Microheterogeneity of Adenosine Cyclic Monophosphate-Dependent Protein Kinases from Mouse Brain and Heart

By ALVIN M. MALKINSON,*† ANTHONY J. GHARRETT†§ and LORA HOGY*
Departments of *Psychiatry and †Genetics and Cell Biology, University of Minnesota, Minneapolis, MN 55455, U.S.A.

(Received 19 December 1977)

1. DEAE-cellulose chromatography of mouse brain cytosol indicated the presence of only the type II isoenzyme of cyclic AMP-dependent protein kinase. Mouse heart cytosol contained approximately equal amounts of the type I and type II isoenzymes. 2. Both brain and heart type II isoenzymes reassociated after a transient exposure to cyclic AMP, but the heart type I isoenzyme remained dissociated. 3. Elution of brain cytosol continuously exposed to cyclic AMP resolved multiple peaks of protein kinase and cyclic AMP-binding activities. A single peak of kinase and multiple peaks of cyclic AMP-binding activities were found under the same conditions with heart cytosol. Various control experiments suggested that the heterogeneity within the brain type II isoenzymic class had not been caused by proteolysis. 4. Kinetic experiments with unfractionated brain cytosol showed that the binding of cyclic AMP, the dissociation of cyclic AMP from protein and the rate of heat denaturation of the cyclic AMP-binding activity gave results consistent with the presence of multiple binding species. 5. It is concluded that the type II isoenzymic peak obtained by DEAE-cellulose chromatography of mouse brain cytosol represents a class of enzymes containing multiple regulatory and catalytic subunits. The two heart cytosol isoenzymes contain a common catalytic subunit. The degree of protein kinase 'microheterogeneity', defined as the presence of multiple regulatory and/or catalytic subunits within a single isoenzymic class, appears to be tissue-specific.

The diverse actions of cyclic AMP in eukaryotes are probably mediated by the activation of protein kinase enzymes (EC 2.7.1.37) (Kuo & Greengard, 1969). The mechanism by which a specific endogenous protein substrate is phosphorylated to effect the physiological response appropriate to the original stimulus is unknown. Langan (1973) has postulated that the apposition *in situ* of a kinase enzyme with an available protein substrate determines the nature of the phosphorylation event. Compartmentalization of cyclic nucleotide pools (Bloom *et al.*, 1973; Steiner *et al.*, 1975) and of protein kinase enzymes (Corbin *et al.*, 1977; Steiner *et al.*, 1978) support this hypothesis.

Cyclic AMP-dependent protein kinases from diverse sources have similar substrate specificities (Langan, 1973) and phosphorylate identical sites within a polypeptide substrate (Kemp *et al.*, 1975). Multiple forms of kinase enzymes have been found within a single tissue (Tao *et al.*, 1970; Chen & Walsh, 1971; Corbin *et al.*, 1975) and within homogeneous

cell populations (Costa *et al.*, 1976; Gharrett *et al.*, 1976; Byus *et al.*, 1977). Protein kinase heterogeneity may reflect subtle differences in the intracellular location of kinase-substrate complexes, or may imply that the regulation of kinase activity *in vivo* may involve more than just the cyclic AMP signal. Careful study of the nature of this heterogeneity therefore seems warranted.

Two major protein kinase isoenzymes have been classified by Corbin *et al.* (1975) on the basis of differences in chromatographic behaviour on ion-exchange columns, association-dissociation properties of the regulatory (R) and catalytic (C) subunits, and effects of MgATP. Other laboratories have confirmed these properties and have also found differences between the isoenzymes with respect to antigenicity (Fleischer *et al.*, 1976), autophosphorylation (Hofmann *et al.*, 1975) and subcellular distribution (Uno *et al.*, 1976). The relative amounts of the two isoenzymes among different tissues of a single species vary considerably (Corbin *et al.*, 1975). Different species vary with respect to the kinase isoenzymic composition found in a particular organ (Corbin & Keely, 1977). The isoenzymic distribution of a single tissue can also vary during ontogeny (Lee *et al.*, 1976; Malkinson *et al.*, 1978) or during

† Present address: School of Pharmacy, University of Colorado, Boulder, CO 80303, U.S.A.

§ Present address: University of Alaska, Southeast Division of Fisheries, Juneau, AL 99802, U.S.A.

changes in growth (Costa *et al.*, 1976; Byus *et al.*, 1976, 1977).

