Chemical heterogeneity as a result of hydroxylamine cleavage of a fusion protein of human insulin-like growth factor I

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Recombinant DNA techniques were used to biosynthesize human insulin-like growth factor I (hIGF-I) as a fusion protein wherein the fusion polypeptide is an IgG-binding moiety derived from staphylococcal protein A. This fusion protein is produced in *Escherichia coli* and secreted into the fermentation broth. In order to release mature recombinant-derived hIGF-I (rhIGF-I), the fusion protein is treated with hydroxylamine, which cleaves a susceptible Asn–Gly bond that has been engineered into the fusion protein gene. Reversed-phase h.p.l.c. was used to estimate the purity of the rhIGF-I preparations, especially for the quantification of the methionine sulphoxide-containing variant. It was determined that hydroxylamine cleavage of the fusion protein produced, as a side reaction, hydroxamates of the asparagine and glutamine residues in rhIGF-I. Although isoelectric focusing was effective in detecting, and reversed-phase h.p.l.c. for producing enriched fractions of the hydroxamate variants, ion-exchange chromatography was a more definitive procedure, as it allowed quantification and facile removal of these variants. The identity of the variants as hydroxamates was established by *Staphylococcus aureus* V8 proteinase digestion, followed by m.s., as the modification was transparent to amino acid and N-terminal sequence analyses. The biological activity of rhIGF-I was established by its ability to incorporate [³H]thymidine into the DNA of BALB/c373 cells and by a radioreceptor assay utilizing human placental membranes. Both assays demonstrate that the native, recombinant and methionine sulphoxide and hydroxamate IGF-I variants are essentially equipotent.

INTRODUCTION

The peptide somatostatin was among the first peptides to be produced by recombinant DNA-derived technology (Itakura *et al.*, 1977). Initial attempts were unsuccessful, possibly owing to intracellular degradation of the small protein by endogenous proteolytic enzymes. Attachment to a large protein, namely β -galactosidase, provided a stabilizing environment. Moks *et al.* (1987) considered this observation when they designed their large-scale process for human insulin-like growth factor I (hIGF-I). In addition, they incorporated an IgG-binding function into the fusion protein, utilizing a fragment of protein A from *Staphylococcus aureus* (Nilsson *et al.*, 1987) which was useful during purification. Since the N-terminus of human IGF-I is glycine, they engineered an asparagine–glycine peptide bond into the fusion protein as a linker that could be specifically cleaved by hydroxylamine (Bornstein & Balian, 1977).

Previously, methionine (Itakura *et al.*, 1977) and tryptophan (Forsberg *et al.*, 1989) were commonly used as linkers. The former linker can be specifically cleaved with CNBr (Gross & Witkop, 1962) and the latter with N-chlorosuccinimide (Shechter *et al.*, 1976). These linkers were not ideal for the fusion of human IGF-I, since the protein contains a methionine residue that can be cleaved with CNBr and oxidized to the sulphoxide with N-chlorosuccinimide (Shechter *et al.*, 1976).

Forsberg *et al.* (1990) have utilized the above-mentioned fusion system to produce recombinant-derived human IGF-I (rhIGF-I) and have characterized a number of modified variants. They have identified a misfolded form containing presumably mismatched disulphide bonds, an oxidized form with the single

methionine residue at position 59 converted into methionine sulphoxide, a variant in which the methionine was substituted by norleucine during biosynthesis, and a form proteolytically cleaved at the Arg³⁶–Arg³⁷ bond. In addition, we have found a variant produced by the cleaving reagent hydroxylamine. Its detection, isolation and identification are described here.

MATERIALS AND METHODS

Materials

Recombinant hIGF-I was purified from *Escherichia coli* broth which contained the secreted fusion protein (Moks *et al.*, 1987). Recombinant-derived human growth hormone (hGH) and transforming growth factor β_1 (TGF- β_1) are Genentech products. Insulin was purchased from Calbiochem and insulin-like growth factor II (IGF-II) from KabiGen.

