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# cAMP-dependent protein kinase from brown adipose tissue: temperature effects on kinetic properties and enzyme role in hibernating ground squirrels

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**Abstract** Arousal from hibernation requires thermogenesis in brown adipose tissue, a process that is stimulated by β-adrenergic signals, leading to a rise in intracellular 3′,5′-cyclic adenosine monophosphate AMP (cAMP) and activating cAMP-dependent protein kinase A (PKA) to phosphorylate a suite of target proteins and activate lipolysis and uncoupled respiration. To determine whether specific adaptations (perhaps temperature-dependent) facilitate PKA kinetic properties or protein-phosphorylating ability, the catalytic subunit of PKA (PKAc) from interscapular brown adipose of the ground squirrel *Spermophilus richardsonii*, was purified (final specific activity = 279 nmol phosphate transferred per min per mg protein) and characterized. Physical properties of PKAc included a molecular weight of 41 kDa and an isoelectric point of 7.8 ± 0.08. A change in assay temperature from a euthermic value (37 °C) to one typical of hibernating body temperature (5 °C) had numerous significant effects on ground squirrel PKAc including: (a) pH optimum rose from 6.8 at 37 °C to 8.7 at 5 °C, (b) $K_m$ values at 37 °C for Mg.ATP (49.2 ± 3.4 μM) and for two phosphate acceptors, Kemptide (50.0 ± 5.5 μM) and Histone IIA (0.41 ± 0.05 mg/ml) decreased by 53%, 80% and 51%, respectively, at 5 °C, and (c) inhibition by KCl, NaCl and $NH_4Cl$ was reduced. However, temperature change had little or no effect on $K_m$ values of rabbit PKAc, suggesting a specific positive thermal modulation of the hibernator enzyme. Arrhenius plots also differed for the two enzymes; ground squirrel PKAc showed a break in the Arrhenius relationship at 9 °C and activation energies that were 29.1 ± 1.0 kJ/mol for temperatures >9 °C and 2.3-fold higher at 68.1 ± 2.1 kJ/mol for temperatures <9 °C, whereas the rabbit enzyme showed a breakpoint at 17 °C with a 13-fold higher activation energy over the lower temperature range. However, fluorescence analysis of PKAc in the absence of substrates, showed a linear change in fluorescence intensity and wavelength of maximal fluorescence over the entire temperature range; this suggested that the protein conformational change indicated by the break in the Arrhenius plot was substrate-related. Temperature change also affected the Hill coefficient for cAMP dissociation of the ground squirrel PKA holoenzyme which rose from 1.12 ± 0.18 at 37 °C to 2.19 ± 0.07 at 5 °C, making the release of catalytic subunits at low temperature much more responsive to small changes in cAMP levels. Analysis of PKAc function via in vitro incubations of extracts of ground squirrel brown adipose with $^{32}$P-ATP + cAMP in the presence versus absence of a PKA inhibitor, also revealed major differences in the patterns of phosphoproteins, both between euthermic and hibernating animals as well as between 37 and 5 °C incubation temperatures; this suggests that there are both different targets of PKAc phosphorylation in the hibernating animal and that temperature affects the capacity of PKAc to phosphorylate different targets. Both of these observations, plus the species-specific and temperature-dependent changes in ground squirrel PKAc kinetic properties, suggest differential control of the enzyme in vivo at euthermic versus hibernating body temperatures in a manner that would facilitate a rapid and large activation of the enzyme during arousal from torpor.

**Key words** Mammalian hibernation · PKA catalytic subunit · *Spermophilus richardsonii* · Brown adipose thermogenesis

**Abbreviations** *BAT* brown adipose tissue · *cAMP* 3′,5′-cyclic adenosine monophosphate · [*cAMP*] concentration of cAMP · *IEF* · isoelectrofocusing · $I_{50}$ inhibitor concentration that reduces enzyme velocity by 50% · $H_3PO_4$ phosphoric acid · *Ka* concentration of activator producing half-maximal activation of enzyme

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rate · *NE* norepinephrine · *NST* nonshivering thermogenesis · *PKA* cAMP-dependent protein kinase A · *PKAc* PKA catalytic subunit · *PKA-I* PKA inhibitor peptide · *pI* isoelectric point · *PMSF* phenylmethylsulphonyl fluoride · *TCA* trichloroacetic acid

## Introduction

In most mammals, exposure to temperatures below the thermoneutral zone causes a quick activation of heat production via both shivering and non-shivering thermogenesis. However, harsh winter environments present intense thermoregulatory challenges to small mammals and for many of these entry into hibernation is the key to winter survival. By depressing metabolic rate, reducing body temperature to near ambient, and entering a state of torpor, these animals conserve stored fuels and minimize energy expenditures at a time when food is scarce. Torpor may last for several weeks during which time numerous physiological functions are adjusted to accommodate hibernation (Wang 1989).

Arousal from torpor is an explosive event during which substrate mobilization for energy production and non-shivering thermogenesis for heat production are at their maxima (Wang 1989). Non-shivering thermogenesis in brown adipose tissue (BAT) is the primary source of heat output for rewarming the animal during arousal (Nedergaard and Cannon 1990). Thermogenesis in BAT can be increased by the administration of thyroxin, norepinephrine (NE) or epinephrine (Wunder 1979). Although both adrenal epinephrine and neuronal NE seem equipotent in activating metabolic responses from brown fat cells in vitro, NE is now accepted as the primary physiological effector of BAT thermogenesis in vivo (Jansky 1973; Wunder 1979). The effects of NE are mediated via stimulation of $\beta_3$-adrenergic receptors (Dicker et al. 1996), which in turn activate adenylate cyclase leading to the release of 3′,5′ cyclic adenosine monophosphate (cAMP) into the cytoplasm. This cAMP allosterically activates cAMP- dependent protein kinase A (PKA) causing catalytic and regulatory subunits of the enzyme to dissociate, and freeing the catalytic subunits to phosphorylate a variety of target proteins (Taylor et al. 1990). One of the targets in BAT is hormone-sensitive lipase. PKA-mediate phosphorylation activates the enzyme to increase the hydrolysis of stored triglycerides. This provides free fatty acids as fuel for mitochondrial oxidation, or as uncouplers of oxidative phosphorylation through the GDP (guanosine diphosphate)-binding protein (Nicholls and Locke 1984). Numerous studies have established that lipid oxidation, uncoupled from oxidative phosphorylation, is the primary thermogenic pathway in BAT (Cannon and Nedergaard 1985; Klaus et al. 1991; Nicholls and Locke 1984; Nicholls et al. 1986). β-Adrenergic signal transduction is also involved in the stimulation of gene expression that results in adipocyte proliferation and differentiation (Cannon et al. 1996).

Information regarding the role of protein kinase cascade systems in the hibernation process is still limited, although some studies have investigated reversible phosphorylation control of glycolytic enzymes during hibernation (Storey 1987a,b; Brooks and Storey 1992a; El Hachimi et al. 1990). In the present study, kinetic and structural studies of the purified catalytic subunit of cAMP-dependent protein kinase (PKAc) from BAT of the Richardson's ground squirrel *Spermophilus richardsonii*, were undertaken at both 37 °C and 5 °C to explore both the enzyme properties and the possible role of temperature in modulating enzyme function in hibernation. Kinetic studies included a comparison of the ground squirrel BAT enzyme with purified muscle PKAc from rabbit (a non-hibernating mammal), to determine if a specially adapted enzyme is present in the hibernator with kinetic properties optimized for low-temperature function.