'Microheterogeneity' refers to the presence of different *R* and/or *C* subunits within a single isoenzymic class. Two different *C*₁ subunits have been found in rabbit skeletal muscle, on the basis of differences in isoelectric focusing (Yamamura *et al.*, 1973; Bechtel *et al.*, 1977). By a similar technique two different *C*_{II} subunits have been described in bovine liver (Sugden *et al.*, 1976). Different *R*₁ subunits have been found in rabbit skeletal muscle as determined by gel filtration (Corbin *et al.*, 1972). Microheterogeneity within the type II kinase class was inferred by Tao & Hackett (1973) and by Knight & Skala (1977), who found that multiple holoenzyme forms could be derived on further purification of type II isoenzymes from erythrocytes and brown adipose tissue respectively. We report here that multiple *C* and *R* subunits are contained within the type II isoenzymic class from mouse brain cytosol. Mouse heart cytosol contained a single *C* subunit and multiple *R* subunits.

Materials and Methods

Materials

Carrier-free [³²P]phosphoric acid and cyclic [³H]-AMP were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). [^γ-³²P]ATP was prepared as described by Post & Sen (1967). Cyclic AMP and cyclic GMP were purchased from Boehringer Mannheim Corp. (New York, NY, U.S.A.). Other nucleotides, bovine serum albumin, histone type IIA and phenylmethanesulphonyl fluoride were obtained from Sigma (St. Louis, MO, U.S.A.). Bio-Solv BBS-3 solubilizer was purchased from Beckman Instruments (Irvine, CA, U.S.A.). All other chemicals used were reagent grade. Sephadex G-25 was purchased from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). DE-52 DEAE-cellulose and CM-52 CM-cellulose were obtained from H. Reeve-Angel (Clifton, NJ, U.S.A.). Both resins were resuspended in 10mM-potassium phosphate buffer, pH 7.0, titrated with 1M-KH₂PO₄ to pH 7.0, and then rinsed with 10mM-potassium phosphate, pH 7.0, in a Buchner funnel.

Randomly bred Swiss-Webster mice obtained from Biolab Corp. (St. Paul, MN, U.S.A.) were used in the chromatography experiments. The kinetic experiments on brain cytosol were done on A/Umc mice obtained from the University of Minnesota Department of Laboratory Medicine and Pathology Inbred Mouse colony. The chromatographic profile of brain cytosol protein kinase holoenzymes from A/Umc mice was identical with that obtained with Swiss-Webster mice. The cyclic AMP-binding specific activities of brain cytosol from Swiss-Webster mice were the same as that of A/Umc mice. It is probable

that results obtained from these strains are interchangeable. All mice were 6–10-week-old males, which were given food and water *ad libitum*.

Methods

Assays. Protein kinase was assayed by the method of Corbin & Reimann (1974) as modified by Gharrett *et al.* (1976). The reaction mixture (150 μl final volume) contained 6.7mM-MgCl₂, 20mM-2-(*N*-morpholino)ethanesulphonate (pH 6.5), 300 μg of histone type IIA, 0.2mM-[^γ-³²P]ATP (15–20 c.p.m./pmol) and 25 μl of a column fraction, with or without 3.3 μM-cyclic AMP. After incubation for 10 min (cyclic AMP present) or 20 min (cyclic AMP absent) at 30°C, the reaction was terminated by transferring a 100 μl sample on to Whatman 3MM filter paper (2cm×2cm) and immediately immersing the paper in 10% (w/v) trichloroacetic acid (10 ml/filter paper). The papers were washed, dried and counted for radioactivity as described by Corbin & Reimann (1974). One unit of enzyme activity corresponded to the incorporation of 1 pmol of ³²P from [^γ-³²P]ATP into histone/min at 30°C.

Cyclic AMP-binding activity was assayed by the method of Gilman (1970) as modified by Gharrett *et al.* (1976). The reaction mixture (200 μl final volume) contained 20 nM-cyclic [³H]AMP (16 Ci/mol), 50mM-sodium acetate, pH 4.0, and 75 μl of a column fraction or 45–70 μg of cytosol protein. After incubation for 5 h at 4°C, 0.8 ml of cold 20mM-potassium phosphate, pH 6.0, was added to each mixture. Protein-bound cyclic [³H]AMP was separated from unbound cyclic [³H]AMP by filtration through cellulose ester Millipore filter discs. After washing of the filters with potassium phosphate buffer, the filters were dried and then their radioactivities counted with a toluene-based scintillant that contained 10% (v/v) Bio-Solv BBS-3 solubilizer (Mackenzie & Stellwagen, 1974).

