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ELUTION OF THE CATALYTIC SUBUNITS OF THE TYPE I AND TYPE II FORMS OF CYCLIC AMP-DEPENDENT PROTEIN KINASE WITHIN THE TYPE I CHROMATOGRAPHIC PEAK

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SUMMARY

The purpose of this investigation was to determine the elution location upon ion-exchange chromatography of the catalytic subunits of both the type I and type II isozyme forms of cAMP-dependent protein kinase. We show that ion-exchange chromatograpohy with DEAE-Sepharose CL-6B yields an apparent type I chromatographic peak of cAMP-dependent protein kinase which can represent not only type I holoenzyme activity but also catalytic activity derived from either the type I or type II enzyme forms. Such knowledge of the elution location of the dissociated catalytic subunits can prevent incorrect identification of the distribution of cAMP-dependent protein kinase isozymes in studies which estimate the isozyme concentrations based on the relative proportions of the kinase activity peaks.

INTRODUCTION

cAMP-dependent protein kinases are the only known receptors for cAMP in mammalian cells[1–4]. cAMP binds to the regulatory subunits of cAMP-dependent protein kinase, freeing the active catalytic subunits from the holoenzyme. The free catalytic subunit then catalyzes the phosphorylation of cellular proteins, modifying cellular functions in a manner characteristic of the agonist which raised cAMP levels in the cell.

Two forms of cAMP-dependent protein kinase have been shown to exist in tissues, designated type I and type II[5–7]. Both enzyme forms consist of two regulatory and two catalytic subunits. The catalytic subunits appear to be very similar proteins, based upon their molecular weights, amino acid compositions and substrate specificities[8–10]. The regulatory subunits, R^I and R^{II} for the type I and type II forms of cAMP-dependent protein kinases, on the other hand, exhibit different molecular weights, different ionic properties, different affinities for cAMP, and behave differently towards ATP[6,11–16].

An additional criterion to distinguish the two forms of cAMP-dependent protein kinase is based upon their separate elution from anion-exchange chromato-

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graphy. Using DEAE-cellulose or DEAE-Sephacel, the type I holoenzyme elutes with 0.05–0.1 *M* salt and the type II form elutes with approximately 0.2 *M* salt[5,7]. We report in this study that ion-exchange chromatography with DEAE-Sepharose CL-6B results not only in the conventional separate elution of the two holoenzyme forms of cAMP-dependent protein kinase but also, upon dissociation of the type II isozyme form, in the elution of catalytic activity derived from the type II enzyme form with the type I chromatographic peak. Appropriate identification of the elution location of dissociated catalytic subunits is necessary in order to avoid artifactual identification of the isozymic distribution of cAMP-dependent protein kinases in tissue extracts.

MATERIALS AND METHODS

Materials

Most chemicals were obtained from Sigma (St. Louis, MO, U.S.A.). Electrophoresis reagents were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.) [γ-^{32}P]ATP (25 Ci/mmole) and 8-azido adenosine cAMP (8-N_3[^{32}P]cAMP) were obtained from ICN Chemical and Radioisotope Division (Irvine, CA, U.S.A.) [2,8-^{3}H]cAMP (38 Ci/mmole) was obtained from Amersham (Arlington Heights, IL, U.S.A.) DEAE-Sepharose CL-6B was purchased from Pharmacia, (Piscataway, NJ, U.S.A.).

Preparation of supernatant fractions for chromatography

Heart tissue (0.8 g), was excised from male rats minced with a pair of scissors, suspended in ten volumes of ice-cold 10 m*M* Tris–HCl, 0.32 *M* sucrose, 3 m*M* magnesium chloride (pH 7.0), and homogenized with 10–15 strokes using a ground glass homogenizer. The homogenate was centrifuged at 19,400 *g* from 10 min at 4°C, the resultant supernatant retained for chromatography studies.

Lyophilized beef heart protein kinase (lot 50F-9550) was obtained from Sigma (St. Louis, MO, U.S.A.). An amount of 2 mg was reconstituted with 2 ml distilled water for chromatography studies.

