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REDUCTION STUDIES ON BACTERIAL RECOMBINANT SOMATOMEDIN C/INSULIN-LIKE GROWTH FACTOR-1

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SUMMARY

N-Met-Somatomedin C/insulin-like growth factor-1 (rSmC) had been produced in recombinant *Escherichia coli* in monomeric form. The intact rSmC peptide is initially synthesized in *E. coli* cells in denatured form as inclusion bodies. The rSmC peptide in these inclusion bodies was found in reduced form. Isolation of this rSmC peptide was accomplished by separation and dissolution of the inclusion bodies, with dissociation of non-covalently aggregated species. The reduced rSmC was converted to a metastable state, termed un-refolded rSmC. Further processing of this rSmC generated two other isomers, termed refolded rSmC. The transitions of the peptide among these different states, reduced rSmC, un-refolded rSmC, and refolded rSmC can be readily monitored by reversed-phase high-performance liquid chromatography. By reduction and re-oxidation of the purified individual isomers we found that they are likely to be related to each other as conformation isomers which appear to be stabilized by disulfide bonds.

INTRODUCTION

Somatomedin C/insulin-like growth factor-1* has extensive sequence similarity to proinsulin[1], and is probably also closely related to proinsulin in its globular tertiary structure. The structural similarity of insulin-like growth factor-1 (IGF-1) to insulin is also reflected by crossover-binding to their respective receptors at reduced affinity[2], with a corresponding crossover of biological activities at high concentrations, both in cell culture and *in vivo*[3].

This proinsulin family of small protein hormones is characterized by a shared arrangement of three disulfide bridges. These three disulfide bridges probably stabilize a native tertiary structure for each hormone which binds with high affinity to the receptor and is biologically active. When these disulfides are reduced under denaturing conditions in solution, the peptide relaxes into a undefined conformation, and if allowed to reoxidize randomly under the denaturing conditions, can generate fifteen possible monomeric combinations. Mismatched disulfides might therefore sta-

* Somatomedin C and insulin-like growth factor-1 were determined by the Endocrine Society to be the same substance.

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bilize multiple isomeric forms of somatomedin C; it is possible that some of these forms might be stable as globular monomers in solution.

Recombinant somatomedin C/IGF-1 (rSmC) was produced by using a specific strain of genetically engineered *Escherichia coli*[4]. This special strain of *E. coli* over-produces rSmC, leading to an accumulation of the heterologous protein as "protein granules" or "inclusion bodies" in the cytoplasm of the cells. Since the discovery of these inclusion bodies in recombinant bacteria, the recovery of recombinant protein in a bioactive form from these inclusion bodies has been an important industrial research topic[5].

General operation steps for achieving this recovery are:

(i) to dissolve the inclusion, usually by using strong chaotropes, (ii) to refold the protein by exchange of the strong chaotrope solution into a dilute, buffered aqueous solution,

(iii) to purify the refolded, renatured species using chromatographic and other separation methods.

We have designed a process for the recovery and production of rSmC[6]. During the research leading to this process, we isolated several different forms of rSmC. Four distinguishable forms have been identified by reversed-phase high-performance liquid chromatography (HPLC). These are: reduced rSmC, un-refolded rSmC, and two forms of refolded rSmC. Each of these forms has a characteristic retention time in reversed-phase HPLC. In this study, the general structural relationship amongst these forms, and their interconversion have been studied.

EXPERIMENTAL

Reversed-phase HPLC

The chromatographic equipment consisted of dual-solvent delivery pumps (Model 114, Beckman Instruments, Fullerton, CA, U.S.A.), a Model 210 solvent gradient mixer (Beckman Instruments) a Model 7010 sample injector (Rheodyne Cotati, CA, U.S.A.), a Model 421 gradient controller (Beckman Instruments), a Model 160 multiple-wavelength UV detector (Beckman Instruments), and a Model R2990-10 pen recorder (Kipp & Zonen, Bohemia, NY, U.S.A.).

Solvents used in reversed-phase HPLC were: solvent A, 0.1% trifluoroacetic acid (TFA, Sequenal grade, Pierce, Rockford, IL, U.S.A.) in water; solvent B, 0.1% TFA in water–acetonitrile (20:80) (HPLC grade, Mallinkrodt, St. Louis, MO, U.S.A.). The gradient program routinely used for separation of the rSmC isomers was: initial elution with 30% in A followed by a linear change to 50% B in A in 20 min.

