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Photoreactive insulin derivatives for the detection of the doubly labeled insulin receptor☆

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Abstract

Two different photoinsulins, the radioactive $N^{\epsilon B29}$-(4-azidosalicyloyl) insulin and the novel biotinylated $N^{\epsilon B29}$-(4-azidotetrafluorobenzoyl-biocytinyl) insulin, were synthesized in order to study the binding stoichiometry of insulin and the insulin receptor in a direct approach. Both derivatives were cross-linked simultaneously to the $(\alpha\beta)_2$ receptor. Insulin-receptor conjugates were formed that carry a radioactive label as well as a biotin label. The doubly labeled complexes were isolated by streptavidin-affinity chromatography. Analysis of both markers, the radioactive ^{125}I marker and the biotin marker, proved the existence of a covalent complex of one receptor molecule and two ligands. Thus, orthogonal photocross-linking is introduced as a method for the isolation and analysis of bivalent receptor complexes.

Keywords: Insulin; Insulin receptor; Photoaffinity labeling; Biotin; Bivalence

1. Introduction

The action of insulin is mediated by the insulin receptor, which is important for glucose, lipid, and other metabolic pathways. The insulin receptor is a functional dimer containing two identical (α,β)-subunits. The α-subunits are located in the extracellular space and they contain the insulin binding sites. The β-subunits anchor the receptor molecule in the membrane and they activate intracellular kinase activity upon insulin binding. The kinase activity in the cytosol is directly correlated to the concentration of insulin in the extracellular space. Because the action of the hormone is initiated by the formation of the insulin-receptor complex, the binding stoichiometry of the insulin receptor is important for the understanding of insulin function.

The symmetrical architecture of the insulin receptor suggests a functional role for both insulin binding sites. However, under physiological conditions, i.e. at insulin concentrations in the range 10^{-9}–10^{-11} M insulin, only one insulin molecule is bound to the receptor. At higher concentrations, the binding of a second insulin molecule seems to be partially suppressed by the first [6].

There is still some controversy about the number of ligands that can be bound to one receptor molecule. Information about binding stoichiometry is mostly derived from binding assays using receptors of different cell lines. Competitive binding assays [11] and quantitative amino acid analysis of complexed receptors [12] indicated that soluble insulin receptor ectodomain (IR921) binds two molecules. However, based on experiments of binding kinetics, a maximal number of four ligands per receptor was proposed [6]. Studies with receptors in human placental membranes, using a combination of affinity labeling and antibody recognition, indicated a 2:1 stoichiometry of the insulin-receptor complex [16]. On the other hand, a 1:1 stoichiometry was found in studies with solubilized human placental insulin receptors [1]. To demonstrate directly the existence of doubly occupied IR921, we used a combination of photoaffinity labeling and the biotin-avidin technique. Photoaffinity labeling of IR921 has been proven to be a valuable tool for the analysis of the insulin-IR921 complex [8,14] and for a wide range of other ligand-receptor complexes [3,4]. Photoaffinity labeling uses the intrinsic affinity of a ligand-receptor pair to form an intermolecular covalent bond via a photoreactive group. Ideally, the photogroup should be placed

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close to the contact surface of both molecules without disturbing the pattern of local interactions. Markers at position B29 in insulin have been shown to fulfill this requirement [14]. Once the covalent crosslinked complex is formed, isolation and identification of the labeling site can be accomplished by means of the biotin-avidin technique. Here, we used a combination of two novel photoinsulins carrying different markers for the detection and isolation of the doubly labeled insulin receptor.

2. Materials and methods

2.1. Chemicals

The soluble ectodomain of the human insulin receptor (IR921) is the hetero-tetrameric $(\alpha\beta_0)_2$ receptor, which is truncated following residue 921 [12]. Human insulin was a donation from Hoechst (Frankfurt, Germany). Carrier-free ^{125}iodine was purchased from Amersham (Braunschweig, Germany). Salts and chemicals were purchased from Fluka (Deisenhofen, Germany). Streptavidin, and 4-azidotetrafluorobenzoyl-biocytinyloxy-succinimide ester were purchased from Roche Diagnostics (Mannheim, Germany). The 4-azidosalicyloyl-N-hydroxysuccinimide ester was synthesized as described elsewhere [7].