Measurement of protein concentration was made by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin used as a standard.

Column chromatography of protein kinase holoenzymes. Tissue samples were minced in a buffered salt solution containing 128mM-NaCl, 1.4mM-CaCl₂, 1.4mM-MgSO₄, 5mM-KCl and 10mM-KH₂PO₄, pH 7.0, and incubated with shaking at 37°C for 20 min. Tissues were then homogenized in 3 vol. (w/v) of ice-cold 10mM-potassium phosphate buffer containing 1mM-EDTA, pH 7.0. Homogenates were centrifuged at 27000g for 40 min, and supernatant fractions were retained. To the supernatant were added ATP and MgCl₂ to final concentrations of 0.15 and 3.5mM respectively. After 60 min at 30°C, the supernatant was chromatographed (descending) on a column (30cm×0.9cm) of Sephadex G-25 and eluted at 4°C with homogenization buffer at a flow

rate of 30 ml/h. Proteins eluted in the void volume, as determined by A_{280} , were pooled for DEAE-cellulose chromatography. Heart cytosolic protein (8–10 mg) or 15 mg of brain cytosolic protein was applied to a column (8 cm $\times$ 0.9 cm) of DE-52 DEAE-cellulose. The column was washed with 30 ml of homogenization buffer, and eluted with 30 ml of a linear NaCl gradient consisting of homogenization buffer containing 0–0.4 M-NaCl at a flow rate of 10 ml/h; 60 fractions (1.0 ml) were collected from each column. The conductivity of the fractions, which had been diluted 1 : 70 with water, was determined with a Labline conductivity meter to determine NaCl concentrations.

Results

Reassociation of type II isoenzymes

Corbin *et al.* (1975) have described two major isoenzyme classes, types I and II. Type I isoenzymes were eluted at low NaCl concentrations (below 0.1 M-NaCl), and type II enzymes were eluted at higher concentrations (above 0.1 M-NaCl). Type I enzymes were readily dissociated by high NaCl concentrations and required MgATP to reassociate. Type II enzymes were less rapidly dissociated by high NaCl and reassociated in the absence of MgATP. To ensure that the kinase isoenzymes were in the

holoenzyme form, cytosol samples were pretreated with MgATP before chromatography (see under 'Methods'). Thus any dissociation which had occurred *in vivo* owing to endogenous cyclic AMP or after killing as a result of post-mortem cyclic AMP increases (e.g. see Breckenridge, 1964; Anderson *et al.*, 1977) was reversed by this procedure. Gel filtration removed both the endogenous cyclic AMP and the exogenously added ATP before ion-exchange chromatography. Omission of these steps decreased the apparent amount of heart type I isoenzyme.

The distribution of protein kinase holoenzymes from heart and brain cytosol are shown in Figs. 1(a) and (b) respectively. Heart cytosol showed apparently equal activities of type I and type II isoenzymes, which eluted at 0.03 M- and 0.18 M-NaCl respectively. Both peaks of activity were stimulated by cyclic AMP (approx. 3-fold) and all the cyclic AMP-binding activity eluting from the column was contained in these peaks. Brain kinase holoenzyme was eluted at 0.21 M-NaCl as one peak (type II), which contained all the cyclic AMP-binding activity and was stimulated by cyclic AMP.

A transient exposure to cyclic AMP had a considerable effect on the chromatographic properties of heart protein kinase (Fig. 1c). The peak of heart type I isoenzyme was absent and a peak of cyclic AMP-independent kinase, presumably free C_1 sub-

Fig. 1. DEAE-cellulose chromatography of protein kinase holoenzymes and of protein kinases briefly exposed to cyclic AMP from brain and heart cytosols

Brain (a) and heart (b) protein kinase holoenzymes were prepared and chromatographed as described in the Materials and Methods section. Brain (c) and heart (d) extracts were prepared similarly, except that the cytosols were incubated for 2 h at 0°C with 1 μ M-cyclic AMP rather than with MgATP. Fractions were assayed for protein kinase activity with (●) or without (○) cyclic AMP, for cyclic [^{3} H]AMP-binding activity (Δ) and for NaCl concn. (▲).

unit, was eluted in the breakthrough fractions. A peak of cyclic AMP-dependent kinase activity was eluted at the same NaCl concentration as the 'associated' type II heart isoenzyme. The cyclic AMP-binding activity was eluted as a broad peak around the type II position. This peak probably contained type II holoenzyme and free R_1 subunits. The profile of brain kinase transiently exposed to cyclic AMP (Fig. 1*d*) is identical with that of brain cytosol that had undergone prior treatment with MgATP, i.e. a single type II holoenzyme was present. Apparently the subunits from both heart and brain type II isoenzymes reassociated after removal of cyclic AMP (perhaps during gel filtration), whereas the heart type I kinase remained dissociated even in the absence of cyclic AMP.

