MONITORING THE RESOLUTION OF PROTEINS AND PEPTIDES IN THE COURSE OF THEIR ELECTROPHORESIS

Use in studying the degradative inactivation of cAMP-dependent protein kinase

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1. Introduction

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate [1,2] has now become one of the most widely used analytical tools in biochemistry for the identification of proteins, for the determination of their molecular weight, and for detecting protein degradations correlated with an altered physiological function (e.g., zymogen activation, activation and inactivation of polypeptide hormones, removal of 'anchoring' or 'signal' peptides). After the electrophoretic resolution is completed, the relative migration of the various proteins in the mixture is usually revealed by staining overnight with a solution of Coomassie blue (0.02%) in acetic acid (7%), then removal of the excess staining dye either electrophoretically or by extensive washing.

We wish to describe here the usefulness of prelabeling the constituents of a mixture to be analyzed with fluorescamine [3] which makes it possible to monitor continuously (under UV light) the resolution of proteins and peptides in the course of their electrophoresis. This is exemplified in the case of the degradative inactivation of catalytic subunit of cAMP-dependent protein kinase by a specific membranal proteinase from the brush border membrane of the rat small intestine.

2. Materials and methods

Free catalytic subunit of cAMP-dependent protein kinase was purified from rabbit muscle as in [4]. Brush border microvillus membranes were prepared from small intestines of 6–8-week-old male Wistar rats (Experimental Animal Unit, Weizmann Institute of Science, Rehovot) following the procedure in [5]. These membranes were further purified by the method in [6]. The C-subunit inactivating factor which brings about degradation of C was solubilized as in [7]. The low M_r calibration kit of proteins for SDS–polyacrylamide gel electrophoresis was purchased from Pharmacia (Uppsala); fluorescamine was obtained from Sigma.

SDS–polyacrylamide gel electrophoresis was done either with a homogeneous gel (15%) or using a linear polyacrylamide gradient gel (7–20%) [8]. Pre-labeling with fluorescamine was done in a 0.05 M $NaHCO_3$ buffer (pH 8.0). The reaction was initiated by addition of fluorescamine solution (0.3% (w/v) in acetone) so as to achieve 0.03% (w/v) final conc. of fluorescamine in the reaction mixture (0.2–1 mg protein/ml). The reaction (and subsequent decomposition of excess reagent [5]) was allowed to proceed for ~1 min at 23°C. At various stages of the electrophoresis (15 V/cm) the gel was illuminated with a UV lamp (Raytech, Stafford CT) and photographed using a green filter and Kodak-X film (~1 min exposure).

Protein concentrations were determined as in [9].

3. Results and discussion

We have shown [7] that upon exposure of the free catalytic subunit (C) of cAMP-dependent protein kinase to brush border membranes from the rat small intestine, the kinase loses its catalytic activity in a time-dependent process. In addition the factor

Abbreviations: C, the catalytic subunit of cAMP-dependent protein kinase (EC 2.7.1.37); M_r, relative molecular mass; SDS, sodium dodecylsulfate

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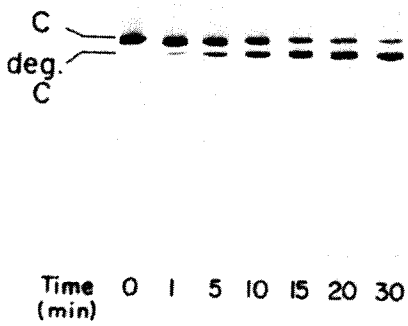

Fig.1. Time-dependent degradation of C by an enzyme extracted from brush border membranes through solubilization in octyl-β-D-glucopyranoside [7]. A reaction mixture containing pure C (final conc. 100 μg/ml) and the membranal enzyme (final conc. 15 μg/ml) was incubated in a 50 mM Hepes buffer (pH 7.5) at 23°C. At the indicated times, aliquots (40 μl) were removed and mixed with 10 μl of a solution containing SDS (10%) and 2-mercaptoethanol (7.5 M), and boiled for 5 min at 100°C. The samples were then subjected to SDS–polyacrylamide gradient gel electrophoresis as in section 2.

responsible for this inactivation can be further purified and solubilized in octyl-β-D-glucopyranoside [7]. When C is incubated with the solubilized enzyme preparation and aliquots of the reaction mixture are removed and subjected to SDS–polyacrylamide gradient gel electrophoresis, it becomes evident that the inactivation is accompanied by a degradation of C into a distinctly separable product (deg. C, fig.1). While the C was 40 000 M_r, the degraded product was found to have 34 000 M_r* (fig.2).

Since there is no indication in the literature suggesting that C may contain a fairly large non-proteinaceous constituent and since the experimentally determined M_r of C has been accounted for by the amino acid sequence of the enzyme which was completed recently [10], it seemed reasonable to assume that the specific, time-dependent degradation observed in the experiment depicted in fig.1 was due to a limited proteolysis of C. However, there was no indication for the presence of a peptide with ~6000 M_r in the gel (fig.1). This fact could be attributed to several reasons. For example, such a peptide, if released, might not become stained by the dye used or it could be further degraded into small peptides and amino acids which would diffuse readily out of the gel.