2.2. Preparation of ^{125}I-N$^{\epsilon B29}$-(4-azidosalicyloyl)-insulin (^{125}I-Asa-insulin)

First, 10.4 mg of $N^{\alpha A1}$,$N^{\alpha B1}$-bis(Msc)-insulin (Msc-, methylsulfonyloxycarbonyl-) were dissolved in 330 μl N,N-dimethylformamide, and 3.3 μl N-methylmorpholine were added. To this solution, 11.8 mg 4-azido-salicyloyloxysuccinimide ester and 4.6 mg 1-hydroxybenzotriazole were added. The reaction was monitored by reversed phase high-performance liquid chromatography (RP-HPLC). After 2 h the reaction was stopped by acidification with 2 ml of 10% (v/v) acetic acid. The product was purified by gel filtration on Sephadex G-25f and lyophilized. Cleavage of protection groups was performed in 10% (v/v) piperidine within 1.5 h at 0°C. The reaction was stopped by addition of 2 ml of 10% (v/v) acetic acid. The product was isolated by gel filtration on Sephadex G-25f. Final purification was accomplished by preparative RP-HPLC on Nucleosil $10C_{18}$.

The photoinsulin was dissolved in iodination buffer (0.3 M phosphate, 6 M urea, pH 7.8) at a concentration of 1 μg/1 μl; 2 μl of this photoinsulin solution were pipetted into the reaction tube and 2 μl of iodination buffer were added. After pipetting 1 μl of ^{125}I-solution (100 mCi, $3.7\cdot10^9$ Bq/ml) into the mixture, the reaction was started by addition of 500 ng chloramine T, dissolved in 5 μl iodination buffer. After 5 min of gentle stirring, the incorporation of iodine was stopped by precipitation using trichloroacetic acid and dissolving in chromatography buffer. The iodinated product was purified on SepPak C_{18} cartridges [9].

2.3. Preparation of N$^{\epsilon B29}$-(4-azidotetrafluorobenzoyl-biocytinyl)-insulin (Atf-Bct-insulin)

First, 10 mg (1.7 μmol) of $N^{\alpha A1}$,$N^{\alpha B1}$-bis(Msc)-insulin were reacted with 3.4 mg (5 μmol) of azidotetrafluorobenzoyl-biocytinyloxysuccinimide ester in 330 μl of N,N-dimethylformamide/N-methylmorpholine (1/0.01, v/v) at room temperature for 3 h with gentle shaking. The solution was acidified with acetic acid, then subjected to gel filtration on Sephadex G-25f in 10% (v/v) acetic acid. Finally, the protein was lyophilized. The crude material was dissolved in 2.5 ml of 10% (v/v) aqueous piperidine at 0°C and kept for 90 min in the dark. The solution was acidified with acetic acid, purified on Sephadex G-25f in 10% (v/v) acetic acid. The collected protein fraction was lyophilized. The yield was 6.4 mg of raw material, containing 43% acylation product according to RP-HPLC. Before use, the protein was further purified by preparative RP-HPLC to $>$90% purity.

2.4. Receptor binding assay

The receptor binding test on IM9-lymphocytes was performed according to the protocol from Ref. [5]. Tracer solution (A14-^{125}I-insulin, 50 μl, 10^4 cpm) and insulin solution (50 μl) were pipetted into the cell suspension in 400 μl 120 mM NaCl, 45 mM KCl, 1.2 mM $MgSO_4$, 1 mM ethylenediamino-tetraacetic acid (EDTA), 10 mM D-glucose, 15 mM sodium acetate, 100 mM 4-(2-hydroxyethyl)-1-piperazinethane-sulfonic acid (HEPES), and 1% (m/v) bovine serum albumin (BSA). Samples were incubated for 2 h at 15°C in a water bath and after centrifugation, the supernatant was removed. The radioactivity of the pellet was counted using a γ-counter.

