Separation between modulator-dependent protein kinases I and II

W.N. Kuo

Division of Science and Mathematics, Bethune-Cookman College, Daytona Beach (Florida 32015, USA), 12 May 1980

Summary. The separation of modulator-dependent protein kinase I from modulator-dependent protein kinase II obtained from the lungs of sexually premature male mice was accomplished by Sephadex G-200 gel filtration. After preincubation of a mouse lung cytosol fraction with arginine-rich histone, theophylline, cyclic GMP and crude protein kinase modulator, a cyclic GMP-dependent protein kinase activity peak present in a non-preincubated sample completely disappeared and was replaced by a late-eluted modulator-dependent protein kinase II peak. There was a difference in substrate specificity between modulator-dependent protein kinase I and modulator-dependent protein kinase II despite their similar dependence on crude protein kinase modulator or partially purified stimulatory protein kinase modulator for their maximal activities.

It has been shown that the conversion of cyclic GMP-dependent protein kinase holoenzyme from mammalian tissues into its catalytic subunit, presumably modulator-dependent protein kinase II[2], can be accomplished by either preincubation of cyclic GMP-dependent protein kinase in the presence of exceedingly high concentration of cyclic GMP and/or histone[3,4], or storage of cyclic GMP-dependent protein kinase at low temperature for appropriately long periods[2]. Recently, indirect evidence from this laboratory suggested that, in addition to cyclic GMP-dependent protein kinase[5-7] and its catalytic subunit[8-10], yet another enzyme, modulator-dependent protein kinase I[11] could be stimulated either by crude protein kinase modulator[4-6,13,14] or by partially purified stimulatory protein kinase modulator[5]. In this study, the author reports not only the conversion of cyclic GMP-dependent protein kinase into modulator-dependent protein kinase II under relatively low concentrations of cyclic GMP and histone, but also the direct separation between modulator-dependent protein kinase I and modulator-dependent protein kinase II.

Materials and methods. [γ-^{32}P] ATP was purchased from New England Nuclear. Cyclic AMP and cyclic GMP were obtained from Boehringer Mannheim (BRD); arginineerich histone (HA) was obtained from Worthington; histone (type II-S), and protamine chloride (grade V), were obtained from Sigma. Sephadex G-100 and G-200 were from Pharmacia.

Sexually premature male ICR mice (each weighing 10.0±2.6 g) were used exclusively. Crude protein kinase modulator was prepared from liver extracts of ICR mice by boiling and trichloroacetic acid-precipitation[5,12,13]. Stimulatory protein kinase modulator was partially purified by Sephadex G-100 gel filtration[5]. Preparation of modulator-dependent protein kinase activity was as follows: Fresh lungs (19.0 g) from 15 IRC mice were homogenized in 7.0 ml of ice cold 50 mM potassium phosphate buffer, pH 7.0, using a glass-teflon homogenizer. The homogenate was centrifuged for 15 min at 30,000 × g. The supernatant fluid (crude extract) was filtered through 2 layers of glass wool to remove fat, and then divided equally into 2 portions, A and B. To portion B was added 3.5 ml of 50 mM potassium phosphate buffer (pH 7.0), containing 35 µmoles of $MgCl_2$, 700 µg of arginine rich histone (HA), 8.7 µmoles of theophylline, 1000 pmoles of cyclic GMP and 600 µg of crude protein kinase modulator. The mixture was preincubated at 4 °C for 60 min with constant gentle stirring followed by filtration through 5 layers of glass wool. Both portions A and B were then applied to 2 separate Sephadex G-200 columns (3.5×46 cm). Potassium phosphate (50 mM, pH 7.0) was used to equilibrate and elute each column. The fraction size in each case was 2.5 ml, and the flow was 0.5 ml/min.

The standard assay system[6] for protein kinase activity contained, in a final volume of 0.2 ml, potassium phosphate buffer, pH 7.0, 10 µmoles; theophylline, 0.5 µmoles; substrate protein, 40 µg; MgCl, 2 µmoles [γ-^{32}P]ATP 1 nmole containing about 1.2×10^6 CPM; with or without cyclic nucleotide, cyclic AMP or cyclic GMP, 60 pmoles; with or without modulator, protein kinase modulator (50.6 µg) or stimulatory protein kinase modulator (40 µg); and appropriate amounts (1.3-1.4 µg) of protein kinase preparations. The reaction was carried out for 10 min at 30 °C. 1 µunit of enzyme activity was defined as that amount of enzyme that transferred 1 pmole of ^{32}P from [γ-^{32}P]ATP per min in recovered substrate protein under the assay conditions.

