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In vitro folding, functional characterization, and disulfide pattern of the extracellular domain of human GLP-1 receptor

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Abstract

The N-terminal, extracellular domain of the receptor for glucagon-like peptide 1 (GLP-1 receptor) was expressed at a high level in *E. coli* and isolated as inclusion bodies. Renaturation with concomitant disulfide bond formation was achieved from guanidinium-solubilized material. A soluble and active fraction of the protein was isolated by ion exchange chromatography and gel filtration. Complex formation with GLP-1 was shown by cross-linking experiments, surface plasmon resonance measurements, and isothermal titration calorimetry. The existence of disulfide bridges in the N-terminal receptor fragment was proven after digestion of the protein with pepsin. Further analysis revealed a disulfide-binding pattern with links between cysteines 46 and 71, 62 and 104, and between 85 and 126. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: G-protein coupled receptor; Inclusion bodies; Renaturation; Ligand binding; Disulfide connectivity

Abbreviations: CD, circular dichroism; DMP, dimethylpimelidate; DTNB, 5,5′-dithio-bis(2-nitrobenzoate); DTT, dithiothreitol; DSC, differential scanning calorimetry; EDC, *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide; EDTA, ethylenediaminetetraacetate; GdmHCl, guanidinium hydrochloride; GLP-1, Glucagon-like peptide 1; GPSRs, G-protein coupled receptors; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high performance liquid chromatography; IPTG, isopropylthiogalactoside; ITC, isothermal titration calorimetry; LDAO, *N*,*N*-dimethyldodecylamine *N*-oxide; nGLP1R, *N*-terminal domain of the human GLP-1 receptor; NHS, *N*-hydroxysuccinimide; NIDDM, non-insulin dependent diabetus mellitus; nPTHR, *N*-terminal domain of the human PTH receptor; NTB, 2-nitro-5-thiobenzoate; NTSB, 2-nitro-5-thiosulfobenzoate; PDEA, 2-(2-pyridinyldithio)ethaneamine hydrochloride; RU, resonance units; SDS, sodiumdodecylsulfate; TCA, trichloroacetic acid; TFA, trifluoracetic acid; TRIS, tris(hydroxymethyl)aminomethane.

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1. Introduction

The paracrine hormone GLP-1 is secreted by the duodenum in response to high glucose levels, which subsequently triggers the release of insulin from pancreatic cells [1,2]. The ability of GLP-1 to induce insulin secretion in dependence on high glucose levels, renders this component potentially useful in the treatment of non-insulin dependent diabetus mellitus (NIDDM) [3,4]. However, the peptide nature of GLP-1 prevents its direct application due to its short half-life in vivo [4]. A rational design of new agonists will therefore greatly benefit from structural knowledge of the interaction between GLP-1 and its receptor.

The receptor protein for GLP1 was first cloned by Thorens [5] in 1992; it belongs to class B of G-protein coupled receptors. The protein has a large N-terminal extracellular domain that was shown to be involved in the ligand binding [6–8]. As for other peptide–hormone receptors such as receptors for parathyroid hormone (PTH) [9], vasoactive intestinal peptide (VIP) [10], calcitonin [11] or pituitary adenylate cyclase activating peptide (PACAP) [12], the N-terminal domain contains six conserved cysteine residues, which are assumed to form three disulfide bonds. It was shown for the PTH-receptor [13–15] and the VIP-receptor [16] that intact disulfide bonds are essential for functionality.

Unfortunately, receptors of this class are not yet available in amounts that will allow their structural analysis. To date, only the receptors for vasoactive intestinal peptide (VIP) [17] and pituitary adenylate cyclase activating peptide (PACAP) [18] have been purified in the sub-milligram range from overexpressing eucaryotic cell lines.

As the extracellular domain of the GLP-1 receptor can specifically bind its peptide [19], we set out to generate large amounts of that portion of the molecule for functional and structural analysis.

2. Materials and methods

2.1. Construction of expression plasmid p(nGLP1R)

A gene fragment, coding for the extracellular part of the human GLP-1 receptor, nGLP1R, was amplified by PCR from a cDNA encoding the human GLP-1 receptor using primers 5′-AAAGAGCTCGCCGGCCCCCGCCCC-3′ and 5′-TTTAAGCTTTTATTTGCGTTTGCGTTTGC-GTTTGCGACCGTAGAGGAACAGGAGCTG-3′. The fragment was inserted into pQE30 (Qiagen) using *Sph*I and *Hind*III restriction sites, and in a second step the codons for four amino acids Ala, Cys, Glu, and Leu between the hexahistidine tag and the coding region of the N-terminus of the GLP-1 receptor, were deleted using the QuikChange site-directed mutagenesis kit of Stratagene with primers 5′-CACCATCACCATGGATCCGC-CGGCCCC-CGCCCCCA-3′ and 5′-TGGGGGCG-GGGGCCGGCGGATCCATGGTGATGGTG-3′. Both strands of the resulting construct were sequenced and found to encode the desired protein.

2.2. Expression and isolation of protein

E. coli strain M15 (pREP4) (Qiagen) bearing p(nGLP1R) was grown exponentially in a 5-l fed-batch process on minimal medium [20]. The growth rate was restricted by constant feeding with glucose, and protein expression was induced with 0.4 mM IPTG when cells had reached an optical density of 50 at 600 nm. Cells were cultivated for an additional 4 h and 700 g of wet cells were harvested by centrifugation. Lysis of the cells and isolation of the inclusion bodies were performed as described by Rudolph et al. [21]. Approximately 10 g of inclusion body protein was prepared from a single fermentation.

Inclusion bodies were solubilized in 50 ml of 6 M GdmHCl, 0.1 M Tris, 1 mM EDTA, 100 mM DTT, pH 8.0 for 2 h at room temperature at a protein concentration of 10 mg/ml. After adjusting the pH of the solution to pH 4 with HCl, insoluble material was removed by centrifugation at 20 000 rpm for 15 min. The supernatant was dialyzed twice against 1 l of 4 M GdmHCl, pH 3.0 to remove DTT. The dialyzed protein solution was centrifuged as above and frozen in aliquots for further use.