Hydroxylamine cleavage reaction

A 1.7-times concentrate of the cleavage buffer (described below) was transferred to a reaction vessel and sparged with nitrogen or argon for 15 min while the solution temperature was increased to 45 °C. The protein solution was added to the vessel and, after adjusting pH and sparging as described above, the cleavage reaction was allowed to proceed for 5–6 h at 45 °C. The final reaction conditions included protein at 6 mg/ml, 2 M-hydroxylamine/HCl, 1 mM-EDTA and 10% (v/v) ethanol in a buffer containing 0.2 M-Tris, pH 9.2. After cleavage had been performed, the solution was cooled to 25 °C and adjusted to pH 6.0 by the addition of acetic acid. The cleavage buffer was separated immediately from the protein using a G-25 Sephadex

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; f.a.b.-m.s., fast-atom-bombardment m.s.; hGH, human growth hormone; hIGF-I, human insulin-like growth factor I; i.e.f., isoelectric focusing; PEI, polyethyleneimine; rhIGF-I, recombinant-derived human insulin-like growth factor I; TGF- β_1 , transforming growth factor β_1 ; TFA, trifluoroacetic acid; IGF-II, insulin-like growth factor II.

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column (Pharmacia) equilibrated with 10 mM-sodium citrate, pH 6.0.

Amino acid analysis

Protein samples (25 µg) were dried under vacuum in a Savant Speed-Vac. Triplicate aliquots were treated with 6 M-HCl (Pierce) at 110 °C under reduced pressure for 24, 48 or 72 h. For cysteine determination samples were treated with performic acid before a 24-h hydrolysis (Hirs, 1967). Analyses were performed using a Beckman 6300 Amino Acid Analyzer with ninhydrin detection. Data were collected at 440 and 570 nm and stored in a Nelson Analytical M6000 data system. Quantification was effected by peak area comparison with an external standard containing 2.0 nmol of each component (Sigma). Residues per molecule were normalized to six leucine residues plus six alanine residues/molecule of rhIGF-I.

N-Terminal sequence analysis

Samples of rhIGF-I (Moks *et al.*, 1987; Forsberg *et al.*, 1990) were subjected to Edman degradation on an Applied Biosystems 477A protein sequencer, with online phenylthiohydantoin detection using an Applied Biosystems 120A analyser. Quantification was performed by the Applied Biosystems 900A data system using peak height comparison with an external standard.

Isoelectric focusing (i.e.f.)

A preformed PAGplate (pH range 3.5–9.5; LKB) was rolled on to the electrophoresis plate chilled at 2–8 °C. Samples (10–30 µg) and pl standards (Serva) were applied to sample wicks approx. 2 cm from the anode. Focusing was carried out at 50 mA, 30 W and 1500 V limits for 2.5 h. An additional 5 µl of purified water per wick was applied and run at constant voltage (1500 V) for 15–30 min. The wicks were removed and constant voltage (1500 V) continued for 30 min. The gel was stained and fixed in 0.08 % Coomassie Brilliant Blue G-250/12 % (w/v) trichloroacetic acid in 0.4 M-K₂SO₄ (pH less than 1) at room temperature overnight. The gel was then washed five times with water within 20 min and dried between two sheets of dialysis membrane soaked in 2 % (v/v) glycerol.

Reversed-phase h.p.l.c.

These separations were performed using a Hewlett-Packard 1090M liquid chromatograph with column temperature control, ternary pumping capabilities, automated sample injector and a variable-wavelength diode array detector controlled by an HP79994A Analytical Workstation. The instrument was equipped with a reversed-phase column (Vydac C₁₈; 4.6 mm × 250 mm; 5 µm particle size, 30 nm pore size) packed by The Separations Group. Analytical chromatograms were obtained using an isocratic mobile phase [0.1 % trifluoroacetic acid (TFA) in 28 % (v/v) acetonitrile] for 19 min followed by a linear gradient of acetonitrile of 0.3 %/min for 5 min and 0.6 %/min for the next 15 min. The flow rate was 0.5 ml/min; the column temperature was controlled at 50 °C, and the absorbance was monitored at 214 nm.