2.5. Analytical detection of biotinylated IR921

Two hundred ng IR921 were incubated with 200 ng Atf-Bct-insulin in 30 μl Krebs-Ringer HEPES (KRH) binding buffer (50 mM HEPES, 130 mM NaCl, 5.1 mM KCl, 1.3 mM $MgSO_4\cdot 7\,H_2O$, 0.1% BSA, pH 7.4) for 90 min at room temperature in the dark. Control experiments contained an additional 1000-fold excess of unlabeled insulin. The covalent crosslinking was performed by irradiation with six 1000 Ws of UV flash light. The labeled receptor was separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (4.5% T for nonreducing conditions and 7.5% T for reducing conditions) [10]. The protein was electroblotted onto a polyvinylidenedifluoride (PVDF) membrane in 25 mM tris(hydroxymethyl)aminomethane (Tris), 192 mM glycine and 20% methanol (pH 8.3) at 250 mA, 100 V, using a semidry transfer chamber (Biorad, Muenchen, Germany). The chemiluminescence detection kit from Roche Diagnostics was used in biotin detection. Nonspecific binding sites were blocked by incubation with Tris buffered saline Tween buffer (TBST) (50 mM

Tris, 0.1% Tween 20, 150 mM NaCl, pH 7.4) containing 1% blocking reagent. The blotting membrane was then incubated with streptavidin-conjugated peroxidase for 30 min. The peroxidase was washed off with four aliquots of TBST buffer after shaking for 10 min. Addition of the chemiluminescence reagent led to an enzymatically catalyzed light emission, which was detected by imaging on an X-ray film (Hyperfilm MP, Amersham).

2.6. Photoaffinity labeling of the IR921

One μg of IR921 was incubated in the dark with 3.6 ng ($2 \cdot 10^6$ cpm) of radioactive photoinsulin (^{125}I-Asa-insulin) in 100 μl KRH binding buffer for 16 h at 6°C. After irradiation with three 1000 Ws of UV flashes, excess insulin was removed by chromatography on Sephadex G-50f in 50 mM NH_4HCO_3 buffer. The fraction containing the receptor material was incubated under the same conditions as described above with 360 ng of biotinylated photoinsulin (Atf-Bct-insulin). After irradiation, the complex was separated by means of a nonreducing 4.5% SDS-PAGE. The receptor was eluted from the gel (50 mM Tris, 0.5 mM EDTA, 0.1% SDS, pH 7.4) for 24 h and the eluted material was incubated for 16 h at 4°C with immobilized streptavidin. After thorough washing of the streptavidin gel, the supernatant (of the incubation) as well as the eluted fraction were separated by means of nonreducing 4.5% and reducing 7.5% SDS-PAGE. Protein bands were electroblotted onto a PVDF membrane. The biotin and ^{125}iodine content of the receptors were determined by chemiluminescence detection and autoradiography, respectively.

3. Results and discussion

Two different photoinsulins, ^{125}I-labeled Asa-insulin and Atf-Bct-insulin (Fig. 1), were used for the synthesis and analysis of a doubly photoaffinity-labeled IR921.

3.1. Synthesis and characterization of photoinsulins

Both bifunctional photoinsulins were synthesized by acylation of the ϵ-amino group of Lys^{B29} of A1,B1-bis(Msc)-protected insulin [13], using the activated esters of the 'building blocks' 4-azidosalicylic acid (Asa) and 4-azidotetrafluorobenzoyl-biocytin (Atf-Bct). Products were obtained by gentle deprotection of the base-labile Msc groups by an aqueous piperidine solution and subsequent chromatographic purification. Both photoinsulins are known to show high crosslinking yields (about 30% of total receptor amount), thereby showing high specificity for the binding site (2%–3% unspecific binding). The receptor binding affinities for the insulin receptor in vitro are 25% (Asa-insulin) and 70% (Atf-Bct-insulin) compared to native insulin. In spite of the lower binding affinity of Asa-insulin,