2.3. Renaturation and purification

For renaturation of nGLP1R a folding reactor (FairMenTech, Göttingen) was used, which allows

repeated addition of denatured protein from a cooled reservoir into a stirred and temperature controlled vessel containing the refolding solution. Six 5-ml pulses of solubilized protein were injected in intervals of 6 h into the vessel containing 500 ml of 100 mM Tris pH 8.5, 500 mM L-arginine, 1 mM GSH, 5 mM GSSG, and 1 mM EDTA (buffer A). The stirrer was set to 400 rpm at a temperature of 10 °C.

After 36 h the vessel was emptied and the refolding solution was centrifuged at 20 000 rpm for 30 min. The supernatant was subsequently diluted with 1500 ml of 20 mM Tris buffer, 1 mM EDTA, pH 7.4 (buffer B) and formed precipitate was again removed by centrifugation for 1 h at 10 000 rpm. For concentration and crude purification, 20 ml of SP-Sepharose Fast Flow (Pharmacia) equilibrated in buffer B was added and binding was allowed for 30 min. The material was poured into a column, washed with buffer B and the receptor fragment was eluted with 1 M NaCl in the same buffer.

The eluted protein was diluted 10-fold into buffer B and applied onto a 5-ml HiTrap SP-Sepharose (Pharmacia) column. After washing with buffer B, receptor fragment was eluted using a salt gradient from 150 mM to 1 M NaCl. The N-terminal fragment of the GLP-1 receptor eluted at approximately 550 mM NaCl. Finally, the protein was loaded onto a gel filtration Superdex 75 prep grade column (Pharmacia) in 0.7 M NaCl, 20 mM Tris, 1 mM EDTA, pH 7.4 and eluted at a flow rate of 1 ml/min.

Protein concentration was determined spectrophotometrically using an adsorption coefficient of $\varepsilon_{280nm} = 46\,325\ M^{-1}\ cm^{-1}$, calculated according to Gill and von Hippel [22]

2.4. Spectroscopic analysis by circular dichroism (CD)

CD spectra were measured using an AVIV 62A DS Spectropolarimeter (AVIV, Lakewood, NJ, USA) in a 1-mm quartz cuvette at 22 °C at a protein concentration of 5.8 μM ($M_w = 17131.9$) in 0.7 M NaCl, 20 mM Tris, pH 7.4 or 7 μM in 6 M GdHCl, 20 mM Tris, pH 7.4. Data were collected at 1-nm intervals over the range 260–200 nm for native protein, and 260–211 nm for denatured protein with a collection time of 5 s/data point. The spectra were accumulated five-fold, corrected for buffer contributions, and converted to mean residue ellipticity according to Schmid [23] using a calculated molecular mass of M_r (nGLP1R) = 17131.9. The secondary structure content was calculated using the program CDNN [24].

2.5. Differential scanning calorimetry

A VP-DSC Micro Calorimeter (MicroCal, Northampton, MA, USA) was used for the calorimetric experiments. The temperature scans were performed at a scan rate of 1 °C/min on samples containing 0.2–0.4 mg/ml of nGLP1R in 0.7 M NaCl, 50 mM Na-Phosphate, 1 mM EDTA, pH 7.4. Prior to the measurements, solutions were degassed. The reversibility of the DSC transitions was verified by reheating the sample in the calorimetric cell after cooling from the first scan. Data were analyzed with MicroCal Origin 4.1 software (Microcal Software, Northampton, MA, USA).

2.6. Multiple peptide synthesis

GLP-1[7–36]-NH_2 and [Ahx_4 37–40,C41]-GLP-1-[7–41]-NH_2 (cysteinylated GLP-1) were synthesized by solid-phase methodology on a SyRo II multiple peptide synthesizer (MultiSynTech, Bochum) applying Fmoc/tBu strategy. The 4-(2′,4′-dimethoxyphenyl-aminomethyl)-phenoxy-group (Rink amide anchor [25]) was used as an anchor molecule on polystyrene-(1%)-divinylbenzene resin (RAM©-resin, Rapp Polymere, Tübingen; 40 mg, 28 μmol/peptide) in order to obtain the peptide amides. The Fmoc-protected amino acids (Nova Biochem, Bad Soden) were coupled in a five-fold excess for 30 min. 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) (1 eq.) was used as activating reagent. Deprotection was carried out in piperidine/dimethylformamide (1:1 v/v) for 3 min and (1:3 v/v) for 5 min. Cleavage was performed in trifluoroacetic acid/ethanedithiol/dimethylsulfide/*m*-cresol/water (80:5:5:5:5) within 2 h. Yields of crude GLP-1 analogues after cleavage

from the resin were approximately 60%. The peptides were purified by preparative HPLC on a nucleosil C_{18} column (5 μ; 25×250 mm; Grom, Ammerbuch). The resulting purity was above 95%. The peptides were characterized using electrospray mass spectrometry. Mass spectra were recorded on an AutoSpec T mass spectrometer equipped with an Electrospray interface (Fisons Instruments, Manchester). The mass values for all synthetic peptides were identical with the calculated values within the limits of the method.

2.7. Cross-linking of receptor and ligand

nGLP1R (0.87 μM in 100 mM borat, 300 mM NaCl, pH 8.5) was mixed with GLP-1-[7-36]-NH_2 or with insulin. After incubation for 20 min at room temperature, dry dimethylpimelidate was added to a final concentration of 8 mM. Samples were incubated for an additional hour, precipitated with TCA, boiled with electrophoresis buffer, and analyzed on SDS-PAGE.

2.8. Determination of binding constants using surface plasmon resonance

In order to determine the dissociation constant of refolded nGLP1R with respect to ligand binding, surface plasmon resonance measurements using a BIAcore™ X-System (BIACORE AB, Uppsala, Sweden) were carried out.

nGLP1R in 20 mM phosphate, 150 mM NaCl, and 1 mM EDTA, pH 7.4 was immobilized onto the CM5 chip using NHS/EDC activation and amine coupling with a derivatization level of 1200 RU. Binding experiments with GLP-1[7–36]amide were performed in buffer B containing 250 mM L-arginine and 0.05% Tween 20. The chip was regenerated by 3 M KCl. Any unspecific binding was monitored on a second surface that was blocked with ethanolamine.