Ion-exchange chromatography

A Bakerbond polyethyleneimine (PEI) column (4.6 mm × 250 mm, 5 µm, 30 nm) was purchased from J. T. Baker Co. The column was equilibrated with 20 mM-Tris/acetate, pH 8.6 (buffer A). After sample injection, the column was run for 3 min before initiating a salt elution with 20 mM-Tris/acetate,

pH 8.6, in 200 mM-sodium acetate (buffer B). The ratio of buffer A to buffer B was held at 17:3 (v/v) for 32 min at a flow rate of 1 ml/min. The column temperature was controlled at 40 °C, and the absorbance was monitored at 280 nm.

Staphylococcus aureus V8 proteinase digestion

Protein samples (50 µg) were exchanged into 100 mM-phosphate, pH 6.5, using a Centricon 10 purchased from Amicon. The samples were digested with the V8 enzyme (Endoproteinase Glu-C; Boehringer-Mannheim) at an enzyme/substrate ratio of 1:3 (w/w) for 30 h at 37 °C. The digested protein was analysed by reversed-phase h.p.l.c. in 0.1 % TFA/acetonitrile-containing mobile phases. The initial mobile phase was 0.1 % TFA for 5 min, followed by a linear acetonitrile gradient of 1.24 %/min for 18 min and then 0.17 %/min for 21.5 min. The flow rate was 0.5 ml/min and the column temperature was controlled at 40 °C. The elution profile was monitored at 214 nm. The peptide-containing fractions were collected and dried in a Savant Speed-Vac.

Fast-atom-bombardment (f.a.b.)-m.s. of V8-proteinase peptides

The peptides were dissolved in 0.1 % TFA/30 % acetonitrile, transferred to the m.s. probe, dried and then dissolved in a liquid matrix of 1-thioglycerol acidified with formic acid. The spectra were obtained on a JEOL JMS HX110HF/HX110HF four-sector instrument equipped with a Cs⁺ LSIMS ion source. The Cs⁺ primary beam energy was 15 keV with an acceleration voltage of 10 kV.

Tandem m.s.

The collision-induced dissociation daughter spectrum was also obtained with the JEOL JMS HX110HF/HX110HF. The precursor ion from 1.0 nmol of peptide, formed by f.a.b. with 15 keV Cs⁺, was selected by MS-1 (the first two sectors of the instrument) at a resolution of 1:1000. The ¹²C isotope peak was selected to ensure that the daughter ions were isotopically pure. The selected ion leaving MS-1 was fragmented with helium in a grounded collision cell; the helium pressure was sufficient to attenuate the precursor intensity to 25 % of its original value. The daughter ions were measured with MS-2, using a linked scan at constant B/E (magnetic field strength/electrostatic analyser voltage) ratio produced by the ACM software program of the JEOL DA-5000 data system, with resolution 1:1000. Calibration of MS-2 was performed with a mixture of alkali metal iodides (Sato *et al.*, 1987).

Bioassay

Mouse-embryo fibroblast BALB/c3T3 cells were seeded into poly-D-lysine-coated 96-well tissue-culture plates in Dulbecco modified Eagle's medium (DMEM) containing 0.1 % bovine calf serum. After incubation for 20–24 h at 37 °C in 10 % CO₂ humidified atmosphere, different concentrations of IGF-I or diluent (DMEM with 0.1 % BSA) were added to replicate wells and incubated for an additional 16 h. [³H]Thymidine was then added to the wells and incubated for another 4 h. Radioactivity incorporated into DNA was measured by lysing the cells, filtering the well contents through glass-fibre filters, washing with 0.1 % Tween-20 and ethanol, and measuring the radioactivity by liquid-scintillation counting.

Radioreceptor assay

Cell membranes containing IGF-I receptors were prepared from term human placenta by homogenization and differential centrifugation (Hall *et al.*, 1974; Marshall *et al.*, 1974). Aliquots of the membrane suspension were stored frozen at –70 °C. Approx. 20 µg of the membrane protein was incubated with ¹²⁵I-

rhIGF-I (approx. 20000 c.p.m.) in the presence or absence of sample rhIGF-I in 50 mM-Tris/HCl, pH 7.5, containing proteinase inhibitors and 0.5 % BSA. After overnight incubation at 5–8 °C the receptor-bound IGF-I was separated by filtration through glass-fibre filters and counted in a γ -radiation counter.

