Purification of Rat Liver Nuclear Protein Kinase NII*  

Rat liver nuclear protein kinase activity (NII), which is eluted from DEAE-Sephadex columns, has been purified approximately 1000-fold from solubilized nuclear protein. The method of purification involved chromatography of protein eluted from DEAE-Sephadex successively on phosvitin-Sepharose, mixed histone-Sepharose, and histone H2b-Sepharose followed by gel filtration on Sephadex G-200. Resulting preparations are homogeneous by polyacrylamide gel electrophoresis. The enzyme consists of three polypeptides with molecular weights of 42,000 (α), 39,000 (α'), and 26,000 (β) which are present in the ratio 1:1:2 indicating that the enzyme has a minimum tetrameric subunit composition of αααβ. The molecular weight and sedimentation coefficient of the purified enzyme were 123,000 and 7.0, respectively, as determined by sucrose density gradient centrifugation in 0.4 M NaCl. The enzyme has maximal activity with phosvitin as substrate and is not stimulated by 10 ms to 10 μM CAMP or cGMP using H2b as substrate.

Extracts of rat liver, like those from other sources, contain protein kinases which are found both in cytoplasmic and nuclear fractions. For comprehensive surveys, see Rubin and Rosen (1) and Krebs (2). Langan (3) has detected a cytoplasmic enzyme from rat liver which utilizes histone as substrate and is stimulated by CAMP. Rat liver nuclear protein kinases have also been previously partially purified and characterized (4-6). Like nuclear protein kinases from other sources, these enzymes prefer phosvitin and casein to histone as substrate and do not require cyclic nucleotides for maximal activity. There are, however, phosvitin kinases in the cytoplasm (7) and some have reported the detection of cAMP-stimulated histone kinase activity in nuclei (8, 9). It is not known whether this atypical distribution is due to cross-contamination or actual subcellular localization. In some systems, it is believed that the nuclear protein kinase activity represents the catalytic subunit of the cAMP-dependent cytoplasmic enzyme which has been translocated to the nucleus (10-12).

Nuclei also contain a number of non-histone or acidic proteins which are phosphorylated by the endogenous nuclear protein kinases (13, 14). Recent evidence suggests that nuclear non-histone protein phosphorylation may be involved in the regulation of transcription (15-19), possibly at the level of RNA polymerase itself (20-25). Since the location of these enzymes and the identification of their endogenous substrates are of major importance, we have begun the purification and characterization of these nuclear protein kinases. Protein kinase NII has been purified using its preferred substrate (phosvitin) and histone (mixed and H2b) immobilized on Sepharose. The purification procedures employed provide a homogeneous preparation of the enzyme which will allow further characterization with regard to endogenous substrates and the potential role of this enzyme in cellular regulation.

MATERIALS AND METHODS

Buffers and Reagents—Chemicals were purchased from the following sources: phosvitin, histone H2b, bovine serum albumin, and DEAE-Sephadex (A-25), Sigma; mixed histone, Worthington; Sepharose 4B-CL and Sephadex G-200-120, Pharmacia; [γ-32P]ATP (10 Ci/mmol), New England Nuclear; catalase, Boehringer, Mannheim; brilliant blue R-250, Bio-Rad. The standard buffer consisted of the following: 0.05 M Tris/HCl, 0.1 mM EDTA, 5.0 mM MgCl₂ (pH 7.9), 1 mM dithiothreitol, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride as a possible precaution against proteolytic degradation.

Protein Kinase Assay—Protein kinase NII assays were performed in a final volume of 100 μl according to the method of Desjardins et al. (5) as previously described (26) except that phosvitin (2 μg/ml) was employed as substrate unless otherwise indicated. Phosvitin and other substrate solutions were routinely prepared by adjusting the pH to 9.0 with 1 N NaOH, placing in a boiling water bath for 10 min, and neutralized with 1 N HCl. All assays contained 0.2 M NaCl to optimize activity and were initiated by the addition of 50 μl of enzyme unless otherwise indicated. When necessary, endogenous phosphorylation was assessed by performing assays in the absence of substrate. Endogenous incorporation was then subtracted from that using exogenous substrate. For assays presented in Figs. 1 to 3 under "Results," 15 to 30 pmol of 32P, are incorporated/10⁶ cpm.

