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A DETERGENT-INDUCED CHARGE SHIFT AS A PREREQUISITE FOR THE ELECTROCHEMICAL ANALYSIS OF THE ADENINE NUCLEOTIDE TRANSLOCATOR, A BASIC MEMBRANE PROTEIN

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The adenine nucleotide translocator is a hydrophobic, basic protein of the inner mitochondrial membrane which is solubilized by the non-ionic detergent Triton X-100. For immunochemical characterization of this membrane-protein by crossed immunoelectrophoresis a charge shift of the protein-Triton X-100 micelle by the introduction of an ionic detergent (deoxycholate) was necessary as a prerequisite to avoid unspecific precipitation of the protein. Beside the charge shift of the protein-detergent micelle, the selection, concentration and ratio of the detergents used and the choice of the agarose with different degrees of electroendosmosis should be carefully considered. The principle derived from these results provides a new methodological possibility for the immunochemical characterization of hydrophobic, basic membrane proteins.

Introduction

Crossed immunoelectrophoresis is a convenient technique for analysis of membrane proteins after solubilization [1]. A variety of membrane proteins have been investigated using different solubilization techniques including ionic and non-ionic detergents, sonication, or pH and ionic strength manipulations [2]. Solubilization of an intrinsic membrane protein for electrochemical investigation of its structural and functional properties is best achieved using non-ionic detergents such as Triton X-100. The detergent should affect neither the quarternary structure of the protein nor its antigenicity to guarantee an undisturbed antigen-antibody reaction. The intactness of the adenine nucleotide translocator (for review, see Ref. 3) after solubilization with Triton X-100 was proved by the intact binding of the highly specific inhibitor carboxy[^{3}H]atractylate [4]. As a reproducible precipitation pattern of immunological analysis of this intrinsic, transmembraneous protein was not obtained in crossed immunoelectrophoresis under the usually applied conditions [1,2], this paper describes the conditions necessary to avoid unspecific precipitation. The principle of a charge-shift of the Triton X-100-protein micelle by the introduction of the ionic detergent deoxycholate can be of overall significance when dealing with very hydrophobic proteins and offers new possibilities for the immunological characterization of these proteins.

Abbreviation: Mops, 4-morpholinepropanesulfonic acid.

Materials and Methods

Purification of the ADP / ATP carrier from heart. Bovine heart mitochondria isolated according to Smith [5] were loaded with carboxy[^{3}H]atractylate

to the point of saturation of all binding sites. Solubilization and purification of the ADP/ATP carrier as a ligand-protein complex were performed as described previously [6,7]. The noncovalent ligand carboxyatractylate does not only protect the protein against denaturation but also provides a quantitative assay for the intactness of the protein under different conditions. For this purpose the stability of the carboxyatractylate binding was monitored by gel filtration (G 75) after incubation of the protein under the various detergent-buffer conditions used in immunoelectrophoresis (for details, see Ref. 8).

Production of the antiserum. Antibodies were raised in rabbits against the purified carboxyatractylate-protein complex. Initially the antigen (0.1 mg) was suspended in Freund's complete adjuvant and the emulsion was injected subcutaneously at multiple sites in the back of rabbits. Blood samples were collected ten days after the last of four booster injections (0.2 mg) with Freund's incomplete adjuvant given at 4-week intervals. The serum was collected biweekly over a 6-week period after which the rabbits were killed and bled for collection of total serum. The blood was allowed to clot at room temperature, centrifuged at 15 000 rpm and stored at −40°C. Control serum collected from the same animals prior to injection was prepared in the same manner.

