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Electric properties of photoaffinity-labelled pancreatic A-subtype cholecystokinin

J. JIMÉNEZ[a], M. DUFRESNE, S. POIROT, N. VAYSSE and D. FOURMY*
INSERM U 151, Bat L3, Avenue Jean Poulhès, CHU Rangueil, 31054 Toulouse Cédex (France)
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ABSTRACT

Although the isoelectric point of a protein is very important, electric focusing of intrinsic membrane proteins in polyacrylamide or agarose gels often fails. The recently introduced Bio-Rad Rotofor cell allowed isoelectric focusing of such a protein, cholecystokinin (CCK) receptor. Both the isoelectric point and the molecular weight (M_r) of pancreatic CCK receptor were determined. For this purpose, membrane CCK receptor was photoaffinity labelled by a cleavable agonist probe, subsequently prepurified on immobilized wheat germ agglutinin and analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis and isoelectrofocusing in solution in the presence of Nonidet P-40. CCK receptor was identified at M_r 85 000–100 000, whereas its deglycosylated product was shifted to M_r 42 000. Further, the isoelectric points of the glycosylated and deglycosylated forms of CCK receptor were pH 4.8 and 4.3, respectively. A knowledge of the isolelectric point should help in characterizing better CCK receptor heterogeneity and/or in purifying CCK receptor proteins.

INTRODUCTION

Cholecystokinin (CCK) is a major neurohormonal peptide with effects on various tissues within the gut[1] and the nervous system[2]. CCK receptor subtype diversity as revealed by pharmacological studies is now well established[3]. However, the molecular basis of such a diversity remains unclear. Rat pancreatic CCK receptor, which is of A-subtype, has been pharmacologically and functionally characterized[4]. Its biochemical knowledge was provided essentially by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of affinity-labelled membrane proteins using radioiodinated CCK probes. In particular, CCK-8 based probes covalently linked to CCK receptor through their N-terminus[5] or C-terminus[6] led to the identification of a glycoprotein of molecular weight (M_r) 85 000–95 000 having a

[a] Present address: Department of Medical Biochemistry and Molecular Biology, School of Medicine, University of Seville, Avda. Sanchez Pizjuan 4, 41009 Seville, Spain.

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protein core of M_r 42 000[7]. Recently we synthesized and characterized a cleavable ligand which was used to photoaffinity label CCK receptor with minor modification of its structure[8]. The probe was [^{125}I]azidosalicylaminodithiopropionate-(Thr^{28},-Nle^{31})-CCK-25-33, [^{125}I]ASD-(Thr^{28},Nle^{31})-CCK-25-33, the peptide moiety of which was released from photoaffinity-labelled CCK receptor by reduction.

In an effort to design a purification strategy for pancreatic CCK receptor, we determined the isoelectric point of its glycosylated and deglycosylated photoaffinity-labelled forms. This new criterion should provide a better understanding of the molecular nature of CCK receptor and thus facilitate its purification.

To achieve isoelectric focusing of CCK receptor, several methods were tried, including focusing in polyacrylamide and agarose gels. As none of them yielded a satisfactory amount of focused CCK receptor, we decided to use the Rotofor cell[9].

EXPERIMENTAL

Membrane preparation

Enriched pancreatic plasma membranes were prepared from male Wistar rats by homogenization of 24 pancreases in 0.3 *M* sucrose according to the method described previously[5].

Photoaffinity labelling of CCK receptor

The synthesis of the photoactivatable ligand [^{125}I]ASD-(Thr^{28},Nle^{31})-CCK-25-33 and its binding to pancreatic membranes were described previously[8]. Briefly, binding of [^{125}I]ASD-(Thr^{28},Nle^{31})-CCK-25-33 (100 p*M*) to plasma membranes (5–10 μg) was carried out at 22°C for 60 min in HEPES buffer (50 m*M*, pH 7.0) containing 115 m*M* NaCl, 5 m*M* $MgCl_2$, 0.01% soybean trypsin inhibitor, 0.1% bacitracin, 1 m*M* ethylene glycol tetraacetate, 0.1 m*M* phenylmethylsulphonyl fluoride and 0.2% bovine serum albumin. Membranes were centrifuged and resuspended in cold buffer without albumin for a 5-min photolysis under a 125-W mercury lamp. Photoaffinity-labelled membranes were solubilized at 4°C for 1 h in binding buffer containing 5% Nonidet P-40. Soluble membrane proteins were recovered by ultracentrifugation at 100 000 *g* for 30 min.