Isolation of Nuclei and Preparation of Soluble Nuclear Protein—Nuclei were isolated from the livers of male Sprague-Dawley rats weighing 200 to 400 g by the method of Blobel and Potter (27) as modified by Guan and Linde (28). Nuclei were routinely frozen in liquid-N₂ prior to use. Protein was solubilized from freshly thawed nuclei by sonication in the presence of 0.3 M (NH₄)₂SO₄ according to the method of Roeder and Rutter (28). The preparation was then diluted to 0.1 M (NH₄)₂SO₄ by the addition of 2 volumes of standard buffer and centrifuged at 48,000 × g for 10 min in a Sorvall RC2B to rid the preparation of insoluble chromatin. The supernatant was then further diluted to 0.05 M (NH₄)₂SO₄ by the addition of an equal volume of the same buffer. This preparation was then directly applied to a DEAE-Sephadex column equilibrated with standard buffer and centrifuged at 48,000 × g for 10 min in a Sorvall RC2B to rid the preparation of insoluble chromatin. The supernatant was then further diluted to 0.05 M (NH₄)₂SO₄ by the addition of an equal volume of the same buffer. This preparation was then directly applied to a DEAE-Sephadex column equilibrated with standard buffer containing 0.05 M (NH₄)₂SO₄ of an appropriate size to accommodate 1 g of protein from a preparation of 150 ml of nuclei. No more than 4 mg of protein/ml of bed volume was applied to DEAE-Sephadex columns. Protein kinase NII was found in the flow-through fraction.
and NII was eluted from DEAE-Sephadex with a linear gradient from 0.05 to 0.7 M (NH₄)₂SO₄ in standard buffer as described by Desjardins et al. (5) and Gano and Lindell (20). Fractions corresponding to 0.673 M to 0.27 M (NH₄)₂SO₄ were routinely pooled for the collection of NII.

Preparation of Substrate-Sepharose Columns—Sepharose 4B-CL was activated with cyanogen bromide and coupled to the desired protein ligand by the method of Cuatrecasas (29) as described by Ryan and Vestling (30). The quantities of Sepharose 4B-CL (wet weight), protein, and coupling buffer used to make the columns described were as follows: phosvitin, 30 g, 1.5 g, 50 ml; mixed histone, 41 g, 0.5 g, 20 ml; and H₂b, 10 g, 0.05 g, 2 ml, respectively. Phosvitin and other proteins coupled to Sepharose were not treated by boiling as previously described for substrate. After coupling, preparations were extensively washed with 1 M NaCl to remove all unbound ligand. The per cent of protein bound was then estimated from the amount remaining in the 1 M NaCl wash. The final yields of bound ligand were as follows: phosvitin, 9, mixed histone, 9, and H₂b, 2 mg of protein/ml of packed Sepharose, respectively.

Affinity Chromatography—Chromatography with derivatized Sepharose was conducted under a standard set of conditions. The salt concentration of starting material was determined by conductance measurements and then adjusted to 0.2 M NaCl or 0.067 M (NH₄)₂SO₄ in the case of pooled fractions eluted from DEAE-Sephadex by diluting with standard buffer prior to loading. After loading, columns were washed with 1 to 3 column volumes of the same buffer containing 0.2 M NaCl or until protein returned to base-line when monitored with an ultraviolet detector. Columns were then eluted with a gradient of 0.2 to 1.5 M NaCl at a rate of 10 ml/h. Fractions of approximately 2 ml were collected and assayed for activity. Enzyme activity is routinely collected from the respective columns by pooling fractions corresponding to the following salt ranges: phosvitin-Sepharose, 0.24 to 0.64 M NaCl; mixed histone-Sepharose, 0.44 to 0.82 M NaCl; and H₂b-Sepharose, 0.34 to 0.75 M NaCl. An overall yield of 13% was obtained by selecting activity strictly on the basis of salt without performing assays of individual columns.