Complete dissociation of kinase enzymes

To keep the subunits dissociated from each other after exposure to cyclic AMP, all elution buffers con-

tained cyclic AMP. In five such experiments brain cytosol gave multiple peaks of *C* subunit activity, either four peaks (Fig. 2*a*) or three peaks with a trailing shoulder to the first peak. The first peak of *C* subunit activity was always largest, but in two experiments the third peak of *C* subunit activity was almost as large. Free cyclic [^{3} H]AMP, chromatographed in the absence of cytosol, was eluted just before the beginning of the NaCl gradient. The other ^{3} H-labelled peaks were retained on Millipore filters and thus represented protein-bound ^{3} H. In one experiment, brain cytosol was heated at 65°C for 30 min before the addition of cyclic [^{3} H]AMP and then subjected to DE-52 DEAE-cellulose chromatography. The ^{3} H-labelled peaks normally eluted at 0.09–0.10M- and 0.20–0.24M-NaCl with unheated cytosol were absent and all ^{3} H appeared just before the start of the NaCl gradient.

In contrast with the results with brain, dissociated heart cytosol contained only a single peak of *C* subunit

Fig. 2. DEAE-cellulose chromatography of brain (a) and heart (b) protein kinases eluted in the presence of cyclic AMP. Heart and brain were homogenized in homogenization buffer without a prior MgATP treatment. The cytosols were incubated with 20 nM-cyclic [^{3} H]AMP for 2 h at 0°C and loaded on to DE-52 DEAE-cellulose columns. All buffers contained 1 μ M-cyclic AMP. Fractions were assayed for protein kinase activity in the absence of cyclic AMP ($\circ$), for ^{3} H (Δ) and for NaCl concentration ($\blacktriangle$).

activity (Fig. 2*b*). Both heart isoenzymes contained *C* subunits that did not bind to DE-52 DEAE-cellulose columns under the conditions of ionic strength and pH used. When heart cytosol was chromatographed on CM-52 CM-cellulose resin a single symmetrical peak of *C* subunit activity was eluted after the NaCl gradient began (results not shown). This suggests that the *C* subunits of the heart I and II holoenzymes are similar or identical in their charge properties.

The ^3H distribution of dissociated heart cytosol was similar to that of dissociated brain cytosol. Two main peaks of material eluted at 0.01–0.05*M*- and 0.15–0.19*M*-NaCl were observed in each of four separate experiments. The ^3H label in these peaks was retained on Millipore filters, but was eluted before the start of the NaCl gradient if the heart cytosol had been heat-denatured. A ^3H -labelled peak eluted immediately after sample application was observed with both brain and heart cytosols. This peak was retained on Millipore filters, but was not affected by heat treatment. The identity of this material is unclear. The slight differences in NaCl concentration required to elute the heart and brain protein-bound cyclic [^3H]AMP (i.e. 0.01–0.05*M* versus 0.09–0.10*M* and 0.15–0.19*M* versus 0.20–0.24*M* respectively) were consistently observed. These peaks of cyclic [^3H]AMP from heart presumably correspond to the R_1 and R_{II} forms studied by Hofmann *et al.* (1975) in type I rabbit skeletal muscle and type II bovine heart respectively. The identity of the protein-bound ^3H from dissociated brain extract, however, is not apparent. In some experiments with dissociated brain extracts the material eluted at 0.20–0.24*M*-NaCl comprised up to 30% of the total protein-bound ^3H label, whereas in no experiment with brain holoenzyme was more than 10% of the cyclic AMP-binding activity eluted outside of the type II region. These

peaks of material probably correspond to different cyclic AMP-binding species located within the brain type II isoenzyme class, i.e. different R_{II} proteins, as suggested below.