Charge shift crossed immunoelectrophoresis. Crossed immunoelectrophoresis was performed according to Clarke and Freeman [9]. The standard buffer system was 0.1 M glycine, 0.038 M Tris, pH 8.7. Electrophoresis runs were conducted in a water-cooled (16°C) flat-bed immunoelectrophoresis apparatus supplied by LKB (Sweden). The final detergent-agarose-system used in the first dimension was 0.08% (w/v) Triton x-100, 0.06% (w/v) deoxycholate present in the agarose-buffer system including the wicks. In the second dimension only 0.08% Triton X-100 was used. Other buffer-detergent mixtures employed are given in the legends to the figures. Unless otherwise stated, 0.75% (w/v) agarose gels were prepared from HSC-agarose Litex (Glostrup, Denmark) with very little electroendosmotic flow ($m_r = 0.02$). The protein was not preequilibrated [10]. The Triton-solubilized protein was applied to the gel and a partial detergent exchange was reached during electrophoresis in the first dimension [11]. In the first dimension electrophoresis was performed at 10 V/cm for 30 min, in the second dimension at 1.5 V.cm for 20 h. After washing in 0.1 M NaCl the gels were pressed, dried and stained with 0.5% Coomassie brilliant blue R in ethanol/water/acetic acid (45 : 45 : 10, v/v). Destaining was performed with the same solvent.

Results and discussion

The carrier protein located in the inner mitochondrial membrane is characterized by its hydrophobicity and a high content of basic amino acids such as lysine and arginine, giving the protein an isoelectric point of −10.3 as determined by isoelectric focusing [4]. A reproducible precipitation pattern could not be obtained by varying the electroendosmotic properties of the agarose (m_r values 0.0–0.13), the ionic-strength (0.002–0.4) and the pH (5.0–11.0) of the electrophoresis buffer. Although Triton X-100 was used in the agarose buffer system, unspecific precipitation of the ADP/ATP carrier developed already in the first dimension (Fig. 1a). This unspecific precipitation may be due to an interaction between the basic protein and SO_4^{2-} and COO^- groups present in the agarose [15] or to a removal of the detergent from the detergent-protein complex by the electrophoresis procedure, resulting in an aggregation. This tendency is presumably due to the hydrophobic domains on the amphiphilic protein coming uncovered as the detergent disappears. Also it was not a simple entrapment of large ADP/ATP carrier molecules in the agarose network because the solubilized molecules could not be sedimented at $100\,000 \times g$ for 1 h. After such a treatment soluble proteins move freely in agarose gels [1].

Therefore, to make the carrier-protein mobile in the electrophoresis system, a charge shift of the protein was induced by addition of detergent in analogy with the principle introduced by Hellenius and Simons [10] to distinguish between hydrophobic and hydrophilic proteins. By limited additions of the anionic detergent deoxycholate or the cationic detergent cetyltrimethylammoniumbromide (CTAB) to the Triton X-100-protein micelles the charges of these detergents were transfered to the protein-detergent micelle complex. Careful

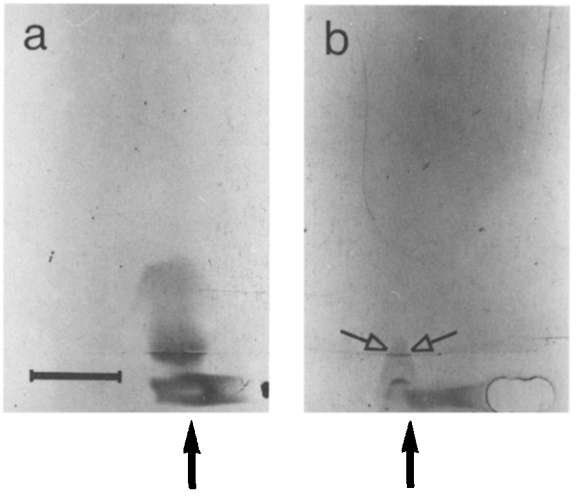

Fig. 1. Crossed immunoelectrophoresis of 10 μl Triton-solubilized carrier protein (10 μg protein). The first-dimension electrophoresis was performed at pH 8.7 in HSC agarose in the presence of: 1%(w/v) Triton X-100 (a); 0.5%(w/v) Triton X-100 plus 0.0125%(w/v) CTAB (b). The second-dimension electrophoresis was performed in agarose of the same type containing 1%(w/v) (a) or 0.5%(w/v) (b) Triton X-100 and 12.5 μl/cm^2 of anti-ADP/ATP carrier antiserum. Anode to the right and bottom. The filled arrow points to unspecific precipitation in the first dimension, the open arrow to the immunoprecipitate in the second dimension. The bar represents 1 cm.

titration of the detergent concentrations in the gel during the first dimension was carried out and the final concentration of Triton X-100 and the ionic detergent was chosen in dependence on migration velocity, intactness of the protein, and the undisturbed formation of the antigen-antibody complex.