## Materials and methods

### Chemicals and animals

Richardson's ground squirrels *S. richardsonii*, were captured in the foothills of the Rocky Mountains near Calgary, Alberta in September 1995. All animals were individually housed in rat cages at the animal care facility of the University of Calgary, Calgary, Alberta and maintained at 22 °C on a fall (10L:14D) photoperiod. At the end of an 8-week feeding period, animals were induced to hibernate by placing their cages in a cold room at 4 °C on a photoperiod of 8L:16D. After a 24-h habituation period, all food (but not water) was withheld. During the 1st week of fasting, the activity of the animals was monitored; those that did not hibernate were removed from the cold room at the end of the week. Those that were hibernating at the end of 7 days were maintained at 4 °C for an additional 2 days so that the minimum time that all animals were torpid before sampling was 2 days and the maximum time of torpor before sampling was 6–7 days. Control animals were kept at 22 °C and killed by decapitation. Tissues from both euthermic and hibernating squirrels were immediately excised, immersed in liquid nitrogen, and then stored at −80 °C until required.

[$\gamma^{32}$P] ATP (3000 Ci/mmol) was obtained from New England Nuclear (Montreal, Quebec, Canada). Lavendustin A, KT-5823, Calphostin C and H-89 were from Biomol (Plymouth Meeting, Pa., USA). Protein kinase C inhibitor peptide (19–36; PKC-I) and PKA inhibitor peptide (5–24; PKA-I) were purchased from Peninsula Labs (Belmont, Calif.). Phosphocellulose paper (P81) was obtained from Whatman (Canlab; VWR, Mississauga, Ont.). Rabbit muscle "Peak 1" PKA was from Sigma Chemical (St. Louis, Mo.). Before use in kinetic studies, regulatory subunits were removed by passing the preparation through a column of Sephadex DE-52 previously equilibrated in PEM buffer (20 mM potassium phosphate, pH 6.8, 2 mM EDTA, and 10 mM 2-mercaptoethanol); regulatory subunits stick to this column and catalytic subunits are eluted with 0.1 mM cAMP in PEM buffer. All other chemicals and column materials were purchased from Sigma Chemical (St. Louis, Mo.) or Boehringer Mannheim (Montreal, Quebec).

### In vitro phosphorylation incubations with endogenous PKAc

Studies were designed to determine the effect of temperature on phosphorylation events specific to PKAc in ground squirrel BAT. Frozen samples of interscapular BAT (0.3–0.5 g) were homogenized 1:4 (w/v) in ice-cold dephosphorylation buffer (20 mM potassium phosphate, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, pH 7.2) containing 1 μg/ml aprotinin, 10 μg/ml

leupeptin and a few crystals of phenylmethylsulphonyl fluoride (PMSF). The homogenate was centrifuged at 20 000 *g* for 20 min at 4 °C. The supernatant was removed and stored on ice for 5 h to allow dephosphorylation by protein phosphatases to occur. The samples were then desalted by centrifugation through a column of Sephadex G-25 (1 × 5 cm) pre-equilibrated in incubation buffer (40 mM imidazole-HCl, 10 mM potassium phosphate, 0.1 mM EDTA, 15 mM 2-mercaptoethanol and 20% v/v glycerol, pH 7.2) (Helmerhorst and Stokes 1980). Aliquots (100 µl) of desalted supernatant were then added to each of four incubation solutions to give a final volume of 200 µl. The four incubation solutions all contained 10 mM ATP, 60 µCi/ml $^{32}$P ATP, 20 mM $MgCl_2$ and 30 mM NaF. Other additives were: (a) solution I, 20 mM EDTA, (b) solution II, incubation solution only with no additions, (c) solution III, 10 mM cAMP, and (d) solution IV, 10 mM cAMP plus 2.5 µM PKA-I inhibitor peptide. The final calculated ATP species concentrations in each incubation solution were determined by computer program (Brooks and Storey 1992b): solution I, 5.2 mM free ATP, 0.03 mM free $Mg^{2+}$ and 1.4 mM Mg.ATP; solution II-IV, 0.13 mM free ATP, 5.0 mM free $Mg^{2+}$ and 9.7 mM Mg.ATP. Incubation mixtures were vortexed and left overnight (16 h) at either 5 °C or 37 °C. Protein bound $^{32}$P was separated from free $^{32}$P-ATP by passage through Sephadex G-25, as above. An aliquot of the incubation sample was then removed, mixed 1:1 with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and run on SDS-PAGE by the method of Laemmli (1970) with 12% acrylamide separating gels and 5% stacking gels. Protein standards were rabbit muscle myosin (205 kDa), *E. coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa). Gels were dried using a Bio-Rad Model 583 gel dryer and exposed to X-ray film for 12 days at −80 °C. Developed autoradiograms were converted into densitometry scans using a Hewlett Packard Scanjet 3c with Corel Photo-Paint v5.0 and Molecular Dynamics ImageQuant v3.22 software.

## Enzyme assay

PKA activity was assayed in a 60 µl volume as described by Corbin and Reimann (1974). The standard reaction mixture contained 20 mM potassium phosphate (pH 6.8), 0.15 mM Kemptide, 50 mM KCl, 1 mM EDTA, 2.08 mM magnesium acetate, 0.237 mM unlabelled ATP, 10 µCi/ml radioactive $^{32}$P-ATP (0.6 µCi per assay) and a 10–20 µl aliquot of enzyme (or enzyme + water). When holoenzyme was being assayed, the reaction mixture also contained 10 µM cAMP. These concentrations gave approximately 0.2 mM Mg.ATP and 0.74 mM free $Mg^{2+}$ in the assay mixture (Brooks and Storey 1992b), values that were determined to be optimal for the ground squirrel interscapular BAT enzyme. When measuring the Michaelis constant for Mg.ATP, the total $Mg^{2+}$ concentration was adjusted to keep the free $Mg^{2+}$ concentration at an optimal level. For determination of the pH dependence of PKAc, activity at different pH values was measured by adding 20 mM potassium phosphate, pre-adjusted to the appropriate pH value. The pH of control reactions containing all components except the radioactive $^{32}$P-ATP, was measured at 22 ± 1 °C and corresponding pH values at 37 °C or 5 °C were calculated based on the known change in buffer pH with temperature. Assays were run for 5–10 min and were linear under these conditions, as determined by preliminary time courses and by specific activity curves. Reactions were stopped by spotting 40 µl aliquots of each assay mixture onto individual 2 × 2 cm pieces of Whatman P81 filter paper. The papers were washed for 5 min intervals with four changes (75 ml each) of 75 mM phosphoric acid ($H_3PO_4$). Papers were then counted using Cerenkov methodology on a Packard Model 1900CA scintillation counter. When measuring activity with histone IIA, incubations were stopped by adding 1 ml of ice-cold 5% trichloroacetic acid (TCA) + 10 mM $H_3PO_4$ and incubating for 10 min on ice. Samples were then filtered through Whatman GF/c filter papers, washed 4 times with the TCA/$H_3PO_4$ solution and counted using Cerenkov methodology. One unit of PKAc activity was defined as the amount of enzyme that catalyzes the incorporation of 1 µmol of $^{32}$P from ATP onto Kemptide per min. Routine assays such as those used to find enzyme activity during purification procedures, were done at 22 °C; assays for kinetic analysis were conducted at either at 37 °C or 5 °C, or over a range of temperatures (Arrhenius plots).

