Compartmentalization of Adenosine 3':5'-Monophosphate and Adenosine 3':5'-Monophosphate-dependent Protein Kinase in Heart Tissue*

(Received for publication, November 18, 1976)

JACKIE D. CORBIN,‡ PETER H. SUGDEN,§ THOMAS M. LINCOLN, AND STANLEY L. KEELY

From the Department of Physiology, School of Medicine, Vanderbilt University, Nashville, Tennessee

In rabbit heart homogenates about 50% of the cAMP-dependent protein kinase activity was associated with the low speed particulate fraction. In homogenates of rat or beef heart this fraction represented approximately 30% of the activity. The percentage of the enzyme in the particulate fraction was not appreciably affected either by preparing more dilute homogenates or by aging homogenates for up to 2 h before centrifugation. The particulate enzyme was not solubilized at physiological ionic strength or by the presence of exogenous proteins during homogenization. However, the holoenzyme or regulatory subunit could be solubilized either by Triton X-100, high pH, or trypsin treatment. In hearts of all species studied, the particulate-bound protein kinase was mainly or entirely the type II isozyme, suggesting isozyme compartmentalization.

In rabbit hearts perfused in the absence of hormones and homogenized in the presence of 0.25 M NaCl, at least 50% of the cAMP in homogenates was associated with the particulate fraction. Omitting NaCl reduced the amount of particulate-bound cAMP. Most of the particulate-bound cAMP was probably associated with the regulatory subunit in this fraction since approximately 70% of the bound nucleotide was solubilized by addition of homogeneous catalytic subunit to the particulate fraction. The amount of cAMP in the particulate fraction (0.16 nmol/g of tissue) was approximately one-half the amount of the regulatory subunit monomer (0.31 nmol/g of tissue) in this fraction. The calculated amount of catalytic subunit in the particulate fraction was 0.18 nmol/g of tissue. Either epinephrine alone or epinephrine plus 1-methyl-3-isobutylxanthine increased the cAMP content of the particulate and supernatant fractions. The cAMP level was increased more in the supernatant fraction, possibly because the cAMP level became saturating for the regulatory subunit in the particulate fraction. The increase in cAMP was associated with translocation of a large percentage of the catalytic subunit activity from the particulate to the supernatant fraction. The distribution of the regulatory subunit of the enzyme was not significantly affected by this treatment. The catalytic subunit translocation could be mimicked by addition of cAMP to homogenates before centrifugation. The data suggest that the regulatory subunit of the protein kinase, at least that of isozyme II, is bound to particulate material, and the active catalytic subunit is released by formation of the regulatory subunit-cAMP complex when the tissue cAMP concentration is elevated. A model for compartmentalized hormonal control is presented.

The adenosine 3':5'-monophosphate-dependent protein kinase has been found in both solubile and particulate fractions of mammalian tissues (1-6). The latter fraction of protein kinase activity comprises a large percentage of the total in some tissues. Rubin et al. (3) found more than 70% of the total erythrocyte cAMP-dependent protein kinase in the particulate fraction, presumably associated with the plasma membrane. Menon (4) reported that 50% of the total enzyme in bovine corpus luteum was associated with the particulate fraction. The presence of cAMP-dependent protein kinase has been noted in preparations of sarcoplasmic reticulum (7-9) and in membrane-enriched fractions (10-12) of heart tissue. The interpretation of many of the early studies is complicated by the presence of the free catalytic subunit in particulate fractions prepared in low ionic strength buffers (13). Furthermore, there have been few detailed studies of either the physiological regulation of the particulate enzyme or the nature of the binding of the enzyme to particulate material. Investigations of this type using heart tissue are presented in this paper.

Although there has been speculation concerning the possible compartmentalization of cAMP in cells for several years (14), definitive evidence for it has been lacking. Since the cAMP-dependent protein kinase is a major receptor for cAMP in heart tissue, the finding of a considerable proportion of this enzyme in the particulate fraction suggested the existence of particulate-bound cAMP. The discovery of a large amount of particulate-bound cAMP in heart tissue is reported here. Proof of the association of this cAMP fraction with the regulatory subunit of protein kinase is also presented. Possible physiological roles for particulate-bound cAMP and cAMP-dependent protein kinase are discussed.

* This work was supported by Program Project Grant AM 07462 and Research Grant AM 15888, and by Postdoctoral Fellowship 1 F32-CA 05128 to Thomas M. Lincoln from the National Institutes of Health.
‡ Investigator of the Howard Hughes Medical Institute.
§ Postdoctoral Fellow of the United Kingdom Science Research Council-NATO. Present address, Nuffield Department of Clinical Biochemistry, Radcliffe Infirmary, Oxford, OX 26 HF, United Kingdom.

3854
Heart Perfusion — Fed male rats (150 to 200 g) or rabbits (500 to 700 g) were used. Removal of hearts and perfusion were carried out as described earlier (15, 16). Perfused and unperfused hearts, and beef hearts which were obtained from a local slaughterhouse were frozen and powdered before use unless indicated otherwise. Since in rabbit hearts the amount of particulate-bound protein kinase was found to increase with increasing age of the animal, the size of the rabbits used is indicated in the text.

Homogenization — Powdered tissue was suspended at 4° in the indicated buffer and homogenized with three up and down turns of a motor-driven Teflon pestle at high speed in a glass tube. In some cases a plastic tube was used without noticeable difference in results.

Protein Assay — The protein determination assay was based on the phosphorylation of histone (Sigma type II-A) and was carried out essentially as described earlier (17). The assay reaction was started by adding 10 μl of the diluted (60 ml per g of tissue) sample to 50 μl of reaction mixture in the presence or absence of 2 mM CAMP. The incubation was carried out at 30° for 5 min unless indicated otherwise. Under these conditions the assay was linear with time and concentration of tissue extract. In some cases the kinase activity is expressed as the protein kinase activity ratio, i.e. the ratio of activity in the absence of CAMP to that in the presence of CAMP (2 mM CAMP).