Skeletal muscle (1.15 g), excised from leg muscle of male rats, was suspended in ten volumes of ice-cold 10 m*M* Tris–HCl, 0.32 *M* sucrose, 3 m*M* magnesium chloride (pH 7.0), and homogenized on ice with a Sorvall Omni-mixer (Ivan Sorvall, Norwalk, CT, U.S.A.) using four, 30-sec bursts. The homogenate was centrifuged at 38,000 *g* for 15 min, retaining the supernatant.

Chromatography

Supernatant fractions were immediately loaded onto columns 4 × 0.9 cm I.D. containing DEAE-Sepharose CL-6B pre-equilibrated within 24 h of use with 10 m*M* potassium phosphate buffer (pH 7.0). Supernatant fractions were loaded, and the columns were washed with equilibration buffer until the eluate was free of material that adsorbed at 280 nm, collecting 2.7 ml/tube with a Gilson microfractionater (Model FC 080 M) at a flow-rate of 0.6 ml/min. Aliquots (10 μl) of the wash fractions were assayed for protein kinase activity in the absence and presence of 0.45 μM cAMP and for [^{3}H]cAMP-binding activity. Protein kinases were eluted from the columns with a linear gradient between solutions of 10 m*M* and 0.5 *M* potassium

phosphate buffer (pH 7.0) using a Pharmacia gradient mixer (GM-1), collecting 0.7-ml fractions at a flow-rate of 0.4 ml/min. Aliquots (50 μl) of the odd fractions were assayed for protein kinase activity in the absence and presence of 0.45 μM cAMP, and 100-μl samples were assayed for [^{3}H]cAMP-binding activity. Aliquots (40 μl) of the fractions containing peak protein kinase and/or peak cAMP-binding activities were labeled with 8-N_3[^{32}P]cAMP for subsequent gel electrophoresis and autoradiography studies. Animal sacrifice, column chromatography and protein kinase and cAMP binding determinations were performed on the same day. Conductivity determinations were made on even-numbered fractions using a Radiometer conductivity meter (Copenhagen, Denmark). Recovery of protein kinase activity with DEAE-Sepharose Cl-6B chromatography was consistently 80–90%.

Protein kinase and cAMP-binding assays

Protein kinase activity was determined in a total volume of 0.225 ml containing 44 μM [γ-^{32}P]ATP, 100 mg protamine sulfate, 44 mM α-glycerol phosphate, 8.8 mM sodium fluoride, 8.8 mM magnesium acetate, 1.8 mM theophylline, and 0.88 mM dithiothreitol (pH 7.0) in the absence and presence of 0.45 μM cAMP. Incubations were at 37°C for 10 min and were stopped with the addition of 1 ml of 11% trichloroacetic acid (TCA) containing 1% sodium dodecylsulphate (SDS). The samples were filtered using Millipore filters (0.3 μm), and filters were washed with 20 ml of TCA–SDS and counted with 5 ml of Beckman Ready-Solv. (Beckman, Fullerton, CA, U.S.A.) in a packard Tri-carb liquid scintillation counter. Results are expressed as picomol ^{32}P incorporated per aliquot.

cAMP-binding activity was determined in a total volume of 0.3 ml containing 8.2 mM magnesium chloride, 58 mM Tris–HCl, 83 nM [^{3}H]cAMP and 5.8 mM theophylline (pH 7.4). Incubations were at 4°C for 1 h and were stopped by the addition of 2.5 ml of wash buffer consisting of 25 mM Tris–HCl and 10 mM magnesium chloride (pH 7.4). Samples were filtered using Millipore filters (0.3 μm) and washed with 20 ml ice-cold was buffer. Filters were counted in 5 ml of 3a-70B (Research Products International, Elk Grove Village, IL, U.S.A.) as described above. Results are expressed as picomol ^{3}H bound per aliquot.