## Purification of BAT PKAc

The method of Rubin et al. (1974) was used with modifications. Frozen interscapular BAT (1.6 g) from control animals was homogenized 1:7 w/v in ice-cold buffer A (10 mM $KH_2PO_4$, 10 mM $K_2HPO_4$, 2 mM 2-mercaptoethanol, 1 mM EDTA, pH 6.8) with 1 µg/ml aprotinin and 10 µg/ml leupeptin and a few crystals of PMSF added. The homogenate was centrifuged at 20 000 *g* for 20 min; the supernatant was saved and applied directly onto a Sephadex DE-52 column (1.5 × 5 cm) which had been previously equilibrated in buffer A. The column was washed with 50 ml of buffer A and then the enzyme was eluted using a linear gradient of 0–1.0 M KCl in buffer A. One ml fractions were collected and assayed for PKA activity. Peak fractions were pooled and concentrated to a volume less than or equal to 2 ml. The concentrated sample was loaded onto a Sephacryl S-300 gel filtration column (2 × 70 cm) equilibrated in buffer A; 1 ml fractions were collected and those containing peak enzyme activity were pooled. The pooled sample was then applied to a second DE-52 column (1 × 5 cm) in buffer A. The column was washed with 50 ml of buffer A and then the PKAc was eluted with 10 ml of 0.1 mM cAMP solution. Fractions (1 ml) were collected and those containing peak enzyme activity were pooled and concentrated to a final volume of 0.75 ml. The enzyme was dialyzed for 2 h against 2 l of buffer A and then used for kinetic analysis. Purified PKAc was stored for up to 3 weeks at 5 °C in buffer A plus 200 mM $K_2HPO_4$, pH 7.0. Purity was judged by SDS-PAGE (as described above) with staining by Coomassie blue. Protein concentrations were determined by the method of Bradford (1976) using the Bio-Rad Laboratories prepared reagent with bovine serum albumin as the standard.

## PKA holoenzyme isolation and activation studies

A sample of previously frozen euthermic ground squirrel interscapular BAT (0.25 g) was homogenized 1:5 (w/v) in PEM buffer containing 1 µg/ml aprotinin and 10 µg/ml leupeptin with a few crystals of PMSF. The homogenate was centrifuged at 22 000 *g* for 20 min in a Sorvall RC2-B refrigerated centrifuge. The supernatant was removed and applied to a Sephadex DE-52 column (1.5 × 5 cm) pre-equilibrated in PEM buffer. The column was washed with 40 ml of PEM buffer and the peak of PKA holozyme was eluted with 40 ml of a 0–750 mM KCl gradient. Aliquots of column fractions were assayed under standard conditions ±1 µM cAMP to locate the peak fractions containing holoenzyme. These fractions were then pooled and stored for up to 2 h at 5 °C until use. Activation constants for cAMP dissociation of the holoenzyme were determined at both 5 °C and 37 °C by incubating aliquots of PKA holoenzyme with different concentrations of cAMP (0.1 nM–20 µM) for 30 min. After incubation, the percentage of enzyme present as the free catalytic subunit in each sample was determined via standard assays (±10 µM cAMP) with Kemptide as the substrate.

## CM-cellulose chromatography and isoelectrofocusing

Isoforms of ground squirrel PKAc were assessed using chromatography on CM-52 cellulose and isoelectric focusing. We found that fully purified BAT PKAc did not survive isoelectrofocusing and so a partially purified enzyme was used for both of these studies. Tissue samples (0.3 g) from euthermic control animals were homogenized 1:5 w/v in buffer A containing protease inhibitors (leupeptin, aprotinin and PMSF – as described previously) and then centrifuged for 20 min at 20 000 *g* at 4 °C. The super-

natant was saved and applied directly onto a DE-52 column (1.5 × 5 cm) which had been previously equilibrated in buffer A. The column was washed with 50 ml of buffer A and then the PKAc was eluted with 10 ml of 0.1 mM cAMP solution in buffer A. One ml fractions were collected and assayed for PKA activity; peak fractions were pooled and aliquots were used for isoform analysis. For CM-52 cellulose chromatography, an aliquot of enzyme (2 ml) was applied to a 2 × 1 cm column of CM-cellulose equilibrated in PEM buffer, pH 7.5. After washing with PEM buffer, the enzyme was eluted with 30 ml of a 0–300 mM KCl gradient in PEM buffer. Fractions (1 ml) were collected and assayed for PKA activity. For isofocusing, a 2 ml aliquot was placed in the middle of a 110 ml isofocusing column containing pH 3.5–10 ampholines stabilized by a 0–30% w/v linear sucrose gradient (Vesterberg, 1971). The column was developed for 12 h at 300 V at 5 °C and then 2 ml fractions were collected, assayed for activity, and pH measured.

### Kinetic studies of purified *S. richardsonii* BAT PKAc and rabbit muscle PKAc

All kinetic studies involving purified ground squirrel BAT or rabbit muscle PKAc were performed at 37 °C and 5 °C using the basic assay procedure outlined above. The former temperature represents the euthermic body temperature of both animals, whereas the latter temperature is typical of body temperature in hibernating ground squirrels (Wang 1989). Water baths set at 37 °C or 5 °C (or other temperatures for Arrhenius plots) were used to maintain constant assay temperature and reaction mixtures were pre-incubated at each temperature for 10 min prior to the addition of enzyme to ensure thermal equilibration. Substrate affinity constants ($K_m$ values) for Kemptide, histone IIA, and Mg.ATP, as well as the inhibitory effects ($I_{50}$ values) of different neutral salts were determined under these conditions with PKAc from both sources. All kinetic data for substrate binding was initially analyzed using Hill plots; however, when the Hill coefficient was equal to 1 the data was described using Michaelis-Menten hyperbolic kinetics. $I_{50}$ values for inhibitors were determined by Job plots (Vo/Vi versus [I]; where Vo is the enzyme velocity in the absence of inhibitor, Vi is the enzyme velocity in the presence of inhibitor and [I] is the concentration of inhibitor used) (Brooks and Storey 1992c). Arrhenius plots and pH curves for both enzymes were determined under $V_{max}$ (enzyme maximal velocity) assay conditions. Kinetic data was analyzed using the Student's *t*-test (2-tailed).

### Fluorescence studies of *S. richardsonii* PKAc

Fluorescence spectra were recorded on a Perkin-Elmer LS-50 fluorescence spectrophotometer. Cuvette temperature was controlled with a water jacketed cell holder attached to a Neslab RTE-210 water bath. Assay conditions included an enzyme concentration of 8 μg/ml in 10 mM potassium phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol in a 3 ml quartz cuvette with a 1 × 1 cm path length. The excitation monochromator was set at 280 nm and the emission monochromator was set to scan the wavelength from 300–450 nm at a speed of 72 nm/min. Temperature-induced conformational measurements were performed on aliquots of purified hibernator PKAc; emission spectra were recorded and corrected for the corresponding blank activity (minus enzyme) by subtraction. Spectral titrations were carried out by adding increasing amounts of 8 M guanidine hydrochloride to the same enzyme solution. The solution was incubated for 30 min with stirring at 37 °C or 5 °C before recording the spectrum after each addition. The volume increase due to the addition of the denaturant solution was held constant for all trials at both temperatures investigated. PKAc activity loss with increasing concentration of guanidine hydrochloride was measured by sampling a small aliquot (2 μl) of the incubations and measuring enzyme activity as described above.