Adenylyl Cylase and Phosphodiesterase Assays — Adenylyl cyclase activity was determined essentially as described by Drummond and Duncan (18). After incubation at 30° for 30 min, the reaction was terminated by placing the tubes in a boiling water bath for 30 s. The CAMP produced was measured from a standard curve by means of the cAMP-binding assay described later, except that 50 μl of the standard buffer sample was added to 110 μl of assay mixture. Phosphodiesterase activity was determined by a modification of the method of Wells et al. (19). Reaction mixtures contained 50 mM Tris buffer (pH 7.5), 25 mM MgCl₂, 0.33 mM/ml of bovine serum albumin, 0.1 μM [H]CAMP (150,000 cpm), and enzyme in a final volume of 150 μl. Hydrolysis of CAMP by phosphodiesterase occurred 50% of the initial level. After incubation at 30° for 10 min, the reaction was terminated by placing the tubes into a dry ice/ethanol bath followed by a boiling water bath for 90 s. The [H]CAMP formed was converted to [H]adenosine by adding 0.02 ml of 0.1 M trichloroacetic acid and centrifuged at 12,000 X g for 5 min. The supernatant or resuspended particulate fraction was added 0.1 pmol of [3H]CAMP (50,000 cpm) to estimate the CAMP recovery. The mixture was incubated for 60 to 90 min at 20°, and then 1 ml of ice-cold 10 mM potassium phosphate (pH 6.8) containing 1 mM EDTA was added. The CAMP was removed by addition of 4 ml of 20% ammonium acetate (pH 7.5), and all 5 ml was collected and counted in a toluene/Triton X-100 scintillant.

Cyclic AMP Assay — The buffers used for preparation of supernatant and particulate fractions always contained 10 mM EDTA and 0.5 mM 1-methyl-3-isobutylxanthine. These agents inhibit the hydrolysis of CAMP by phosphodiesterase. To 1-ml aliquots of supernatant or resuspended particulate fraction was added 0.1 pmol of [3H]CAMP (50,000 cpm/pmol) to estimate the CAMP recovery. Immediately thereafter, the suspension was deproteinized by addition of 100 μl of 30% trichloroacetic acid and centrifuged at 12,000 to 20,000 X g for 20 min. The supernatant fraction was then pipetted onto a Dowex 50 column (0.9 x 10 cm, 100 to 200 mesh) equilibrated with 0.1 N HCl. The column was then eluted with 30 ml of 0.1 N HCl, and the 10- to 30-ml fraction was collected and lyophilized to dryness. The residue was resuspended in 1 ml of 50 mM sodium acetate (pH 4.0). Cyclic AMP was then determined by the protein-binding assay of Gilman (21).

CAMP-binding Protein (Regulatory Subunit) Assay — Binding of CAMP to proteins was routinely assayed at neutral pH by a method similar to that of Gilman (21). An aliquot (20 μl) suitably diluted (60 mgl of tissue) in buffer was added to 55 μl of a solution of 50 mM potassium phosphate (pH 6.8), 1 mM EDTA, 0.5 mM/mg of histone (Sigma type II-A), 2 mM NaCl, and 1 μM [3H]CAMP (6000 cpm/pmol). The mixture was incubated for 60 to 90 min at 20°, and then 1 ml of ice-cold 10 mM potassium phosphate, 1 mM EDTA at pH 6.8 was added. The CAMP was removed by filtration through a Whatman No. 3 filter paper. The 60-μl (15 μm) previously moistened with the same buffer. The reaction tube was rinsed with another 1 ml of the same buffer. The filter was rinsed with 8 ml of buffer and dried in an oven at 150°. Radioactivity retained by the filter was estimated by counting in 10 ml of a toluene/Triton X-100 scintillant.

Partial Purification of Regulatory Subunits — The regulatory subunits of rat heart protein kinase were purified on DEAE-cellulose columns essentially as described earlier (22). The method is similar to that for purification of the supernatant fraction holokinase as described in Fig. 4. A homogenate (4 ml/g of tissue) of 15 fresh rat hearts (no perfusion or freezing) was prepared in 10 mM potassium phosphate (pH 6.8) containing 1 mM EDTA. After centrifugation at 27,000 X g for 30 min, the supernatant fraction (57 ml) was incubated at 0° in the presence of 10 μM CAMP for 30 min and then applied to a DEAE-cellulose (Whatman No. DE 15) column (2.5 x 25 cm) equilibrated with the homogenization buffer. The column was washed with 200 ml of buffer containing 10 μM CAMP, followed by 1 liter of buffer containing 1 mM CAMP, and finally with 1 liter of buffer. The column was eluted with a 500-ml linear (0 to 0.5 M) gradient of NaCl, and the fractions were assayed for CAMP-binding activity. Two peaks of CAMP-binding activity eluted at 0.1 and 0.25 M NaCl, and were presumably derived from type I and type II protein kinases, respectively (22, 23). The peak fractions were pooled separately, concentrated by (NH₄)₂SO₄ precipitation (80% saturation), and re-suspended in and dialyzed against 10 mM potassium phosphate (pH 6.8).

Sucrose Density Gradient Centrifugation — Sucrose gradients were prepared by the method of Martin and Ames (24) as described earlier (25) in 5 mM Tris, pH 7.5, containing 1 mM EDTA. Bovine hemoglobin (4.6 S) and rabbit muscle phosphorylase b (8.2 S) were used as markers to calculate sedimentation coefficients.