The column used in the reversed-phase HPLC was Aquapore RP-300 cartridge (100 × 3.9 mm I.D.) obtained from Brownlee Labs. (Santa Clara, CA, U.S.A.). The eluent flow-rate was kept at 1.0 ml/min and the detector was kept at 280 nm throughout the analysis. The sample volume injected was 200 μl.

Samples were usually injected with out pre-treatment. Alkaline solutions were adjusted to pH 3.0 with TFA before injection. Turbid samples were filtered through a 0.22-μm filter (Nylon 66 type, Alltech, Deerfield, IL, U.S.A.) before injection.

Size-exclusion chromatographic analysis

A prepacked Superose 12 column (300 × 10 mm I.D.), obtained from Pharmacia (Piscataway, NJ, U.S.A.), was used for size exclusion analysis. The FPLC instrument (Pharmacia) was used in this analysis. The eluent used for size exclusion chromatography was composed of 50 m*M* ethanolamine, pH adjusted with hydrochloric acid to 9.0, 0.5 *M* sodium chloride, and 0.05% (w/v) sodium azide (Sigma, St. Louis, MO, U.S.A.). The flow-rate was kept at 1.0 ml/min.

The UV detector (Model UV-1, Pharmacia) was set at 280 nm. Sample injection was made through a seven port valve (Model MV-7, Pharmacia) and the sample loop was 200 μl.

Cation-exchange purification of metastable rSmC

A cation-exchange column (150 × 44 mm I.D.) was slurry packed with Sp-Sephadex resin (fine grade, Pharmacia), and equilibrated with a low ionic strength eluent. The low ionic strength eluent was composed of 50 m*M* sodium acetate, pH adjusted to 6.5. The oxidized, dialyzed rSmC solution (see below) was pH adjusted to 6.5 with glacial acetic acid, and applied to the SP-Sephadex column. The column was continuously eluted with 50 m*M* acetate buffer until the eluent showed low UV absorbance. The purified metastable rSmC was eluted from the column when the eluent was changed to a buffer composed of 50 m*M* acetate pH 6.5 with 0.1 *M* sodium chloride added. Metastable rSmC was collected in this fraction, subsequently concentrated, and diafiltered on a YM-2 ultrafiltration membrane (Amicon, Danvers, MA, U.S.A.) using a stirred pressure cell (Model 2000, Amicon). The dialyzed solution was freeze dried to a white fluffy powder.

Oxidation and reduction studies

Purified refolded rSmC (single reversed-phase HPLC peak isomer) (1 mg) was dissolved in 2 ml of a solution containing 6 *M* urea, 0.1 *M* Tris, pH 9.0. To this solution, we added 15.4 mg (50 m*M*) dithiothreitol (DTT) (Sigma), and the reaction vessel stopper sealed. The reduction reaction was quenched by addition of conc. hydrochloric acid to bring the solution to pH 3.0. The reaction solution was then injected onto a HPLC column for analysis. Oxidation of the reduced rSmC was achieved by refolding dialysis as discussed below.

Refolding dialysis studies

Reduced and denatured rSmC solution from the above experiment was placed in a dialysis bag (Spectrapor No. 6, mol. wt. cut off, 3.5 kilodalton, Spectrum Medical Industries, Los Angeles, CA, U.S.A.) and dialyzed against 10 volumes of low concentration of chaotrope, 0.1 *M* Tris, pH 9.0. This dialysis was allowed to proceed at ambient temperature for 24 h. The dialysis medium was changed to 0.1 *M* acetic acid for the next 24 h. The refolded rSmC was monitored by reversed-phase HPLC.

Dissolution of inclusion bodies, and time sequence study of redox state of dissolved inclusion bodies

Washed inclusion bodies (1 g wet weight) was dispersed in 200 ml of a solution containing 0.1 *M* sodium carbonate, 0.1 *M* sodium bicarbonate, and 0.25% (w/v) sodium dodecylsulfate (SDS). The suspension was homogenized with a Polytron

homogenizer (Brinkmann Instrument, Westbury, NY, U.S.A.) to achieve solubilization. An aliquot of the clarified solution was passed through a 200 × 4.4 mm I.D. column, packed with AG11A8 resin (Biorad Labs.) The column was eluted with 40 m*M* sodium bicarbonate buffer pH 9.8. This chromatographic process removed the excess detergent (SDS) used for inclusion solubilization[7]. The solution, after detergent removal, was ready for HPLC analysis. The solution was acidified with TFA before injection onto a reversed-phase HPLC column. At 6, 12, 15, 18 and 24 h after the initial solution, an aliquot of the dissolved inclusion was taken, the detergent removed by an AG11A8 column, acidified, and analyzed by HPLC. This sequence of samples revealed the kinetics of the oxidation of the detergent-dissolved inclusion bodies.