## Results

### In vitro phosphorylation incubations with endogenous PKAc

To assess the patterns of PKA-mediated phosphorylation of BAT proteins and the influence of temperature on these, extracts of interscapular BAT from both euthermic and hibernating animals were incubated in vitro under four different conditions and at temperatures representing euthermic (37 °C) and hibernating (5 °C) body temperatures. $^{32}$P-labeled protein products were then separated by one-dimensional SDS-PAGE, followed by autoradiography and densitometry scanning to reveal the relative intensity of radiolabeled bands. Extracts from both hibernating and enthermic animals incubated under incubation I conditions (+EDTA to chelate the $Mg^{2+}$ needed for kinase function) showed very few bands of very low intensity labeling on autoradiograms from either hibernating or euthermic animals at either incubation temperature; hence, very little protein phosphorylation occurred under these conditions. Autoradiograms of gel lanes containing samples from incubation II conditions (representing endogenous PKAc activity) also showed very little $^{32}$P incorporation into proteins. Extracts incubated under incubation III conditions (+cAMP) showed strong labeling of multiple bands, whereas when the specific inhibitor, PKA-I, was added (incubation IV, representing non-PKA mediated phosphorylation) fewer bands were labeled in a manner similar to the incubation II band intensity. Figure 1 shows the net result when densitometry scans from incubation IV autoradiograms were subtracted electronically from the scans of incubation III autoradiograms. This provides the net pattern of cAMP-stimulated, PKA-mediated protein phosphorylation in BAT extracts. Several peaks representing phosphoproteins were seen, generally centered at 210, 110, 66, 45, 30, and 20 kDa. Comparisons of extracts from euthermic versus hibernating animals revealed several instances of differential labeling. A small amount of $^{32}$P incorporation was observed at 210 kDa in BAT extracts from euthermic animals incubated at either temperature. However, this peak was absent in extracts prepared from BAT of hibernating individuals, suggesting that the protein involved may not be present while the animals are hibernating. In incubations at 5 °C (Fig. 1a), a marked difference in $^{32}$P-labeling pattern was seen for proteins of 40–50 kDa in extracts from euthermic versus hibernating animals. Pronounced labeling of this peak was found in extracts from hibernating individuals whereas in euthermic animals only two small peaks were seen in this molecular weight range. At 37 °C, however, both hibernating and euthermic extracts showed the same overlapping peak at 41 kDa but a small peak at 50 kDa was present in BAT extracts from euthermic but not hibernating animals. Incubations at both temperatures also showed small differences in the labeling patterns of

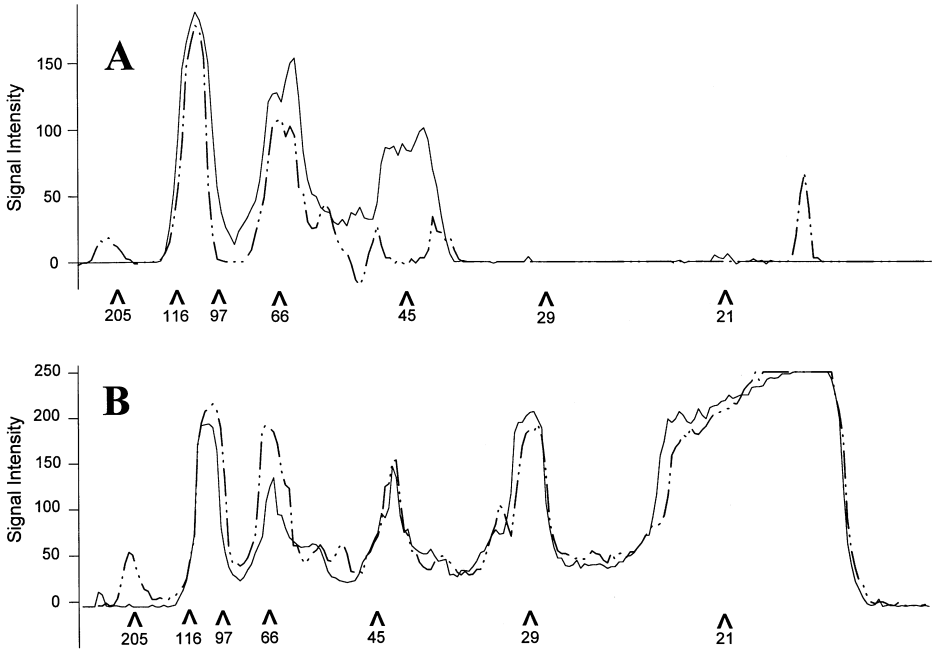


**Fig. 1A, B** Densitometry scanning of autoradiograms showing $^{32}P$ incorporation into cytosolic proteins in vitro in extracts of ground squirrel brown adipose tissue (BAT) catalyzed by endogenous protein kinases at **A** 5 °C and **B** 37 °C. Phosphoproteins in extracts of euthermic (*broken line*) and hibernating (*solid line*) animals are presented. Samples from in vitro phosphorylation incubations were run on SDS-PAGE, autoradiographed and scanned; relative signal intensity of bands is shown. Scans of autoradiograms representing incubation IV [+cAMP-dependent protein kinase A inhibitor peptide (PKA-I)] were subtracted electronically from those of incubation III [+3′5′-cyclic adenosine monophosphate (cAMP)] to correct for protein phosphorylation that was not cAMP-dependent protein kinase A (PKA)-mediated. The data shown represent a single trial but essentially the same results were achieved with duplicate incubations. Protein standards shown on the *x-axis* are: rabbit muscle myosin (205 kDa), *E. coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa)

60–70 kDa proteins with proportionally greater labeling of the peaks in this region at 37 °C for euthermic extracts and at 5 °C for extracts from hibernating individuals. The relatively greater labeling in this peak when body temperature and incubation temperature were matched may suggest that there are hibernation-related changes in either the properties of PKA or of its protein targets. Finally, a large smear of $^{32}P$ incorporation was present at low molecular weights (< 20 kDa) in the gel lanes containing extracts from 37 °C incubations; this may represent protein degradation products.

## PKA activity in vivo and holoenzyme dissociation studies

Total activity of PKA in BAT of euthermic squirrels was 1.2 ± 0.05 mU per mg soluble protein (mean ± SEM, $n = 4$) with a very low percentage present as the free catalytic subunit, just 1.0 ± 0.2%. In BAT from hibernating specimens, total activity was 33% higher at 1.5 ± 0.06 mU/mg protein ($P < 0.05$, Student's *t*-test) but the PKAc percentage was still low at 2.7 ± 0.8%. Calculated per gram wet weight of tissue, these values were 126 ± 10 and 154 ± 5 mU/gww ($P < 0.05$, Student's *t*-test), for tissue from euthermic and hibernating animals, respectively.

The PKA holoenzyme consists of two regulatory and two catalytic subunits that dissociate in vivo upon cAMP binding to the regulatory subunits. The effect of temperature (5 °C versus 37 °C incubation) on the dissociation of the holoenzyme type II from euthermic BAT, was investigated by determining the cAMP concentration at which 50% of the holoenzyme was dissociated. At 37 °C, the $K_a$ value for cAMP was calculated to be $0.582 \pm 0.029$ μM cAMP and the Hill coefficient was 1.12 ± 0.18, a near hyperbolic relationship. A similar $K_a$ value was determined at 5 °C ($0.591 \pm 0.022$ μM), but the Hill coefficient in this case was distinctly sigmoidal at 2.19 ± 0.07 and significantly different from the value at 37 °C ($P < 0.01$, 2-tailed Student's *t*-test).

