IDENTIFICATION OF PHOTOAFFINITY LABELED INSULIN RECEPTOR PROTEINS BY LINEAR POLYACRYLAMIDE GRADIENT GEL ELECTROPHORESIS UNDER NON-DENATURING CONDITIONS

L. KUEHN, H. MEYER, M. RUTSCHMANN and P. THAMM

Biochemical Department, Diabetes Forschungsinstitut an der Universität Düsseldorf, Düsseldorf, and *Deutsches Wollforschungsinstitut, Aachen, FRG*

Received 14 February 1980

1. Introduction

Photoaffinity probes have been used to selectively label functional components in a number of membrane systems [1–10]. To identify the labeled molecules, membranes were subsequently solubilized with sodium dodecyl sulfate (SDS) and analyzed by SDS-polyacrylamide gel electrophoresis. A major disadvantage of this technique is that non-covalent interactions among proteins are abolished, precluding the detection of proteins whose functional state depends on a complex subunit structure. On the other hand, it has been shown that a number of membrane proteins can be extracted from the membrane under non denaturing conditions, thus affording their functional integrity [11]. This observation has prompted us to examine the possibility of performing the photolabeling reaction in the presence of non-ionic detergent.

In this communication we report the covalent linking of a photosensitive, radioactive insulin analogue to proteins previously solubilized from porcine liver membranes. Electrophoresis of the reaction mixture on linear polyacrylamide gradient gels showed that proteins migrating at positions corresponding to an MW of 300 000 and 600 000, respectively, were specifically labeled, suggesting these proteins to be the non-denatured binding components of the insulin receptor.

Abbreviations: BSA, bovine serum albumin; Napa, 4-azido-2-nitrophenylacetyl-; SDS, sodium dodecyl sulfate; Tris, Tris-(hydroxymethyl)aminomethane

2. Materials and methods

Porcine monocomponent insulin was a gift from Hoechst AG, FRG. Reagents for polyacrylamide gel electrophoresis, Triton X-100 and Coomassie Blue G 250 were purchased from Serva, Heidelberg, FRG. Enzymobeads were from Bio-Rad, München, FRG and ^{125}I-NaI was from New England Nuclear, Boston, MA, USA. The following MW standards were used: thyroglobulin (MW 660 000) and catalase (MW 230 000), both from Sigma, München; ferriton (MW 345 000), BSA (MW 68 000) and ovalbumin (MW 43 000) from Serva, Heidelberg. All other reagents were of the highest quality available. Protein was determined according to Lowry et al. [12] with BSA as a standard. The photoreactive insulin analogue, B2-Napa-des-Phe^{B1}-insulin was synthesized and chemically characterized as described elsewhere [13]. The biological activity in the in vitro fat cell assay was 78% that of native insulin (C. Diaconescu, personal communication). Radioiodination of native insulin and of photoinsulin to a specific activity of 200–250 μCi/μg was performed with ^{125}I-NaI and Enzymobeads according to the instructions supplied by the manufacturer. The iodinated products were 98–99% trichloroacetic acid precipitable and 90–93% immunoprecipitable. Insulin receptor protein was solubilized and purified about 30-fold over crude homogenate as described [14]. Hormone receptor binding studies were performed in triplicate with solubilized membrane protein (35 μg/ml) and iodinated hormone in 0.1 M sodium phosphate buffer, pH 7.8, 0.1% (w/v) BSA and 0.1% (v/v) Triton X-100. In a parallel set of tubes, 10^{-6} M native insulin was included to determine and correct for

nonspecific binding. Samples were incubated for 5 h at 15°C in the dark and the insulin receptor complex was quantitatively determined as described by Cuatrecasas [15]. Photolysis of the reaction mixture was carried out for 90 min at 4°C, using a 25 W Philips UV lamp (366 nm) at a distance of about 2 cm. Electrophoresis was performed on linear polyacrylamide gradient slab gels (110 × 110 × 1.5 mm). The following electrophoretic conditions were found to give optimal resolution and sharpness of bands: gels had a uniform cross-linkage of 3% with respect to *N,N'*-methylenebisacrylamide and contained 0.3 M Tris · HCl, pH 9.2 and 0.1% (v/v) Triton X-100; 50 ml gel solution contained 3.2 ml of a 1% (w/v) solution of ammonium persulfate and 0.018 ml *N,N,N',N'*-tetramethylethylenediamine, and the electrophoresis buffer contained 0.15 M Tris-glycine, pH 8.8 and 0.1% (v/v) Triton X-100. 50 μl samples containing either 50 μg protein of the photolyzed reaction mixture or 4–10 μg of the molecular weight standards in electrophoresis buffer with 10% sucrose were applied to individual gel slots. Electrophoresis was performed in a vertical slab gel apparatus (Desaga, Heidelberg) for 7 h at 4°C, applying a constant current of 70 mA. After staining in a solution of 0.1% Coomassie Blue and destaining in 7.5% acetic acid, gels were dried and subsequently autoradiographed on Kodak X-ray film, type X-Omat R. The results from gel electrophoresis were analyzed by plotting the computed acrylamide concentration reached by a protein and its molecular weight on a bilogarithmic scale [16]. Linear regression analysis was performed with the aid of a Hewlett Packard programmable calculator.