SDS polyacrylamide gel electrophoresis and isoelectric focusing gel analysis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using 15% cross-linked polyacrylamide gel following the Laemmli method[8], and stained by Coomassie blue for detection. The staining procedure was modified for this protein by omitting the gel prewash in acid–methanol, because rSmC is soluble in this solution and is leached from the gel; after electrophoresis, the gel was immediately immersed in the stain solution. Isoelectric focusing (IEF) gel analysis was done using Ampholine pre-cast gels (LKB, Gaithersburg, MD, U.S.A.) and silver staining for detection.

Radioreceptor assays

Radioreceptor binding assays were done using a particulate membrane preparation from fresh human placental tissue, following the method of Marshall *et al.*[9] with minor modifications. The assay buffer was 50 m*M* Tris–HCl, 0.25% (w/v) bovine serum albumin (RIA Grade, IGF-free, Sigma), pH 7.4. To each tube, in a total volume of 5600 μl, were added 50 μl placental membrane protein, determined by the method of Bradford[10], [^{125}I]IGF-1 tracer (iodinated in-house, by the chloramine-T method[11], specific activity 100–120 μCi/μg, 12 000 cpm), and standard or unknown IGF-1 doses. The tubes were incubated for 16–20 h at 4°C, and centrifuged at 6000 *g* for 30 min, and the supernatants removed by aspiration. The pellets were washed by resuspending in 1.0 ml of cold assay buffer recentrifuged and counted in a Micromedics γ radiation counter (Model 28019, ICN Biomedical, Horsham, PA, U.S.A.).

Cell culture bioactivity assays

Bioactivity of the IGF-1 preparations was measured as stimulation of Madden Darby bovine kidney (MDBK) cell[12] proliferation in a minimal medium [DME–F-12 (1:1), *i.e.* Dulbecco's modified eagles's medium (DME) mixed 1:1 with Ham's F-12 nutrient mixture], supplemented with 0.1% fetal bovine serum. Briefly, the assay protocol was as follows: stationary monolayer MDBK cultures were treated with trypsin, pelleted, and resuspended in medium without serum supplement. The cell suspension was diluted with medium to $1.0 \cdot 10^4$ cells/ml, with the appropriate concentration of standard or unknown IGF-1 preparation added. A 2-ml volume of the suspension was seeded into 35-mm plastic culture wells (9.62 cm^2), and the cultures incubated at 37°C. At appropriate time points, the resulting monolayer cultures were

treated with trypsin, suspended, and counted using a Coulter cell counter (Model ZM, Hialeah, FL, U.S.A.).

RESULTS AND DISCUSSION

The structure of rSmC

The *E. coli* system we have used for production of rSmC employs a perfect construction with a chemically synthesized gene having codons optimized for *E. coli*[13]. The resulting product is N-Met-SmC/IGF-1, a 71-residue peptide having the authentic 70-residue sequence[1], with the addition of Met at the amino terminus as a result of the bacterial start codon. This system has been optimized for efficient expression of the relatively small perfect construction peptide, allowing the direct production of rSmC instead of producing a larger intermediate fusion protein, and avoiding the complications introduced by chemical cleavage.

Redox state of inclusion bodies

The redox state of peptides in bacterial inclusion bodies has been shown to be fully reduced[14]. However, the recombinant peptides are tightly packed at high concentration in the inclusion bodies; once the cell is broken and the inclusion bodies are exposed to oxygen, their sulfhydryl groups rapidly oxidize to form intra- and intermolecular disulfide bonds with neighboring thiol groups. Reduced peptides may be separated from oxidized forms by gel electrophoresis because of their hydrodynamic differences: the reduced forms occupy a larger hydrodynamic volume than the more compact oxidized forms and their electrophoretic migration is relatively retarded.