Materials — Type II-A histone from calf thymus, Fraction V bovine serum albumin, Triton X-100 (bovine pancreas type III), soybean trypsin inhibitor, phosphorylase b, and CAMP were obtained from Sigma; γ-32PATP was prepared by a modification of the method of Glynn and Chappell (25). Epinephrine was obtained from Parke-Davis as the HCI salt. 1-Methyl-3-isobutylxanthine was a gift from Searle and Co. The bovine liver catalytic subunit was purchased as described elsewhere (26). The preparation was homogenous as judged by sodium dodecyl sulfate disc gel electrophoresis, sedimentation velocity centrifugation, and sedimentation equilibrium centrifugation. Casein and crystalline hemoglobin were from Nutritional Biochemical Co. Crystalline bovine serum albumin was obtained from International Chemical and Nuclear. Rats and rabbits were purchased from Harlan Industries.

RESULTS

Supernatant and Particulate-bound Protein Kinase — The distribution of protein kinase activity and CAMP binding protein (regulatory subunit) in supernatant and particulate fractions of homogenates of hearts from different species is shown in Table I. Rat, rabbit, and beef hearts were selected for these experiments because supernatant fractions from them exhibited the broadest variation in protein kinase isozyme patterns (see Ref. 27 and Fig. 6). There was a good correlation between the ratio of concentrations of protein kinase to CAMP-binding activities in all fractions and species. From the specific activity of the pure catalytic subunit (3 x 10⁷ units/mg) of molecular weight 40,000 (28) it was calculated from the data of Table I that the molar ratio R/C is 0.80, 0.94, and 0.80, respectively, in rat, rabbit, and beef heart homogenates. In agreement with Hofmann et al. (28), this suggests equimolar distribution of the two subunits in this tissue, as would be expected for the holoenzyme (R₂C), i.e. neither the regulatory nor the catalytic subunit is in excess. From these experiments and other studies which will be presented later, it was concluded that the CAMP-binding activity represents the regulatory subunit of protein kinase. Beef heart contained the largest total amount of both activities in homogenates, approximately double that of rabbit heart. Although beef heart also contained the highest absolute amount of particulate-bound kinase and CAMP-binding activity, the percentage of these activities in the particulate fraction was highest in rabbit and lowest in rat heart. The kinase activity was CAMP-stimulated in all fractions as indicated by the protein kinase activity ratio. This also indicated the presence of a small amount of free catalytic
Compartmentalization of CAMP and Protein Kinase

3856

animals were used. Results are the mean of three experiments.

\( \text{Fraction} \)
\( \text{Rat} \)
\( \text{Rabbit} \)
\( \text{Beef} \)
\( \text{Rat} \)
\( \text{Rabbit} \)
\( \text{Beef} \)
\( \text{Rat} \)
\( \text{Rabbit} \)
\( \text{Beef} \)

| Protein kinase activity | cAMP-binding protein | Protein kinase activity ratio |
|------------------------|----------------------|-----------------------------|
| Homogenate             | 78,700               | 0.52                        | 0.17                        |
| Supernatant            | 77,000               | 0.51                        | 0.14                        |
| Particulate            | 22,300               | 0.15                        | 0.26                        |
| Particulate/homogenate | 0.28                 | 0.28                        | 0.29                        |

Table I

Distribution of protein kinase activity and cAMP-binding protein in supernatant and particulate fractions of homogenates of hearts from different species

Supernatant and particulate fractions (3 washes) were prepared as described in Fig. 1 in the absence of NaCl. For the rabbit preparation, 0.5-kg animals were used. Results are the mean of three experiments.

The particulate fractions were routinely prepared by centrifugation at \( 20,000 \times g \) for 15 min followed by washing and centrifugation at the same speed. The percentage of the protein kinase or cAMP-binding activity in the particulate fraction was not significantly different, however, even when centrifugation was carried out at \( 3000 \times g \) for 5 min, suggesting the presence of large particles. Approximately 90% of the protein kinase and cAMP-binding activities remained associated with the particulate fraction after centrifugation in linear sucrose density gradients (30 to 70% w/v) at \( 1900 \times g \) for 30 min at 4°C (not shown). Likewise, approximately 90% of either the adenylate cyclase or phosphodiesterase activities in the gradient fractions was also associated with this peak, which was about one-third from the bottom of the gradient tube after centrifugation.

Several experiments examined whether the cAMP-dependent protein kinase was bound in a specific manner to particulate material. The percentage of the kinase or cAMP-binding activities in the particulate fraction was not affected appreciably by tissue dilution during homogenization, suggesting that binding to the particles is not a second order process which occurs during preparation (not shown). The percentage was also not affected by aging the homogenate at 25°C before centrifugation. The data of Fig. 1 indicated that the percentage of the cAMP-binding activity in the particulate fraction was only slightly decreased by increasing the KCl concentration to 150 mM. Higher ionic strengths caused a larger decrease in this fraction, and a concomitant and proportional increase in the supernatant fraction. The protein kinase activity in the particulate fraction was decreased more than the cAMP-binding activity in this fraction when homogenates were prepared at low ionic strength (<0.5 M). This could be caused by slight dissociation of the holoenzyme into particulate-bound regulatory and free catalytic subunits in the absence of MgATP (23) at these ionic strengths. No further decrease in particulate-bound kinase activity occurred from 0.5 to 1.5 M KCl. Disappearance of kinase or binding activity from the particulate fraction was accompanied by their appearance in the supernatant fraction. In several of the subsequent experiments approximately physiological ionic strength buffers were used for homogenization and washing. In such media the binding of the regulatory subunit or holoenzyme to particulate material is prevented (13).