3. Results and discussion

The binding activity of ^{125}I-labeled B-2-Napa-des-Phe^{B1}-insulin in the dark was determined in order to quantitate subsequent photolabeling experiments. The specific binding of the photoderivative and of insulin to solubilized membrane proteins as a function of hormone concentration is shown in fig.1. The results indicate that the insulin analogue binds in an almost identical fashion as native insulin.

To demonstrate the photoreactivity of the insulin analogue, the following experiments were performed: 10^{-9} M radioactive insulin analogue was equilibrated with 35 μg/ml solubilized membrane protein in the dark. Subsequent addition of high concentrations of

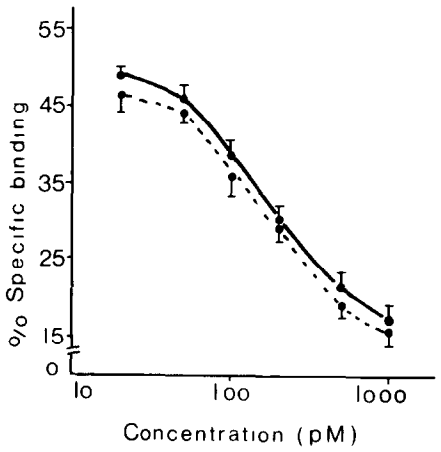

Fig.1. Specific binding in the dark of ^{125}I-insulin (•——•) and ^{125}I-labeled B-2-Napa-des-Phe^{B1}-insulin (•– – –•) to proteins solubilized from porcine liver membranes. Binding studies were performed as described under materials and methods. Binding is expressed as per cent of the total ^{125}I-radioactivity present in the incubation mixture. Each value is the mean ± SEM of triplicate determinations.

Triton X-100 (2%, v/v) to non-irradiated samples resulted in the complete dissociation of the radioactive complex. In contrast, following exposure to light, 10–15% of the complex preformed in the dark had become resistant to treatment with the detergent (data not shown). Controls also showed that the complex formed between ^{125}I-insulin and solubilized membrane protein was fully dissociable under these conditions.

For the analysis of photolabeled proteins, electrophoresis on linear polyacrylamide gradient gels appeared to be an especially suitable technique. It has been recently shown that, in the presence of SDS, electrophoresis on such gels allows accurate molecular weight determination of proteins and glycoproteins with a substantial content of carbohydrate over a wide range of molecular weights from 10^4 to 10^6 daltons. A linear relationship has been obtained by plotting the log of the molecular weight against the log of the acrylamide concentration reached by a protein [16]. In order to test the validity of this relationship when gels contained nonionic detergent instead of SDS, the migration pattern of several well-established molecular weight standards in Triton X-100 containing gels was examined. As indicated in fig.2, a linear relationship is obtained both for the 5–20% and 6–12% linear gradient gels tested. Linear regres-

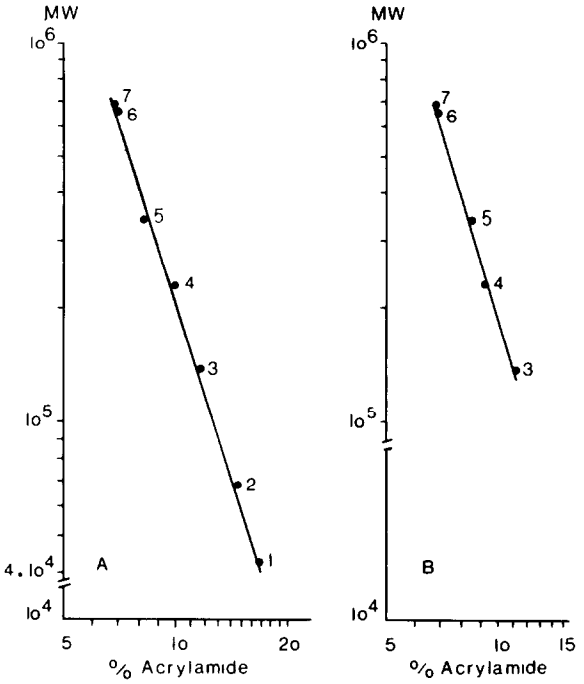

Fig.2. Plots obtained with standard proteins after electrophoresis in a 5–20% linear gradient gel (A) and a 6–12% linear gradient gel (B) in the presence of 0.1% Triton X-100. For experimental details, see materials and methods. (——), best linear fit. 1, ovalbumin; 2, BSA monomer; 3, BSA dimer; 4, catalase; 5, ferritin monomer; 6, thyroglobulin; 7, ferritin dimer.