Partial proteolysis of protein kinase samples can result in the formation of different chromatographic species of holoenzymes (Talmadge *et al.*, 1977) and of free *R* subunits (Sugden & Corbin, 1976). Three different types of experiments were done to test whether the multiple *C* and *R* species observed in brain cytosol were due to limited proteolysis during dissociation (results not shown). The presence of 1.2*mM*-phenylmethanesulphonyl fluoride (a serine protease inhibitor) during homogenization did not alter the chromatographic profile of brain cytosol from that seen in Fig. 2(*a*). Any proteolytic activity causing the multiplicity of active species must have appeared as a result of dissociation, otherwise multiple holoenzyme peaks would have been observed as well. Incubation of brain cytosol with 20*nM*-cyclic [^3H]AMP for different lengths of time (1–4 h) did not change either the number of ^3H -labelled peaks or their relative distribution. Finally, if dissociated brain cytosol contained a proteolytic activity absent from the dissociated heart cytosol, then mixing the two cytosols during dissociation might affect the profile of dissociated heart cytosol. It did not.

We thus conclude that brain and heart cytosol each contain two different *R* subunits. Additionally, the brain type II holoenzyme contains three or four chromatographically separable *C* subunit activities and the two heart isoenzymes share a common *C* subunit activity.

Kinetic studies of unfractionated brain cytosol

The experiments described above suggest that the multiple peaks of subunits *R* and *C* from brain cytosol did not result as a consequence of proteolysis

Table 1. *Effect of various compounds on cyclic [^3H]AMP binding in mouse brain cytosol*

Data shown are the relative amounts of 20*nM*-cyclic [^3H]AMP bound at different concentrations of inhibitor, the amount bound in the absence of inhibitor being used as a reference. All values represent the mean of at least two experiments. Values for inhibitors at 10 μM are the means $\pm$ S.E.M. from four experiments.

Compound	Concn. of inhibitor (μM)	...	Cyclic [^3H]AMP bound relative to control			
			0.1	1	10	100
Adenosine			1.04	1.00	0.74 $\pm$ 0.02	0.66
AMP			1.09	1.03	0.74 $\pm$ 0.06	0.65
ADP			1.08	0.97	0.73 $\pm$ 0.11	0.46
Cyclic AMP			0.29	0.04	0.005	0.002
2':3'-Cyclic AMP			1.02	0.93	0.72 $\pm$ 0.07	0.72
Dibutyl cyclic AMP			1.04	0.86	0.38 $\pm$ 0.05	0.08
8-Bromo cyclic AMP			0.80	0.16	0.02	0.004
Cyclic GMP			1.12	0.77	0.23 $\pm$ 0.03	0.05
Cyclic CMP			1.17	0.98	0.45	0.14
Cyclic IMP			0.70	0.23	0.05	0.008

during dissociation of the holoenzyme. However, if proteolysis had been an immediate consequence of dissociation or if the putative proteinase did not have serine at the active site, these experiments would not have been conclusive. Therefore a series of experiments was done on the cyclic AMP-binding activity of unfractionated brain cytosol in an attempt to demonstrate heterogeneity of *R* subunits without prior dissociation.

The binding assay used in these experiments was quite specific for cyclic AMP, as indicated by the relative inability of structurally related compounds to compete for binding (see Table 1). Only cyclic AMP, 8-bromo cyclic AMP and cyclic IMP showed significant inhibition of the binding of 20 nM-cyclic [^{3} H]AMP at inhibitor concentrations below 1 μ M.

The affinity for binding cyclic [^{3} H]AMP to unfractionated brain cytosol was determined by Scatchard (1949) analysis (Fig. 3). Curvature in such a plot results either from heterogeneity of binding components or from a more complicated equilibrium reaction (e.g. co-operativity). Both high- and low-affinity components were found. Least-squares estimates of K_d values and binding capacities (R_T) from five separate experiments were made. The K_d of the

Fig. 3. Scatchard plot of cyclic [^{3} H]AMP binding to brain cytosol

Brain cytosol was prepared by homogenizing brains in 5 vol. (w/v) of unbuffered 0.32M-sucrose and using the final supernatant of three consecutive centrifugations: 3000g, 5 min; 27000g, 15 min; 105000g, 40 min. The cyclic AMP-binding assay was as described in the Materials and Methods section. Each point is the mean of duplicate determinations. Cyclic [^{3} H]AMP concentration was varied from 0.2 nM to 125 nM. This curve is representative of five different experiments.

high-affinity component was 2.35 ± 0.62 nM and R_T equalled 5.65 ± 1.32 pmol of cyclic AMP/mg of protein.