In an attempt to locate the small, clipped-off part of C, we labeled with fluorescamine the constituents of the reaction mixture at different times and subjected these samples to electrophoresis. During these experiments we observed that with such labeled samples it becomes possible to monitor continuously the

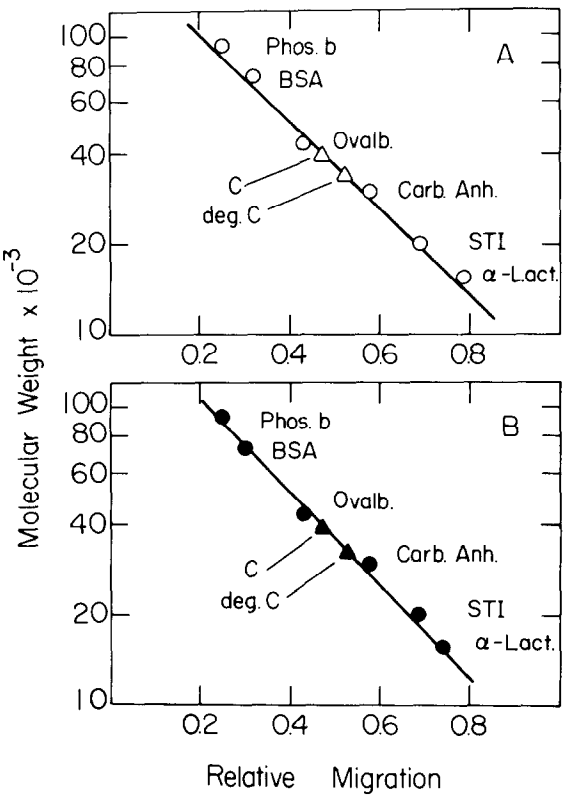

Fig.2. Determination of the M_r of C and of its high M_r degradation product (deg. C) by SDS–polyacrylamide gradient gel electrophoresis. The calibration curves were obtained using a M_r calibration kit containing: rabbit muscle glycogen phosphorylase (Phos. *b*, 94 000); bovine serum albumin (BSA, 67 000); ovalbumin (Ovalb., 43 000); bovine erythrocyte carbonic anhydrase (Carb. Anh., 30 000); soybean trypsin inhibitor (STI, 20 000) and bovine α-lactalbumin (α-Lact., 14 400). C and degraded C (deg. C) were obtained from the reaction mixture in the legend to fig.1. (A) Calibration curve obtained with unlabeled markers (after staining with Coomassie blue); (B) calibration curve obtained with fluorescamine-labeled markers (photographically recorded under UV light), C and deg. C were also labeled with fluorescamine, prior to electrophoresis.

* We had reported ~30 000 M_r for the degraded C [7]. On the basis of repeated experiments with further purified proteinase preparations, the degraded C seems to be closer to 34 000 M_r

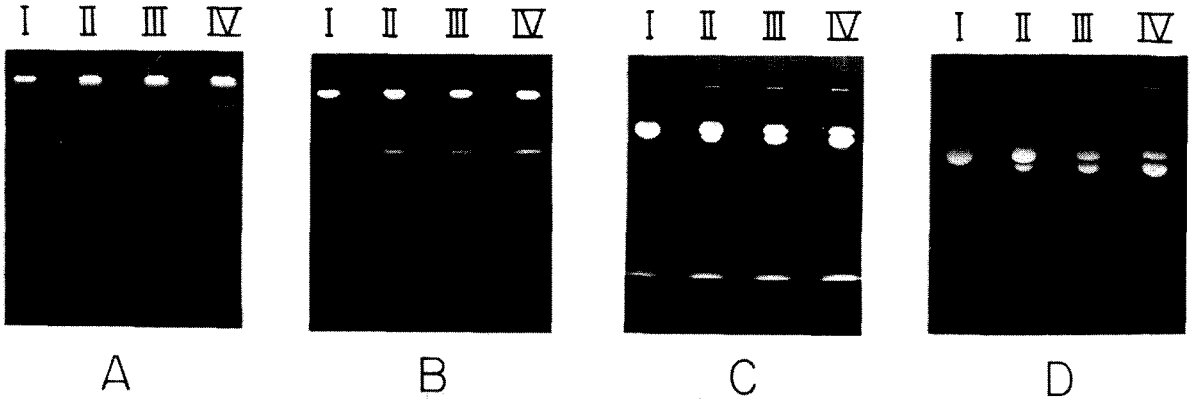

Fig.3. Use of fluorescamine labeling and electrophoresis for the continuous monitoring of the reaction between C and the membranal enzyme which causes its degradative inactivation. A reaction mixture containing C (final conc. 0.9 mg/ml) and the solubilized membranal enzyme (final conc. 45 μg/ml) in 50 mM $NaHCO_3$ buffer (pH 8.0) was incubated at 23°C. The following times (I, 0.5 min; II, 10 min; III, 40 min; IV, 180 min) samples of 40 μl were removed, labeled with fluorescamine, mixed with SDS and 2-mercaptoethanol and boiled as in the legend to fig.1. The samples were then applied on a 15% polyacrylamide gel and the course of electrophoresis was followed and recorded photographically under UV light. (A–D) Photographs of the same gel taken at various stages of the electrophoresis: (A) upon entry of the samples into gel; (B) after 20 min; (C) after 50 min; (D) after 120 min.

course of the electrophoresis by simply illuminating the gel with an UV lamp.