RESULTS

Amino acid and N-terminal sequence analysis

The amino acid composition of purified rhIGF-I (results not shown) demonstrated correlation with the theoretical values (Rinderknecht & Humbel, 1978). The N-terminal sequence of rhIGF-I was determined by automated Edman degradation (results not shown). It was possible to identify 57 of the 70 residues (Fig. 1). The observed sequence was identical with that of the native peptide (Rinderknecht & Humbel, 1978), and confirmed that the fusion peptide was removed. No secondary sequences were detected at a sensitivity of approx. 1 %, suggesting that no additional cleavages were present.

Isoelectric focusing (i.e.f.)

The results from i.e.f. are shown in Fig. 2. A major band is

Fig. 1. Primary sequence of IGF-I

Disulphide bonds (–S–S–) and V8-proteinase cleavage sites ($\blacktriangleright \equiv \blacktriangleleft$) are indicated. The arrow ($\rightarrow$) denotes a non-specific cleavage site. The fragments are numbered consecutively from the N-terminus.

Fig. 2. Isoelectric focusing of rhIGF-I and pI standards

The lanes were loaded as follows: 1, pI standards; 2, 3 and 4, rhIGF-I (30, 20 and 10 μ g respectively); 5, 6 and 7, rhIGF-I after ion-exchange chromatography (30, 20 and 10 μ g respectively).

visible at an estimated pI of 8.5 (on the basis of standards run on the same gel). A minor more acidic band with a pI of approx. 8.3 can be seen.

Reversed-phase h.p.l.c.

Isocratic conditions were utilized as described in the Materials and methods section to improve the resolution of the major peak of rhIGF-I and closely eluted components of slightly greater hydrophilicity. The analysis of rhIGF-I by reversed-phase h.p.l.c. is shown in Fig. 3. The major peak comprises approx. 83 % of the total integrated area. All peaks in this preparation were collected for characterization as described below.

Staphylococcus aureus-V8-proteinase mapping

The reversed-phase h.p.l.c. elution profile obtained from a V8-proteinase digest of rhIGF-I is shown in Fig. 4(a). Peptide fractions were collected manually and analysed for amino acid composition, amino acid sequence by Edman degradation (results not shown) and molecular mass by f.a.b.-m.s. (Table 1). All of the predicted V8-proteinase fragments were found. They were numbered from the N-terminus as expected from the predicted cleavages after the acidic residues aspartic and glutamic acid (Fig. 1). A non-specific cleavage after glycine which has been observed before (Allen, 1981; Francis *et al.*, 1989) occurred in V_5 . The disulphide Cys¹⁸-Cys⁶¹ was unequivocally established. The positions of the two disulphides in V_2 - V_7 , required further analysis to confirm their sequence similarity to insulin (Ryle *et al.*, 1955). This was accomplished by treatment of the V_2 - V_7 peptide pair with trypsin to eliminate a Cys¹⁷-Cys⁴⁸/Cys⁶-Cys⁵² structure which would result in the formation of two peptides. Such a digestion yielded one peptide with a mass of 1412.7 mass units, an increase of 18 mass units. Its retention time on reversed-phase h.p.l.c. decreased by approx. 2 min, consistent with the increase in polarity due to the generation of an amino and a carboxy group. The other possible structure, Cys⁶-Cys⁴⁷/Cys⁴⁸-Cys⁵², could be differentiated by a manual one-cycle Edman degradation (Schwabe & McDonald, 1977; Raschdorf *et al.*, 1988), which would release two peptides. A f.a.b.-m.s. analysis of the degradation products illustrated that only threonine was released. These results are consistent with the disulphide linkages (Cys⁶-Cys⁴⁸/Cys⁴⁷-Cys⁵²) reported by Raschdorf *et al.* (1988) and Iwai *et al.* (1989).

The individual peaks isolated by reversed-phase h.p.l.c. of intact rhIGF-I (Fig. 3) were also analysed by V8-proteinase mapping. The maps of the respective peaks are shown in Fig. 4. Peak 1 (Fig. 3) was identified as IGF-I containing a methionine sulfoxide residue by f.a.b.-m.s. analysis of the V8-proteinase digest fragments. The f.a.b.-m.s. data for the two peaks eluted at approx. 33 min (Fig. 4b) are shown in Table 2. The new peak, II, is identified as a V_4 - V_9 methionine sulfoxide-containing peptide, since the extra 16 mass units are associated with the methionine-containing V_9 fragment.