The rates at which cyclic [^{3} H]AMP dissociated from the binding components in brain cytosol were determined at three different temperatures (Fig. 4). The rate of dissociation at 0°C was very low. Half of the bound cyclic [^{3} H]AMP was replaced by unlabelled cyclic AMP within 1 h when the dissociation reaction was run at 20°C, and only a single rate of dissociation was observed. At 30°C the cyclic AMP dissociation curve indicated the presence of at least two major components. Imposed on this finding, however, is the fact that nearly half of the cyclic [^{3} H]AMP dissociated from the control sample (in which water rather than excess of unlabelled cyclic AMP was added) within 1 h at 30°C. This suggested that denaturation of the binding activity had occurred at 30°C. No such loss of activity occurred at 20°C. Pretreatment at 20°C may release binding activity in complex with endogenous cyclic AMP and provide a means for studying both total binding sites and available (not in complex) binding sites, as has been done by Khac *et al.* (1973) and Dufau *et al.* (1977) in other systems.

The rate at which a protein loses its biological activity as a function of temperature is a sensitive

Fig. 4. Dissociation of cyclic [^{3} H]AMP from brain cytosol

Brain cytosol (45–70 μ g, prepared as in Fig. 3) was incubated with 20 nM-cyclic [^{3} H]AMP and 50 nM-sodium acetate in 200 μ l final volume for 5 h at 0°C; 10 μ l, containing either 4 nmol of unlabelled cyclic AMP or water, was added to each tube. The tubes' contents were mixed and quickly placed either on ice or in a water bath maintained at 20 or 30°C for various times. The samples were then filtered as described. The relative amount of cyclic [^{3} H]AMP bound to the samples that had been incubated at 0 (▲) and 20°C (●) was compared with that bound after the initial 5 h incubation. The points at 30°C (○) represent the relative amounts of cyclic [^{3} H]AMP bound to samples to which unlabelled cyclic AMP had been added compared with samples to which water had been added.

means of detecting structural differences among species of protein having the same activity (Paigen, 1971). Thermal lability follows first-order kinetics; hence a plot of the logarithm of binding activity remaining against the length of time that the sample was subjected to heat provides a criterion for determining heterogeneity. We have previously demonstrated a correspondence between heat denaturation and the number of chromatographically separable cyclic [^3H]AMP-binding sites in normal and virus SV40-transformed 3T3 cells (Gharrett *et al.*, 1976). The effect of preincubation at three different temperatures on the cyclic AMP-binding activity is shown in Fig. 5. Heating at 45°C increased cyclic AMP-binding activity 25–30% above that in unheated control experiments. This may have been due to a heat-induced dissociation of endogenously bound cyclic AMP. A rapid initial loss of activity was followed by a gradual decrease in activity at 55°C. Only 10% of the activity remained after 15 min at 60°C. The 60°C inactivation curve clearly indicated the presence of at least two binding components. The loss of activity that occurred at 30°C in the dissociation experiments probably resulted from the different incubation conditions used. Cytosols were heated in unbuffered sucrose for the heat-denaturation experiments, whereas more highly diluted cytosol samples were heated at pH4 in the dissociation experiments. Cyclic AMP-binding activity is

Fig. 5. Heat denaturation of cyclic AMP-binding activity of brain cytosol

Brain cytosol (4–6 mg of protein/ml of unbuffered 0.32 M-sucrose, prepared as described in Fig. 3) was heated at 45 (●), 55 (▲) or 60°C (○) in tightly capped tubes. At intervals, samples were removed and added to a cyclic AMP-binding reaction mixture kept on ice. Untreated cytosol was kept on ice for use as a reference (100% activity). The cyclic AMP-binding activity of all samples was determined.

more labile at acidic pH (Mackenzie & Stellwagen, 1974).

These experiments support the conclusions of the chromatographic analyses of dissociated brain cytosol, namely that multiple *R* subunits are contained within the type II enzyme peak.

Discussion

Earlier studies by Corbin and colleagues (Soderling *et al.*, 1973; Corbin *et al.*, 1975; Corbin & Keely, 1977) have shown that rat heart type II enzyme reassociates rapidly after dissociation by various agents, whereas type I reassociates slowly. Both isoenzymes were activated by agents that raised cyclic AMP concentrations in heart extracts (Corbin & Keely, 1977). The length of time that heart extracts could remain activated after removal of cyclic AMP by gel filtration was inversely related to the relative amount of type II isoenzyme present. When extracts from intact SV3T3 cells that had been treated with dibutyryl cyclic AMP were chromatographed, the type I isoenzyme was completely dissociated, whereas only partial dissociation of the type II isoenzyme was observed (Gharrett *et al.*, 1976). Treatment of extracts from these cells with cyclic AMP caused total dissociation of both isoenzymes. Chromatographic profiles of rat testes (Means *et al.*, 1976) and human peripheral lymphocytes (Byus *et al.*, 1977) after addition of follitropin or of dibutyryl cyclic AMP respectively showed that type I kinase had dissociated and the type II isoenzyme had remained in holoenzyme form. The interpretation of these results was that in the intact tissue the type I isoenzyme had responded to the agonist and the type II isoenzyme had not. It could also be suggested, however, that the type II isoenzymes may have dissociated at least partially but then reassociated during subsequent homogenization and chromatography.