Using this procedure we were able to detect a time-dependent appearance of low M_r materials which did react with fluorescamine and thus became visible under UV light. These low M_r compounds were clearly seen at the initial stages of the electrophoretic migration but later became diffuse and finally migrated out of the gel. Fig.3 shows 4 pictures of the same gel (homogeneous polyacrylamide, 15%), taken under UV light at different times in the course of the electrophoresis. While panels B and C clearly show the low M_r materials that rapidly migrate into the gel, panel D (where the low M_r materials have already diffused out) clearly shows the resolution between C and its high M_r degradation product (deg. C).

The major advantage of being able to continuously monitor the course of electrophoresis is obvious here, since it becomes possible to carry out an analysis (of the very same samples and on the same gel) of both the low M_r constituents which separate out first, and the high M_r constituents which are resolved only at a much later stage (fig.3).

Fig.4 illustrates a follow-up of the resolution of 6 proteins (on SDS–polyacrylamide gradient gel, 7–20%) as recorded photographically. It is clearly evident from this figure how the procedure proposed here makes it possible to find out (without having to

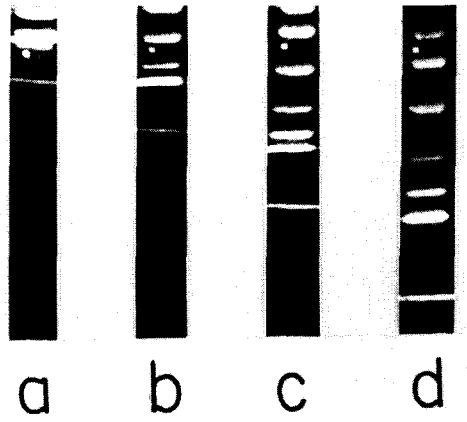

Fig.4. Monitoring the course of electrophoresis of a mixture of marker proteins. A sample containing 6 proteins dissolved in 50 mM $NaHCO_3$ buffer (pH 8.0) (phosphorylase *b*, 0.12 mg/ml; bovine serum albumin 0.17 mg/ml; ovalbumin, 0.3 mg/ml; carbonic anhydrase, 0.17 mg/ml; soybean trypsin inhibitor, 0.17 mg/ml; α-lactalbumin, 0.25 mg/ml) was reacted with fluorescamine and subjected to SDS–polyacrylamide gradient gel electrophoresis as in section 2. The resolution of this mixture with time was recorded photographically under UV light. The 4 panels are photographs of the same electrophoresis track taken at different times: (a) upon entry of the sample into the gel; (b) after 20 min; (c) after 60 min; (d) after 120 min.

terminate the electrophoresis and stain the gel) at what stage a desired separation has already been achieved.

It should be emphasized, however, that when proteins are labeled with fluorescamine their M_r is increased somewhat, depending on the number of amino acids in the molecule which become labeled. As a result, the relative migration of the various proteins in a mixture may be slightly affected. Since proteins which have been labeled with fluorescamine under the conditions used here can still be stained with Coomassie blue, we attempted to evaluate the extent to which fluorescamine labeling may interfere with M_r determinations.

Panels (A,B) in fig.2 represent a comparison of a molecular weight determination of C and degraded C using a calibration kit of 6 proteins (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soy-bean trypsin inhibitor and α-lactalbumin). While in (A) the marker proteins as well as C and degraded C were subjected to electrophoresis without labeling, in (B) all proteins were pre-labeled with fluorescamine. It can readily be seen that in spite of the fact that the relative migration of 2 of the markers (bovine serum albumin and α-lactalbumin) decreases slightly in the case of the labeled proteins, the linearity of the calibration curve is still preserved and the M_r-values obtained for C and degraded C are very close (within 3%) to those determined for the non-labeled C and deg. C.

In conclusion, the useful features of the procedure recommended here are:

(i) Since the course of electrophoresis is continuously monitored, it becomes possible to record photographically also the initial stages of the process, before some of the low M_r constituents have diffused within the gel, migrated out, or decomposed during electrophoresis, staining or destaining;

(ii) Since there is no need to arrest the electrophoresis at each stage for staining, it becomes possible to proceed further on the same gel until a desired resolution between closely migrating constituents has been secured;

(iii) The modified procedure is rapid (pre-labeling is instantaneous and the staining/destaining steps are unnecessary) and exceptionally sensitive [3]. Furthermore, since fluorescamine reacts with primary amines which are widespread in peptides and proteins, the sensitivity of this procedure does not depend on the presence of SH groups (cf. [11]);

(iv) The constituents of a tested sample can in some cases be completely removed out of the gel, by continuing the electrophoresis and since this can be ascertained visually it provides for a repeated use of the same gel.

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