## Purification of ground squirrel PKAc

Overall, the purification scheme developed for BAT PKAc from control ground squirrels resulted in a 197-fold purification with a yield of 16% (Table 1); the scheme was highly reproducible with an approximately 200-fold purification occurring in all trials ($n = 3$). The final specific activity of purified PKAc when assayed with Kemptide as the substrate (without cAMP) was 279 mU/mg protein at 22 °C (range was 246–279 in different trials). SDS-PAGE of the purified catalytic subunit showed a single protein band with a molecular

**Table 1** Purification of cAMP-dependent protein kinase catalytic subunit (PKAc) from brown adipose tissue (BAT) of euthermic ground squirrels

| Step | Total protein (mg) | Total activity (mUnits) | % Yield | Fold-purification | Specific activity (mUnits/mg) |
|---|---|---|---|---|---|
| Crude | 109.4 | 155 | 100 | 1 | 1.41 |
| DE-52 (KCl) | 1.79 | 116 | 75.2 | 46 | 65.1 |
| Gel filtration | 0.26 | 66.0 | 42.9 | 180 | 255 |
| DE-52 (cAMP) | 0.09 | 25.1 | 16.2 | 197 | 279 |

weight of 41 ± 3 kDa ($n = 3$). Specific protein kinase inhibitors were tested to confirm the identification of the ground squirrel enzyme as a PKAc. PKA-I (Cheng et al. 1986) and H-89 (Chijiwa et al. 1990) strongly decreased enzyme activity with $I_{50}$ values of 26.0 ± 2.4 nM and 45.8 ± 3.2 nM, respectively. The specific inhibitor of cGMP-dependent protein kinase, KT-5823 (Kase et al. 1987) and the inhibitor of protein kinase C, PKC-I (House and Kemp 1977) were much less effective, with $I_{50}$ values of greater than 80 μM and 50 μM, respectively. Purified ground squirrel PKAc lost activity rapidly over time during storage at 5 °C and was not stabilized by the addition of either glycerol (up to 50% v/v) or Triton X-100 (0.1% v/v). However, in the presence of high phosphate (200 mM) the enzyme retained stability for at least 3 weeks.

## Isoforms

The initial step in the purification procedure, DE-52 chromatography with salt gradient elution, is commonly used to separate the two PKA holoenzyme isoforms (Corbin et al. 1975; Reimann et al. 1971). However, DE-52 chromatography of BAT PKA produced only a single peak of enzyme activity eluting at about 0.25 M KCl indicating the presence of just a single holoenzyme type in this tissue. Isoforms of the catalytic subunit have also been detected and these are separable by CM-52 chromatography (Kinzel et al. 1987). Partially pure BAT PKAc was prepared by DE-52 chromatography with elution by a cAMP gradient and then subjected to CM chromatography. BAT PKAc eluted as a single peak at 130 mM KCl with only a very minor shoulder seen at 195 mM (Fig. 2a). By contrast, when a sample of PKAc was prepared from a mixture of ground squirrel organs (samples of equal tissue mass from liver, kidney, brain, skeletal muscle, BAT, and heart), the presence of two major types of catalytic subunit in other tissues was clear (Fig. 2b). One peak was centered at 130 mM KCl and the second at 170–180 mM; a third minor peak was also seen at 245 mM. The same result was confirmed by column isoelectrofocusing. Partially purified BAT PKAc (prepared as above for CM chromatography) showed only a single peak of activity after isofocusing and the isoelectric point was the same in samples from either euthermic (Fig. 3a) or hibernating animals (7.8 ± 0.08, $n = 2$, 1 euthermic and 1 hibernating sample), indicating that no change in isoform occurred while hibernating. By contrast, isofocusing of a comparably prepared liver sample from euthermic ground squirrels showed two distinct peaks with pI values of 6.5 and 7.2 (Fig. 3b).

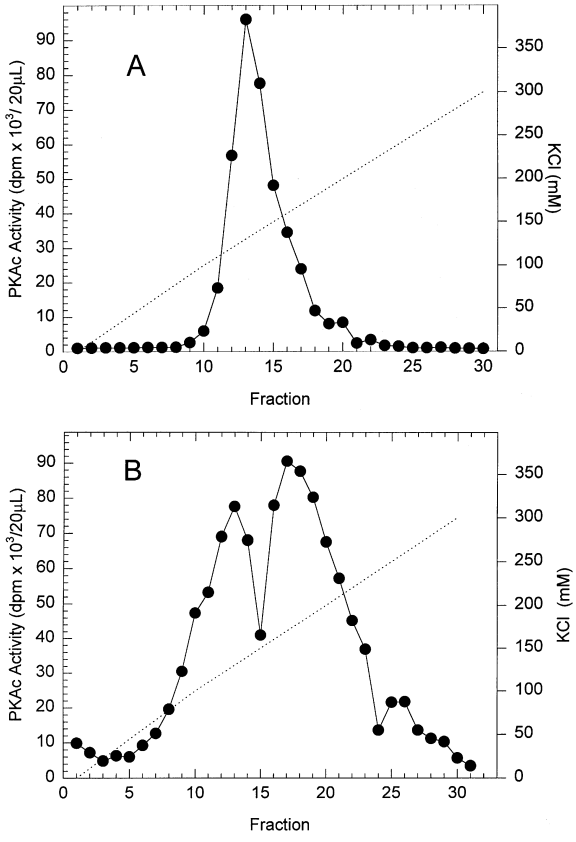


**Fig. 2A, B** Elution profiles of ground squirrel PKA catalytic subunit (PKAc) from CM-52 Cellulose. **A** shows BAT PKAc, and **B** shows a multi-tissue extract made with equal masses of liver, kidney, brain, heart, muscle and BAT tissues. The column was eluted with a 0–300 mM KCl gradient in PEM buffer pH 7.5. Activity was measured in 20 μl aliquots of column fractions (1 ml) and is plotted relative to the maximum activity in the peak tube which was 90–95,000 dpm/20 μl in both cases

## Kinetic characterization of ground squirrel PKAc

The substrate preference of purified PKAc was assessed by monitoring incorporation of $^{32}P$ onto different PKAc substrates (Kemptide, histone IIA, histone IIIS, myelin basic protein, and histone VIS); enzyme activity with Kemptide (LRRA*S*LG, representing the phosphorylation site in pyruvate kinase; Kemp et al. 1977) proved to be the greatest so this was used as the preferred substrate

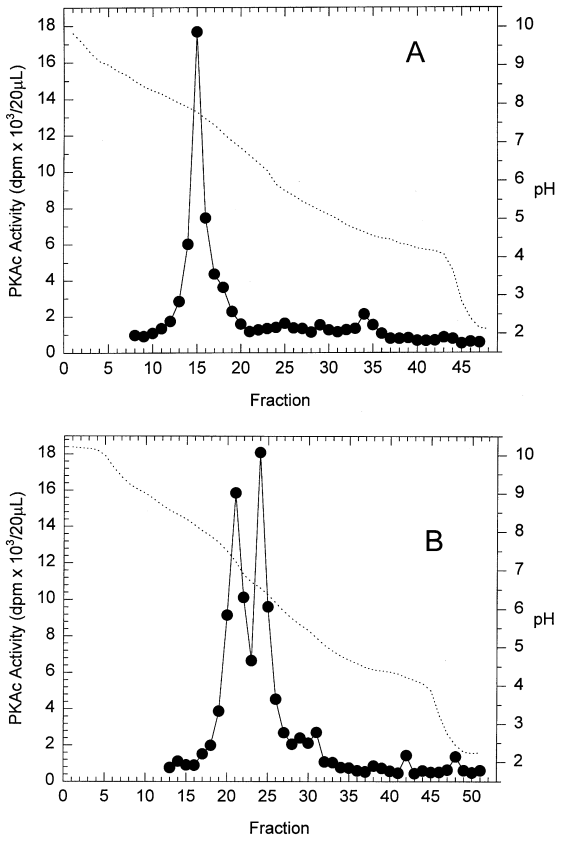


**Fig. 3A, B** Isoelectrofocusing of BAT **A** and liver **B** PKAc in a pH 3.5–10 gradient of ampholines. Activity was measured in 20 µl aliquots of column fractions (2 ml) and is plotted relative to the maximum activity in the peak tube which was about 18,000 dpm/20 µl in both cases. The pH gradient is shown by the *dashed line*