Separation of reduced and oxidized peptides may also be accomplished by reversed-phase HPLC. The reduced forms are retained longer on reversed-phase

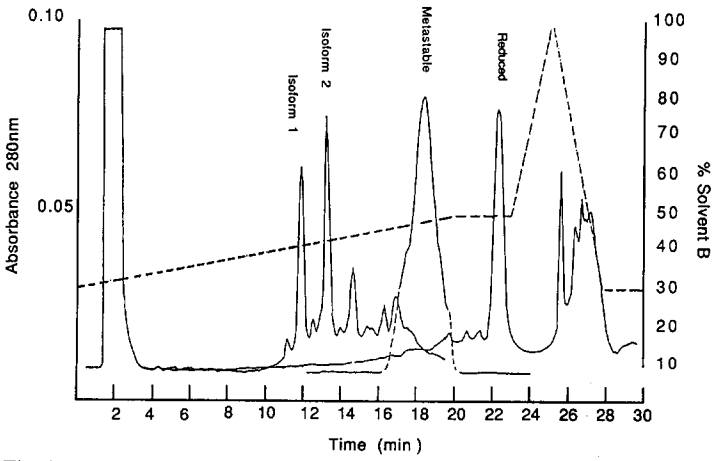

Fig. 1. Reversed-phase HPLC separation of different forms of rSmC: reduced rSmC, metastable rSmC, and oxidized-refolded rSmC (two isomer forms). Column 100 × 4.6 mm I.D. Aquapore RP-300; solvents, A = 0.1% aq. TFA, B = acetonitrile–water–TFA (80:19.9:0.1); UV detection at 280 nm; sample loop size, 200 μl; flow rate, 1.0 ml/min; gradient composition, 30 to 50% solvent B in A over 20 min.

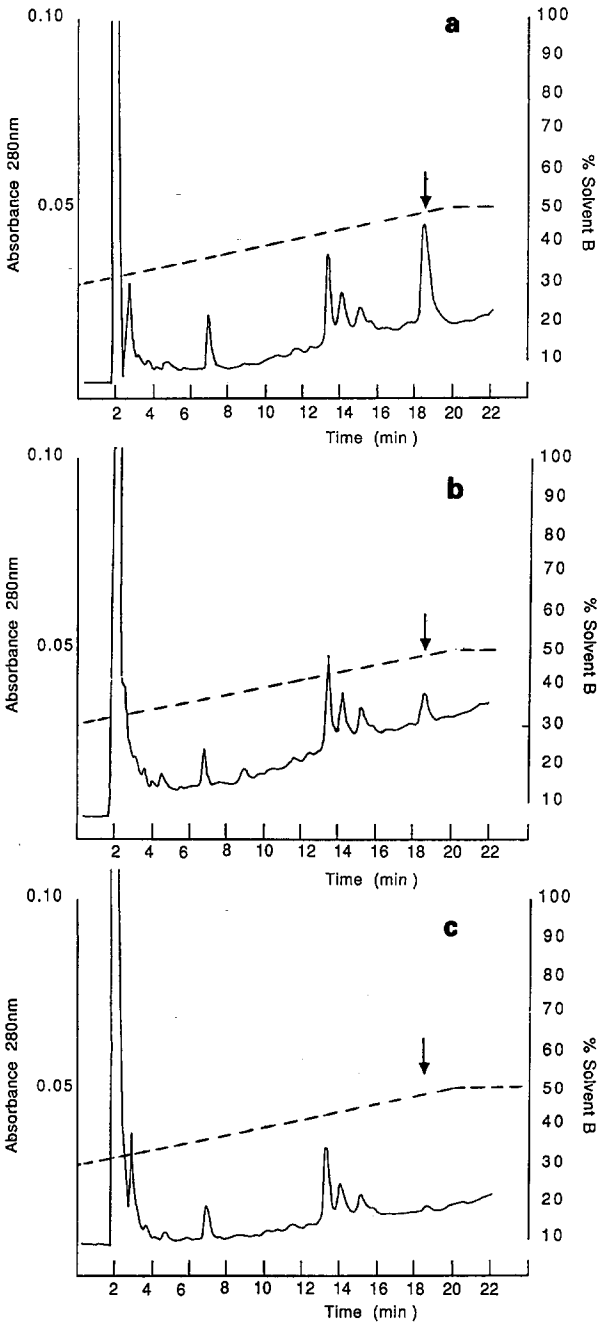

Fig. 2. Time sequence study of rSmC oxidation states in a dissolved inclusion preparation. (A) Chromatograms 12 h after dissolution; (B) chromatogram 15 h after dissolution; (C) chromatogram 18 h after dissolution. See Fig. 1 for chromatographic conditions. The arrows indicate where reduced rSmC eluted under the same conditions.