Similarly, peak 2 (Fig. 3) was identified as an IGF-I hydroxamate variant, a side reaction of the hydroxylamine cleavage. It is known that glutamine and asparagine residues can be converted into hydroxamates (Bornstein & Balian, 1977). This reaction results in the addition of 16 mass units, an oxygen atom, to the amide-containing peptides. If the additional 16 mass units seen in peak V_4 - V_9 (Table 2, Fig. 4c) had been the result of the oxidation of the Met⁵⁹ residue in the V_9 peptide, the elution time of the modified peptide would have been earlier, as in Fig. 4(b). The molecular ion, 868.2, suggests that the additional 16 mass units are associated with Gln¹⁵ in the V_4 fragment. Selection of this ion and sequencing by the mass spectrometer confirmed that the oxygen atom was on the Gln¹⁵ residue (Fig. 5).

Fig. 3. Analysis of rhIGF-I by reversed-phase h.p.l.c.

Chromatography was performed on a Vydac C_{18} column and the sample was eluted with TFA/acetonitrile-containing mobile phases as described in the Materials and methods section.

Table 1. M.s. analysis of V8-proteinase peptides from rhIGF-I

V8 peptide	Mass	
	Theoretical*	Observed
1	302.14	—
3	346.20	346.4
8	686.43	686.3
5 _b ,6	1502.76	1502.7
2-7	1421.53	1421.4
5a	1406.69	1406.6
4-9	2129.04	2129.2

* Monoisotopic.

Table 2. M.s. analysis of V9-proteinase peptides of individual rhIGF-I fractions isolated by reversed-phase h.p.l.c. (Fig. 3)

Fig.	Peak	Observed mass	Assignment
4(b)	I	1406.5	V _{5a}
		2891.6	V _{5,6}
	II	852.2	V ₄
		1295.5	V ₉ +16
		2145.0	V ₄ -V ₉ +16
4(c)	V ₄ -V ₉	868.2	V ₄ +16
		1279.5	V ₉
		2129.9	V ₄ -V ₉
		2146.0	V ₄ -V ₉ +16
4(d)	V _{5b,6}	1502.5	V _{5b,6}
		1518.6	V _{5b,6} +16
	V _{5a}	1406.5	V _{5a}
		2891.7	V _{5,6}
		2908.3	V _{5,6} +16

The V8-proteinase map (Fig. 4d) of the main peak from Fig. 3 suggests that the V_{5b,6} peptide, which is under-recovered, has been partially modified. M.s. analyses of peaks V_{5b,6} and V_{5a} are tabulated in Table 2. It can be seen that an additional 16 mass units are associated with the amide-containing peptides in the V8-proteinase cleavage fragments V_{5b,6} and the intact V_{5,6} peptide. Hence, reversed-phase h.p.l.c. of the intact molecule can resolve the Gln¹⁵ hydroxamate variant, but not the Asn²⁶ or Gln⁴⁰ hydroxamates from the main peak material.

Fig. 4. *Staphylococcus aureus*-V8-proteinase maps of rhIGF-I fractions

Peaks are numbered according to the cleavages shown in Fig. 1. (a) Unfractionated rhIGF-I; (b) rhIGF-I peak 1 from Fig. 3; (c) rhIGF-I peak 2 from Fig. 3; (d) rhIGF-I peak 3 from Fig. 3. Chromatography was performed on a Vydac C_{18} column and the sample was eluted with an acetonitrile gradient as described in the Materials and methods section. Abbreviation: Met-SO, methionine sulfoxide.

Ion-exchange chromatography

Since it is possible that the slightly more acidic band seen on i.e.f. (Fig. 2) may be the hydroxamate-containing variants, a

Fig. 5. Tandem m.s. analysis of molecular ion 868.2
Peaks are labelled in accordance with Biemann's (1988) adaptation of the Roepstorff-Fohlman (1984) ion designations.

separation was attempted using chromatography on a PEI column which should be effective in separating differentially charged variants from rhIGF-I itself. Such a separation is shown in Fig. 6. The minor peaks at approx. 16 and 19 min were identified as the hydroxamate variants, as shown below.