Gel Filtration and Sucrose Gradient Centrifugation—Sephadex G-200 columns of either 130 ml (14 x 1.2 cm) or 240 ml (13 x 1.5 cm) were equilibrated and developed with standard buffer containing 0.4 M NaCl. Gradients (5.0 ml) of 5 to 20% sucrose in standard buffer containing 0.4 M NaCl without glycerol were run in a Spinco SW 50.1 rotor. Prior to centrifugation, preparations of enzyme were briefly dialyzed against the same buffer to remove glycerol. After centrifugation at 3°C for 14 to 16 h at 37,000 rpm (120,000 x g), tubes were punctured at the bottom, and 10-drop fractions were collected. Protein kinase activity was assayed as previously described. Molecular weights and sedimentation coefficients were calculated by the method of Martin and Ames (31).

Polyacrylamide Gel Electrophoresis—Native gels consisted of 6.5% acrylamide and 0.15% N,N'-methylenebisacrylamide in 0.37 M Tris (pH 8.8) (32). After polymerization, gels were pre-electrophoresed in running buffer (2.5 mm Tris, 20 mm glycine, pH 8.2) for 2 or more h. Samples were applied and subjected to electrophoresis at 1 mA/gel at 4°C. To assess protein kinase activity, native gels were cut into 2 mm slices with a razor blade and put into individual assay vials containing 50 μl of phosvitin substrate (10 mg/ml). After incubating for 1 to 72 h at 4°C, 30 μl of the regular assay mixture minus acceptor protein substrate was added and incubated at 37°C for 1 to 2 h. Aliquots of 50 μl were withdrawn, spotted to filters, and washed as described above. Electrophoresis in sodium dodecyl sulfate gels (7.5% acrylamide, 0.2% N,N'-methylenebisacrylamide), staining, and destaining were performed according to the method of Weber and Osborne (33), except that destaining was also performed by diffusion at 60°C. Gels were scanned with a Gilford gel scanning attachment at 550 nm.

Protein Determination—Protein was determined by the method of Lowry et al. (34) after the addition of an equal volume of cold 10% trichloroacetic acid and centrifugation in a Brinkmann microcentrifuge to remove glycerol. Bovine serum albumin was used as a protein standard.

Ionic Strength—All measurements of ionic strength were performed with a Radiometer model CDM2r conductivity meter. Conductivity measurements were converted to molarity by interpolation from standard curves of NaCl and (NH₄)₂SO₄, neglecting the slight contribution of standard buffer which gives a conductivity equivalent to 0.04 M NaCl.

RESULTS

The following is a description of the purification of protein kinase NII from a representative preparation starting with 120 ml of nuclei obtained from 280 g of rat liver. To date, this procedure has been used 15 times.

DEAE-Sephadex Chromatography—The profile of protein and protein kinase activity routinely seen has been previously described by Desjardins et al. (5). Protein kinase N1 does not adhere to DEAE-Sephadex under the conditions of chromatography employed. The DEAE-Sephadex flowthrough fraction containing N1 was concentrated by precipitation with solid (NH₄)₂SO₄ to 85% and centrifuged at 19,600 x g in the Sorvall GSA rotor. The resulting pellet was resuspended in a minimal volume of standard buffer containing 0.2 M NaCl and stored frozen in liquid N₂.

Phosvitin-Sepharose Chromatography—Fractions containing protein kinase NII, eluted from the DEAE-Sephadex column, were pooled and subjected to chromatography on phosvitin-Sepharose. The results are shown in Fig. 1. Seventy percent of the protein kinase activity bound<sup>2</sup> and was eluted at approximately 0.5 M NaCl. The remaining activity was recovered after rechromatography of the flowthrough fractions on the same column. In subsequent preparations from a similar number of nuclei, 40 to 100 ml of column material was sufficient to bind 80 to 97% of the activity after a single passage.