Lectin prepurification of CCK receptor

As the yield of photoaffinity labelling was relatively low (4% of initial binding on membranes), we enriched the membrane proteins in photoaffinity-labelled CCK receptor by taking advantage of its glycosylation. Chromatography on immobilized wheat germ agglutinin (WGA) was performed according to Rosenzweig *et al.*[10]. Retained glycoproteins were eluted with 0.5 *M* N-acetylglucosamine. Fractions corresponding to the radioactive peak were ultraconcentrated (Centricon 30, Amicon) and, to avoid protein aggregation, free sulphydryl groups were carboxymethylated with iodoacetic acid as described by Waxdal *et al.*[11]. Finally, proteins were precipitated with methanol–chloroform–water (4:1:3)[12] and dried under vacuum. Alternatively, aliquots of CCK receptor eluted from the wheat germ agglutinin column were directly subjected to isoelectric focusing and SDS-PAGE.

Isoelectric focusing of CCK receptor

A Rotofor cell (Bio-Rad Labs.) was used[9]. The focusing medium was composed of a solution containing 2% Nonidet P-40, 1.5% (w/v) of ampholytes (1%, pH 3.5–9.5, and 0.5%, pH 2.5–4.5, Ampholines, LKB). The electrode solutions were 0.1 *M* NaOH and 0.1 *M* H_3PO_4. Focusing was achieved at 4°C in two steps: (i) prefocusing at 12 W constant power for 1 h, then the Nonidet P-40 solubilized sample was loaded into the chamber; (ii) isoelectric focusing at 12 W constant power for 3 h. The contents of the focusing chamber were collected for pH and radioactivity determinations as recommended by Bio-Rad Labs. Fractions corresponding to the focused radioactive peak were ultraconcentrated for subsequent SDS-PAGE analysis.

Other methods

Prepurified and isoelectric focused CCK receptor were analysed by SDS-PAGE in a 10% acrylamide gel according to Laemmli[13]. Apparent molecular masses were calculated using prestained standard proteins (Bethesda Research labs.). Deglycosylation of CCK receptor was performed by treating precipitated proteins (10-50 μg) with 50 μl of dried trifluoromethane sulphonic acid (TFMSA) for 5 min at 0°C according to the modified method of Edge *et al.*[14]. The reaction was stopped by adding 500 μl of iced 1 *M* Tris solution. Deglycosylated proteins were recovered and desalted by ultrafiltration (Centricon 10, Amicon).

RESULTS

Photoaffinity labelling and prepurification of CCK receptor

Photoaffinity labelling of pancreatic plasma membranes with [^{125}I]ASD-(Thr^{28},Nle^{31})-CCK-25-33 identified a component migrating as a broad band at M_r 85 000–100 000 in SDS-PAGE. The covalent labelling of this component was removed by incubating [^{125}I]ASD-(Thr^{28},Nle^{31})-CCK-25-33 with the membranes in the presence of 10^{-6} *M* CCK prior to photolysis (Fig. 1, lanes 1 and 2). Treatment with dithiothreitol prior to electrophoresis did not affect the labelling pattern (not shown). This confirms data obtained with other probes[5,6] and establishes that the cleavable probe [^{125}I]ASD-(Thr^{28},Nle^{31})-CCK-25-33 specifically identified CCK receptor. Photoaffinity-labelled CCK receptor was fully retained on immobilized wheat germ agglutinin and eluted with N-acetyl-β-D-glucosamine[8]. Using such a lectin affinity chromatography provided a purification rate of 17–20-fold from starting membrane proteins, and yielded prepurified CCK receptor samples of specific activity 0.7 μCi/mg proteins. Prepurified CCK receptor was identified again at M_r 85 000–100 000 (Fig. 1, lane 3).

Isoelectric focusing of photoaffinity-labelled CCK receptor

Previous attempts to focuse CCK receptor in either polyacrylamide or agarose gels failed. These results were attributed to the biochemical nature of CCK receptor, an integral membrane protein which is glycosylated and probably very hydrophobic. As an alternative, we performed focusing in solution in the presence of 2% Nonidet P-40. As illustrated in Fig. 2, prepurified CCK receptor was focused as a single radioactive peak at pH 4.8 $\pm$ 0.1 (n = 5). The content of this peak was identified as being CCK receptor which migrated at M_r 85 000–100 000 in SDS-PAGE (Fig. 2,