Reversed-phase h.p.l.c. and *Staphylococcus aureus*-V8-proteinase mapping of hydroxamate-containing fractions

The peaks were collected from the ion-exchange-chromatography step (Fig. 6) as two pools and analysed by reversed-phase h.p.l.c. (Fig. 7). The major peak was shown to consist of rhIGF-I and its methionine sulphoxide-containing variant. The minor peaks appeared to be the hydroxamate-containing variants, the peak at approx. 21 min in the reversed-phase separation (Fig. 7) being modified at Gln¹⁵ and the peak co-eluted with main peak material being modified at Asn²⁶ and Gln⁴⁰, confirming the results from the section above.

The V8-proteinase map of the hydroxamate-containing peaks isolated by ion-exchange chromatography is shown in Fig. 8. The peaks designated a-e were collected and analysed by f.a.b.-m.s. Hydroxamate groups were found at all three amide-containing V8-proteinase peptides as shown in Table 3.

Biological activity

IGF-I is known to stimulate DNA synthesis and cell proliferation in 3T3 fibroblasts (Leof *et al.*, 1982; Riss *et al.*, 1988). In the bioassay using BALB/c3T3 cells, rhIGF-I caused a dose-dependent stimulation of [³H]thymidine incorporation into

Table 3. M.s. analysis of V8-proteinase peptides generated from hydroxamate-containing rhIGF-I fractions isolated by ion-exchange chromatography

Peak	Observed mass	Assignment
a	1518.6	V _{5b,6} + 16
b	1502.7	V _{5b,6}
c	2906.1	V _{5,6} + 16
	2922.1	V _{5,6} + 32
d	1406.7	V _{5a}
	1422.8	V _{5a} + 16
e	2129.8	V ₄ -V ₉
	2145.9	V ₄ -V ₉ + 16

Fig. 6. Ion-exchange chromatography of rhIGF-I
The chromatography was performed on a PEI column and the sample was eluted with sodium acetate as described in the Materials and methods section. The analog output to the integrator was scaled at 1.0 Volt/0.5 A unit.

Fig. 7. Reversed-phase h.p.l.c. of fractions separated by ion-exchange chromatography
a, Main peak from PEI column; b, pooled minor peaks from PEI column. Chromatography was performed on a Vydac C₁₈ column and the sample was eluted with TFA/acetonitrile-containing mobile phases as described in the Materials and methods section.

Fig. 8. *Staphylococcus aureus*-V8-proteinase mapping of hydroxamate-containing fractions isolated by ion-exchange chromatography
Chromatography was performed on a Vydac C₁₈ column, and the sample was eluted with an acetonitrile gradient as described in the Materials and methods section.

nuclear material (Fig. 9). The biological activity of rhIGF-I and the two variants is essentially equal. Insulin and recombinant human insulin-like growth factor II (rhIGF-II), two mitogens that are known to have structural similarity to IGF-I, exhibited

Fig. 9. Activity of rhIGF-I in the bioassay in comparison with the rhIGF-I derivatives and other polypeptide growth factors

Mouse-embryo fibroblast BLAB/c3T3 cells were incubated with the agonist as described in the materials and methods section. $\square$, rhIGF-I; $\blacksquare$, rhIGF-I hydroxamates; $\times$, rhIGF-I methionine sulfoxide; $\circ$, insulin; $\bullet$, rhIGF-II; $\triangle$, rhTGF- β_1 ; $\blacktriangle$, rhGH.

Fig. 10. Activity of rhIGF-I in the radioreceptor assay in comparison with the rhIGF-I derivatives and other polypeptide growth factors

Agonists were incubated with receptor preparations as described in the Materials and methods section. $\blacksquare$, rhIGF-I; $\square$, rhIGF-I methionine sulfoxide; $\triangle$, rhIGF-I hydroxamate; $\bullet$, rhIGF-II; $\circ$, insulin.

less than 10% activity in comparison with rhIGF-I, whereas rhGH and TGF- β_1 were inactive. Since other polypeptide growth factors such as platelet-derived, fibroblast and epidermal growth factors, are also mitogenic to BLAB/c3T3 cells, this bioassay is mainly used to measure IGF-I activity in highly purified preparations.

