THE PURIFICATION OF NUCLEAR PROTEIN KINASE BY AFFINITY CHROMATOGRAPHY

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1. Introduction

Owing to their putative involvement in the regulation of gene expression, the nuclear phosphoproteins have been studied extensively in recent years [1–6]. There is much evidence to suggest that the turnover of protein-bound phosphate in the nucleus is carried out in situ, i.e., by nuclear protein kinases and phosphoprotein phosphatases [7–10] that are distinct from enzymes carrying out similar functions in the cytoplasm. Attempts to purify the nuclear protein kinase activity have led to the demonstration that it exists in multiple molecular forms [9,11,12].

Fractionation of nuclear extracts on phosphocellulose has yielded from two to twelve fractions containing protein kinase activity in different laboratories. In our laboratory ten individual fractions are reproducibly obtained from rat liver nuclei, six of which are present in significant quantities [9]. Fractionation on phosphocellulose, while separating the various protein kinases from one another does not, however, separate the kinase activities from the proteins that serve as their phosphate acceptor substrates, as attested by the fact that the kinases can be assayed in the column effluent without addition of phosphate acceptor proteins. It is for this reason that attempts to study the substrate specificity of individual protein kinases toward their physiological substrates, the chromatin-associated proteins, have remained inconclusive [11,13].

The activity of the nuclear protein kinases is manifold greater when casein serves as phosphate acceptor. Taking advantage of the affinity of the nuclear kinases for casein we have prepared a casein–Sepharose column on which the enzymes can be cleanly separated from their endogenous phosphate acceptors. The substrates as well as the protein kinases are obtained in high yield, the latter in a highly purified state.

2. Materials and methods

Sepharose 4B was obtained from Pharmacia Fine Chemicals. Electrophoretically pure DNAase I and pancreatic RNAase were obtained from Worthington Biochemical Corp. All other reagents were reagent grade purchased from Sigma Chemical Co. [^{32}P]ATP was prepared by the method [14].

Nuclei were prepared by the method [15]. All operations were carried out at 0–4°C unless otherwise noted. Purified nuclei from 15 g rat liver were extracted once with 15 ml 0.14 M NaCl. This extract contains most of the nuclear phosphoprotein phosphatase activity. The extracted nuclei were suspended in 4 ml 0.5 M NaCl containing 10 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, and digested with 20 μg DNAase I and 10 μg pancreatic RNAase for 30 min at 20°C while kept in suspension by occasional homogenization. The suspension was centrifuged at 7000 × g for 10 min and the pellet extracted once more without addition of nucleases. The combined supernates were dialyzed against 100 vol. 0.1 M NaCl, 50 mM Tris–HCl, pH 7.5, 0.05% Nonidet P-40 (buffer I) for 16 h. The precipitate formed was removed by centrifugation and the soluble proteins were fractionated on a 20 ml casein–Sepharose column as described in the text.

2.1. *Preparation of casein–Sepharose*

Casein was coupled to Sepharose 4B according to the method of [16] as modified [17]. Thoroughly

washed Sepharose 4B was centrifuged at 700 × *g* for 15 min and 20 ml packed beads were suspended in an equal volume of distilled water, placed in an ice bath and kept in suspension by means of a magnetic stirrer. The pH was brought to 11 with a drop of 10% NaOH. Solid CNBr 59/40 ml suspension were added and the pH was kept between 10.8 and 11.2 by dropwise addition of 10% NaOH until the reaction was completed as evidenced by stabilization of the pH. The activated Sepharose was washed rapidly on a coarse sintered glass filter under gentle suction with 10 vol. cold coupling buffer (0.01 M Na phosphate, 0.10 M NaCl, pH 7.5). Casein 300 mg/20 ml coupling buffer were mixed with the activated Sepharose and transferred to an Erlenmeyer flask. The mixture was agitated in a rotary shaker bath at 20°C for 16 h, passed over a coarse sintered glass filter and the degree of coupling was determined by measuring the protein content in the filtrate by the method [18]. Coupling efficiency was routinely between 95% and 100%. After thorough washing with cold coupling buffer the casein–Sepharose was suspended in 0.2 M ethanolamine and left for 3 h in the cold. It was then washed with several volumes of buffer I and could be stored in the cold in the presence of 10^{-5} M merthiolate. A 20 ml column will bind all of the nuclear protein kinase contained in 15 g rat liver, but determination of the capacity of individual preparations by small batch absorption is recommended. The column can be regenerated twice by thorough washing with 2 M NaCl and re-equilibration with buffer I.