If the regulatory subunit and holoenzyme bind in a nonspecific manner to particulate material either during or following homogenization of tissues, it might be expected that addition of competing proteins to the homogenization buffer would prevent the binding. However, the addition of exogenous proteins to the buffer at high concentration did not reduce the amount of cAMP-binding activity in the particulate fraction.
(Table II). Histone slightly increased the activity in this fraction. Addition of excess regulatory subunits which had been partially purified from rat heart homogenates did not alter the amount of particulate-bound cAMP-binding activity. The latter experiment suggested either that the particulate-binding sites for the regulatory subunits were saturated or that the protein kinase and regulatory subunit bound to particulate material were different from the soluble forms.

**Solubilization of Regulatory Subunit and Protein Kinase**

As will become clearer later (see Table VI), when cAMP is added to homogenates or particulate fractions, the particulate-bound holoenzyme dissociates into its regulatory and catalytic subunits. In media of physiological ionic strength the regulatory subunit remains bound to particulate material, but the catalytic subunit is released into the supernatant fraction. This property can be used to prepare particulate fractions containing the regulatory subunit but essentially free of the catalytic subunit. Comparisons can then be made between the solubilization of the particulate-bound regulatory subunit (cAMP-washed) and holoenzyme. Triton X-100 solubilized the regulatory subunit and holoenzyme of the rabbit heart particulate fraction assayed by cAMP binding (Fig. 2). At a concentration of 1% almost all (>90%) of the particulate fraction regulatory subunit or holoenzyme was solubilized. The disappearance of either the regulatory subunit or holoenzyme from the particulate fraction was accompanied by a corresponding increase of activity in the supernatant fraction. There was no significant difference in solubilization of the regulatory subunit as compared with the holoenzyme. Triton X-100 treatment also solubilized the cAMP-dependent protein kinase activity (see Fig. 4). The regulatory subunit and holoenzyme could also be solubilized by partial proteolysis with the use of low concentrations of trypsin (Fig. 3). Regardless of whether the starting material was the particulate-bound holoenzyme or the regulatory subunit, trypsin treatment effectively solubilized the cAMP-binding activity. As before, disappearance of the activity from the particulate fraction was associated with its appearance in the supernatant fraction. Trypsin treatment also solubilized the cAMP-dependent protein kinase activity when the starting material was particulate-bound holoenzyme (not shown). It was also found that, using the holoenzyme as the starting material, most of the cAMP-binding and kinase activities were solubilized by increasing the pH above pH 8 (not shown). The activities lost from the particulate fraction again appeared in the supernatant fraction. Since the cAMP-binding and kinase activities were solubilized at the same pH, and the kinase activity was cAMP-dependent (not shown), it is assumed that it was the holoenzyme which was solubilized by this procedure.

The particulate enzyme solubilized by Triton X-100 exhibited a sedimentation coefficient of 7.5 ± 0.1 S as compared with 7.3 ± 0.1 S for the supernatant enzyme. The high pH and

![Fig. 2](left). Effect of Triton X-100 on solubilization of particulate fraction protein kinase holoenzyme and regulatory subunit. A homogenate (16 mg/ml) of frozen, powdered heart muscle (no perfusion) from a 7-kg rabbit was made in 10 mM potassium phosphate (pH 6) containing 0.25 M NaCl as described under "Experimental Procedures." The homogenate was divided into two equal parts for preparation of particulate fractions containing either bound protein kinase holoenzyme (no additions) or regulatory subunit. Centrifugation and washing were done as described in Fig. 1, with the same volume and buffer as above. For preparation of particulate fractions containing bound CAMP-binding protein, the first wash contained 1 µM CAMP and the second wash contained 0.1 µM CAMP. The third wash contained no CAMP. The particulate fractions were suspended in the same buffer as above (15 ml/g of original tissue) containing the indicated concentrations of Triton X-100. The particulate fractions were incubated at 3100 g for 15 min and the supernatant and resuspended particulate fractions diluted to 60 ml/g of original tissue in buffer not containing Triton X-100 for assay of cAMP-binding protein. Under the conditions used, the Triton X-100 carverrover did not significantly affect the CAMP-binding assay.

![Fig. 3](right). Trypsin solubilization of particulate fraction protein kinase holoenzyme and free R subunit. Particulate fractions containing either holoenzyme or free R subunit were prepared as described in Fig. 2. The particulate fraction was suspended (16 mg/ml) in 10 mM potassium phosphate and 0.25 M NaCl containing the indicated concentrations of trypsin which had been freshly dissolved (1 mg/ml) in 10 mM acetate buffer at pH 4. After incubation for 10 min at 25° a solution (10 mg/ml) of soybean trypsin inhibitor was added to a final concentration of 150 µg/ml. After mixing and centrifugation, the supernatant and resuspended particulate fractions were assayed for cAMP-binding protein activity.

**Table II**

*Effects of homogenization in presence of exogenous regulatory subunits and other proteins on presence of cAMP-binding protein in particulate fraction*

The preparation of the first (R₁) and second (R₂) DEAE-cellulose peaks of supernatant protein kinase regulatory subunit of rat heart was as described under "Experimental Procedures." The exogenous regulatory subunits (R₁ and R₂) were 4.1- and 3.3-fold, respectively, in excess of the endogenous regulatory subunit activity.