Photoaffinity labelling, gel electrophoresis and autoradiography

Photoaffinity labeling of 40-μl aliquots of column fractions was done in a final volume of 50 μl containing 1 mM magnesium chloride, 0.1 mM ATP, 0.5–1 μM 8-N_3[^{32}P]cAMP, in the absence and presence of 0.1 mM cAMP. The reaction mixture was preincubated in darkness for 60 min at room temperature and then exposed to UV mineralight (Ultra Violet Products, San Gabriel, CA, U.S.A.) Model R-52 for 10 min at a distance of 10 cm. Reactions were stopped by the addition of 20 μl stop solution containing 30% glycerol, 3% SDS, 150 mM Tris–HCl (pH 8.75), 0.04% bromophenol blue and 15% β-mercaptoethanol, and tubes were placed in a boiling-water bath for 3 min. Proteins were separated by SDS–polyacrylamide gel electrophoresis as described by Rudolph and Krueger[17] using an SE 500 series electrophoresis unit (Hoeffer Scientific, San Francisco, CA, U.S.A.). After electrophoresis, gels were stained, destained, dried using a Hoeffer slab-gel drier, and placed with Kodak X-Omat XRP-5 X-ray film for 12 h to 7 days. Molecular weights (MW) were estimated on each gel from the migration rates of protein standards (chymotryposinogen,

MW = 25,000; bovine serum albumin, MW = 68,000; ovalbumin, MW = 45,000; catalase, MW = 60,000; fumarase, MW = 48,000; lactate dehydrogenase, MW = 35,000; glutamate dehydrogenase, MW = 56,000) relative to the dye front. Background labeling of the gels was higher with some batches of the label.

Miscellaneous techniques

Heat-stable protein kinase inhibitor was prepared as previously described[18]. Saturating levels of the inhibitor were used in all studies. Final concentrations of all solutions are indicated in the text.

RESULTS AND DISCUSSION

Ion-exchange chromatography of rat heart extract, previously shown to contain exclusively the type I form of cAMP-dependent protein kinase[5,7], on DEAE-Sepharose CL-6B columns resulted in the elution of a single peak of protein kinase activity with 0.05–0.1 *M* potassium phosphate (Fig. 1A). The protein kinase peak was stimulated approximately 2.5-fold by exogenous cAMP in the assay. This rather low degree of stimulation is probably attributable to the dissociating effects of protamine[19,20]. Inclusion in the assay of a saturating concentration of the heat-stable protein kinase inhibitor resulted in a 97% reduction in protein kinase activity (not shown). cAMP-binding activity co-eluted exactly with the protein kinase activity peak (Fig. 1A). Identification of the cAMP-binding activity shown in Fig. 1A, as R^I was confirmed by the specific labeling (in the presence and absence of exogenous cAMP) of a protein band labeled with 8-N_3[^{32}P]cAMP which migrated on gel electrophoresis (Fig. 2A) with a relative molecular weight of 47,000 (refs. 21 and 22). No additional specifically labeled cAMP-binding proteins were detected. It is concluded that this peak of cAMP-stimulated protein kinase activity represents the holoenzyme form of the type I isoenzyme of cAMP-dependent protein kinase.

Incubation of this rat heart extract with 5 μM cAMP (4°C, 30 min) followed by chromatography on DEAE-Sepharose CL-6B also resulted in the elution of a single peak of protein kinase activity with 0.03–0.08 *M* potassium phosphate (Fig. 1B). Although the protein kinase activity in this peak was not stimulated by exogenous cAMP (Fig. 1B) it was inhibited 82% by the heat-stable protein kinase inhibitor (not shown). However, cAMP-binding activity no longer eluted with the peak of protein kinase activity. Rather cAMP-binding activity eluted as a separate peak with 0.13–0.18 *M* potassium phosphate (Fig. 1B). Identification of the cAMP-binding activity (Fig. 1B) as R^I and confirmation of the absence of R^I from the peak of protein kinase activity (Fig. 1B) was achieved using the cAMP-photoaffinity analog (Fig. 2B). In addition to the R^I, a specifically labeled lower molecular weight band (MW = 39,000) was also detected on the autoradiograph, presumably representing a proteolytic breakdown product of R^I (refs. 22–24). Previous studies have shown that preincubation of a crude supernatant fraction from rat hearts with 5 μM caused 100% dissociation of the enzyme, based upon protein kinase meaurements in the absence *versus* the presence of 0.45 μM cAMP (kinase activity measured in absence divided by that measured in the presence of cAMP = 1.0). It is therefore concluded that the peak of kinase activity (Fig. 1B) represents dissociated catalytic subunits derived from the type I isozyme and that the separate peak of cAMP-binding activity