Mixed Histone-Sepharose—Protein kinase activity eluted from phosvitin-Sepharose was then applied to a mixed histone-Sepharose column. Fig. 2 shows a typical pattern of elution of activity from this column. The peak of protein kinase activity was eluted at approximately 0.6 M NaCl. This column bound 100% of the starting activity.

<sup>2</sup>The performance of individual protein-Sepharose columns was evaluated from a determination of the per cent activity bound. This is calculated by subtracting the total activity of the flowthrough and wash from that of the starting material. Per cent binding does not imply per cent yield from these columns.
Histone H2b-Sepharose – Protein kinase activity eluting from the mixed histone-Sepharose column was subsequently applied to a column of H2b-Sepharose, which was eluted batchwise with 1 M NaCl as shown in Fig. 3. Subsequent preparations have employed a 10-ml H2b-Sepharose column which resulted in the binding of 90 to 100% of starting protein kinase activity. Its capacity has not been exceeded in any preparation of up to 1000 units. Histone H2b-Sepharose columns are now routinely eluted with gradients as discussed under "Materials and Methods."

Sephadex G-200 Gel Filtration – Fractions collected from the H2b-Sepharose column were pooled and concentrated with an Amicon ultrafiltration apparatus with a UM-10 membrane. Two to four milliliters of concentrated protein were applied to a 240 ml G-200 column equilibrated with standard buffer containing 0.4 M NaCl. As shown in Fig. 4, the protein kinase activity is partially separated from the bulk of the protein, which appears near the void volume in a single peak. Enzyme activity elutes in a total of approximately 20 2-ml fractions. The last 15 fractions of activity are routinely pooled, concentrated to 1 to 2 ml by ultrafiltration, and stored in liquid N2. The enzyme remains active after several months of storage as well as after repeated freezing and thawing.

Purification – Table I depicts the level of purification obtained using the described sequence of purification steps. The data presented are typical and this method is currently being repeated freezing and thawing.

Approximately 4 ml (1.1 mg of protein) of concentrated pooled fractions from H2b-Sepharose was applied to a 240-ml column (136 x 1.5 cm) of Sephadex G-200 which had previously been equilibrated with standard buffer containing 0.4 M NaCl. Fractions (1.7 ml) were collected and assayed as described in the legend of Fig. 1. Protein was monitored by an ISCO UV analyzer. Fractions 47 to 64 were pooled for chromatography on a histone H2b-Sepharose column.

Histone H2b-Sepharose – Protein kinase activity eluting from the mixed histone-Sepharose column was diluted to 131 ml. This was applied to a 4.5-ml column of H2b-Sepharose. The column was then washed and batch eluted with standard buffer containing 1 M NaCl. Fractions were collected and assayed for protein and activity as described in the legend of Fig. 1. ---, NaCl (m); ---, protein kinase activity; ---, protein concentration (μg/ml). Fractions 47 to 64 were pooled for chromatography on a histone H2b-Sepharose column.

Histone H2b-Sepharose – Protein kinase activity eluting from the phosvitin-Sepharose column were diluted to 280 ml. This was applied to a 50-ml mixed histone-Sepharose column and eluted with a 200-ml linear gradient of 0.2 to 1.2 M NaCl. Fractions were collected and aliquots were withdrawn and assayed as described in the legend of Fig. 1. ---, NaCl (m); ---, protein kinase activity; ---, protein concentration (μg/ml). Fractions 47 to 64 were pooled for chromatography on a histone H2b-Sepharose column.