| Experiment no. | Addition to homogenizing medium | cAMP-binding protein | Supernatant | Particulate |
|----------------|---------------------------------|----------------------|-------------|-------------|
|                |                                 | nmol/g tissue        |             |             |
| 1              | None                            | 0.68                 | 0.56        |
|                | R₁                              | 2.79                 | 0.32        |
|                | R₂                              | 2.26                 | 0.52        |
| 2              | None                            | 0.67                 | 0.60        |
|                | Histone (5 mg/ml)               | 0.34                 | 0.81        |
|                | Casein (5 mg/ml)                | 0.64                 | 0.50        |
|                | Serum albumin (5 mg/ml)         | 0.66                 | 0.70        |
|                | Hemoglobin (5 mg/ml)            | 0.57                 | 0.60        |
The existence of two classes (isozymes I and II) of CAMP-activated Particulate-bound CAMP-dependent Protein Kinase-bound enzyme. The particulate-bound enzyme was solubilized by trypsin (Type II-A), protamine, and casein as substrates, the relative substrate preferences for histone and protamine being different. The homogenate was then centrifuged at 20,000 × g for 15 min. The pellet was resuspended by homogenization in the original volume of buffer (no NaCl) and Triton X-100 before DEAE-cellulose chromatography. It can be seen in Table III that a large amount of the total CAMP of perfused rabbit heart was associated with the particulate fraction. The concentration of CAMP in the particulate fraction was less than the concentration of CAMP in the supernatant fraction. For preparation of the supernatant fractions (lower panels), homogenization was performed as described above in the absence of NaCl. After the first centrifugation, the supernatant was diluted three times and chromatographed as described for the particulate fraction.

Substrate Specificity—Uno et al. (30) have reported that soluble and membrane-bound kinases of various tissues exhibit different substrate preferences for histone and protamine. Using the protein kinase assay conditions described under "Experimental Procedures," we have found no significant difference in the substrate specificity of the rabbit heart supernatant protein kinase as compared with the particulate-bound enzyme. The particulate-bound enzyme was solubilized with 0.2% Triton X-100 before testing. Using histone (Sigma Type II-A), protamine, and casein as substrates, the relative rates of phosphorylation were 11:3:2, respectively, for the supernatant enzyme and 12:3:1 for the particulate enzyme.

DEAE-cellulose Chromatography of Supernatant and Solubilized Particulate-bound CAMP-dependent Protein Kinase—The existence of two classes (isozymes I and II) of CAMP-dependent protein kinases in supernatant fractions of hearts from different species. For preparation of the enzyme from particulate fractions (upper panels), approximately 1 g of heart tissue was frozen, powdered, and homogenized (3 ml/g) in 10 mM potassium phosphate (pH 6.8), 10 mM EDTA, 0.5 mM 1-methyl-3-isobutylxanthine, and 100 mM NaCl as described under "Experimental Procedures" for perfused hearts. The 100 mM NaCl prevents non-specific binding of the catalytic subunit to particulate material (13). The homogenate was then centrifuged at 20,000 × g for 15 min. The pellet was resuspended by homogenization in the original volume of buffer (no NaCl) and Triton X-100 before DEAE-cellulose chromatography. It can be seen in Table III that a large amount of the total CAMP of perfused rabbit heart was associated with the particulate fraction. The concentration of CAMP in the particulate fraction was less than the concentration of CAMP in the supernatant fraction. For preparation of the supernatant fractions (lower panels), homogenization was performed as described above in the absence of NaCl. After the first centrifugation, the supernatant was diluted three times and chromatographed as described for the particulate fraction.

Hormonal Effects on Supernatant and Particulate Protein Kinase and CAMP—It can be seen in Table III that a large amount of the total CAMP of perfused rabbit heart was associated with the particulate fraction. The concentration of CAMP in the particulate fraction was less than the concentration of the regulatory subunit, the regulatory subunit of the protein kinase. If the hearts were perfused with either epinephrine alone or epinephrine plus 1-methyl-3-isobutylxanthine, the CAMP level increased in the supernatant and particulate fractions. In the presence of the two agents together, the CAMP level increased more in the supernatant than in the particulate fraction. This could be explained by the saturation of the regulatory subunit in the latter fraction at the high concentrations of tissue CAMP. The measured level of the regulatory subunit monomer (0.31 nmol/g) was about the same as the level of CAMP (0.33 nmol/g) in this fraction. The concentrations of regulatory subunit (RC + RCAMP) shown in Table III were calculated from the CAMP-binding activity, assuming binding of 1 mol of CAMP per regulatory subunit monomer (31). The concentrations of total catalytic subunit (RC + C) were calculated from the specific activity of homogeneous catalytic subunit (26). The total amount of regulatory subunit (supernatant plus particulate) was not always exactly the same as the total amount of catalytic subunit, probably because of the errors inherent in calculation of catalytic subunit amounts from specific activity of pure enzyme. Perfusion of hearts either with epinephrine alone or epinephrine plus 1-methyl-3-isobutylxanthine did not significantly alter the regulatory subunit level in either frac-
Homogenates (60 mg/g of tissue for Experiment 1 and 5 ml per g of tissue for Experiment 2) were prepared from frozen hearts from 0.5-kg rabbits which had been perfused for 2 min in the absence or presence of epinephrine (Epi) or epinephrine plus 1-methyl-3-isobutylxanthine (MIX) as indicated. The homogenization buffer was 10 mM potassium phosphate (pH 6.8), 10 mM EDTA, 0.5 mM 1-methyl-3-isobutyaxanthine, and 0.25 M NaCl. In Experiment 1 the particulate fraction was washed three times in the same volume and buffer in the absence of NaCl. In Experiment 2 the homogenate was centrifuged once at 20,000 × g for 10 min at 4°. The particulate fraction was resuspended in the respective buffer indicated and centrifuged again. All particulate fractions were resuspended (10 ml/g of tissue) in the buffer above in the presence of 0.25 M NaCl and placed in a boiling water bath for 1 min. After centrifugation, the clear supernatant fraction was assayed for CAMP as described under "Experimental Procedures." Results are mean ± S.E. for six determinations.