HPLC columns. We speculated that more hydrophobic domains are exposed in the reduced form than in the oxidized forms. This separation for rSmC is shown in Fig. 1.

Fully reduced rSmC was analyzed by reversed-phase HPLC. Purified refolded rSmC can be completely reduced by the addition of excess DTT. This reduced form, when analyzed by reversed-phase HPLC, showed a single peak at a retention time of 23 min. Isomer 1 and isomer 2 of the refolded rSmC, when reduced, showed the same reduced peak, as shown in Fig. 1.

Oxidation studies of dissolved rSmC peptide from inclusion bodies

For reduced, denatured peptides in solution, the oxidation of thiols to disulfides by molecular oxygen is catalyzed by the presence of externally introduced mercaptide anions[15]. The conformations assumed by the peptide are in dynamic equilibrium with constant interchange, allowing rearrangement to the globular monomer.

To follow reoxidation of rSmC, we monitored the time course of disappearance of the reduced form of rSmC by reversed-phase HPLC, after dissolution of inclusions. Fig. 2 shows the results. After 12 h, the initial predominant peak corresponding to the fully reduced form began to diminish in size. After 15 h the peak corresponding to the fully reduced form had diminished to one fourth its original size. After 18 h, the fully reduced peak had diminished to a very small peak. This study indicated that oxidation of the sulfhydryl bonds progressed in the liquid state, and was essentially complete in 18 h.

Metastable rSmC

A metastable form of rSmC was generated by dissolving inclusion bodies, and allowing oxidation to proceed in the liquid state. The solution, after oxidation and denaturant removal, can be further purified by cation exchange chromatography. The protein obtained by the process, shown in Fig. 1, appears homogeneous by reversed-phase HPLC.

This metastable form of rSmC was unstable in alkaline solution, and tended to aggregate; we refer to this form as metastable rSmC. Metastable rSmC, when analyzed under the usual conditions by reversed-phase HPLC, showed a retention time of 18 min as in Fig. 1. Reduction of this metastable rSmC by DTT addition shifted the HPLC peak to a retention time of 23 min, identical to a fully reduced rSmC peak.

Refolding–renaturation of metastable rSmC

A protein assumes a random coil structure when dissolved in a strong chaotrope solution, *e.g.* 6 *M* guanidine chloride or 8 *M* urea, due to the competition of the chaotrope for the intramolecular non-covalent interactions, the hydrogen bonds and salt bridges which stabilize the folded structure. By slowly diluting the chaotrope concentration in solution, these intramolecular interactions will re-establish and the folded structure will be regenerated. If the process is gradual, the most stable form of the protein will tend to predominate. The less stable structures usually precipitate from solution because they are not perfect glubular monomers, and have solvent-exposed hydrophobic surfaces which bind intermolecularly.

In the case of rSmC, mismatched disulfides may be transiently formed by ox-

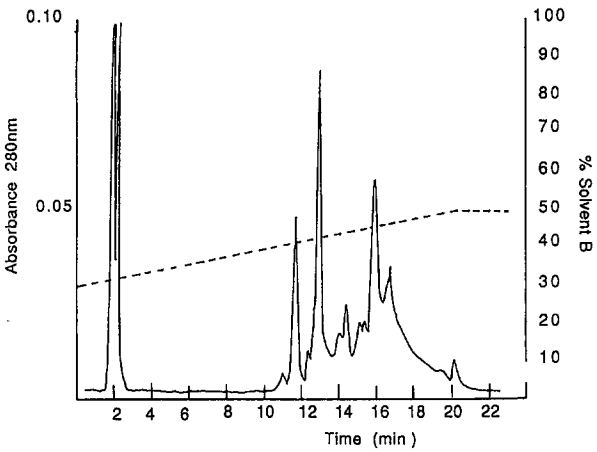

Fig. 3. Reversed-phase HPLC profile showing retention time shift after refolding dialysis. The metastable rSmC peak was observed to shift to a shorter retention time after refolding. See Fig. 1 for chromatographic conditions.

idation during the refolding; residence time in these mismatched states is reduced by the presence of mercaptide ions which catalyze their reduction and rearrangement. For this reason, the refolding process is mediated by addition of small molecule thiols to the solution. Because the mercaptide anion is the effective mediator in this process, and the pK_a of thiols is in the neighborhood of pH 9.1, the refolding solution must be slightly alkaline to be effective.

