF-I analogues the order of potency with concentrations giving 50% of the maximum stimulation achieved by IGF-I was: [Arg³]IGF-I (1.9 ng/ml) > [Gly³]IGF-I (2.5 ng/ml) > IGF-I (14 ng/ml) > [Lys⁹]IGF-I (24 ng/ml). The order of potency for the alternative isomers was the same, but potencies were only 1–2% of those of the native isomers (Figure 4a). Similar potency concentrations of the native isomers of the 83-amino-acid analogues were: Long-[Gly³]IGF-I (3.5 ng/ml) > Long-[Arg³]IGF-I (4.2 ng/ml) > Long-IGF-I (14 ng/ml) > Long-[Lys⁹]IGF-I (15 ng/ml). The alternative folding forms of these Long isomers exhibited a wider range of potency differences than observed with the 70-amino-acid peptides. In particular, the alternative folding isomer of Long-[Arg³]IGF-I was only moderately less potent than the native isomer and was as potent as the native folding isomers of Long-IGF-I and Long-[Lys⁹]IGF-I (Figure 4b).

As the potency of alternative folding isomer Long-[Arg³]IGF-I is only slightly less than that of the native isomer, we considered the possibility that the alternative folding isomer rearranges to the native isomer during the assay. However, this is unlikely, since no native Long-[Arg³]IGF-I could be detected after incubation of the pure alternative folding isomer under the conditions of the receptor-binding assay (Figure 5). This observation is also relevant for the IGFBP-binding assay, where the

lower pH would result in an even lower rate of disulphide rearrangement.

DISCUSSION

Oxidative folding of IGF-I and IGF analogues

The oxidative folding of human IGF-I *in vitro* yields two major products of similar thermodynamic stability, the native isomer and an alternative isomer with two mismatched disulphide bonds [8]. Thus it is apparent that the amino acid sequence of IGF-I does not contain sufficient information to determine uniquely the native globular structure in accord with the classical Anfinsen concept [20]. The proportion of native and misfolded isomers present in the final equilibrium of IGF-I reported here is similar to that achieved when IGF-I is refolded by other techniques [7,10,21,22]. By contrast, the presence of the 13-amino-acid N-terminal extension and a charge reversal at Glu³ in Long-[Arg³]IGF-I affects the folding pathway and the folding outcome (Figure 3). In this case the equilibrium products of Long-[Arg³]IGF-I folding appeared via a fast-forming intermediate containing two disulphide bonds and two free thiols rather than via a multitude of minor folding forms, as observed with IGF-I. A key point is that the changes in the amino acid sequence of Long-[Arg³]IGF-I appear to have the effect of reducing the number of folding options to give a single predominant intermediate. A second point is that the increased time to reach equilibrium by Long-[Arg³]IGF-I compared with IGF-I suggests that the rate-limiting step involves this intermediate in the folding pathway. While it is possible that the Long-[Arg³]IGF-I intermediate contains non-native disulphide bonds, we speculate that it contains a native Cys⁶–Cys¹⁸ disulphide bond formed early in folding, as has been reported for IGF-I [7], as well as another native disulphide bond which guides folding to the correct three-disulphide product.

Although the analysis reported here reveals only part of the folding process, it is clear that modification of the B-domain affects the folding outcome, a situation seen in the equilibrium distribution of folding products for the IGF-I analogues. Moreover, the relative thermodynamic stability of the folding isomers is indicated by the composition of the equilibrium folding mixture [7]. For the charge substitutions at Glu³ in IGF-I the alternative folding isomer and the other minor hydrophobic products of folding are destabilized, leading to preferential formation of the native folding isomer. A different situation is seen for the charge reversal at Glu⁹ in helix 1 of the IGF-I molecule, where the abundance of the native and alternative isomers is decreased. Presumably this amino acid substitution results in destabilization of the helix structure or affects charge interactions with other regions of the IGF-I molecule. The 13-amino-acid extension appears to confer stability to Long-IGF-I, Long-[Arg³]IGF-I and Long-[Lys⁹]IGF-I, resulting in an increased predominance of the native folding form at equilibrium. This effect is most striking for Long-[Lys⁹]IGF-I, where the N-terminal extension overcomes the disruptive effect of the charge reversal near the beginning of helix 1, increasing the yield of the native isomer (Table 1).

The different proportions of native and alternative isomers in the Glu³-substituted IGF-I analogues may perhaps be explained by structural observations based on the solution structure of native IGF-I [2] in which the amino acids Glu³ and Arg⁵⁶ are spatially close to one another [approx. 0.45 nm (4.5 Å)], but too far away to form a salt bridge [1]. However, it is conceivable that these two amino acids form a salt bridge in the alternative folded form of IGF-I, stabilizing the folding isomer. If this salt bridge exists in the alternative isomer of IGF-I, then one would expect

that a charge substitution of Glu³ by a neutral Gly would remove the salt bridge, thus reducing its stability and hence its abundance. Moreover, substitution by Arg would cause charge repulsion, further reducing the stability. Similarly the presence of the hydrophobic 13-amino-acid extension may prevent formation of the proposed salt bridge as a result of steric hindrance.

Thus the artificial 13-amino acid N-terminal extension reported here facilitates correct folding of IGF-I and IGF-I analogues, probably by imparting a steric constraint in the refolding reaction. We hypothesize that this constraint prevents the contact of regions, such as Glu³ and Arg⁵⁶ in IGF-I, that would otherwise draw the folding pathway into stable non-native disulphide arrangements at the time the second disulphide bond is formed. As indicated above, the N-terminal extension also exerts an influence over other disruptive forces, as in the [Lys⁹]IGF-I analogue. The present study confirms that the amino acid sequence of IGF-I contains insufficient information to determine uniquely the native globular structure. However, we propose that when part of the B-domain is prevented from making contact with the rest of the molecule at a crucial point in folding, native disulphide bonds are formed in high yields. This concept is reinforced by the observation that the C-terminal E-peptide extension of pro-IGF-I does not affect the folding outcome in a similar fashion [23]. While undefined accessory proteins may be required to assist the folding of IGFs *in vivo*, it is also possible that the N-terminal signal sequence of pre-pro-IGF-I guides the folding of the emerging nascent polypeptide chain in the endoplasmic reticulum. Hence the hydrophobic signal presequence may function in an analogous way to the hydrophobic artificial N-terminal extension investigated in the present study.

Biological properties of folding isomers

In correctly folded IGF-I, the important residues involved in binding to IGFBPs are Glu³ [24], Phe⁴⁹-Arg⁵⁰-Ser⁵¹ [25] and the α -helix between residues 8 and 18 of the B-domain [26]. However, IGFBP-4, which is the major form of binding protein secreted by L6 cells [27], requires IGF-I residues Phe⁴⁹-Arg⁵⁰-Ser⁵¹ for optimal binding [28,29]. The observed reduced IGFBP-binding properties of the alternative folding isomers is therefore consistent with a model of misfolded IGF-I [30], which shows modification of the IGF-I structure in the region of residues 47–52.

The alternative folding isomers of IGF-I, [Gly³]IGF-I, [Arg³]IGF-I and [Lys⁹]IGF-I show a parallel reduction in potency compared with the correct folding form in each of the biological assays. This shows that the same conformational changes occur in each of these alternative folding isomers, affecting the same residues on the receptor-binding and the IGFBP-binding faces [2]. However, the 'Long' 83-amino-acid IGF-I analogues do not exhibit this uniform reduction in potency between the alternative

and native isomer forms, suggesting that these alternative isomers differ in their surface topology.

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