### Table IV

**Effect of 0.25 M NaCl on appearance of CAMP in particulate fraction**

Perfusion and homogenization (5 ml/g of tissue) were done as described in Table III. The homogenization and wash buffer was 10 mM potassium phosphate (pH 6.8), 10 mM EDTA, 0.5 mM 1-methyl-3-isobutylxanthine (MIX) in the presence or absence of 0.25 M NaCl as indicated. The homogenates were centrifuged at 20,000 × g for 10 min at 4°. The supernatant fraction was discarded, and the particulate fraction was resuspended in the respective buffer indicated and centrifuged again. All particulate fractions were resuspended (10 ml/g of tissue) in the buffer above in the presence of 0.25 M NaCl and placed in a boiling water bath for 1 min. After centrifugation, the clear supernatant fraction was assayed for cAMP as described under "Experimental Procedures." Results are mean ± S.E. for six determinations.

| Experiment | Addition to perfusate | NaCl in homogenization and wash buffer | cAMP in particulate fraction | nmol/g tissue |
|------------|-----------------------|---------------------------------------|-----------------------------|----------------|
| 1          | None                  | 0.07 ± 0.01                           | 0.09 ± 0.01                 | 0.27 ± 0.03   |
|            | Epi (1 μM)            | 0.11 ± 0.01                           | 0.15 ± 0.01                 | 0.24 ± 0.00   |
| 2          | None                  | 0.08 ± 0.02                           | 0.16 ± 0.03                 | 0.33 ± 0.02   |
|            | Epi (1 μM) + 2 MIX    | 0.82 ± 0.13                           | 0.33 ± 0.02                 | 0.30 ± 0.02   |

### Table V

**Removal of CAMP from particulate fraction by addition of homogeneous bovine liver catalytic subunit**

As described in Fig. 1, approximately 600 mg of rabbit heart which had been perfused with epinephrine, frozen, and powdered was homogenized (60 mg/g of tissue) in 10 mM potassium phosphate (pH 6.8), 10 mM EDTA, 0.5 mM 1-methyl-3-isobutylxanthine, and 250 mM NaCl. The particulate fraction was washed three times as described in Fig. 1 in the absence of NaCl. The final suspension, which was in the same volume as the original, was divided into four equal aliquots (9 ml each) which were treated for 30 min as indicated. The suspensions were centrifuged again and resuspended in 1 ml of buffer (no NaCl) containing 5% trichloroacetic acid and a small amount of [3H]cAMP (for recovery purposes). Chromatography and cAMP assay were performed as described under "Experimental Procedures." The final concentration of the C-subunit was 10,000 units/ml. Values are mean ± S.E. for four determinations.

| Treatment | Temperature | cAMP nmol/g tissue |
|-----------|-------------|-------------------|
| None      | 0°          | 0.17 ± 0.04       |
| + C subunit | 0°         | 0.08 ± 0.01       |
| None      | 25°         | 0.17 ± 0.03       |
| + C subunit | 25°        | 0.05 ± 0.01       |

The data of Table III were suggestive that the finding of particulate-bound cAMP was due to the presence of the regulatory subunit-cAMP complex in the particulate fraction. Further evidence for this conclusion was the finding that the addition of homogeneous liver catalytic subunit followed by centrifugation caused disappearance of most of the particulate-bound cAMP (Table V). This would be expected since reassociation of the regulatory and catalytic subunits causes release of free cAMP (31, 32), which would be removed during centrifugation.

The effect of epinephrine on redistribution of the catalytic subunit shown in Table III was probably mediated by cAMP since cAMP addition to homogenates produced effects similar to that of epinephrine (Table VI). Although the regulatory subunit distribution was only slightly changed after addition of cAMP, a large portion of the protein kinase activity was shifted to the supernatant fraction. In some experiments (e.g. Table VI, Experiment 2) slight decreases in cAMP-binding activity in both supernatant and particulate fractions were observed after addition of high concentrations of cAMP to homogenates. This was probably due either to the carryover of some cAMP from the homogenate to the cAMP-binding pro...
Compartmentalization of cAMP and Protein Kinase

Transfer of protein kinase activity from particulate to supernatant fraction after addition of cAMP

Original supernatant and washed resuspended particulate fractions were prepared from homogenates (3 ml/g of tissue) in 10 mM potassium phosphate (pH 6.8), 10 mM EDTA, 0.5 mM 1-methyl-3-isobutylxanthine, and 0.25 M NaCl. Unperfused frozen heart powder from 7-kg rabbits was used. After homogenization the samples sat on ice for 60 min before centrifugation. Three washes were done in the same volume and buffer as the original for Experiment 1. As would be expected from the results of Fig. 2, in Experiment 1 a lower percentage of the particulate protein kinase was found due to removal of kinase during successive washes in 0.25 M NaCl. Where indicated, homogenization and first wash were done in the presence of 1 mM cAMP in Experiment 1. In Experiment 2, the particulate fraction was resuspended and assayed without washing. Results are mean ± S.E. for three determinations.

| Experiment no. | Addition to homogenate | Protein kinase activity | cAMP-binding protein |
|----------------|------------------------|-------------------------|----------------------|
|                |                        | Supernatant             | Particulate          | Supernatant | Particulate |
| 1 (7-kg rabbit)| None                   | 99,000 ± 4,600          | 38,700 ± 3,100       | 0.37 ± 0.03 | 0.48 ± 0.04 |
|                | cAMP, 1 µM             | 127,500 ± 3,000         | 13,500 ± 3,900       | 0.39 ± 0.06 | 0.41 ± 0.05 |
| 2 (0.5-kg rabbit)| None                  | 39,500 ± 2,900          | 31,290 ± 4,400       | 0.33 ± 0.03 | 0.29 ± 0.06 |
|                | cAMP, 1 µM             | 65,000 ± 2,600          | 13,563 ± 1,800       | 0.26 ± 0.02 | 0.22 ± 0.03 |