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Materials and Methods. Assay of protein kinase activity. (b) a parallel gel was stained and assayed as described under Materials and Methods. Fractions from the single peak of protein kinase activity were pooled and 200 μl (30 μg) was applied to a native gel (0.6 (inside diameter) x 8 cm). This gel was stained and a parallel gel was assayed as described under "Materials and Methods." Fractions from the region of the gel as stain. A parallel gel was sliced and assayed for enzyme activity as described under "Materials and Methods." Fig. 5a shows that the peak of enzyme activity is observed in a ratio of 1:1:2 corresponding to a native minimum molecular weight of 133,000 which is near that (123,000) calculated from sedimentation data. The conditions of assay were as specified under "Materials and Methods" except those assays containing the cyclic nucleotides also contained 0.8 mM aminophylline and 10 mM NaF. All values are corrected by subtraction of endogenous incorporation in the absence of exogenous substrate. Blank values indicate either no additions to the basic assay or no determination.

**Table I**

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* Percentages are expressed relative to activity obtained with the preferred substrate or in the absence of cyclic nucleotides.

**Fig. 5.** Gel electrophoresis of protein kinase NII. (a) Pooled fractions from G-200 were concentrated to 0.4 ml by ultracentrifugation, layered over a 5 to 20% sucrose gradient, centrifuged, and fractionated as described under "Materials and Methods." Fractions from the single peak of protein kinase activity were pooled and 200 μl (30 μg) was applied to a native gel (0.6 (inside diameter) x 8 cm). This gel was stained and a parallel gel was assayed as described under "Materials and Methods." Fractions from the region of the gel as stain. A parallel gel was sliced and assayed for enzyme activity as described under "Materials and Methods." Fig. 5a shows that the peak of enzyme activity is observed in a ratio of 1:1:2 corresponding to a native minimum molecular weight of 133,000 which is near that (123,000) calculated from sedimentation data. The conditions of assay were as specified under "Materials and Methods" except those assays containing the cyclic nucleotides also contained 0.8 mM aminophylline and 10 mM NaF. All values are corrected by subtraction of endogenous incorporation in the absence of exogenous substrate. Blank values indicate either no additions to the basic assay or no determination.

**Table II**

**Substrate specificity and effects of cAMP and cGMP on protein kinase NII**

Appropriate aliquots of protein kinases were dialyzed against standard buffer containing 0.4 M NaCl for 2 to 4 h. Fifty-microliter aliquots of each enzyme were assayed in triplicate using the indicated substrates. The conditions of assay were as specified under "Materials and Methods" except those assays containing the cyclic nucleotides also contained 0.8 mM aminophylline and 10 mM NaF. All values are corrected by subtraction of endogenous incorporation in the absence of exogenous substrate. Blank values indicate either no additions to the basic assay or no determination.

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ence. Relative substrate preference of the purified protein kinase NII can be found in Table II. The enzyme prefers phosvitin > casein > H2b as substrate while an impure preparation of protein kinase NI prefers casein > phosvitin > H2b. Some protein kinases, especially histone kinases, are activated by cAMP. Table II also shows that the pure enzyme is not activated by cAMP at concentrations which stimulate a rise in intracellular concentrations of cGMP also have no stimulatory effect on NII activity. Table II also shows that the pure enzyme is not activated by cAMP at concentrations which stimulate a rise in intracellular cGMP activity.

DISCUSSION

Pure preparations of protein kinase NII are not stimulated by cAMP or cGMP and the activity of the enzyme is maximal with phosvitin substrate rather than histone. These characteristics appear to indicate that it may be unique from the cAMP-dependent histone kinases (2, 3, 35). These results corroborate previous studies with the partially purified enzyme (4, 5). Substrate specificity and other studies also confirm the previous conclusion obtained with impure enzymes that several properties of NII are distinguishable from those of protein kinase NI (4, 5). A detailed comparison will be presented separately.