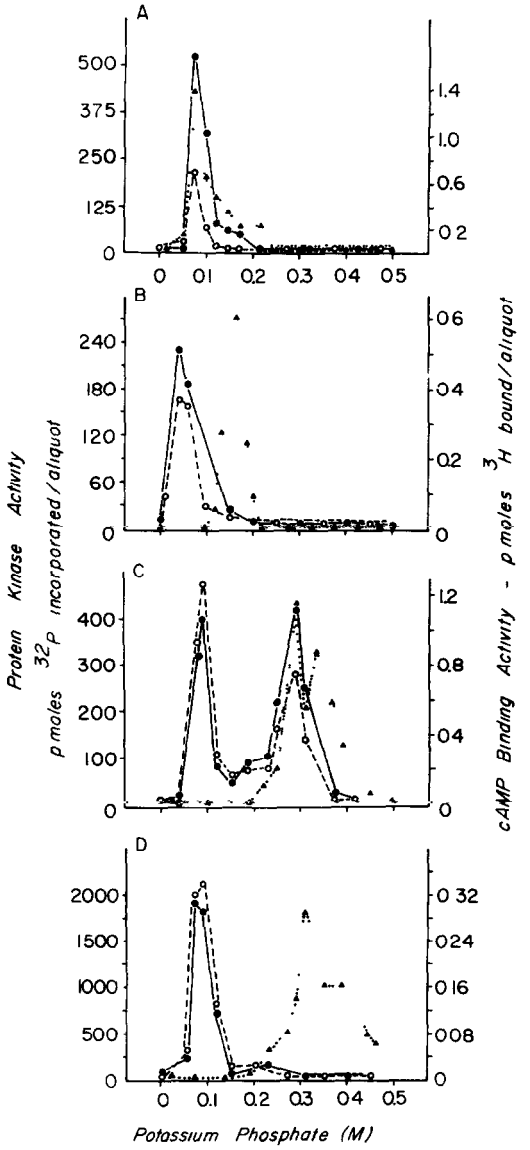

Fig. 1. DEAE-Sepharose CL-6B elution profiles of rat and beef heart extracts. Chromatography was conducted as described in Materials and Methods, eluting protein kinases with a linear potassium phosphate gradient. Aliquots of column fractions were assayed for protein kinase activity in the absence (○) and apresence (●) of 0.45 μM cAMP and for cAMP-binding activity (△) as indicated. Results are representative of a minimum of two or three experiments for each panel. Neither protein kinase nor cAMP-binding activities were detected in column load or wash volumes. For comparison purposes among panels, enzyme activities in the eluted column fractions are graphed against the measured conductivities, expressed on the abscissa as the molarity of potassium phosphate. (A) Elution profile control rat heart extract. The equivalent of 400 mg wet weight was loaded onto the column. (B) Elution profile of rat heart extract incubated at 4°C for 30 min with 5 μM cAMP. The equivalent of 400 mg wet weight was loaded onto the column. (C) Elution profile of control beef heart extract [2 mg beef heart protein kinase (Sigma)]. (D) Elution profile of beef heart extract (2 mg) incubated at 4°C for 30 min with 5 μM cAMP. For the rest of the details see Materials and Methods.