The biological effects of IGF-I are believed to be mediated through specific receptors on the cell surface. The ability of IGF-I to bind to its receptor, therefore, can be used as an indirect measure of its biological activity. Recombinant hIGF-I competed with ^{125}I -IGF-I for binding sites on the placental membranes in a dose-dependent manner (Fig. 10). The order of biological activity of various analogues in this assay is similar to that in the

bioassay, except that IGF-II is relatively more active in the former. Natural IGF-I purified from Cohn fraction IV of human serum as described by Svoboda *et al.* (1980) exhibited dose responses that are identical with those of the rhIGF-I in both the assays (results not shown).

DISCUSSION

A battery of analytical techniques are at our disposal to chemically characterize recombinant DNA-derived proteins. When a number of these were used to analyse rhIGF-I, including amino acid composition, N- and C-terminal sequence analyses, peptide mapping, reversed-phase h.p.l.c. and SDS/PAGE no heterogeneity was observed. Not until analysis by isoelectric focusing was performed did such evidence arise. As can be seen in Fig. 2, a minor more acidic impurity was observed at a level of about 15%, as estimated by densitometry. More acidic bands on i.e.f. gels are usually associated with deamidation of the protein (Hancock *et al.*, 1988). This was not likely in the present situation, since the difference in pI from the native form is only 0.2 pH unit. Theoretically, the pI expected for monodeamidated human IGF-I is 6.5, calculated as described by Shire (1983). The observed difference of 0.2 pH unit was consistent with the presence of two free cysteine thiol groups. However, analysis with 5,5'-dithiobis-(2-nitrobenzoic acid) [Ellman (1959) reagent] revealed no such functional groups. Fractions enriched in the acidic component were isolated by a reversed-phase h.p.l.c. protocol at an elevated temperature as described in the Materials and methods section. F.a.b.-m.s. analyses of the V8-proteinase fragments generated from digestion of peak 2 (Fig. 3) revealed that peptide V₄ was modified, resulting in a 16-mass-unit increase of its molecular mass, suggesting the presence of an additional oxygen atom. However, the amino acid composition of this peptide does not contain amino acid residues which are susceptible to oxidation. The literature from 30 years ago provides a reasonable explanation (Ramachandran & Narita, 1958). The first sentence of their manuscript begins: 'Amides on reaction with hydroxylamine are converted into hydroxamic acids...'. These workers successfully used hydroxylamine to quantify the asparagine and glutamine content of proteins. Inadvertently, we were converting these residues, Gln¹⁵, Asn²⁶ and Gln⁴⁰, to hydroxamates.

The data generated from a similar analysis of peak 3 (Fig. 3) demonstrated that, in addition to residue Gln¹⁵ in peptide V₄, residue Gln⁴⁰ in peptide V_{5b,6} had been converted into a hydroxamate. The data (Table 2) did not definitively establish that Asn²⁶ had also been modified. Hence, since i.e.f. was seen to be so effective in separating the hydroxamate-containing material from IGF-I itself, an ion-exchange chromatography method was developed as described in the Materials and methods section. The data (Table 3) generated from the analysis of the peptide map (Fig. 8) of the acidic fractions (Fig. 6) verified that the Asn²⁶ residue was indeed converted into a hydroxamate. Attempts to prevent production of hydroxamate-containing residues by optimizing the cleavage-reaction pH, duration, hydroxylamine concentration and other components were unsuccessful. Every condition tested produced various amounts of hydroxamate.

Even though both bioassays indicated that the hydroxamate-containing rhIGF-I protein is biologically active, it is desirable to remove this variant from rhIGF-I preparations before undertaking clinical studies. This can be accomplished by including an ion-exchange-chromatography step in the purification procedure. Perhaps an even more desirable approach is to avoid constructing fusion proteins with a hydroxylamine-sensitive cleavage site if the protein of interest contains asparagine or glutamine residues,

especially in view of the known mutagenicity of hydroxylamine (Phillips & Brown, 1967).

We thank Dr. John Ogez for the purification of IGF-I from human Cohn fraction IV, and Dr. John Stults for the tandem m.s. analysis.

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Received 30 October 1991/24 December 1991; accepted 6 January 1992