Protein kinase activity was measured for 10 min at 30°C in 0.2 ml reaction vol. containing 25 mM Tris–acetate, pH 7.5, 10 mM $MgCl_2$, 200 μg casein or 100 μg endogenous substrate, 10 μM ATP (5×10^5 cpm/nmol), 0.3 mM EGTA and varying amounts of nuclear extracts. When purified protein kinase was assayed, 2–4 μg protein was added to the incubation.

One unit of protein kinase activity is defined as the amount of enzyme required to transfer 1 nmol ^{32}P from ATP to acceptor protein under the conditions of the assay.

2.2. *Electrophoresis*

Electrophoretic separation of phosphoproteins was carried out in slabs of 5–15% polyacrylamide gradient gels containing 0.1% sodium dodecyl sulfate (SDS) in the buffer system described [19]. Purified, washed nuclei were incubated as described above, pelleted by centrifugation, washed once in 25 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$ and incubated in the same buffer at 30°C for 10 min with 5 μg DNAase I and 2.5 μg RNAase. SDS to a final concentration of 2%, β-mercaptoethanol to 4 mM were added, the samples were boiled for 2 min, cooled and sucrose to 20% was added. Soluble substrates incubated with or without enzyme were precipitated with 1/10 vol. 100% trichloroacetic acid, redissolved in 0.1 N NaOH, neutralized to approx. pH 7.5–8 with 0.1 N acetic acid and treated in identical fashion as the nuclei, except that nuclease digestion was omitted. Gels were subjected to a current of 20 mA, stained with Coomassie blue, dried and exposed to Kodak NS.T5 film.

3. Results and discussion

When rat liver nuclei are extracted with 0.5 M NaCl, 10 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$ in the presence of DNAase I and pancreatic RNAase at 20°C, 80% of the protein and most of the protein kinase activity are solubilized. Although recovery of protein kinase activity in this extract is usually greater than 100%, measurable amount remain in the pellet. Upon dialysis of the 0.5 M NaCl extract against 0.1 M NaCl, 50 mM Tris–HCl, pH 7.5, containing 0.05% Nonidet P-40 (buffer I) more than 50% of the proteins are recovered in soluble form while some precipitate as the salt concentration is lowered. The precipitate redissolves completely in 0.5 M NaCl. Analysis of this pellet by electrophoresis in polyacrylamide gel (data not shown) indicates that primarily three protein species are insoluble in buffer I. Their solubility properties and estimates of their molecular weights suggest that they may be the contractile proteins described [20].

The proteins remaining soluble in buffer I were applied to a 20 ml casein–Sepharose column equilibrated with the same buffer. The majority of the proteins are not bound to the column and are recovered in the buffer I effluent, apparently in the undenatured state. This is inferred from the efficiency with which they serve as phosphate acceptors for the kinase. When monitored with casein as phosphate acceptor a very small amount of protein kinase activity can be detected in the buffer I effluent, but activity with endogenous substrate (table 1, column 2, line 4) can

Table 1
Purification of protein kinase from rat liver nuclei

	Volume	Substrates (total units)		Yield (%)	Total protein (mg)	Specific activity[a]
		Endogenous	Casein			
Purified nuclei	10.0	6.0	13.2	100	22.7	0.58
0.5 M NaCl extract	8.0	7.1	17.0	129	18.0	0.94
Proteins soluble in 0.1 M NaCl	5.3	4.4	12.1	92	9.7	1.25
Casein–Sepharose effluent at 0.1 M NaCl (substrate)	18.0	0.23	0.7	–	7.3	–
Casein–Sepharose effluent at 0.6 M NaCl (enzyme)	3.3	nil	10.4	79	0.24	43.5

[a]Based on activity measured with casein as substrate

only be measured after the pooled fractions have been concentrated at least 10-fold.

The column is then washed with 20 vol. buffer I and the enzymes are eluted with the same buffer containing 0.6 M NaCl. The active fractions are located with casein as substrate. In the absence of casein no activity can be detected even after several-fold concentration (table 1, column 2, line 5) indicating that the enzyme is completely free of substrate.

In the presence of substrate the enzymes can be stored for several days at 0°C without loss of activity, whereas the purified enzymes lose activity rather rapidly. They can be stabilized considerably if collected directly into tubes containing 500 μg/ml bovine serum albumin. This is not practicable when gel analyses are to be performed or for the determination of specific activity. For these purposes we concentrated the active fractions as rapidly as possible by submersing the enzyme, contained in a dialysis bag, in dry Sephadex G-200. The results of a typical purification are summarized in table 1.