DISCUSSION

As is the case for erythrocytes (3) and corpus luteum (4), a large percentage of the total cAMP-dependent protein kinase of heart tissue is associated with the low speed particulate fraction of homogenates. The holoenzyme is attached to particulate material by its regulatory subunit. At physiological ionic strength, the appearance of catalytic activity in the particulate fraction is a consequence of the binding of the catalytic subunit to the particulate-bound regulatory subunit. Most of the free catalytic subunit binding to the particulate fraction of heart homogenates at low ionic strength is probably nonspecific (13). The finding that it is mainly the type II isozyme associated with the particulate fraction is apparent due to the nature of its regulatory subunit. This would be expected since the catalytic subunits of types I and II isozymes are probably identical (22, 23, 27, 31).

Evidence presented here suggests that the protein kinase is bound to particulate material in the cell. This conclusion is derived from studies of dilution and aging of homogenates, from the stability of the bound enzyme at physiological ionic strength, from the lack of removal of the bound enzyme by competing proteins, and from the finding of mainly isozyme II in the particulate fraction. The enzyme is not trapped in a nonspecific manner in vesicles formed during homogenization as indicated by the differences in isozyme distribution between particulate and supernatant fractions. Either the particulate-bound holoenzyme or regulatory subunit can be easily solubilized by Triton X-100, partial proteolysis, high ionic strength, or treatment at high pH. The particulate-bound protein kinase thus appears to represent a "peripheral" as opposed to an "integral" membrane protein according to the criteria set forth by Singer (33). The relatively loose binding of the enzyme to the particulate fraction and the effect of proteolytic enzymes and pH changes imply that the percentage of the bound enzyme could have been underestimated in these studies because of loss of some of the bound enzyme during homogenization and washing. These effects, as well as differences in age of the animal, ionic strength, and number of washes might also explain the variation in amount of particulate enzyme in different experiments. The organelle distribution of the particulate-bound activity is not known. Whether or not the activity is associated with the plasma membrane, as is the case in erythrocytes and corpus luteum (3, 4), remains to be established. Sulakhe et al. (34) reported the presence of cAMP-dependent protein kinase in purified cardiac sarcoplasm, although the percentage yield was low. The association of the enzyme with myofibrils is also possible.

Epinephrine causes dissociation of the particulate enzyme into its regulatory and catalytic subunits in the presence or absence of 1-methyl-3-isobutylxanthine. After dissociation, the regulatory subunit remains bound to particulate material, but the catalytic subunit transfers to the soluble fraction. Addition of cAMP itself to the homogenate also causes this transfer. The lack of observance of this phenomenon by Rubin et al. (3) in erythrocytes and by Horwood and Singhal in rat heart (12) could have been due to their use of low ionic strength buffers which would have allowed binding of the free catalytic subunit to the particulate fraction (13). The finding of a large percentage of the total cAMP in the particulate fraction is not surprising in view of the presence of a high percentage of the protein kinase (regulatory subunit) in this fraction. This fraction of cAMP was elevated in the presence of epinephrine. The finding of increased levels of particulate cAMP when homogenization and washing were performed in the presence of 0.25 M NaCl could be explained by the inhibitory effect of the higher ionic strength on reassociation of the particulate R cAMP complex with the free catalytic subunit.

The occurrence of particulate-bound cAMP and protein kinase could be a part of an overall compartmentalized system for hormonal control as shown in Fig. 5. For simplicity the dimer of the protein kinase is shown. The existence of particulate-bound hormonal receptors [R] and adenylate cyclase [A]
for stimulation and synthesis of cAMP is well known. Much of the cAMP phosphodiesterase activity is also particulate-bound (35). It would seem that the location of the cAMP receptor (regulatory subunit) near the site of synthesis of cAMP could offer a selective advantage for the cell. This might particularly be the case if the free cAMP concentration is higher at the site of its production than it is at more central portions of the cell (36). A smaller diffusion distance for cAMP might allow a more rapid response of the protein kinase. Activation of the holoenzyme by cAMP elevation could result in translocation of the dissociated catalytic subunit. The "mobile" catalytic subunit should then diffuse through the soluble portion of the cell and catalyze phosphorylation of cytoplasmic or other proteins. The reversal of activation would involve decrease in cAMP followed by reassociation of the catalytic subunit with the particulate-bound regulatory subunit. The theory obviously assumes that cAMP causes physical dissociation of the holoenzyme into regulatory and catalytic subunits in the cell. Although such in vitro dissociation has not been established with certainty, its physiological sense is made clear from the above considerations. The theory also assumes that the particulate-bound protein kinase is emplaced in the vicinity of adenylate cyclase in the plasma membrane or in other cell organelles, which remains to be proved. Even if not, however, the "mobile" catalytic subunit hypothesis is still valid. The location of the catalytic subunit near its substrate (indicated by the triangle), which might also be particulate, could be similarly advantageous for the cell since catalysis of phosphorylation would be more efficient.

The theory of Fig. 5 involves three basic, and possibly independent, processes, each of which is amenable to testing: (a) regulatory subunit-adenylate cyclase compartmentalization; (b) catalytic subunit-substrate compartmentalization; and (c) catalytic subunit translocation. The evolution of either, or all, of these processes may have resided in selective binding of the regulatory subunit, particularly that of isozyme II, to particulate material. The distribution of isozymic forms of enzymes in separate cell compartments is well recognized (37).

Acknowledgments—We are grateful to Maureen McRedmond, Tanya Poe, Sheila Shay, and Debra McCarthy for excellent technical assistance. We are also indebted to Dr. Charles R. Park for his encouragement and advice.