First the addition of CTAB to the gel buffer system was tested. The presence of CTAB was expected to change the solubility of the protein and also to neutralize the charged groups of the agarose gel. Although the concentration of CTAB was considerably lowered compared to the original literature [10,11] unspecific precipitation took place in the first-dimension gel (filled arrows) (Fig. 1b). Only little improvement of immunoprecipitation was obtained, as indicated by the open arrows. Apart from the unspecific precipitation a partial denaturation of the carrier protein, as tested by carboxy[^{3}H]atractylate binding, was always observed after addition of CTAB, indicating that the membrane protein is extremely sensitive toward treatment with CTAB (Table I). Similar results were obtained using the ionic detergent cholate (Table I).

The combination of deoxycholate and Triton X-100 was more successful since conditions could be established under which no denaturation of the protein was observed (Table I) and a good migration velocity was reached without unspecific interaction of the antigen with the agarose in the first or second dimension. A well-defined and reproducible precipitation pattern was obtained at a Triton x-100 concentration of 0.08% (w/v) and a deoxycholate concentration of 0.06% (w/v), which means a weight ration Triton X-100 to deoxycholate of −1.25 (Fig. 2d). Data published previously indicate that Triton X-100 resembles deoxycholate in its inability to bind cooperatively and nonspecifically to proteins [14]. Therefore deoxycholate is easily incorporated into the Triton X-100 micelles and its free concentration is thus drastically reduced below the relatively high critical micelle concentration of deoxycholate. However, the ratio and the absolute detergent concentrations are important. At a Triton x-100/deoxycholate ratio of 0.15%/0.1% the precipitation pattern was much weaker (see Fig. 2e) and at 0.25%/0.2% neither an immune precipitate, nor any unspecific precipitation could be seen. As no dissociation of

TABLE I

THE EFFECT OF VARIOUS DETERGENTS ON THE CARBOXYL[^{3}H]ATRACTYLATE RELEASE AS MONITORED BY GEL FILTRATION (G-75)

The adenine nucleotide translocator was incubated under various detergent-buffer conditions at 4°C for 2 h and 24 h, respectively. Afterwards the intactness of the isolated protein was assayed by the stability of carboxy[^{3}H]atractylate ([^{3}H]CAT) binding as monitored by gel filtration (G-75). DOC, deoxycholate.

Detergents, %(w/v)	[^{3}H]CAT released (%)	Incubation time (h)
0.2% Triton X-100	0	2
	0	24
0.06% DOC, 0.08% Triton X-100	0	2
	0	24
0.06% Cholate 0.08% Triton X-100	15%	2
	50	24
0.01% CTAB, 0.2% Triton X-100	20	2
	25	24