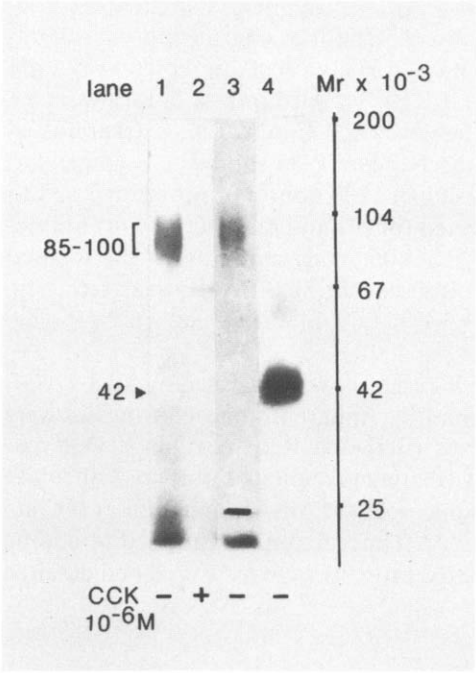

Fig. 1. SDS-PAGE of photoaffinity-labelled CCK receptor. Membranes photoaffinity labelled by [^{125}I]ASD-(Thr^{28},Nle^{31})-CCK-25-33 (lanes 1 and 2) and wheat germ agglutinin-prepurified CCK receptor (lane 3) were identified at M_r 85 000–100 000. This M_r was shifted to 42 000 after TFMSA deglycosylation (lane 4).

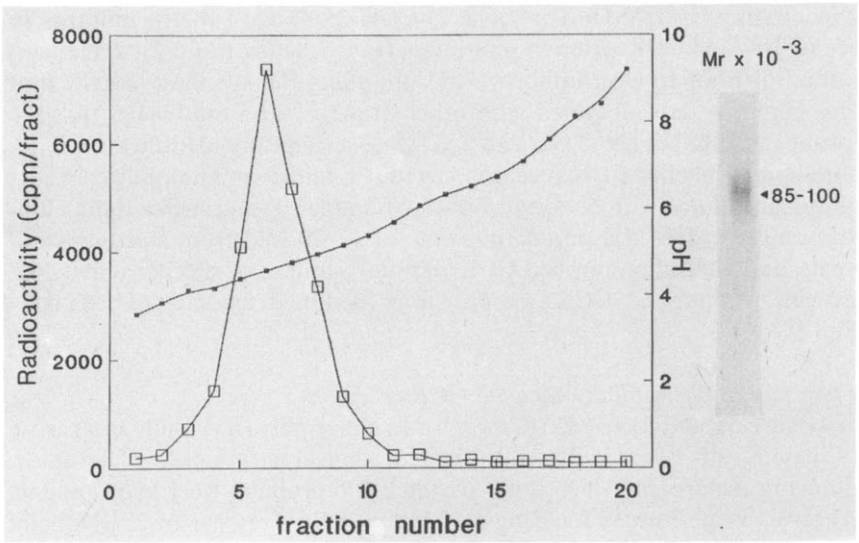

Fig. 2. Isoelectric focusing of photoaffinity-labelled CCK receptor. An aliquot of wheat germ agglutinin-prepurified CCK receptor (50 μg of proteins, 50 000 cpm) was electrically focused in 2% Nonidet P-40 solution for 3 h. The focusing pH was 4.8 ± 0.1 (n = 5). Right: SDS-PAGE of an aliquot of the focused radioactivity showing CCK receptor at M_r 85 000–100 000.

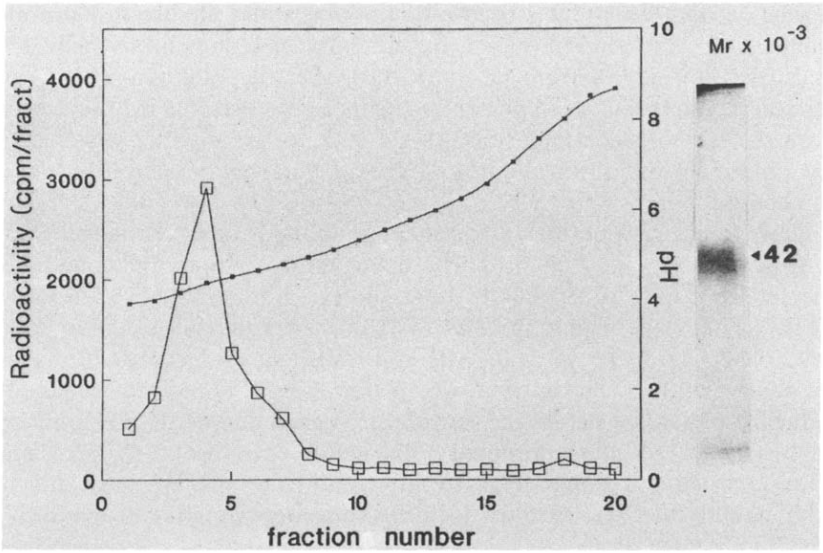

Fig. 3. Isoelectric focusing of deglycosylated photoaffinity-labelled CCK receptor. An aliquot of wheat germ agglutinin-prepurified CCK receptor (50 μg of proteins, 50 000 cpm) was precipitated, treated at 4°C with 50 μl of TFMSA for 5 min and submitted to isoelectric focusing. The focusing pH was 4.3 ± 0.1 (n = 3). Right: SDS-PAGE of the focused radioactivity showing deglycosylated CCK receptor at M_r 42 000.

right). We evaluated whether reduction and carboxymethylation affected the electric properties of CCK receptor by focusing chemically unmodified CCK receptor. We found the focusing pH to be 5.0 (n = 2).