In another set of experiments cysteinylated GLP-1 was coupled to a PDEA-activated Biacore CM5 chip according to the instruction of the supplier. Approximately 50 RU of peptide were routinely immobilized. The second surface was blocked with cysteine. Protein samples were dialyzed against buffer B containing 250 mM L-arginine and 0.05% Tween 20, and injected at a flow rate of 30 μl/min. Regeneration was performed with a pulse of 6 M urea and 100 mM formic acid, pH 2. For analysis, binding curves were fitted using the BIA evaluation 3.0 software (Biacore AB, Uppsala, Sweden) according to 1:1 Langmuir binding.

In competition experiments, pre-incubation of nGLP1R was carried out with different concentrations of GLP-1[7–36]-NH_2 for 20 min at room temperature. The mixture was subsequently injected onto the sensor chip surface at a flow rate of 30 μl/min. Calculation of the dissociation constant was performed according to Eqs. (1) and (2).

$$S = S_0 + \Delta S_{\max} \times \frac{(RL)}{(R)_0} \tag{1}$$

with S as the measured resonance signal after reaching the equilibrium plateau, S_0 as resonance signal without competing peptide, $\Delta S_{\max}$ as difference between S_0 and S in the presence of a saturating concentration of competing peptide, and (RL) and $(R)_0$ as concentrations of receptor–ligand complex and total receptor, respectively. According to the law of mass–action, the concentration of receptor–ligand complex is

$$(RL) = \frac{\left[(R)_0 + (L)_0 + K_d\right]}{2} - \sqrt{\left\{\frac{\left[(R)_0 + (L)_0 + K_d\right]}{2}\right\}^2 - R_0 \times (L)_0}. \tag{2}$$

2.9. Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry experiments were carried out using a MicroCal ITC titration calorimeter (MicroCal. Inc., Nothhampton, MA, USA) using procedures previously described by Wiseman et al. [26]. Titration of GLP-1 (26.3 μM) into a nGLP1R solution (2.04 μM) was performed at 20 °C in 0.25 M L-arginine, 50 mM phosphate, and 1 mM EDTA, pH 7.4. The data were analyzed with ORIGIN software (Microcal Software, Northampton, MA, USA).

2.10. Affinity chromatography

Two milligrams of cysteinylated GLP-1 was coupled to 1 ml of SulfoLink Gel (Pierce) according to the instructions of the supplier. The renatured protein was dialyzed against 0.25 M L-arginine in buffer B and loaded onto the affinity matrix. After incubation at room temperature for 1 h the matrix was washed with five column volumes of buffer B containing 0.25 M L-arginine. Protein was eluted with 5 mg/ml GLP-1[7–36]amide at room temperature. Strongly bound material was removed by 6 M urea, 100 mM formic acid, pH 2 and fractions were analyzed by SDS-PAGE.

2.11. Disulfide pattern analysis

Refolded and purified nGLP1R was transferred into 100 mM Tris, 10 mM $CaCl_2$, pH 7.8 by dialysis. The final protein concentration was 32 μg/ml. To 0.5 ml of this solution 2 μl of a chymotrypsin solution (1 mg/ml of chymotrypsin in 1 mM HCl, Chymotrypsin sequencing grade, Roche Biochemicals) were added and the protein was digested overnight at 37 °C.

The digest was first analyzed and subsequently separated by reversed-phase HPLC using a YMC ODS-AQ C 18 column (pore-size = 120 Å, particle size = 5 μm, length = 25 cm, and inner diameter = 1 mm). One hundred and fifty microliters of the digest was injected and peptides were eluted with a linear gradient (eluent A, 0.1% TFA in water; eluent B, 80% acetonitrile, 20% water, 0.1% TFA; slope of the separating part of the gradient, 0.67% B/min; flowrate, 50 μl/min; and column temperature = 35 °C). For disulfide elucidation, two HPLC runs were performed.

In the first run (referred to as analytical in the following), cyst(e)ine-containing peptides were detected by a post-column reaction employing a methodology previously described by Thannhauser et al. [27]. In this procedure, disulfides (R_1SSR_2) are first cleaved by sulfitolysis:

$$R_1SSR_2 + SO_3^{2-} \xrightarrow{pH>9} R_1SSO_3^- + R_2S^-.$$

The detection is then based on the reaction of the thiolate anions RS^- with NTSB (2-nitro-5-thiosulfobenzoate):

$$NTSB + RS^- \xrightarrow{pH>9} NTB + R_1SSO_3^- \quad (pH > 9).$$

For on-line detection, a solution containing NTSB and sodium sulfite was added between the column and the detector. The time available for the reaction was approximately 1 min. Peptides were detected at 220 nm; the reaction product of cyst(e)in containing peptides, NTB (2-nitro-5-thiobenzoate), was detected at 432 nm.

A stock solution of NTSB was prepared by dissolving 10 g of DTNB [5,5′-dithio-bis(2-nitrobenzoate)] in 800 ml of 1 M Na_2SO_3, pH 7.5. The solution was brought to 38 °C, and oxygen was bubbled through until the solution had turned from bright red to pale yellow. The final reagent solution was prepared by diluting the NTSB stock solution tenfold with 50 mM glycine, 100 mM Na_2SO_3, pH 9.5 (adjusted with NaOH).

In the second run (referred to as collection run in the following), 50-μl fractions were collected without post-column reaction. Fractions containing cyst(e)ine, as identified by the analytical run, were further analyzed by Edman sequencing [instrument: Procise, Applied Biosystems, Foster City, USA, 20 μl of the RP-HPLC eluate were loaded using ProSorb cartridges (Applied Biosystems)].

3. Results

3.1. Expression of recombinant protein

A fragment encoding the N-terminal 125 amino acids of the GLP-1 receptor without the putative signal sequence was amplified by PCR from a plasmid containing the entire coding sequence of the human GLP-1 receptor [28] and inserted into a pQE30-vector. The downstream primer contained a coding region for a stretch of eight consecutive positively charged residues to generate a polyionic tag. Vector insertion led to the addition of a further 16 amino acids at the N-terminus of the receptor fragment including a hexahistidine tag, which was necessary for overexpression of the protein, and one extra cysteine. The hexahistidine tag was necessary for overexpression of the protein. In order to circumvent potential problems with the

analysis of disulfide connectivity, the extra cystein and three neighboring amino acids were removed in the final construct, nGLP1R, which is shown in Fig. 1.