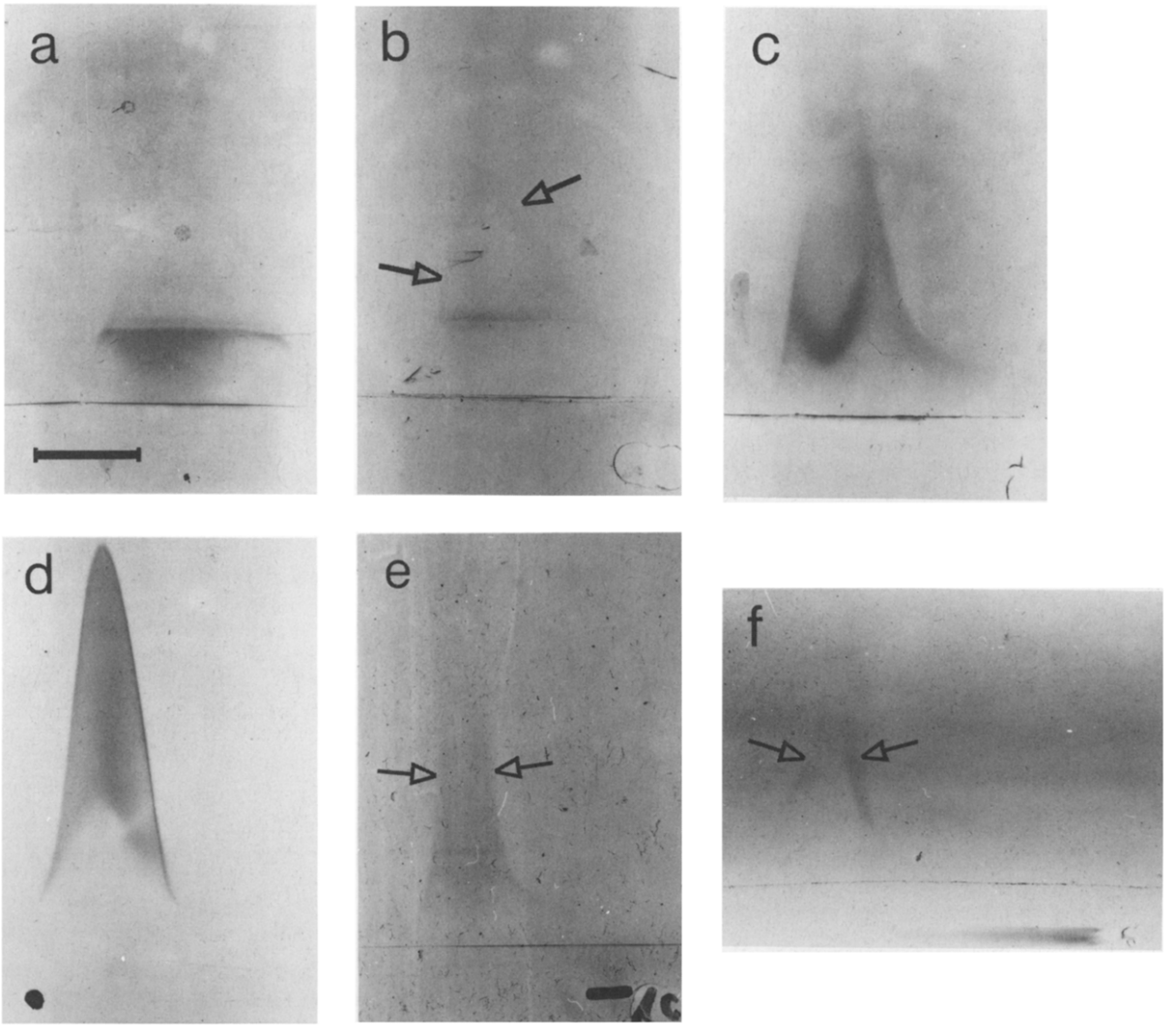

Fig. 2. Crossed immunoelectrophoresis of 10 μl of Triton-solubilized carrier protein (10 μg protein), at pH 8.7 in the presence of 0.06% (w/v) deoxycholate and 2% (w/v) Triton X-100 (a), 0.06% deoxycholate and 1.2% Triton X-100 (b), 0.06% deoxycholate and 0.5% Triton X-100 (c), 0.06% deoxycholate and 0.08% Triton X-100 (d + f), 0.1% deoxycholate and 0.15% Triton X-100 (e) in the first dimension. HSC agarose (a–e) or HSA agarose (f) was used. The second dimension was performed in the same type of agarose at an identical Triton X-100 concentration in the presence of 12.5 μl/cm^2 of anti-ADP/ATP carrier antiserum. Anode to the left and top.

carboxy[^{3}H]atractylate from the protein was observed under all these detergent concentrations, indicating intactness of the antigen, it has to be assumed that the failing precipitation may be caused by an interference of the detergents with the antigen-antibody reaction. In addition to selection and concentration of the detergents the choice of agarose is important. Thus, on using the more charged type of agarose, HSA ($m_r = 0.13$), instead of HSC ($m_r = 0.02$), unspecific precipitation of the protein in the first dimension was observed (Fig. 2f), although the general electrophoretic conditions used were the same. HSC agarose is characterized by a very low content of charged groups leading to a low electroendosmotic flow. This might be responsible for minor interaction of the antigen with the agarose, causing improved mobility of the protein.