Our purification scheme involves the use of some novel and probably selective affinity chromatographic steps, each of which provides modest but reproducible purification with good yield. Yields obtained from these columns are lower than the respective binding capacities because select fractions are chosen and also because they reflect any losses of activity which occur during the course of the purification. The basis for the selective action of these columns is unknown. While all of the substrate affinity ligands are phosphoproteins, their effectiveness in chromatography has not yet been correlated with the degree of their phosphorylation. The enzyme binds efficiently to columns of histone (mixed or individual H2b). This may or may not be related to the fact that it resides on chromatin and is presumably involved in the phosphorylation of other nuclear proteins. It has been determined that the enzyme also binds to columns of H2a, but with no greater selectivity than to H2b. It is possible that these columns may be useful for the purification of other chromatin-associated proteins from rat liver as well as protein kinases from other sources.

Other investigators have reported the presence of multiple protein kinase activities in their preparations from nuclei (9, 36, 37). In our experience, protein kinase NII preparations exhibited no heterogeneity in all of the steps employed in its purification when phosvitin was employed as substrate. It is possible, however, that additional protein kinase activities are present in the 0.1 M (NH4)2SO4-insoluble chromatin pellet and the flow-through of the phosvitin-Sepharose or other affinity columns but display maximal activity with substrates other than phosvitin.

As isolated from G-200 columns, the sodium dodecyl sulfate-polyacrylamide gel patterns reveal occasional traces of contaminating polypeptides of 97,000, 69,000, 52,000, 34,000, and 19,000 daltons. These contaminants can be minimized by pooling more selective fractions from the G-200 column or subjecting concentrated G-200 preparations to an additional sucrose gradient centrifugation step (data not shown). None of the contaminant bands could be detected on sodium dodecyl sulfate-polyacrylamide gels of the enzyme purified by sucrose gradient centrifugation and no additional protein is seen from native gels of this material (Fig. 3a). On the basis of patterns seen in sodium dodecyl sulfate gels, nuclear protein kinase NII contains only three different subunits of 42,000 (α), 39,000 (α'), and 26,000 (β), respectively. These are present in a αα'β combination which gives a molecular weight of 135,000.

In addition to its cAMP independence and substrate preference, purified kinase NII exhibits many other characteristics previously reported by others for partially purified preparations. The θαw we observe (7.0) is close to that determined by Desjardins (8) (7.3) at a similar ionic strength. From this data, a native molecular weight of 123,000 was calculated. However, estimates of size by gel filtration according to the method of Andrews (38) revealed a molecular weight of 250,000 in the standard buffer containing 0.4 M NaCl. Removal of diithiothreitol and phenylmethylsulfonylfluoride increased the elution volume from the G-200 column and a molecular weight of 240,000 was calculated. This is still larger than that calculated from sucrose gradients even though gel filtration buffer differed from that of sucrose gradients only by the presence of glycerol. We are unable to explain the discrepancy between molecular weights determined by sedimentation and gel filtration but this has also been noted for another phosphitin kinase (39). It is possible that we have isolated an aggregate form of the enzyme by gel filtration. Takeda et al. (4) have observed a similar molecular weight (230,000) of their Form A enzyme on G-200 which may also be the high molecular weight K IVa enzyme described by Rikans and Ruddon (40).

On the basis of our results and others (4, 5), the enzyme is predominantly of nuclear origin. Under the conditions of our protein kinase assay, up to 12% of the total casein kinase activity can be detected in the cytosol. This activity could represent a separate cytoplasmic protein kinase or some of the nuclear enzyme(s) which is lost in the isolation of nuclei. More definitive experiments will be necessary to further characterize the relationship of these activities.

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REFERENCES

13. Langun, T. A. (1967) in Regulation of Nuclear Protein and Protein Biosynthesis (Koningsberger, V. V., and Bosch, L., eds.) pp. 233-242, Elsevier, Amsterdam
15. Wang, T.-Y., and Kostraba, N. C. (1975) in Chromosomal Proteins and Their Role in the Regulation of Gene Expression
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Purification of rat liver nuclear protein kinase NII.
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