REFERENCES

1. Krebs, E. G. (1972) Curr. Top. Cell. Regul. 5, 99–133
2. Lemaire, S., Pelletier, G., and Labrie, F. (1971) J. Biol. Chem. 246, 7303–7310
3. Rubin, C. S., Erlichman, J., and Rosen, O. M. (1972) J. Biol. Chem. 247, 6135–6139
4. Menon, K. M. J. (1973) J. Biol. Chem. 248, 494–501
5. Forte, L. R., Chao, W. H., Walkenbach, R. J., and Ryzington, K. H. (1975) Biochim. Biophys. Acta 389, 84–96
6. Sloboda, R. D., Rudolph, S. A., Rosenbaum, J. L., and Greenberg, P. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 177–181
7. Kirchberger, M. A., Tada, M., and Katz, A. M. (1974) J. Biol. Chem. 249, 676–683
8. LaRaia, P. J., and Mokin, E. (1974) Circ. Res. 35, 285–306
9. Entman, M. L., Kaniike, K., Goldstein, M. A., Nelson, T. E., Boren, E. F., Futch, T. W., and Schwartz, A. (1976) J. Biol. Chem. 251, 3140–3146
10. Krause, E. G., Will, H., Schirpke, B., and Wellenberger, A. (1976) Adv. Cyclic Nucleotide Res. 5, 435–458
11. Hui, C., Drummond, M., and Drummond, G. L. (1976) Arch. Biochem. Biophys. 173, 415–427
12. Horwood, D. M., and Singhal, R. L. (1976) J. Mol. Cell. Cardiol. 8, 29–38
13. Keely, S. L., Corbin, J. D., and Park, C. R. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 1501–1504
14. Butcher, R. W., Sneyd, J. G. T., Park, C. R., and Sutherland, E. W. Jr. (1966) J. Biol. Chem. 241, 1651–1653
15. Morgan, H. E., Henderson, M. D., Reimann, E. M., and Park, C. R. (1961) J. Biol. Chem. 236, 251–261
16. Keely, S. L., Corbin, J. D., and Park, C. R. (1975) J. Biol. Chem. 250, 4832–4840
17. Corbin, J. D., and Reimann, E. M. (1975) Methods Enzymol. 38, 287–290
18. Drummond, G. I., and Duncan, L. (1979) J. Biol. Chem. 245, 976–983
19. Wells, J. N., Baird, C. E., Wu, Y. J., and Hardman, J. G. (1975) Biochim. Biophys. Acta 384, 430–442
20. Cherrington, A. D., Assimacopoulos, F. D., Harper, S. C., Corbin, J. D., Park, C. R., and Exton, J. H. (1976) J. Biol. Chem. 251, 5209–5218
21. Gilman, A. G. (1970) Proc. Natl. Acad. Sci. U. S. A. 67, 306–312
22. Corbin, J. D., Soderling, T. R., Sugden, P. H., Keely, S. L., and Park, C. R. (1976) in Aqueous Cell Functions and Growth (Dumont, J. E., Brown, B., and Marshall, N. J., eds) pp. 221–247, Plenum Press, New York
23. Corbin, J. D., Keely, S. L., and Park, C. R. (1975) J. Biol. Chem. 250, 384–225
24. Martin, R. G., and Ames, B. N. (1961) J. Biol. Chem. 236, 1372–1379
25. Glynn, I. M., and Chappell, J. B. (1964) Biochem. J. 90, 147–149
26. Sugden, P. H., Holladay, L. A., Reimann, E. M., and Corbin, J. D. (1976) Biochem. J. 159, 489–492
27. Corbin, J. D., and Keely, S. L. (1975) J. Biol. Chem. 252, 910–918
28. Hofmann, F., Bechtel, P. J., and Krebs, E. G. (1977) J. Biol. Chem. 252, 1441–1447
29. Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, E. H., and Krebs, E. G. (1971) J. Biol. Chem. 246, 1977–1985
30. Uno, I., Ueda, T., and Greengard, P. (1975) J. Biol. Chem. 250, 4832–4840
31. Hofmann, F., Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1975) J. Biol. Chem. 250, 2181–2195
32. Hofmann, F., Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1975) 250, 7795–7801
33. Brostrom, C. O., Corbin, J. D., King, C. A., and Krebs, E. G. (1976) J. Biol. Chem. 252, 1977–1985
34. Sugden, P. H., Holladay, L. A., Reimann, E. M., and Corbin, J. D. (1976) Biochem. J. 159, 489–492
35. Suh, S. C., Futch, T. W., and Schwartz, A. (1975) Can. J. Biochem. 54, 438–455
36. Robison, G. A., Butcher, R. W., and Sutherland, E. W. (1971) in Cyclic AMP (Robison, G. A., Butcher, R. W., Sutherland, E. W., eds) pp. 72–90, Academic Press, New York
37. Swillens, S., Paiva, M., and Dumont, J. E. (1974) FEBS Lett. 49, 92–95
38. Marks, C. L. (1975) in Isozymes (Markert, C. L., ed) Vol. 1, pp. 1–9, Academic Press, New York
Compartmentalization of adenosine 3′:5′-monophosphate and adenosine 3′:5′-monophosphate-dependent protein kinase in heart tissue.
J D Corbin, P H Sugden, T M Lincoln and S L Keely

*J. Biol. Chem.* 1977, 252:3854-3861.

Access the most updated version of this article at [http://www.jbc.org/content/252/11/3854](http://www.jbc.org/content/252/11/3854)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/252/11/3854.full.html#ref-list-1](http://www.jbc.org/content/252/11/3854.full.html#ref-list-1)