Autoradiography of electrophoretograms in SDS-containing polyacrylamide gels (fig.1) confirms that the substrates obtained from the casein–Sepharose column contain negligible amounts of protein kinase (channel C). When this fraction is incubated with the purified protein kinase, the pattern of phosphorylated proteins (channel B) closely resembles that generated in vivo (channel A), i.e., obtained by the same extraction procedure of nuclei from livers of rats that had been injected with ^{32}P 3 h before sacrifice.

The clean separation of nuclear protein kinase activity from endogenous substrates has not been previously achieved. Attempts to discover whether individual peaks of enzyme activity, as obtained by fractionation on phosphocellulose, phosphorylate specific acceptor proteins remained, therefore, inconclusive.

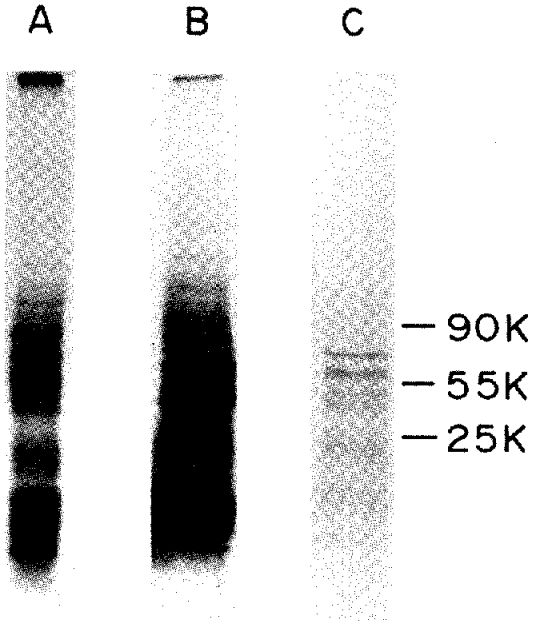

Fig.1. Autoradiogram of the phosphoproteins from rat liver nuclei separated by electrophoresis in acrylamide gel as described in Materials and methods. (A) Pattern of phosphoproteins extracted from liver nuclei of rats injected with 5 mCi ^{32}P 3 h before sacrifice. (B) Pattern of phosphoproteins generated by incubating purified nuclear protein kinase in the presence of substrates prepared as described in the text. (C) Substrates incubated without added protein kinase.

The endogenous protein kinase activity was inactivated by heating an aliquot of their phosphoprotein preparation in order to use it as substrate for individual kinases obtained by fractionation on phosphocellulose [11]. This procedure, however, inactivates the substrates as well, as shown by their poor acceptor capacity (^{32}P incorporated per μg acceptor protein). Electrophoretic analysis in SDS containing acrylamide gels reveals only 6–8 poorly defined bands (data not shown). The fraction not retained on phosphocellulose was used as substrate for protein kinase fraction IVb [13]. Although it was claimed that this flow-through fraction contained little kinase activity, addition of the partially purified enzyme did not materially alter the pattern of phosphorylated proteins upon SDS acrylamide gel electrophoresis, indicating that the fractions, though enriched in substrates and enzyme, respectively, are still cross-contaminated. The clean separation of the nuclear protein kinases from their endogenous substrates on casein–Sepharose should greatly facilitate studies of their specificity toward their physiological substrates.

In summary, a simple procedure for the purification of protein kinase (EC 2.7.1.37) activity from rat liver nuclei is described. Fractionation of crude nuclear extracts on casein–Sepharose yields two fractions: one not bound to the column and eluted with the equilibrating buffer, contains the majority of chromatin-associated proteins which serve as phosphate acceptors for the kinase. The second fraction which is eluted from the column at 0.6 M NaCl contains the enzymes in a highly purified state. Substrates and enzymes are recovered in good yield and incorporate virtually no phosphate when incubated separately. When incubated together the pattern of phosphoproteins generated demonstrates – within the limits of resolution of one-dimensional gel electrophoresis – that all the nuclear proteins that are phosphorylated in vivo are also phosphorylated in vitro. This is inferred from the close similarity of the positions of the majority of the bands; that relative intensities differ ought not to be surprising, if it is born in mind that the phosphate acceptor fraction is extracted in the phosphorylated state (see channel A). It is not known at present whether the fact that the nuclear phosphoprotein fraction can serve as substrate for purified kinase requires the presence of endogenous phosphoprotein phosphatases; whether the acceptor proteins can be 'superphosphorylated' i.e., whether molecules already containing phosphate as extracted, can accept additional phosphate at different sites, or whether a fraction of the acceptor proteins are extracted in the unphosphorylated state. The present work is but the first step in the continuing effort to answer these questions.

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