It is of interest to compare the soluble protein kinases from heart and brain. A widely different distribution of the two isoenzyme classes has been found in the heart cytosol of different species (Corbin & Keely, 1977). In contrast, all or nearly all of the soluble brain protein kinases of bovine (Miyamoto *et al.*, 1973), rat (Corbin *et al.*, 1975), pig (Nesterova *et al.*, 1975) and mouse (Fig. 1) is the type II isoenzyme. The isoenzyme distribution changed as a function of postnatal age in mouse heart cytosol, but underwent no such change in brain cytosol (Malkinson *et al.*, 1978). Fig. 2 suggests that the *R* subunits of heart cytosol are similar to, but not identical with, the *R* subunits of brain cytosol in their chromatographic properties. The heart isoenzymes share a chromatographically identical *C* subunit, whereas the single brain holoenzyme class contains several different types of *C* subunit. The degree of microheterogeneity within the type II isoenzyme class

therefore appears to vary with the tissue source. Immunological studies on bovine brain type II kinase have also suggested the presence of multiple species of protein kinase enzymes (Fleischer *et al.*, 1976). Four different holoenzymes could be observed on polyacrylamide-gel electrophoresis of rat brown-adipose-tissue type II holoenzyme (Knight & Skala, 1977). Finally, soluble and particulate type II isoenzymes within the same tissue may have different structures (Uno *et al.*, 1976).

Although the studies reported here clearly suggest microheterogeneity within an isoenzymic class, the nature of this heterogeneity is not known. Both of the C_1 subunits of rabbit skeletal-muscle protein kinase contained phosphoserine (Bechtel *et al.*, 1977). R_{II} subunits can also exist in both phosphorylated and dephosphorylated states (Hofmann *et al.*, 1975; Walter *et al.*, 1977). Storage of protein kinase from skeletal muscle (Corbin *et al.*, 1972) or heart (Rubin *et al.*, 1972) resulted in the appearance of additional forms of the enzyme. These experiments suggested that additional biologically active species were being generated by partial proteolytic breakdown. Direct evidence for such proteolysis in other systems has been obtained (Sugden & Corbin, 1976; Talmadge *et al.*, 1977). The presence of phosphorylated and unphosphorylated forms of the two subunits, and of catalytically active breakdown products, could give rise to microheterogeneity. In addition to such post-translational events, a multiplicity of R and C gene products may also be contained within a single isoenzymic class.

We thank Dr. J. D. Corbin (Vanderbilt University) for suggesting a pretreatment of cell extracts before chromatography to maintain type I isoenzymes in the holoenzyme state. This work was supported by a National Foundation-Basil O'Connor Starter Grant and United States Public Health Service grant no. GM22492.