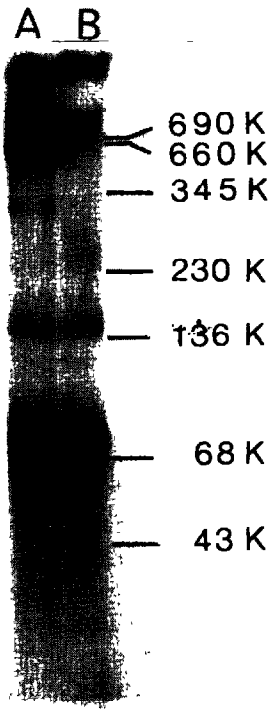

Fig.3. Autoradiographs showing the covalent labeling of solubilized membrane proteins by ^{125}I-labeled B-2-Napa-des-Phe^{B1}-insulin in the absence (A) and the presense of 10^{-6} M native insulin (B). The positions of the MW standards from a parallel run are indicated.

sion analysis of the data yielded correlation coefficients in excess of 0.99.

When solubilized membrane proteins were photolyzed in the presence of the radioactive insulin analogue and electrophorized on 5–20% linear gradient gels, results were obtained as shown in fig.3. The covalent linking of the photoinsulin resulted in the labeling of four bands (fig.3A). However, only two bands were specifically labeled since they were completely suppressed when an excess of native insulin was present in the incubation mixture (fig.3B). The MW of these proteins was estimated to be about 300 000 and 600 000, respectively, with the higher molecular weight species predominating. It is very unlikely that the 600 000 MW species had arisen through crosslinking by the photoderivative of the smaller MW species, since the photoprobe used in these experiments was a monoazidophenyl-derivative of insulin [13], but several other possibilities which might have caused an aberrant migration behaviour of the proteins must be considered, among these nonspecific interactions between the insulin binding components and other solubilized membrane constituents, detergent effects and effects of the gel buffer used being potential sources of error. There are several lines of evidence arguing against this notion: First, heat inactivation of the receptor sample prior to the photoreaction resulted in a gel pattern indistinguishable from that shown in fig.3B, that is a selective suppression of the two specifically labeled bands, while the two bands corresponding to nonspecifically labeled BSA monomer and BSA dimer were unchanged with respect to both their relative gel position and intensity. In the case of nonspecific interactions among proteins, one or more additional nonspecific bands should have been observed. Second, a variation of the Triton concentration between 0.01% and 0.5% again did not change the electrophoretic pattern shown in fig.3, suggesting that within this concentration range, the detergent was without effect on the state of aggrega-

tion of the solubilized membrane proteins. Third, although a somewhat lower binding of the photoprobe to solubilized membrane proteins was observed when the binding buffer was exchanged for the gel buffer, neither the specificity of the reaction nor the migration pattern of proteins in the gel were changed. Taken together, these findings suggest that the specifically labeled bands observed may be the non denatured binding components of the insulin receptor.

Two other studies have been concerned with the characterization of insulin binding proteins by covalent labeling of plasma membranes with photoreactive insulin analogues. However, due to the use of SDS for subsequent solubilization of the membranes, a characterization of the active macromolecule(s) was not possible [8–10]. In another, very recent study, membranes of cultured human lymphocytes were solubilized with Triton X-100 and the crude detergent extract was subjected to quantitative polyacrylamide gel electrophoresis [17]. The authors describe two insulin binding proteins, a major one with an estimated MW of 10^6 and a minor activity with an estimated MW of 200 000. These estimates, obtained with a methodologically different approach compare favourably with those found in the present study. In both cases, the higher MW species is predominant. The differences in MW may be explained simply by the different sources of the receptor activity, the different degree of purity of the preparations analyzed and the different electrophoretic systems used. Clearly, the characterization of a specifically labeled binding protein on the basis of its migration distance in polyacrylamide gels (or in gel filtration or sedimentation experiments) is not sufficient to answer the question whether this protein is responsible for the function in the membrane. It can at best be operationally defined as the species capable of specifically binding a ligand. To prove the identity of the binding protein and the functional hormone receptor will ultimately depend on the purification of the binding activity and the demonstration of bioactivity in a reconstituted system. The method of photoaffinity labeling in conjunction with linear polyacrylamide gradient gel electrophoresis under non denaturing conditions should, by virtue of speed, accuracy and high resolution be a very useful tool to analyze insulin binding proteins or other membrane proteins at various stages during purification.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 113) and the Ministerium für Wissenschaft und Forschung des Landes Nordrhein Westfalen. We wish to thank Drs. H. Reinauer and D. Brandenburg for their helpful suggestions. The expert technical assistance of Ms. Jutta Simon is greatly appreciated.

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