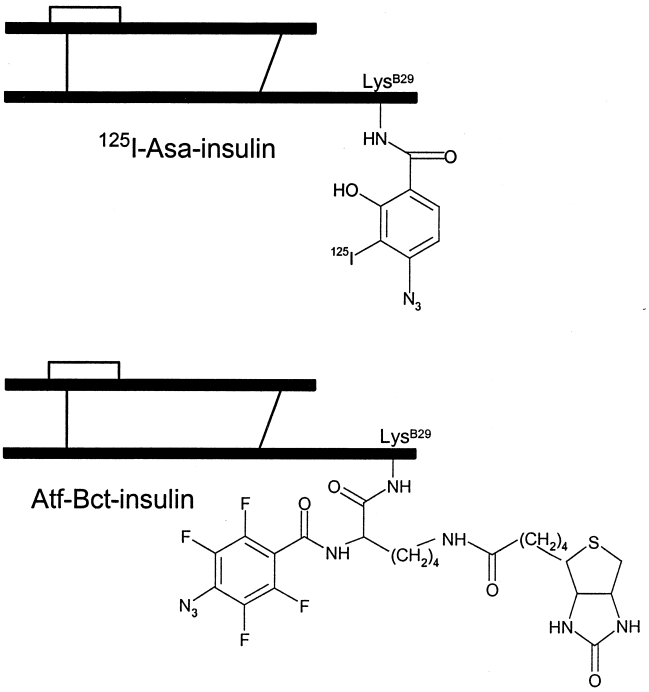

Fig. 1. Scheme of the structure of the two bifunctional photoinsulins that were used for crosslinking of both binding sites of IR921: radioactive N$^{\epsilon B29}$-(^{125}iodo-4-azidosalicyloyl)-insulin (^{125}I-Asa-insulin) and N$^{\epsilon B29}$-(4-azidotetrafluorobenzoyl-biocytinyl)-insulin (Atf-Bct-insulin).

the crosslinking yields were very similar. This is because the low dissociation constant for formation of the insulin-receptor complex (~1 nM) guarantees nearly complete occupation of the receptor under the reaction conditions that were chosen in labeling experiments. Both photolabeled IR921 complexes, the iodinated as well as the biotinylated, could be detected with high sensitivity in the lower nanogram range (Fig. 2). Separation on a SDS-PAGE revealed two bands for the IR921 photocomplex under nonreducing conditions, as a proteolytic side reaction had cleaved one β-subunit, leading to masses of 350 kDa for the intact complex and to 310 kDa for the cleaved complex. Under reducing conditions, a band was found at 123 kDa, corresponding to the IR921 α-subunit that is crosslinked to the photoinsulin. The sensitivity of the biotin detection was determined to be about 1 ng of applied receptor from the crosslinking experiment.

3.2. Detection of the doubly labeled IR921 complex

The bivalence of the IR921 was shown by sequential photo-crosslinking with the radioactive ^{125}I-Asa-insulin and the biotinylated Atf-Bct-insulin. The experimental procedure and potential reaction products are shown in Fig. 3. IR921 was labeled covalently with ^{125}I-Asa-insulin by irradiating a 1:5 (ligand:receptor) mixture of ^{125}I-Asa-insulin and IR921 with three 1000 Ws of UV light flashes. The

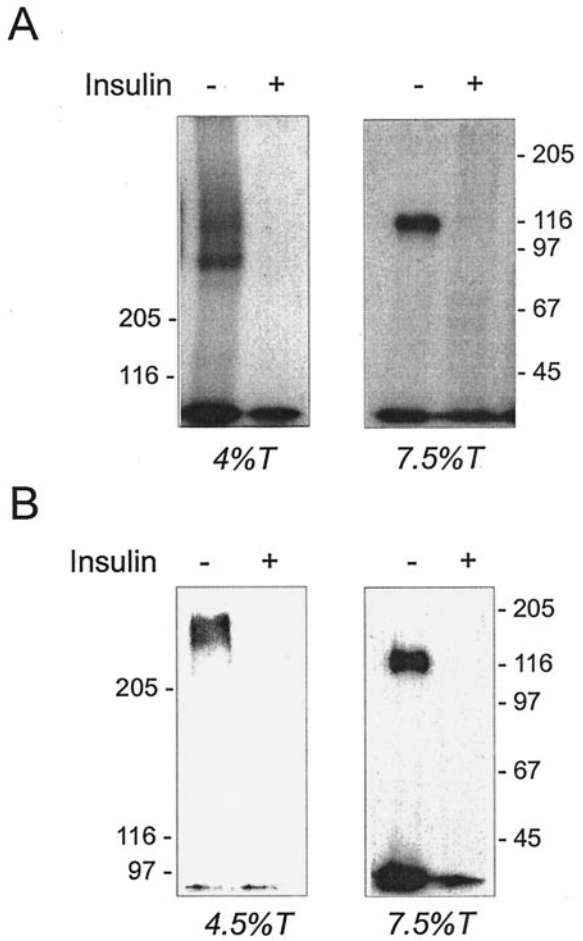

Fig. 2. Photoaffinity labeling of IR921 with ^{125}I-Asa-insulin (A) and with Atf-Bct-insulin (B) under reducing (7.5% T) and nonreducing (4% and 4.5% T) conditions analyzed by SDS-PAGE. Radioactive bands were detected by autoradiography (A) and biotin was detected after blotting by chemiluminescence (B). Lanes marked with + show protein labeling (control) with an excess of native insulin (10^{-5} M).