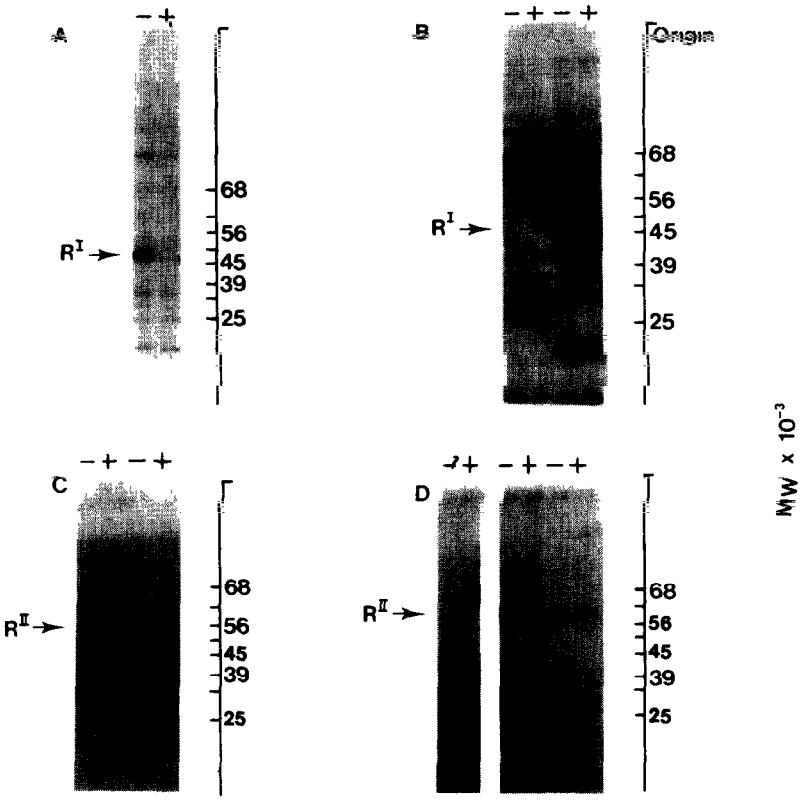

Fig. 2. Autoradiographs showing the incorporation of 8-N_3[^{32}P]cAMP into cAMP-binding proteins contained in aliquots of selected DEAE-Sepharose CL-6B column fractions. Aliquots of column fractions were incubated with 8-N_3[^{32}P]cAMP, in the absence (−) and presence (+) of 0.1 m*M* cAMP as described in Materials and Methods. Subsequent UV irradiation, gel electrophoresis and autoradiography of dried gels are also described in Materials and Methods. Molecular weight (MW × 10^{-3}) determinations for each gel were based upon the relative migration rates of protein standards. (A) DEAE-Sepharose CL-6B fractions obtained from control rat heart tissue. Lanes 1 and 2: aliquots from fractions eluting with 0.075 *M* salt. (B) DEAE-Sepharose CL-6B fractions obtained from rat heart extract incubated with 5 μM cAMP. Lanes 1 and 2: aliquots from fractions eluting with 0.05 *M* salt; lanes 3 and 4: aliquots from fractions eluting with 0.17 *M* salt. (C) DEAE-Sepharose CL-6B fractions obtained from control beef heart extract. Lanes 1 and 2: aliquots from fractions eluting with 0.09 *M* salt; lanes 3 and 4: aliquots from fractions eluting with 0.29 *M* salt. (D) DEAE-Sepharose CL-6B fractions obtained from beef heart extract incubated with 5 μM cAMP. Lanes 1 and 2: aliquots from fractions eluting with 0.09 *M* salt; lanes 3 and 4: aliquots from fractions eluting with 0.31 *M* salt; lanes 5 and 6: aliquots from fractions eluting with 0.385 *M* salt

represents regulatory subunits also derived from the type I isozyme of cAMP-dependent protein kinase.

Beef heart extracts contain exclusively the type II isozyme of cAMP-dependent protein kinase[5,7]. Ion-exchange chromatography of the beef heart extract on DEAE-Sepharose CL-6B resulted in the elution of two peaks of protein kinase activity (Fig. 1C). The first peak of kinase activity eluted with 0.05–0.1 *M* potassium phosphate, was not stimulated by exogenous cAMP (Fig. 1C), and was inhibited 95% by the heat-stable protein kinase inhibitor (not shown). No cAMP-binding activity was as-