Summing up, the charge shift of a protein-detergent micelle containing an intrinsic membrane

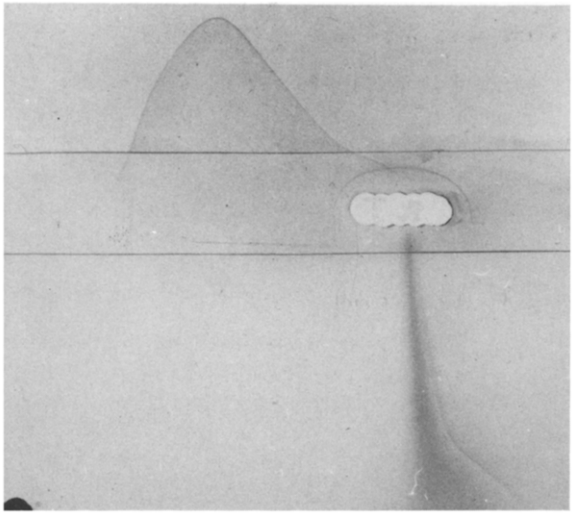

Fig. 3. Crossed immunoelectrophoresis of a Triton X-100 extract of mitochondria preloaded with carboxyatractylate. Mitochondria were suspended in 4% Triton X-100, 100 mM Na_2SO_4 and 10 mM Mops at pH 7.2 and extracted 30 min at 4°C. The mixture was then centrifuged at $143000 \times g$ for 40 min and the precipitate was discarded. First-dimension electrophoresis was performed at pH 8.7 in the presence of 0.06%(w/v) deoxycholate and 0.08%(w/v) Triton X-100, the second dimension at 0.08%(w/v) Triton X-100 with 12.5 $\mu l/cm^2$ of anti-ADP/ATP carrier antiserum. Anode to the left and top.

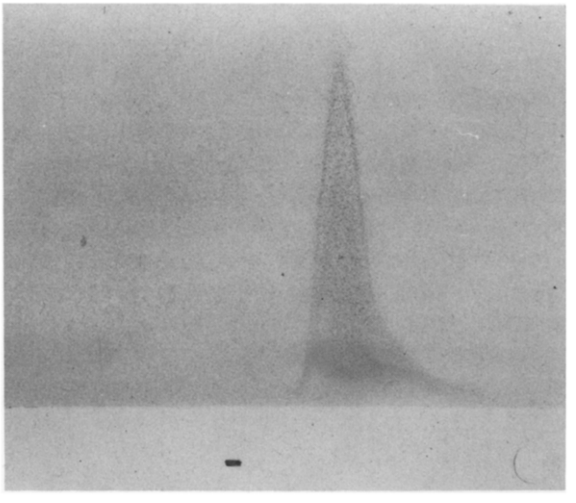

Fig. 4. Crossed immunoelectrophoresis of 10 μl Triton-solubilized GDP-binding protein from brown fat mitochondria (10 μg protein) (for isolation procedure, see Ref. 17). First-dimension electrophoresis was performed at pH 8.7 in HSC agarose in the presence of 0.06%(w/v) deoxycholate and 0.08%(w/v) Triton X-100, second dimension at 0.08%(w/v) Triton X-100 with 15 $\mu l/cm^2$ of a GDP-binding protein antiserum. Anode to the left and top.

protein like the ADP/ATP carrier by introduction of deoxycholate offers the methodological possibility for electroimmunochemical analysis of hydrophobic and basic membrane proteins [16]. Apart from the immunochemical characterisation of isolated proteins this method is also very useful in the study of intrinsic proteins present in an unpurified membrane fraction (Fig. 3). That the principle is of overall significance is obvious from the fact that it could be applied to other membrane proteins like the GDP-binding protein from brown fat mitochondria [17] (Fig. 4). If the detergent concentration and relation of the ionic to nonionic detergent are carefully titrated, a loss of antigenic properties and denaturation of the protein can be avoided and well-defined precipitation patterns can be obtained.

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