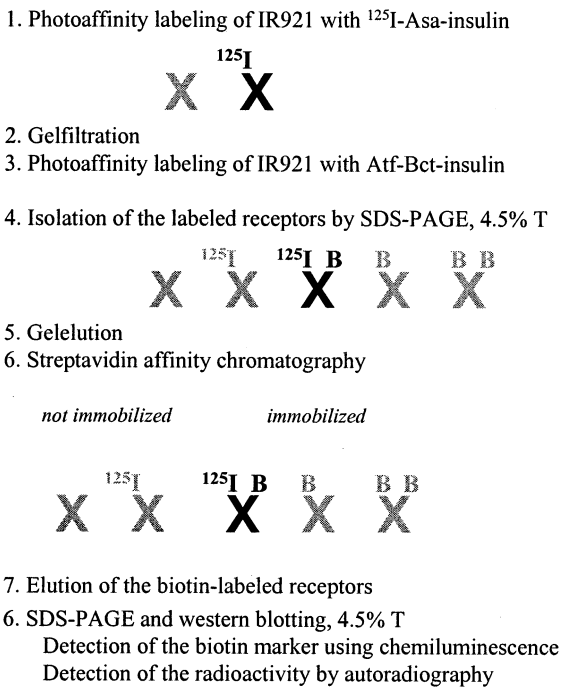

Fig. 3. Scheme of the experimental procedure for the preparation and analysis of the doubly labeled IR921.

covalent receptor complex was separated from excess insulin by gel chromatography. The efficiency of crosslinking with the radioactive insulin derivative was 32%. The purified complex was incubated with a 20-fold excess of Atf-Bct-insulin. Note that a biotin moiety is attached to the Atf-Bct-insulin, giving a biotinylated receptor complex upon crosslinking. Photolabeling was performed by irradiating with three 1000 Ws of UV flash light. A control sample lacking the second labeling step was used as reference. The mixture of various receptor complexes (see Fig. 3) was separated from excess reagents by means of SDS-PAGE. The receptor bands were cut out and the protein material was eluted from the gel with recovery yield of 62% of the applied radioactivity.

Biotinylated receptors were immobilized on a streptavidin column. The nonbiotinylated material was thoroughly washed off, i.e. the streptavidin gel was rinsed until no further radioactivity eluted from the column. A total amount of 14% of the applied radioactivity remained immobilized on the gel. This material represents the doubly labeled IR921, as it carries a biotin group for immobilization and a radioactive marker. The control sample (lacking the labeling with Atf-Bct-insulin) yielded no immobilized radioactivity, indicating that the binding to the streptavidin gel is specific for biotin.

With the aim to underpin the double labeling by further analysis, the receptor complexes were eluted from the streptavidin gel. The protein material was again subjected to a SDS-PAGE separation and electroblotting, in order to detect the markers by chemiluminescence detection and autoradiography, respectively. The detection of biotin was performed by exposure to an X-ray film for 15 min. The radioactivity was detected after washing the blot membrane and exposure of an X-ray film to the membrane for 3 days (Fig. 4).

3.4. Orthogonal photoaffinity labeling

The IR921 is able to bind two insulin molecules. Here, we isolated and characterized a doubly labeled covalent IR921 complex (Fig. 5) using two different photoreactive insulins for a combination of photoaffinity labeling and affinity chromatography. Both photoreactive insulins carried an additional marker group: Asa-insulin carried a ^{125}I radioactive marker, and Atf-Bct-insulin contained a biotin group. This combination of orthogonal markers allowed an independent verification of the two crosslinking steps. The biotin moiety served as a valuable tool for the affinity