Expression of recombinant protein was carried out by fed-batch fermentation of *E. coli* M15 p(nGLP1R). The receptor fragment was expressed in high yield by induction with IPTG. Approximately 10 g of recombinant protein were recovered from 700-g cell paste as inclusion bodies. As shown in Fig. 2 (lane 3), most of the cellular proteins are removed after inclusion body isolation. Inclusion bodies were solubilized using 6 M GdmHCl under strongly reducing conditions. After removal of the reductant DTT by dialysis the nGLP1R solution was frozen and stored at −80 °C.

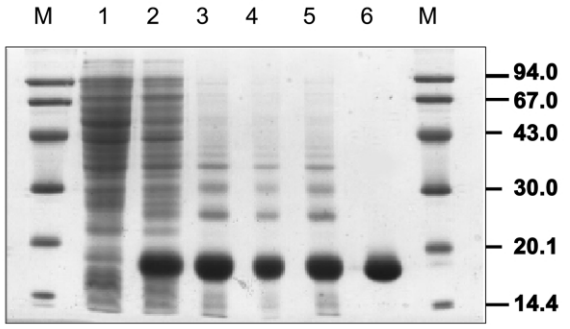

Fig. 2. Isolation of active extracellular GLP-1 receptor fragment. Protein samples were taken at different stages of the purification and analyzed by SDS-PAGE. (M) Marker proteins (lane 1), *E. coli* lysate from non-induced cells (lane 2), *E. coli* lysate from induced cells (lane 3), inclusion bodies (lane 4), insoluble fraction after refolding (lane 5), soluble fraction after refolding (lane 6), eluted fraction from Superdex 75 prep grade column.

3.2. Renaturation, purification and characterization of the receptor fragment

Pulse renaturation [29], which allows refolding of proteins at rather high concentrations while avoiding the formation of aggregates [30], was used for refolding of nGLP1R. After addition of six 5-ml pulses in 6-h time-intervals into a refolding buffer containing 500 mM L-arginine and a redox system of oxidized and reduced glutathione, precipitated material was removed by centrifugation. The L-arginine stabilizes the native state of a protein by preferential hydration [31,32], and it reduces unproductive aggregation of unfolded polypeptides or of folding intermediates by increasing their solubility during folding [33]. The oxido-shuffling system allows rapid reshuffling of incorrect disulfide bonds [21]. The renatured protein solution was concentrated on SP-Sepharose, diluted to lower the salt concentration, and then subjected to chromatography on SP-Sepharose. An additional purification step was performed using size exclusion chromatography. The eluted protein from the gel filtration column was shown to be homogeneous when analyzed by SDS-PAGE (Fig. 2, lane 6). The identity of the renatured and purified receptor fragment was confirmed by partial amino acid sequencing of the first 20 amino acids. The yield of refolded and purified protein

```
                                13
                  MR GSHHHHHHGS AGPRPQGATV SLWETVQKWR EYRRQCQRSL

43   TEDPPPATDL FCNRTFDEYA CWPDGEPGSF VNVSCPWYLP WASSVPQGHV

93   YRFCTAEGLW LQKDNSSLPW RDLSECEESK RGERSSPEEQ LLFLYGRKRK

143  RKRKN
```

Fig. 1. Sequence of the cloned and expressed extracellular fragment of the GLP-1 receptor. Authentic portions of the sequence are shown in bold, the added tags are in italics.

was approximately 10% of starting inclusion body material.

To obtain information about the secondary structure of the refolded protein, far-UV CD spectra were measured under native and denaturing conditions (Fig. 3). The spectrum of the native species showed a high content of secondary structure, corresponding to 30% α-helix and 16% β-strand, as determined by the CDNN software [24]. However, since the CD spectrum of the native protein did not resemble a classical helix-dominated spectrum, one should consider these calculated values only as an estimation. The denatured protein corresponds to the CD-spectrum of a fully extended polypeptide chain [23].

The temperature-induced unfolding of the receptor fragment was studied by differential scanning calorimetry. The midpoint of the thermal transition is 44 °C with an apparent $\Delta H_{\text{unfolding}}$ of 74 kcal/mol (Fig. 4). By performing repeated DSC scans of each sample the transitions were found to be reversible to 60–70%.

Clearly, nGLP1R forms after refolding a homogenous, stable and structured protein domain.

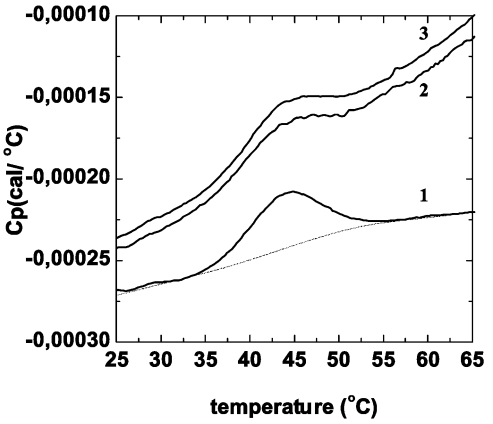

Fig. 4. Differential scanning calorimetry with nGLP-1 receptor fragment. Temperature scans at a scan rate of 1 °C/min were performed from 20 to 80 °C at a protein concentration of 0.3 mg/ml in 0.25 M L-arginine, 50 mM Na-Phosphate, 1 mM EDTA, pH 7.4. By performing repeated DSC scans of each sample (1, first scan; 2, second scan; and 3, third scan), the transitions were found to be reversible to 60-70%. Data were analyzed with MicroCal Origin 4.1 software, yielding a T_m of 44 °C and a $\Delta H_{\text{unfolding}}$ of 74 kcal/mol. The dotted line constitutes the calculated baseline of the first scan.