References

- Anderson, W., Krzanowski, J., Polson, J. & Szentivanyi, A. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 398 (abstract)
- Bechtel, P. J., Beavo, J. A. & Krebs, E. G. (1977) *J. Biol. Chem.* **252**, 2691-2697
- Bloom, F. E., Wedner, H. J. & Parker, C. W. (1973) *Pharmacol. Rev.* **25**, 343-358
- Breckenridge, B. M. (1964) *Proc. Natl. Acad. Sci. U.S.A.* **52**, 1580-1586
- Byus, C. V., Chubb, J. M., Huxtable, R. J. & Russell, D. H. (1976) *Biochem. Biophys. Res. Commun.* **73**, 694-702
- Byus, C. V., Klimpel, G. R., Lucas, D. O. & Russell, D. H. (1977) *Nature (London)* **268**, 63-64
- Chen, L. J. & Walsh, D. A. (1971) *Biochemistry* **10**, 3614-3621
- Corbin, J. D. & Keely, S. L. (1977) *J. Biol. Chem.* **252**, 910-918
- Corbin, J. D. & Reimann, E. M. (1974) *Methods Enzymol.* **38**, 278-299
- Corbin, J. D., Brostrom, C. O., King, C. A. & Krebs, E. G. (1972) *J. Biol. Chem.* **247**, 7790-7798
- Corbin, J. D., Keely, S. L. & Park, C. R. (1975) *J. Biol. Chem.* **250**, 218-225
- Corbin, J. D., Sugden, P. H., Lincoln, T. M. & Keely, S. L. (1977) *J. Biol. Chem.* **252**, 3854-3861
- Costa, M., Gerner, E. W. & Russell, D. H. (1976) *J. Biol. Chem.* **251**, 3313-3319
- Dufau, M. L., Tsuruhara, T., Horner, K. A., Podesta, E. & Catt, K. J. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3419-3423
- Fleischer, N., Rosen, O. M. & Reichlin, M. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 54-58
- Gharrett, A. J., Malkinson, A. M. & Sheppard, J. R. (1976) *Nature (London)* **264**, 673-675
- Gilman, A. G. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 305-312
- Hofmann, R., Beavo, J. A., Bechtel, P. J. & Krebs, E. G. (1975) *J. Biol. Chem.* **250**, 7795-7801
- Kemp, B. E., Bylund, D. B., Huang, T. S. & Krebs, E. G. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3448-3452
- Khac, L., Harbon, S. & Clauser, H. J. (1973) *Eur. J. Biochem.* **40**, 177-185
- Knight, B. L. & Skala, J. P. (1977) *J. Biol. Chem.* **252**, 5356-5362
- Kuo, J. F. & Greengard, P. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **64**, 1349-1355
- Langan, T. A. (1973) *Adv. Cyclic Nucleotide Res.* **3**, 99-153
- Lee, P. C., Radloff, D., Schweppe, J. C. & Jungmann, R. A. (1976) *J. Biol. Chem.* **251**, 914-921
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Mackenzie, C. W., III & Stellwagen, R. H. (1974) *J. Biol. Chem.* **249**, 5755-5762
- Malkinson, A. M., Hogy, L., Gharrett, A. J. & Gundersen, T. J. (1978) *J. Exp. Zool.* in the press
- Means, A. R., Fakunding, J. L. & Tindall, D. J. (1976) *Biol. Reprod.* **14**, 54-63
- Miyamoto, E., Petzold, G. L., Kuo, J. F. & Greengard, P. (1973) *J. Biol. Chem.* **248**, 179-189
- Nesterova, M. V., Sashchenko, L. P., Vasiliev, V. Y. & Severin, E. S. (1975) *Biochim. Biophys. Acta* **377**, 271-281
- Paigen, K. (1971) in *Enzyme Synthesis and Degradation* (Rechcigl, M., ed.), pp. 1-46, University Park Press, Baltimore
- Post, R. W. & Sen, A. K. (1967) *Methods Enzymol.* **10**, 773-776
- Rubin, C. S., Erlichman, J. & Rosen, O. M. (1972) *J. Biol. Chem.* **247**, 6135-6139
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660-672
- Soderling, T. R., Corbin, J. D. & Park, C. R. (1973) *J. Biol. Chem.* **248**, 1822-1829
- Steiner, A. L., Whitley, T. H., Ong, S. H. & Stowe, N. W. (1975) *Metabolism* **24**, 419-428
- Steiner, A. L., Koide, Y. M., Earp, H. S., Bechtel, P. J. & Beavo, J. A. (1978) *Adv. Cyclic Nucleotide Res.* **9**, 691-705

- Sugden, P. H. & Corbin, J. D. (1976) *Biochem. J.* **159**, 423-437
- Sugden, P. H., Holladay, L. A., Reimann, E. M. & Corbin, J. D. (1976) *Biochem. J.* **159**, 409-422
- Talmdage, K. W., Bechtel, E. & Eppenberger, U. (1977) *Eur. J. Biochem.* **78**, 419-430
- Tao, M. & Hackett, P. (1973) *J. Biol. Chem.* **248**, 5324-5332
- Tao, M., Salas, M. L. & Lipmann, F. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 408-414
- Uno, I., Ueda, T. & Greengard, P. (1976) *J. Biol. Chem.* **251**, 2192-2195
- Walter, U., Uno, I., Liu, A. Y.-C. & Greengard, P. (1977) *J. Biol. Chem.* **252**, 6588-6590
- Yamamura, H., Nishiyama, K., Shimomura, R. & Nishizuka, Y. (1973) *Biochemistry* **12**, 856-862