sociated with this first peak of protein kinase activity (Fig. 1C). Incubation of an aliquot from this kinase peak with 8-N_3[^{32}P]cAMP revealed the total absence of R^I and R^{II} in these fractions (Fig. 2C, lanes 1 and 2). It is concluded that this peak of kinase activity consists exclusively of catalytic subunits derived from the type II isozyme of cAMP-dependent protein kinase*. The second peak of kinase activity eluted with 0.25–0.35 *M* potassium phosphate, was partially stimulated with exogenous cAMP (Fig. 1C), and was inhibited 83% by the heat-stable protein kinase inhibitor (not shown). A peak of cAMP binding activity exactly co-eluted with this peak of protein kinase activity (Fig. 1C). Identification of the cAMP binding activity eluting with 0.29 *M* salt and co-eluting with the protein kinase activity as R^{II} was confirmed by the specific labeling with 8-N_3[^{32}P]cAMP of a protein band which migrated on gel electrophoresis with a relative molecular weight of 56,000 (refs. 6, 21, 25, 26; Fig. 2C, lanes 3 and 4). No additional cAMP binding proteins were detected on the autoradiograph. A shoulder or second peak of cAMP-binding activity closely followed the first peak of cAMP-binding activity and eluted with 0.36 *M* potassium phosphate (Fig. 1C). Photoaffinity labeling of aliquots of the shoulder fractions with 8-N_3[^{32}P]cAMP revealed that the labeled band migrated as R^{II} and was indistinguishable from that which co-eluted with the kinase activity (not shown). Therefore, the shoulder fractions of R^{II} cAMP-binding activity do not appear to represent a proteolytic breakdown product of R^{II}.

Incubation of the beef heart extract with 5 μM cAMP (4°C, 30 min), followed by subsequent chromatography on DEAE-Sepharose CL-6B, resulted in the elution of a single peak of protein kinase activity with 0.05–0.1 *M* potassium phosphate and a single peak of cAMP-binding activity with 0.25–0.4 *M* potassium phosphate (Fig. 1D). The peak of protein kinase activity was not stimulated by exogenous cAMP (Fig. 1D), was inhibited 95% by the heat-stable protein kinase inhibitor (not shown), and exhibited no cAMP-binding activity (Fig. 1D). Photoaffinity labeling of aliquots of the kinase peak with 8-N_3[^{32}P]cAMP confirmed the absence of cAMP-binding proteins in these fractions (Fig. 2D, lanes 1 and 2). It is concluded that this peak of kinase activity represents catalytic subunits derived from the type II isozyme of cAMP dependent protein kinase. The peak of cAMP-binding activity was devoid of associated kinase activity (Fig. 1D). Incubation of aliquots from both the peak fractions and the shoulder fractions of cAMP-binding activity with 8-N_3[^{32}P]cAMP revealed the presence of R^{II}, migrating with a relative molecular weight of 58,000 (Fig. 2D, lanes 3, 4, 5 and 6). Also present were specifically labelled, lower molecular weight bands (MW = 39,000), presumed to be the proteolytic breakdown products of R^{II} (refs. 23, 26, 27). It is concluded that the cAMP-binding activity eluting between 0.25 and 0.4 *M* potassium phosphate in Fig. 1D, consists of protein kinase regulatory subunits derived from the type II form of cAMP-dependent protein kinase.

The elution location of the dissociated catalytic subunits from DEAE-Sepharose CL-6B was confirmed by subjecting partially purified catalytic subunits (Sigma) to chromatography on DEAE-Sepharose CL-6B. Catalytic kinase activity eluted with 0.04–0.1 *M* potassium phosphate (not shown).

That catalytic subunits derived from both isozyme forms in a single tissue elute

* Additional experiments confirmed that Sigma Chemical Co.'s beef heart protein kinase lot number 50F-9550 was partially dissociated.

together as a single peak with the type I chromatographic peak is shown in Fig. 3. A supernatant fraction derived from rat skeletal muscle, reported to contain both type I and II isozyme forms of cAMP-dependent protein kinase[5], was incubated with 5 μM cAMP (4°C, 30 min) and subsequently chromatographed on DEAE-Sepharose CL-6B. Catalytic kinase activity eluted as a single peak with 0.1 M potassium phosphate. cAMP-binding activity eluted as two distinct peaks of activity with 0.15 M and with 0.35–0.45 M potassium phosphate. Based upon our previous results, cAMP binding activity eluting with 0.15 M potassium phosphate is presumed to be R^I, and that eluting with 0.35 M potassium phosphate, R^{II}.