Results. Cyclic GMP-dependent protein kinase, modulator-dependent protein kinase I and modulator-dependent protein kinase II activities were well detected in different fractions of Sephadex G-200 gel filtration, when 40 µg of histone (type II-S) was used as the substrate under the assay conditions described (figure). In the non-preincubated sample, the peak activity fraction of modulator-dependent protein kinase I was clearly eluted after that of cyclic GMP-dependent protein kinase. However, after preincubation with arginine-rich histone, cyclic AMP, and theophylline, the cyclic GMP-dependent protein kinase peak was replaced by a late-eluted modulator-dependent protein kinase II peak. This suggests that cyclic GMP-dependent protein kinase had been converted into its catalytic subunit, modulator-dependent protein kinase II which accordingly

Comparison of substrate specificity of modulator-dependent protein kinases from Sephadex G-200 step

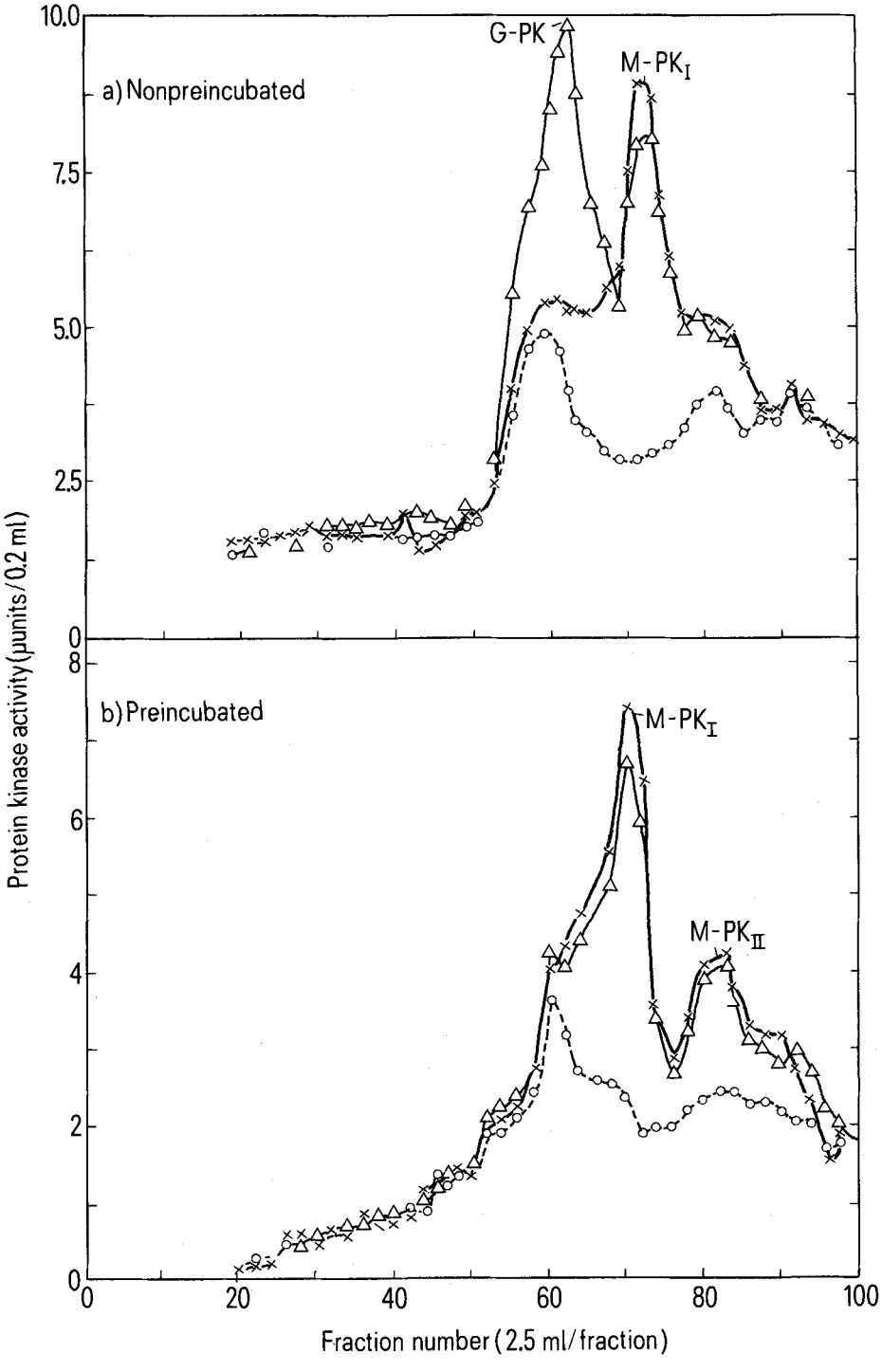
Substrate	PK	None		+cAMP		+cGMP	
		−PKMs	+PKMs	−PKMs	+PKMs	−PKMs	+PKMs
				microunits			
Arginine-rich histone	M-PK_I	0.55	1.34	0.60	1.40	0.58	1.31
	M-PK_{II}	0.52	1.29	0.62	1.29	0.50	1.18
Protamine chloride, grade V	M-PK_I	0.56	2.25	0.55	2.17	0.52	1.99
	M-PK_{II}	0.47	0.92	0.49	0.90	0.45	0.86