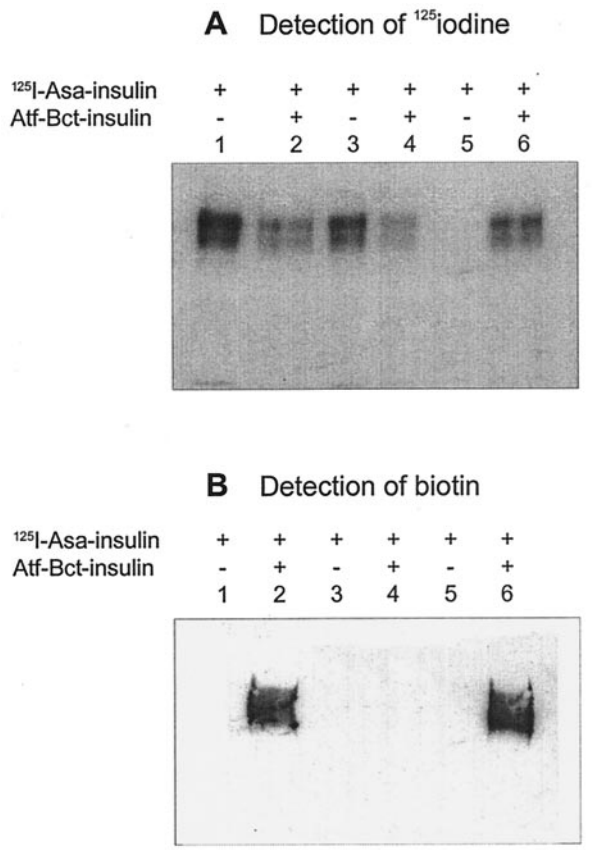

Fig. 4. Detection of the bivalence of the insulin receptor. IR921 was photolabeled as described in Materials and methods. Lanes 1 and 2: Gel-eluted receptor band of the SDS-PAGE after crosslinking (step 5 in Fig. 3). Lane 1: control sample crosslinked with ^{125}I-Asa-insulin only. Lanes 3 and 4: Supernatant of streptavidin immobilization (step 6 in Fig. 3). Lane 3: control sample. Lanes 5 and 6: Eluted fraction from streptavidin affinity column, Lane 5: control sample. Each fraction was tested for radioactivity (A) and biotin content (B). The double positive signal in lane 6 proves the existence of receptor molecules carrying a radioactive label as well as a biotin label.

purification of the labeled receptor, as well as for the sensitive detection of the second crosslinking step.

We noted in the introduction that the insulin receptor probably binds only one insulin molecule under physiological conditions, which is sufficient to trigger the intracellular kinase activity. The question that then arises is, why is the ability of the insulin receptor to bind a second hormone ligand evolutionally conserved, and what are the biologic implications of this binding. The insulin receptor is one of the few members of the protein tyrosine kinase (PTK) family [15] having a dimeric architecture. Most of the other PTK receptors dimerize upon ligand binding. One possible reason for the second binding site would be that the evolution of the kinase function directed the development of a dimeric architecture, thus the bivalence would be gained indirectly without having a major biologic relevance. Otherwise, the insulin receptor dimer could act as an insulin buffer, which binds the first ligand with high affinity, leading to a sensitive dose-response curve at low insulin concentrations. Presuming a local excess of insulin ($>10^{-9}$ M), binding of the second ligand with low affinity at high insulin concentrations could occur without any further stimulation of the kinase activity. This mechanism would smooth the dose-response curve at high insulin concentrations, whereas the diffusion of the second ligand at decreasing insulin levels would lead to a prolonged hormone action. An indication for such a mechanism is given in the work on monomeric insulin receptors [2] that show a noncooperative binding behavior. Further work on functional differences between monomeric receptors and their oligomeric counterparts would be of great value for the understanding of receptor oligomerization. Orthogonal photo-crosslinking of such complexes could be helpful for the analysis of ligand-receptor stoichiometries.

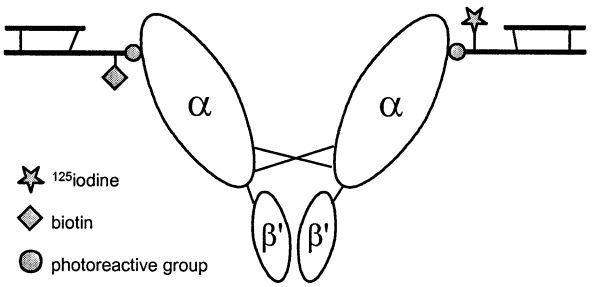

Fig. 5. Model of the doubly crosslinked IR921

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