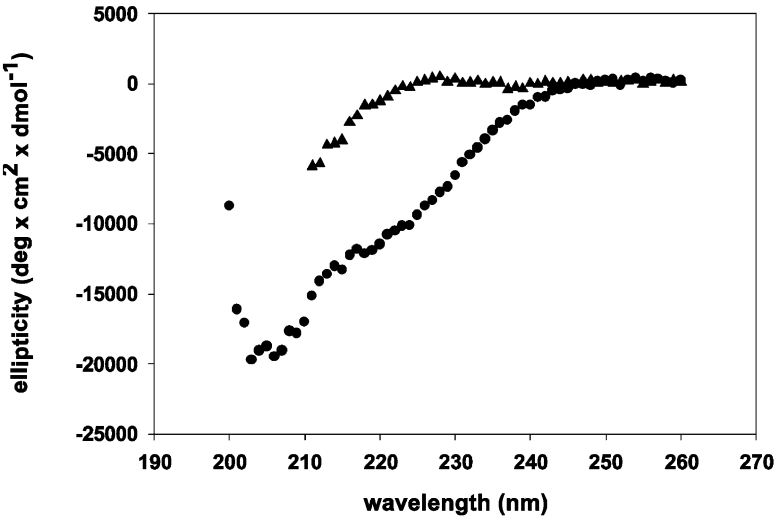

Fig. 3. CD spectra of the extracellular domain of the GLP-1 receptor. CD spectra of the protein under native conditions (●) and under denaturing conditions (▲). Data were collected at 1-nm intervals over the range 260–200 nm for native protein, and 260–211 nm for denatured protein with a collection time of 5 s/data point. The spectra were accumulated five-fold, corrected for buffer contributions, and converted to mean residue ellipticity according to Schmid [23]. The secondary structure content was calculated using the program CDNN [24].

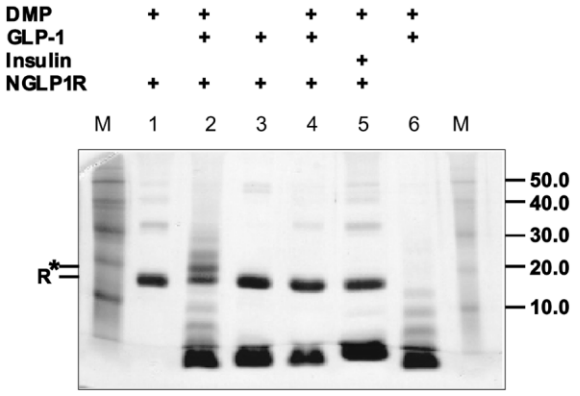

Fig. 5. Cross-linking between the extracellular fragment of the GLP-1 receptor and its ligand. Receptor fragment (0.87 μM) was incubated with GLP-1[7–36]-NH_2 (4.3 μM) or insulin (4.3 μM) for 20 min in 100 mM borate, 300 mM NaCl, pH 8.5. Where indicated, dimethylpimelidate (8 mM) was added for 1 h. The cross-linking reaction was stopped with TCA, the samples were washed with acetone, boiled in sample buffer for 5 min, and analyzed on SDS-PAGE. R indicates the unmodified receptor, and cross-linked complex (lane 2) is indicated by an asterisk. As a control, the receptor was incubated in 50 mM DTT, 2% SDS for 10 min at 50 °C before starting the cross-linking procedure. No additional band was detected on SDS-gel in the appropriate size of a receptor–ligand cross-linked product (lane 4). Also, no receptor–ligand cross-linked product could be detected in a control reaction of the receptor fragment with insulin (lane 5). Multiple bands in lanes 1, 2, 5 and 6 are oligomers of nGLP1R or peptide.

3.3. Binding experiments

The N-terminal, extracellular fragments of group B GPCRs are believed to provide high affinity binding sites for their respective ligands [6–8,19]. To analyze the binding characteristics of nGLP1R we first confirmed the existence of the receptor–ligand complex in a cross-linking experiment. Receptor fragment and GLP-1[7–36]amide were incubated for 20 min and cross-linked using the homobifunctional amine reactive dimethylpimelidate. After cross-linking, a new band appeared on SDS-PAGE with a molecular weight corresponding to the added molecular mass of receptor and ligand (Fig. 5, lane 2). As a control, the receptor was denatured and reduced in 50 mM DTT, 2% SDS for 10 min at 50 °C before starting the cross-linking procedure. No additional band was detected on SDS-gel in the appropriate size of a receptor-ligand cross-linked product (Fig. 5, lane 4). Also, no receptor–ligand cross-linked product could be detected in a control reaction of nGLP1R with the peptide hormone insulin (Fig. 5, lane 5).

As a cross-linking experiment is only a first proof for a specific interaction between two components, we further analyzed binding with surface plasmon resonance.

nGLP1R was immobilized onto a Biacore CM5 sensor chip in random orientation using the amine coupling protocol and the chip was probed with GLP-1, parathyroid hormone and calcitonin (Fig. 6a). Only the authentic ligand, GLP1, was found to bind to the immobilized protein in a concentration-dependent manner indicating a specific interaction. The signal after equilibrium binding of the peptide to the immobilized fragment was lower than expected from the initial derivatization levels. This could be due to partial denaturation of the receptor fragment during the coupling procedure. In addition, binding to the receptor fragments might be affected by steric hindrance, as the amine coupling produces randomly oriented proteins at the surface. For these reasons, we could not calculate a dissociation constant with this set of experiments.

To characterize the binding in a quantitative manner the binding assay was inverted. Cysteinylated GLP-1 peptide was coupled to a Biacore CM5 sensor chip after surface activation with PDEA. Renatured extracellular receptor fragments were used as mobile analyte in the binding experiment. The running buffer contained 250 mM L-arginine, an additive used for in vitro folding of proteins [33], as this amount of additive was shown to stabilize the receptor fragment against aggregation while not affecting the binding properties. However, concentrations of L-arginine of at least 0.5 M abolished the binding completely (data not shown).

The K_d was derived from a series of binding experiments using different concentrations of renatured nGLP1R (Fig. 6b). A single K_d value of 46 ± 17 nM was derived after fitting the data with a 1:1 Langmuir model.