Assay conditions were as described in the text, except for the variations of substrates. Each value shown represents the mean of the triplicate samples. M-PK_I and M-PK_{II} activities were measured by using 1.3 and 1.4 µg fresh enzymes respectively. Abbreviation: PK, protein kinase; M-PK_I, modulator-dependent protein kinase I; M-PK_{II}, modulator-dependent protein kinase II; PKMs, stimulatory protein kinase modulator; cAMP, cyclic AMP; cGMP, cyclic GMP.

might have a much smaller molecular weight. When the non-preincubated cyclic GMP-dependent protein kinase peak fraction was stored at −20 °C for 14 days, it lost its cyclic GMP-dependence but acquired a substrate specificity similar to that of modulator-dependent protein kinase II (data not shown). This observation likewise suggests the in vitro conversion of cyclic GMP-dependent protein kinase into its subunit, modulator-dependent protein kinase II.

The comparison of substrate specificities of modulator-dependent protein kinase I and modulator-dependent protein kinase II is noted (table). Both enzymes were cyclic nucleotide-independent but modulator-dependent, and both accepted arginine-rich histone as a substrate. But protamine chloride was greatly preferred by modulator-dependent protein kinase I. This difference, together with clean separation between these 2 enzyme peaks in gel filtration, conclusively indicates that they are 2 separate enzymes.

Discussion. The introduction of protein kinase modulator to portion B and/or the possible presence of unknown factors in the cytosol fraction, apparently facilitated the complete dissociation of cyclic GMP-dependent protein kinase into its subunits, even when preincubation was with the lower concentration of histone (100 µg/ml) and a much



Protein kinase activity observed in Sephadex G-200 chromatography. ○—○, Basal; ×—×, PKM; △—△, PKM+cGMP.

lower concentration of cyclic GMP (0.14 μM) than those (histone, 400 μg/ml; cyclic GMP, 100 μM) previously reported to have had the same effect[3,4]. The weakness of the modulator-dependent protein kinase II peak indicates the possible loss of enzyme activity during the process of preincubation and dissociation. It proved to be much easier to observe the converted modulator-dependent protein kinase II by using a cyclic GMP-dependent protein kinase-rich tissue, such as lung in this study, as the starting sample for preincubation. When non-cyclic GMP-dependent protein kinase-rich tissue, such as testis, was tried, no modulator-dependent protein kinase II activity was detected[14]. The successful separation between greatly phosphorylated protein substrates, such as protamine, and slightly phosphorylated stimulatory protein kinase modulator, strongly suggests that stimulatory protein kinase modulator was a real stimulatory factor, and not a substrate[14]. With the in vitro verification of 2 forms of modulator-dependent protein kinases, future investigations may study the conditions needed to convert cyclic GMP-dependent protein kinase holoenzyme into its subunit and to reverse the process in vivo, and the possible existence of multiple forms of modulator-dependent protein kinases in vivo and their physiological functions.