In another set of experiments, we chemically deglycosylated prepurified CCK receptor. A component of M_r 42 000 was obtained (Fig. 1, lane 4), demonstrating that removal of carbohydrates from CCK receptor decreased its apparent mass by 60%, as already achieved by endoglycosidase-F treatment of affinity-labelled membrane CCK receptor[7]. Moreover, we evaluated whether these carbohydrates also contribute to the electric charge of the receptor protein. As shown in Fig. 3, chemically deglycosylated CCK receptor was focused at pH 4.3 ± 0.1 (n = 3). Analysis of the content of this radioactive peak demonstrated a component of M_r 42 000 (Fig. 3, right), confirming deglycosylation of CCK receptor.

Finally, we determined the focusing pH of the small chemical residue of the probe ([^{125}I]ASD) which remained on the photoaffinity-labelled receptor. [^{125}I]ASD was focused at pH 4.1 ± 0.1 (n = 5) (not shown), which is close to the isoelectric point of the receptor protein core. This result indicates that only a weak charge modification occurred on CCK receptor protein by affinity labelling with [^{125}I]ASD-(Thr^{28},Nle^{31})-CCK-25-33 and that the isoelectric point of photoaffinity-labelled CCK receptor is representative of that of the native receptor.

DISCUSSION

Since its first introduction, isoelectric focusing, especially when used in two-dimensional electrophoresis, has been applied extensively to the problems of charac-

terizing macromolecules. There have been a number of studies involving hormone receptors where isoelectric focusing is used to yield the isoelectric point of the receptor and as a purification step[15]. More generally, the isoelectric point of a protein is a physico-chemical characteristic as important as its molecular weight. It provides informations on the amino acid composition of the protein, permits the selection of optimum pH values for purification and helps in the development of separation methods based on charge.

In an effort to design a purification strategy for pancreatic CCK receptor, we determined its isoelectric point. For this purpose, numerous methods and conditions of isoelectric focusing were tested but without success. For instance, when using isoelectric focusing in polyacrylamide gel (PAGIF), only a very small amount of CCK receptor was able to enter the gel rod. Attempts with agarose gels revealed that migration of CCK receptor did occur but was so slow that CCK receptor could not reach reproducible pH values before the agarose gel melted and/or the pH gradient drifted dramatically. All solutions proposed in the literature to resolve the problems encountered were tested, but none significally improved the results. We speculatively attributed the inability of CCK receptor focusing either to its high hydrophobicity and/or to the high degree of glycosylation. In addition, in spite of the presence of SDS, CCK receptor is detected as an unusual very broad band in SDS-PAGE. Such abnormal migration may be due both to hydrophobicity and to the degree and /or heterogeneity of glycosylation, as suggested recently[16].

To circumvent these difficulties, we used the Rotofor cell, which consists of a cylindrical focusing chamber divided into twenty compartments by polyester membranes. Because of the absence of a gel matrix, the proteins easily migrate through the membranes to their focusing pH and are obtained in solution with a high recovery[17]. Using this apparatus we focused photoaffinity-labelled pancreatic CCK receptor and its deglycosylated product at pH 4.8 ± 0.1 and 4.3 ± 0.1, respectively.

This study demonstrated that removing the glycoside moiety of CCK receptor shifted its M_r from 85 000–100 000 to 42 000 but weakly decreased its isoelectric point (4.3 *versus* 4.8). Consequently, the 60% decrease in M_r found in SDS-PAGE is unlikely to be due to a charge effect. Further, the results indicated that the acidic property of CCK receptor is more relevant to the amino acid composition of the protein than to the sialic acids ending carbohydrate chains[6].

During the preparation of this paper, Duong *et al.*[18] reported on the purification of CCK receptor. They used chromatography on the strong cation exchanger Mono-S (Pharmacia) as a first step. At their experimental pH of 6.5, a Mono-S column should retain basic proteins but not CCK receptor. The only explanation for this result lies either in a non-specific interaction of CCK receptor with the Sepharose-S matrix or in the interaction of a large aggregate containing several basic proteins in addition to CCK receptor.

The isoelectric point of CCK receptor is a new biochemical characteristic provided by this study that may be used for establishing the molecular basis of CCK receptor heterogeneity and for setting up an additional purification step on the basis of the electric properties of CCK receptor. On the other hand, the Rotofor cell should be helpful in determining the isoelectric point of membrane proteins without decreasing their biological activity.

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