As expected, binding of receptor to the sensor surface was no longer detectable after denaturation

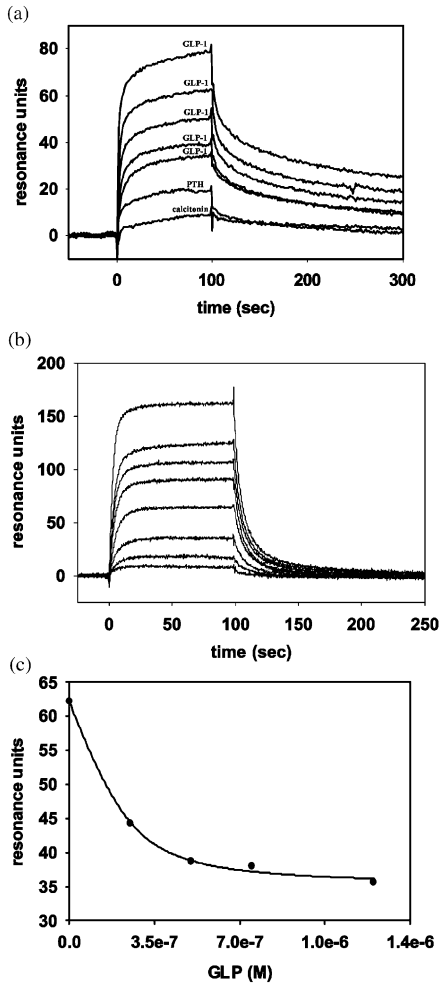

Fig. 6. Kinetics of interactions between GLP-1 and receptor fragment. (a) Binding of different ligands to the immobilized receptor. Receptor fragment was immobilized onto a CM5 chip and probed with different ligands (top to bottom): 28 μM GLP-1; 14 μM GLP-1; 7 μM GLP-1; 3.5 μM GLP-1; 1.75 μM GLP-1; 28 μM PTH; and 28 μM calcitonin. (b) Titration with receptor fragment. Cysteinylated GLP-1 was immobilized onto the CM5 chip and refolded and purified receptor fragment was used as analyte at different concentrations (top to bottom): 200; 100; 80; 60; 40; 20; 10; and 5 nM. A single K_d value of 46 ± 17 nM was derived after fitting the data with a 1:1 Langmuir model. (c) Competition experiment: 0.25 μM receptor fragment was preincubated with increasing concentrations of GLP-1 for 20 min at room temperature and injected onto CM5 chip with immobilized cysteinylated GLP-1. The measured plateau values with respect to the competitor concentration are shown. The dissociation constant determined in the competition experiment was 47 ± 0.79 nM.

of the protein with 2% SDS and 50 mM DTT at 95 °C (data not shown).

In addition, a competition experiment on the GLP1-sensor chip was carried out by preincubation of nGLP1R with increasing amounts of ligand. As expected, the amount of uncomplexed receptor decreased as more GLP-1 is present in the preincubation mixture (Fig. 6c). Data analysis was carried out by non-linear regression, resulting in a dissociation constant of 47 ± 0.79 nM.

As a second method to quantitatively assess the binding of GLP-1 to nGLP1R, isothermal titration calorimetry was used. Titration of GLP-1 with nGLP1R leads to the release of heat upon binding (Fig. 7). Analysis of data revealed a 1:1 stoichiometry of the two binding partners, a K_d value of 144 nM and an apparent ΔH_{ITC} of -24.5 kcal/mol.

The data shown here clearly demonstrate that the refolded receptor fragment nGLP1R exhibits specific binding to its authentic ligand, with an

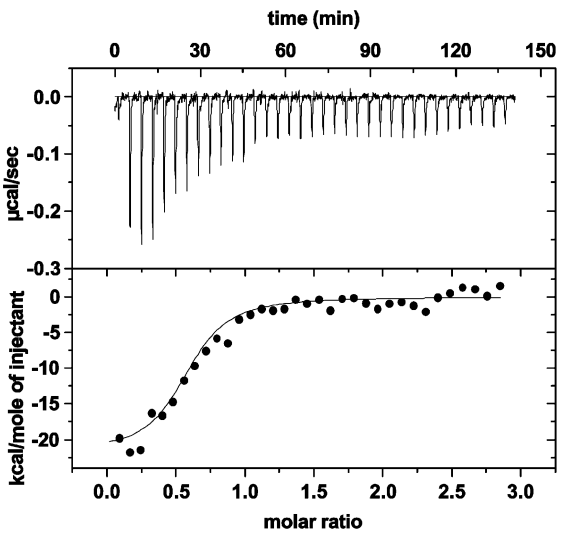

Fig. 7. Isothermal titration calorimetry with nGLP1R and GLP-1. A representative isotherm for binding of GLP-1 to its receptor fragment in 0.25 M L-arginine, 50 mM phosphate, and 1 mM EDTA, pH 7.3 at 20 °C is shown. Top, the baseline subtracted raw data; bottom, the peak-integrated and concentration-normalized enthalpy change vs. GLP-1/nGLP1R ratio. The solid line is the best fit to the experimental data according a 1:1 binding model. The calculated K_d value is 144 nM and ΔH_{ITC} is 24.49 kcal/mol.

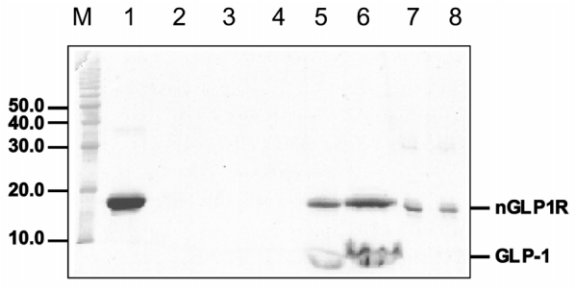

Fig. 8. Affinity chromatography of the receptor fragment. Cysteinylated GLP-1 was immobilized onto SulfoLink Gel and the matrix was probed with refolded and purified receptor fragment. Load (lane 1); flow-through (lane 2); wash (lanes 3 and 4); eluate after addition of 5 mg/ml GLP-1-[6–37]amide (lanes 5 and 6); and eluate with 6 M urea, 100 mM formic acid, pH 2 (lanes 7 and 8).

affinity less than 100–200-fold lower as data previously given in the literature [34–36] for the full-length receptor in the plasma membrane.