for subsequent kinetic studies. The specific activity of the purified ground squirrel enzyme was 71.9 mU/mg protein at 5 °C and 470 mU/mg protein at 37 °C with Kemptide as the substrate. Michaelis constants for substrates of ground squirrel PKAc all decreased dramatically with a decrease in assay temperature (Table 2). $K_m$ values measured at 5 °C as a percentage of the corresponding values at 37 °C were only 20% for Kemptide, 49% for histone IIA, and 47% for Mg.ATP. In contrast, rabbit muscle PKAc showed $K_m$ values that were temperature-independent for Mg.ATP and Kemptide but decreased by one-third for histone IIA, when assayed at 5 °C. $K_m$ values also differed between species and were higher in all cases for the ground squirrel enzyme when compared with the rabbit enzyme at 37 °C. However at 5 °C, $K_m$ for histone IIA of the ground squirrel enzyme was still greater than the rabbit value (by 2.5-fold), but $K_m$ for Kemptide was only about one-third of the rabbit value; Km ATP was similar for the two enzymes. The effects of salts on PKAc activity at the two assay temperatures are also presented in Table 2 as $I_{50}$ values. In general, the ground squirrel enzyme was inhibited at both temperatures by salts in the order: $NaF > NH_4Cl > KCl \sim NaCl$. Fluoride was also the strongest inhibitor of rabbit PKAc, whereas the other salts had similar effects at 37 °C; however, the order of inhibition was reversed at 5 °C ($KCl > NaCl > NH_4Cl$). A decrease in assay temperature to 5 °C reduced the inhibition of both enzymes by NaCl and $NH_4Cl$ ($I_{50}$ values increased) but only significantly raised the $I_{50}$ KCl of the ground squirrel enzyme. Inhibition by fluoride was insensitive to assay temperature. Ground squirrel PKAc had higher $I_{50}$ values than the rabbit enzyme for all salts investigated at 5 °C. The $I_{50}$ values for NaCl and NaF were also higher for the ground squirrel enzyme when assayed at 37 °C.

The effect of assay temperature on the maximum velocity of purified ground squirrel and rabbit PKAc is shown as Arrhenius plots in Fig. 4. Both plots showed two distinct linear segments but with different break points. The break in the plot occurred at 9 °C for ground squirrel PKAc with activation energies of 29.1 ± 1.0 kJ/mol for $T > 9$ °C and 59.9 ± 2.1 kJ/mol for $T < 9$ °C, a rise of 2.1-fold at the lower temperatures (Fig. 4A). For the rabbit enzyme, the break occurred at 17 °C with activation energies of 23.6 ± 0.4 kJ/mol at $T > 17$ °C and 87.1 ± 1.4 kJ/mol at $T < 17$ °C (Fig. 4B), a rise of 3.7-fold at the lower temperatures.

**Table 2** The effect of temperature on ground squirrel BAT and rabbit muscle PKAc kinetic properties: substrate affinities and inhibition by salt. Data are means ± SEM, $n = 3$ separate determinations

| | Ground squirrel | | Rabbit | |
|---|---|---|---|---|
| | 37 °C | 5 °C | 37 °C | 5 °C |
| Substrate $K_m$ values | | | | |
| Mg.ATP (µM) | 49.2 ± 3.4 | 23.3 ± 2.0[a] | 27.1 ± 0.8[b] | 28.6 ± 1.8 |
| Kemptide (µM) | 50.0 ± 4.5 | 10.1 ± 1.1[a] | 34.4 ± 1.6[a] | 34.8 ± 6.3[b] |
| Vmax (mU/mg protein) | 6.85 ± 0.12 | 1.94 ± 0.06 | 4.82 ± 0.21 | 0.57 ± 0.03 |
| Histone IIA (mg/ml) | 0.41 ± 0.05 | 0.20 ± 0.04[a] | 0.12 ± 0.01[b] | 0.08 ± 0.01[a,b] |
| Vmax (mU/mg protein) | 1.56 ± 0.08 | 0.39 ± 0.01 | 1.27 ± 0.10 | 0.19 ± 0.01 |
| Salt $I_{50}$ values | | | | |
| KCl (mM) | 181 ± 9 | 438 ± 23[a] | 158 ± 21 | 198 ± 20[b] |
| NaCl (mM) | 210 ± 9 | 378 ± 30[a] | 133 ± 22[b] | 253 ± 17[a,b] |
| $NH_4Cl$ (mM) | 121 ± 5 | 154 ± 6[a] | 129 ± 17 | 302 ± 7[a,b] |
| NaF (mM) | 65 ± 8 | 52 ± 2 | 41 ± 7[b] | 35 ± 2[b] |

[a] significantly different from the corresponding value at 37 °C using the Student's *t*-test (2-tailed), $P < 0.05$; [b] significantly different from the corresponding value for the ground squirrel enzyme at the same temperature, $P < 0.05$

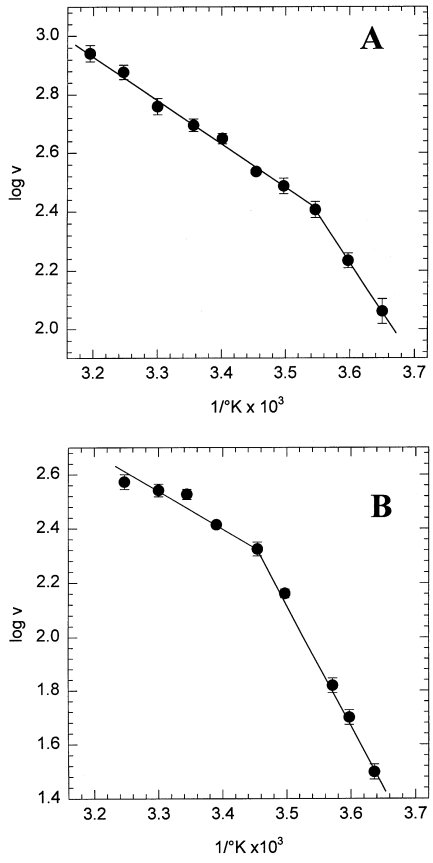


**Fig. 4A, B** Arrhenius plots for **A** ground squirrel and **B** rabbit PKAc. Reaction mixtures were pre-incubated for 10 min to allow temperature equilibration before initiating the reaction with the addition of PKAc. Optimal assay conditions were used. Data points shown are for assay temperature from 1–40 °C for ground squirrel PKAc with the break seen at 9 °C range and from 2–35 °C for rabbit PKAc with the break at 17 °C. Data are means ± SEM, $n = 4$