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2 W.N. Kuo, L.P. Liu, C.F. Duggans, K.M. Foggie, M.J. McNeal and K. Perry, Experientia *36*, 904 (1980).
3 M. Shoji, J.G. Patrick, J. Tse and J.F. Kuo, J. biol. Chem. *252*, 4347 (1977).
4 M. Shoji, J.G. Patrick, C.W. Davis and J.F. Kuo, Biochem. J. *161*, 213 (1977).
5 W.N. Kuo, M. Shoji and J.F. Kuo, Biochim. biophys. Acta *437*, 142 (1976).
6 J.F. Kuo, Proc. natl Acad. Sci. USA *71*, 4037 (1974).
7 J.F. Kuo, W.N. Kuo, M. Shoji, C.W. Davis, V.L. Seery and T.E. Donnelly, J. biol. Chem. *251*, 1759 (1976).
8 M. Shoji, J.G. Patrick, C.W. Davis and J.F. Kuo, Biochem. J. *161*, 213 (1977).
9 M. Shoji, J.G. Patrick, J. Tse and J.F. Kuo, J. biol. Chem. *252*, 4347 (1977).
10 M. Shoji, N.L. Brackett, J. Tse, R. Shapira and J.F. Kuo, J. biol. Chem. *253*, 3427 (1978).
11 W.N. Kuo, K.M. Foggie and L.P. Liu, Experientia *36*, 906 (1980).
12 M.M. Appleman, L. Birnbaumer and H.N. Torres, Archs Biochem. Biophys. *116*, 39 (1966).
13 D.A. Walsh, C.D. Ashby, C. Gonzales, D. Calkins, D.H. Fisher and E.G. Krebs, J. biol. Chem. *246*, 1977 (1971).
14 W.N. Kuo, unpublished data.

Progesterone stimulates energy-dependent contraction of swollen heart mitochondria[1]

D.W. Jung and G.P. Brierley

Department of Physiological Chemistry, Ohio State University, Columbus (Ohio 43210, USA), 1 April 1980

Summary. Progesterone stimulates the rate and efficiency of respiration-dependent contraction of heart mitochondria in nitrate salts at alkaline pH. Ion extrusion under these conditions is normally slow and inefficient due to the elevated permeability of the membrane to monovalent cations through a putative uniport pathway. Progesterone also inhibits passive swelling under these conditions and appears to act by restricting cation influx through the uniport pathway.

Progesterone has been shown to affect the respiration and the permeability properties of mitochondira[2-7]. At low concentrations (approximately 50 μM) progesterone inhibits NADH oxidation at a site between flavoprotein and coenzyme Q[2-4] while higher concentrations (150 μM) inhibit ADP-stimulated succinate oxidation in an uncoupler-sensitive manner[3-5]. At concentrations above 200 μM progesterone acts as a weak uncoupler and the detergent-like properties of the steroid contribute to swelling of mitochondria and the loss of matrix components[6,7].

The energy-dependent contraction of swollen mitochondria appears to be an osmotic response to the electroneutral extrusion of cations on an endogenous cation/H^+ exchanger[8-11]. At neutral pH this extrusion of cations is a rapid and efficient reaction[10], but at pH 8.3 the influx of cations through a putative uniport pathway (alkaline uniport) appears to undermine efficiency and reduce the rate of net cation extrusion and contraction[10,11]. This paper reports that progesterone and other steroids inhibit passive swelling and stimulate respiration-dependent contraction of heart mitochondria at alkaline pH.

Materials and methods. The preparation of beef heart mitochondria by a Nagarse method and the simultaneous monitoring of respiration, pH, and swelling and contraction by changes in absorbance at 540 nm were carried out as previously described[10,12].

Results and discussion. Heart mitochondria swell spontaneously when suspended in nitrate salts at pH 8.3 and 37 °C in the absence of metabolic energy[9-11] (figure 1). Swelling under these conditions represents an osmotic response to the passive influx of nitrate and monovalent cation. Swollen mitochondria extrude accumulated ions and contract when respiration is initiated. Cation extrusion appears to occur via an endogenous cation/H^+ exchanger which is dependent on the ΔpH component of the protonmotive force[9-11]. Progesterone at 160 μM inhibits spontaneous swelling and activates succinate-supported contraction at pH 8.3 (figure 1). This activation of contraction is seen whether progesterone is added initially or immediately before succinate. Respiratory control, much like that associated with contraction at neutral pH[10] is also induced by progesterone under these conditions. The elevated respiration accompanying contraction declines to a low (approximately state 4) rate after contraction is complete and a steady-state volume is maintained (figure 1, B). In the absence of progesterone respiration is elevated to a level approximately that of state 3 and is linear to anaerobiosis (figure 1, B).

Titration of respiration-dependent contraction in Na^+ nitrate at pH 8.3 (figure 2) shows the optimum activation of the rate of concentration is 3.8-fold and occurs at 160 μM progesterone. The rate of respiration during contraction declines with increasing progesterone concentration and at 240 μM the rate of respiration during concentration approximates the controlled rate observed at lower concentrations. The efficiency of contraction (rate of contraction/rate