3.4. Analysis of homogeneity

Although the receptor fragment was found to be homogeneous and monomeric by means of SDS-PAGE, analytical ultracentrifugation and analytical gel filtration (data not shown), and by binding studies, one cannot exclude the existence of incorrectly folded, inactive species. To estimate the portion of active molecules in our refolding product, we decided to introduce an affinity chromatography step. For that, cysteinylated GLP-1[7–36] was covalently fixed onto an activated support. Receptor fragment was then equilibrated in buffer B containing 250 mM L-arginine and loaded onto the affinity matrix. As shown in Fig. 8, most of the material ($\geq$80%) was bound to the affinity matrix. Binding could be competed with GLP-1-[7-36]-amide and resulted in almost complete recovery of bound molecules.

3.5. Analysis of the disulfide pattern

Human GLP-1 receptor possesses six cysteines in the N-terminal, extracellular domain. These cysteines are conserved among class B of G-protein coupled receptors and are believed to form three essential and conserved disulfide bonds. In order to analyze the disulfide bond pattern of nGLP1R, a protein digest was performed and the resulting fragments were analyzed by RP-HPLC, Edman-sequencing and mass spectrometry.

Fig. 9 illustrates the RP-HPLC diagrams obtained for the analytical run (detection at 220 and 432 nm, including on-line cyst(e)ine detection) and the collection run (detection at 220 nm, including fraction collection) of a chymotryptic digest of nGLP1R. Cyst(e)ine-containing peptides, i.e. peptides exhibiting 432-nm signals, were only observed at retention times above 55 min. The cyst(e)ine-containing fractions were further analyzed by Edman sequencing and electrospray mass spectrometry. The results of these analyses are compiled in Table 1, and demonstrate that Cys46 and Cys71, as well as Cys62 and Cys104, are connected by disulfide bonds. The third disulfide linkage is formed by Cys85 and Cys126.

4. Discussion

The receptor for GLP-1 belongs to the class B of GPCRs that bind to oligopeptidic ligands [37]. Due to the poor accessibility of all these receptors from natural sources only two of them, the receptor for vasointestinal peptide and that for pituitary adenylate cyclase activating peptide, have been isolated so far. Still, these quantities do not allow structural characterization of the receptors. We therefore decided to utilize *E. coli* as a high-level expression system and to refold the protein of interest from insoluble and inactive material. The extracellular, N-terminal domain, nGLP1R, was chosen as a target as it was shown to be necessary and sufficient for ligand binding [19].

For expression and purification nGLP1R was equipped with a N-terminal hexahistidine tag, which was necessary for overexpression of the protein, and a polycationic sequence at its C-terminal end, which facilitates purification by ion exchange chromatography. The protein was isolated in the form of inclusion bodies from the host and solubilized using chaotropic agents. Approximately 10% of the initially provided material was refolded into an active conformation, and subsequently concentrated and purified by cation exchange chromatography and size exclusion chro-

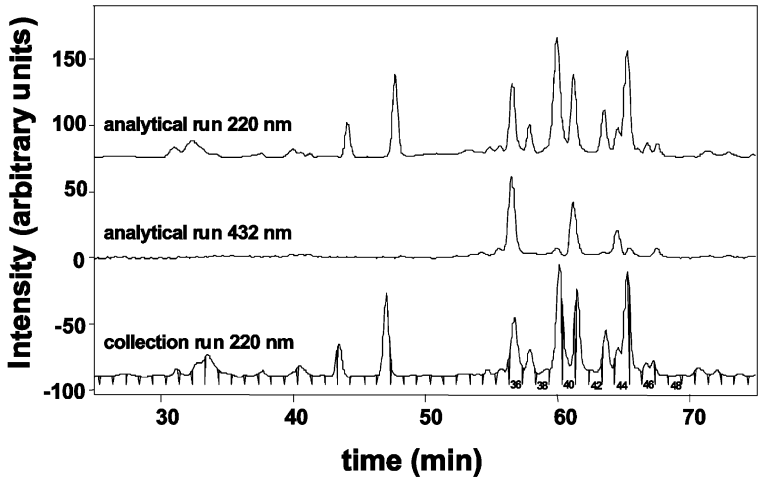

Fig. 9. Analysis of disulfide pattern. RP chromatograms obtained for the analytical run (detection at 220 nm before post-column reaction and at 432 nm after the cyst(e)ine-specific reaction), and the collection run (fractions collected without post-column reaction) of the chymotryptic digest of an nGLP-1 receptor sample. The 432 nm chromatogram was multiplied by 10 to facilitate comparison. The cyst(e)ine-containing fractions were collected and further analyzed by automated Edman sequencing and electrospray mass spectrometry.

Table 1
Edman sequencing of cyst(e)ine containing fractions of the collection run shown in Fig. 9

Fraction No.	Number of sequencing cycles	nGLP1R amino acid sequences determined[a]	Amount (pmol)[b]	Interpretation[c]
36	15	Y|A-WPDGEPGSF	**8**	C_{38}–C_{63} disulfide linkage between
		Y|RRQ-Q	**8**	AC_{38}WPDGEPGSF and RRQC_{63}Q
		F|-N-TF	**4**	C_{54}–C_{96} disulfide linkage between
		F|-TAEGLW	**4**	C_{54}NRTF and C_{96}TAEGLW
39	15	W|LQKDNSSLPW	20	LQKDNSSLPW
		E|Y--WP-GE-GS	**1**	C_{38}–C_{63} disulfide linkage between
		Y|--WP-GE-GSF	**1**	YAC_{38}WPDGEPGS... or AC_{38}WPDGEPGSF
		Y|RR—QR	**2**	and RRQC_{63}QR...
41	15	Y|VNVS-PW	**8**	C_{77}–C_{118} disulfide linkage between
		WvRDLSE-EESKRGERS	**8**	VNVSC_{77}PW and RDLSEEC_{118}EESKRGERS...
		L|TEDPPPATDLF	7	TEDPPPATDLF
44	15	F|-N-TFDEY	**6**	C_{54}–C_{96} disulfide linkage between
		F|-TAEGLW	**6**	C_{54}NRTFDEY and C_{96}TAEGLW
		W|YLPW	20	YLPW

[a] -: Determination of amino acid not possible due to background, carry-over from previous cycle, presence of amino acid with low detection efficiency or presence of cysteine. X|:X indicates the amino acid preceding the sequence determined.