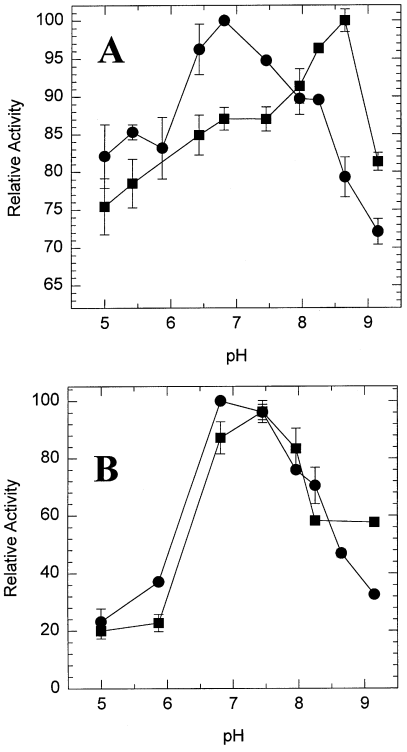


**Fig. 5A, B** Effect of assay pH on purified ground squirrel **A** and rabbit **B** PKAc at two assay temperatures: 5 °C (*squares*) and 37 °C (*circles*). Activities are plotted relative to the maximal activity at the optimal pH. The pH of the assay was changed by adding 20 mM potassium phosphate pre-adjusted to the appropriate pH value. The pH of control reactions containing all components except $^{32}$P-ATP was determined at 22 ± 1 °C. Total $Mg^{2+}$ and ATP concentrations were adjusted to maintain constant concentrations of Mg.ATP and free $Mg^{2+}$ at each temperature and pH (Brooks and Storey 1992a). Data are means ± SEM, $n = 4$

PKAc from both animals displayed similar velocity changes with temperature over the higher temperature range; however, the activation energy of rabbit PKAc at low temperatures was significantly higher than that determined for the ground squirrel enzyme.

The pH optimum of ground squirrel PKAc changed dramatically with a change in assay temperature. Figure 5A shows that the optimum was about pH 6.8 at 37 °C but increased by about 2 full pH units to 8.7 at 5 °C. However, at 5 °C high activity was retained over a broad range (85–90% of maximum between pH 6.5 and pH 8). By contrast, rabbit PKAc showed very little change in pH optimum with temperature with an optimum of 6.8–7.5 at both assay temperatures (Fig. 5B).

## Fluorescence measurements of *S. richardsonii* PKAc

Temperature induced conformational changes in the protein were monitored by following tryptophan fluorescence intensity. Fluorescence is a sensitive probe of localized protein motion and has been used extensively to monitor conformational changes induced by various structural modifiers such as temperature and chemical denaturants (Eftink 1994). The wavelength of maximum fluorescence (λmax) for ground squirrel PKAc shifted to lower values as temperature increased. The change was linear with a calculated slope of −0.047 nm/°C increase in temperature ($r = 0.94$) (Fig. 6A). Fluorescence emission intensity measured at the λmax also changed with temperature (Fig. 6B), decreasing linearly with an increase in temperature; the change was −0.347 fluorescence units/°C ($r = 0.96$).

Protein unfolding induced by guanidine-HCl was also monitored by fluorescence emission spectrum. The λmax of purified ground squirrel PKAc increased with increasing guanidine-HCl concentration at both 5 °C and 37 °C incubation temperatures, with no discernable differences between the results at the two temperatures (Fig. 7A). In both cases, the λmax at 0 M guanidine-HCl was 338 nm and this rose to a denatured value of 350 nm in the presence of 4 M guanidine-HCl. The effect of guanidine-HCl on fluorescence intensity was also examined at 5 °C and 37 °C. A wavelength of 335 nm was selected for this study because the change in fluorescence intensity between native and denatured states was greatest at this wavelength. In this case, incubation

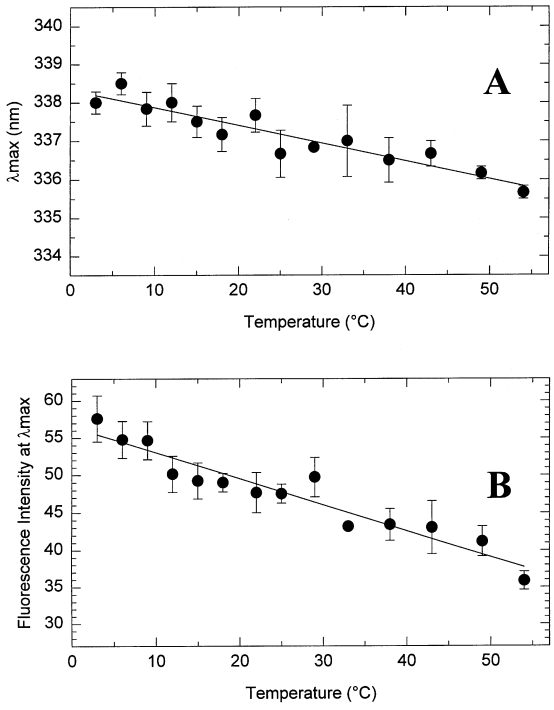


**Fig. 6A, B** The effect of changing temperature on **A** the wavelength of maximum fluorescence emission (λmax) and **B** fluorescence emission intensity at λmax for *S. richardsonii* PKAc (8 μg/ml) in 10 mM potassium phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol. The solution was equilibrated with stirring at each temperature for 30 min prior to fluorescence measurement. Data are means ± SEM, $n = 3$

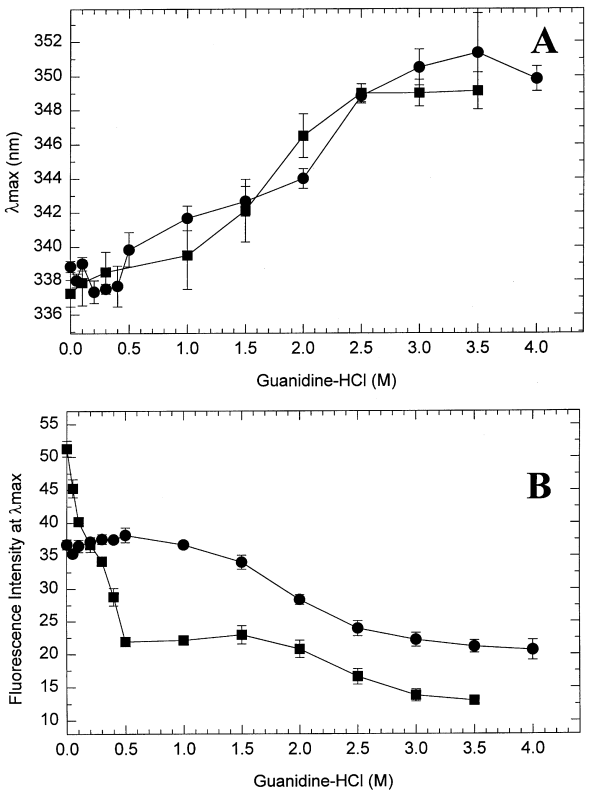


**Fig. 7A, B** The effect of guanidine-HCl concentration at 37 °C (*filled squares*) and 5 °C (*filled circles*) on **A** the wavelength of maximal fluorescence emission (λmax), and **B** the fluorescence emission intensity at 335 nm. Successive aliquots of 8 M guanidine-HCl were added to *S. richardsonii* PKAc (8 μg/ml initial concentration) in 10 mM potassium phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol. The solution was equilibrated with stirring at each denaturant concentration for 30 min prior to fluorescence measurement. Data are means ± SEM, $n = 3$

temperature did influence protein unfolding (Fig. 7B). At 37 °C, there was an initial rapid decrease in fluorescence intensity as guanidine-HCl concentration increased up to 0.5 M, whereas the subsequent addition of more denaturant up to 4 M resulted in a more gradual decline in fluorescence intensity. At 5 °C there was no change in fluorescence intensity up to 1.0 M guanidine-HCl, but above this amount of denaturant a gradual decrease in fluorescence intensity was observed. The changes in PKAc fluorescence characteristics with added guanidine-HCl were also reflected in a loss of enzyme activity with increasing concentrations of guanidine-HCl (Fig. 8). Denaturation occurred at lower guanidine-HCl levels at 37 °C than at 5 °C. At 37 °C, denaturation occurred with 90% of enzyme activity lost by 0.3 M guanidine-HCl whereas 0.9 M guanidine-HCl elicited the same activity loss at 5 °C. The initial rate of enzyme activity loss was 2.4-fold greater at 37 °C than the rate of loss at 5 °C. The guanidine-HCl concentration that gave 50% loss of activity was 274 ± 16 mM at 5 °C and 107 ± 8 mM at 37 °C.