We refolded the metastable rSmC by dissolving the isomer in a solution composed of 6 *M* urea, 0.1 *M* Tris, 10 m*M* β-mercaptoethanol, pH 9.0. This solution was dialyzed against 10 volumes of a buffer composed of low concentration of chaotrope, 0.1 *M* Tris, pH 9.0. The dialysis was allowed to proceed at ambient temperature for 24 h. An aliquot of the dialyzate was taken from the dialysis bag, acidified, and analyzed by reversed-phase HPLC. The chromatogram is shown in Fig. 3. A shift to lower retention time occurred compared with that of the metastable rSmC. This refolding process thus generated a more hydrophilic species with lower affinity for the reversed-phase HPLC column. This affinity difference suggests that the conformation of metastable rSmC has hydrophobic domains exposed to the aqueous solvent environment. Refolded rSmC, in turn, is rearranged to a conformation with hydrophobic domains shielded inside a specific steric structure.

Studies on the chromatographic affinities of different disulfide isomers of IL-2 to reversed-phase HPLC[16], indicated that unnatural disulfide isomers of IL-2 has much longer retention than native-like IL-2 isomers. Thus, refolded rSmC showed similar shift of retention time exhibited by IL-2 isomers.

Reversed-phase HPLC was also demonstrated[17] to be capable of differentiating between different protein conformations. We found that by refolding metastable rSmC, a number of different isomers were generated. These conformational isomers were separated as distinct peaks on reversed-phase HPLC shown in Fig. 3. However, two major peaks predominated, indicating that these two isomers must be of comparable stability.

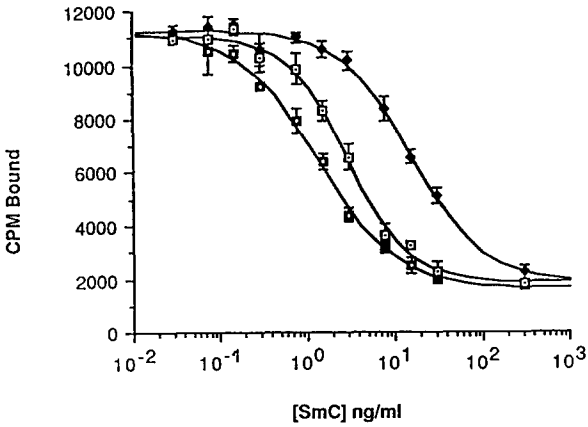

Fig. 4. Radioreceptor assay of separated isomer forms of rSmC. (◆) Isoform 1, (■) isoform 2, (⊡) purified rSmC.

In summary, four major isomers of rSmC peptide could be separated by reversed-phase HPLC: reduced rSmC, metastable rSmC, and two isomers of refolded rSmC, as shown in Fig. 1. The two refolded rSmC isomers showed the shortest retention times and the reduced rSmC showed the longest retention time in reversed-phase HPLC.

Investigation of the two major refolded rSmC isomers

The two major isomers were separated by preparative reversed-phase HPLC as shown in Fig. 1. These separated forms were analyzed by size exclusion chromatography on a Superose-12 column (Pharmacia) by SDS-PAGE, and by IEF. SDS-PAGE indicated that both isomers are monomeric in molecular size. IEF showed that both isomers have a p*I* of 8.3.

A mixture of the two separated isomers was made and its components were not resolved by these methods. Thus, the only analytical method that distinguished the two isomers was reversed-phase HPLC.

The separated isomers have significant but different receptor binding affinities and bioactivities. Specific binding of the separated isomers, and the mixture preparation from which they were separated, in a human placental membrane radioreceptor assay is shown in Fig. 4. The ED-50* values for isomer 1, isomer 2, and the starting mixture were 16 ng, 1.4 ng, and 2.9 ng respectively, showing a clear distinction in the binding affinities of the two isomers.

These receptor binding differences correlate with differences in the potency of the two isomers in stimulating proliferation of MDBK cells in monolayer culture. Taking the potency of the starting mixtures as 100%, isomer 1 had a potency of 81% total stimulation at 25 ng/ml with an ED-50 of 16 ng, while isomer 2 had a potency of 146% total stimulation at 100 ng/ml with an ED-50 at 0.91 ng.

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* ED-50 is the half displacement value of the substrate by radio-labeled rSmC in radioreceptor assay.

These bioactivity results show that, while both of the rSmC/IGF-1 isomers have significantly high receptor binding and biological activity, these activities are clearly different in a defined test system.

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