[b] As estimated from the initial yield of the corresponding amino acid sequence, and **bold** numbers correspond to disulfide linked peptides.

[c] …:C-terminus not defined by the sequencing results.

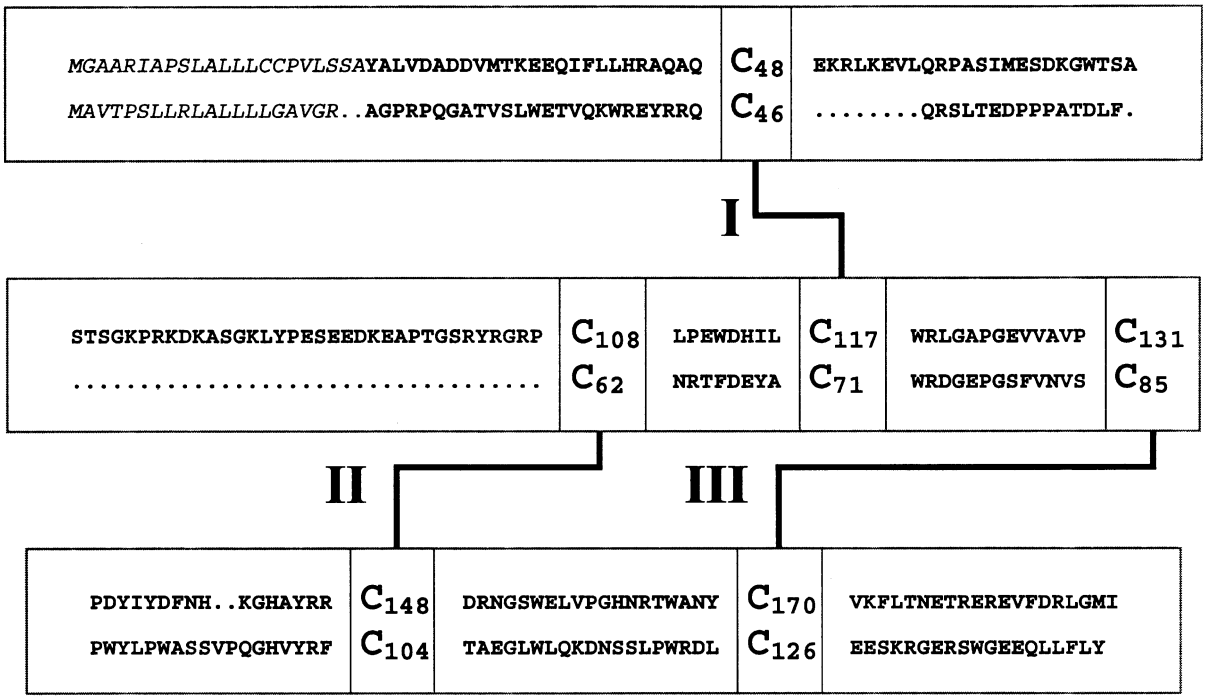

Fig. 10. Disulfide connectivity of the N-terminal fragments of human GLP-1 receptor and PTH receptor. Top, sequence of nPTHR; and bottom, sequence of the nGLP1R. The putative signal sequences of both receptors are shown in italics. Disulfide bridges are numbered with Roman numerals.

matography. The purified product showed homogeneity with respect to molecular mass and behavior on analytical ultracentrifugation, analytical gel filtration, reversed phase HPLC, and binding characteristics.

The refolded fragment was shown to bind its ligand and the accompanying complex formation could be followed by cross-linking, surface plasmon resonance spectroscopy, and isothermal titration calorimetry. The receptor fragment has a lower affinity to its peptide than the complete receptor embedded in the plasma membrane. Assuming functional homogeneity of the protein the dissociation constants of nGLP1R determined by surface plasmon resonance, and isothermal titration calorimetry studies were between 46 and 144 nM. In contrast, the complete GLP-1 receptor, when expressed in eukaryotic cell lines, was found to bind its ligand with a K_d between 0.5 and 1 nM [34–36]. A similar decrease in affinity between complete receptor and the extracellular domain was also observed for the PACAP-receptor [12] and PTH receptor [15]. Possibly, the N-terminal, extracellular portion of these receptors provides one high-affinity binding site for the ligand that will be supplemented by other interactions such as interactions with extracellular loops or transmembrane regions [38–42]. The affinities of some G-protein coupled receptors are dependent on the coupling of heterotrimeric G proteins, as shown for the somatostatin receptor [43] and the latrotoxin receptor [44]. The absence of such a mediator protein could also lead to a lower affinity of nGLP1R for GLP-1.

In addition to quantitative characterization of binding affinities, we demonstrated the homogeneity of the sample with respect to its binding activity, as more than 80% of the material was found to be specifically bound to and eluted from an immobilized ligand. It is very likely that some of the protein adsorbs unspecifically to the column matrix and cannot be eluted, thus lowering recov-

ery from the matrix to only 80%. Hence, these findings do not necessarily indicate that the remaining 20% are non-functional.

Here, we report a procedure that can generate large amounts of the extracellular domain of the GLP-1 receptor. This procedure provides a valuable tool for further structural characterization of the extracellular portion of the human GLP-1 receptor by NMR or X-ray crystallography.

As a first step towards the structural analysis of this receptor domain, we determined the disulfide connectivity of its six cysteine residues. The presence of these extracellular cysteine residues is highly conserved among the members of this family of proteins, thus underlining their structural importance. Mutational analysis of cysteine residues in the extracellular portion of the VIP receptor has identified six cysteine residues essential for ligand binding [45]. Moreover, treatment of such receptors with reductants was shown to completely abolish the binding of their respective ligands [19,14]. Our analysis revealed a disulfide bond pattern with links between cysteines 46 and 71, 62 and 104, and between 85 and 126. The disulfide pattern of the extracellular ligand-binding domain of human parathyroid hormone receptor, which also belongs to class B of GPCRs, was recently published by Grauschopf [15]. The connection of cysteine residues was shown to be the same as in nGLP1R, supporting the hypothesis of a conserved disulfide pattern of this receptor class (Fig. 10). This pattern provides strong sterical constraints on the structure of these domains and will be used as a template for modeling approaches.

Acknowledgments

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