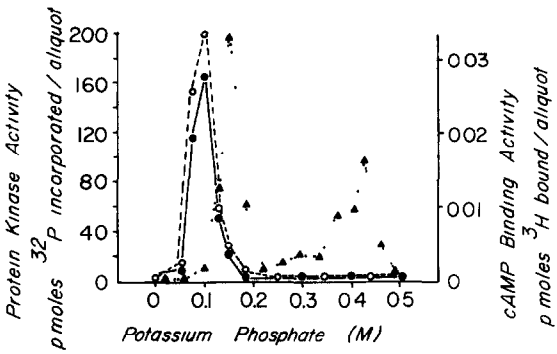

Fig. 3. DEAE-Sepharose CL-6B elution profile of rat skeletal muscle extract. For details, see legend to Fig. 1 and Materials and Methods. The equivalent of 400 mg wet weight of a rat skeletal muscle was applied to the column. Equivalent results were obtained in two separate experiaments.

CONCLUSIONS

The elution location of the holoenzymes of cAMP-dependent protein kinase upon ion-exchange chromatography is well known and reflects the combined isoelectric points of the acidic regulatory and the more basic catalytic subunits [5–8,28–31]. Since the catalytic subunits are essentially identical, the separate elution of the two holoenzymes of cAMP-dependent protein kinase reflects the isoelectric points of R^I and R^{II}.

Upon dissociation of the holoenzymes, one would predict that the regulatory subunits would elute based upon their individual isoelectric points. Experimental results have essentially confirmed these predictions. R^{II} tends to require a slightly higher salt concentration to be eluted from DEAE-cellulose or DEAE-Sepharose CL-6B compared to the type II holoenzyme[32,33] (Fig. 1) and R^I elutes as a sharp peak between the two holoenzymes[21,34,35] (Figs. 1 and 3). Since the isoelectric point of the catalytic subunit is considerably more basic than R^I (refs. 28–31), one would predict that the catalytic subunits should elute either in the elution flow-through or with a very low salt concentration, the exact location depending on the pH of the elution buffer and the ionic strength of the ion-exchange matrix. Although only a few reports have addressed specifically the elution location of the catalytic subunit, experimental results using differing ion-exchange matrices and buffers reveal a variety of elution locations for the catalytic subunit. For example, with extracts prepared in

5 m*M* Tris (pH 7.5) and 1 m*M* EDTA, dissociated catalytic subunits eluted from DEAE-cellulose with the flow-through portion of the profile[5]; with extracts prepared in 10 m*M* potassium phosphate, 0.5 m*M* methylisobutylxanthine (MIX), 0.5 m*M* EDTA, and 0.1% Triton X-100, pH 6.5, catalytic subunits eluted from DEAE-cellulose with the type I chromatographic peak with approximately 50 m*M* potassium phosphate[34]; and with extracts prepared in 10 m*M* potassium phosphate, pH 6.5, 10 m*M* Tris–HCl, 1 m*M* magnesium chloride, 1 m*M* EDTA and 1 m*M* β-mercaptoethanol (pH 7) or 10 m*M* Tris–HCl, 0.32 *M* sucrose, 3 m*M* magnesium chloride (pH 7.0) catalytic subunits eluted diffusely with the column flow-through and wash volumes[34] (not shown).

The results presented herein demonstrate that the catalytic subunits consistently elute as a sharp peak with the type I chromatographic peak with 0.05–0.1 *M* potassium phosphate from DEAE-Sepharose Cl-6B when extracts are made in 10 m*M* Tris–HCl, 0.32 *M* sucrose, 3 m*M* magnesium chloride (pH 7.0). To our knowledge, this is the first report of the elution of dissociated catalytic subunits with the type I chromatographic peak using standard chromatographic conditions (*i.e.*, omitting MIX and EDTA). The basis for the elution location of catalytic subunits from DEAE-Sepharose CL-6B may reflect a tighter association of the catalytic subunits to this matrix compared with DEAE-cellulose. The significance of this rests with the potential for misidentifying the composition of the type I chromatographic peak. For example, ion-exchange chromatography of a tissue extract containing both the type I and type II isozyme forms of cAMP-dependent protein kinase in which the type II holoenzyme became dissociated (either *in vivo* or through *in vitro* manipulations) would yield the apparent presence of only the type I isozyme and "free" cAMP-binding activity eluting in the location of the type II chromatographic peak. Thus, improper identification of the elution location of the catalytic subunits could cloud the interpretation of studies which rely solely on the proportion of the kinase activity peaks to estimate relative isozyme concentrations.

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