## Discussion

Reversible protein phosphorylation by protein kinases and protein phosphatases is an important mechanism used to regulate the actions of many different cellular proteins and the metabolic functions that they control. PKA is undoubtedly the best known and most extensively studied of the protein kinases and is particularly well known for its many roles in the control of intermediary metabolism (Taylor et al. 1990). In hibernating mammals, β-adrenergic signals that are mediated intracellularly via PKA are key to the thermogenic response of BAT (Horwitz 1989; Nedergaard and Cannon 1990). Hence, the regulation and function of PKA at both low and high body temperatures is critical to signal transduction in BAT. The present study was undertaken, therefore, to determine whether adaptive changes were made to the kinetic or physical properties of ground squirrel BAT PKAc to support enzyme function at euthermic versus hibernating body temperatures. One factor that could be important to enzyme function at low temperature was an increase (by 33%) during hibernation in the total activity of PKA present in BAT which raises the scope for enzyme activation during arousal. Both euthermic and hibernating animals also had very low steady state percentages of PKAc (1.0–2.7%) which shows that the potential activation of the

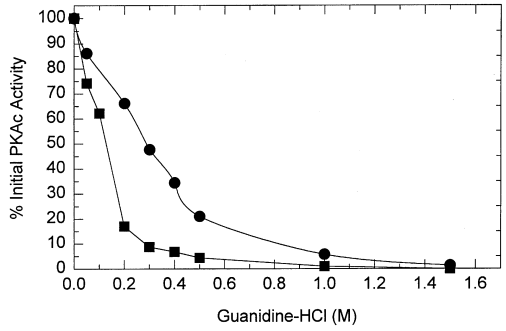


**Fig. 8** The effect of guanidine-HCl on the activity of purified ground squirrel PKAc at 37 °C (*filled squares*) and 5 °C (*filled circles*). After each 30 min incubation of samples described in Fig. 7, a 2 µl aliquot was removed for activity assay under optimal conditions at 23 °C. Measured activities were adjusted for the volume dilution of adding successive aliquots of guanidine-HCl. Data are means ± SEM, $n = 3$

enzyme during arousal can be as much as 30–100 fold. This large potential for PKA activation would be highly beneficial during arousal, for it could elicit very rapid and similarly large increases in the activities of enzymes and functional proteins that are targets of PKAc phosphorylation. It is also of interest that of six major organs tested, total PKA activity in BAT was exceeded only in brain (J. MacDonald and K. Storey, personal observation); this again emphasizes the importance of this signaling system in BAT.

The in vitro incubation studies indicated that a variety of cellular proteins in ground squirrel BAT were targets of PKAc-mediated phosphorylation. Proteins of 40–50 kDa were particularly interesting, for these showed a high level of $^{32}$P-incorporation in extracts from hibernating animals during incubation at 5 °C, whereas phosphorylation of target proteins in this size range was much lower in extracts from euthermic animals. This suggests that target proteins that are lacking in non-hibernating individuals, become available in BAT of hibernating animals. Whether these proteins were induced by cold exposure when ground squirrels were transferred to 4 °C or by the transition into the torpid state cannot be determined from our present data; but clearly significant differences exist in the suite of PKA target proteins present in BAT of warm, euthermic animals compared with cold, torpid ones. Furthermore, phosphorylation of proteins in the 45–50 kDa peak (and to a lesser extent the 60–70 kDa peaks) was relatively more intense after 5 °C incubations than those at 37 °C, suggesting that low temperature can alter either PKAc action towards selected protein targets or protein susceptibility to PKAc-mediated phosphorylation. Hence, these might represent proteins that are targets of PKAc during arousal from low body temperature. If PKAc action towards target proteins changes at low body temperatures, then the properties of ground squirrel BAT PKAc, and in particular the effects of temperature change on the enzyme, are of great interest.

Two isoforms of PKA holoenzyme, type I and II, are routinely found in mammalian tissues and their differences have been attributed to two distinct types of regulatory subunits (Taylor and Radzio-Andzelm 1994). The tetrameric holoenzymes can be separated on DE-52 when eluted with a salt gradient and tissue specific patterns are found (Corbin et al. 1975; Reimann et al. 1971). Most tissues show varying amounts of both holoenzyme forms but white adipose tissue (rat epididymal fat pad) showed only the type II enzyme which elutes at a higher KCl concentration than type I (Corbin et al. 1975). The single peak of PKA holoenzyme detected in ground squirrel BAT is therefore consistent with expectations for an adipose tissue, and its elution at a high salt concentration (0.25 M) suggests that it is probably also a type II holoenzyme.

Although two types of PKAc holoenzyme have been known for many years, defined by differences in their regulatory subunits, it was long believed that there was only one form of PKAc until Kinzel et al. (1987) documented the presence of two catalytic subunit types (PKAcα and PKAcβ) in rat and rabbit skeletal muscle. This was subsequently confirmed by others (Olsen and Uhler 1989) and a third form was reported in one instance (in human testis) (Beebe et al. 1990). PKAcα is ubiquitous whereas PKAcβ is largely restricted to the brain and reproductive tissues (Gamm et al. 1996). The two forms of catalytic subunits differed in pI values and in sensitivities to protease degradation (Kinzel et al. 1987) but their functional significance remained a point of contention. Except for small differences in amino acid sequence which defined the pI and protease susceptibility differences, most other properties of the two forms were the same (Kinzel et al. 1987). Thus, there were no significant differences in subunit migration in SDS gels, $K_m$ values for ATP, Kemptide and other phosphate-accepting substrates, pH dependence, or inhibition by ions, regulatory subunits of holoenzyme I or II, or specific heat and acid-stable inhibitors (Kinzel et al. 1987; Olsen and Uhler 1989). However, new work has at last shown that there may be some differences in enzyme function and properties between the two PKAc forms. Functional differences are indicated from studies with PKAc β1 (splice variant 1) knockout mice, which showed (although total PKA remained constant) that certain specific electrical responses to stimuli were reduced or missing in the brain of these mice (Qi et al. 1996). Differences in enzyme properties were also recently demonstrated by Gamm et al. (1996) who used cDNA fragments to produce PKAcα and PKAcβ as recombinant proteins in *E. coli* and then analyzed their properties. The two PKAc isozymes showed differences in their affinities for and substrate-inhibition by phosphate-accepting substrates, as well as different sensitivities to PKA inhibitor peptides and the RII regulatory subunit.

Kinzel et al. (1987) separated the two catalytic subunit forms on CM-cellulose but comparable trials of CM-cellulose chromatography with the PKAc prepara-

tion from ground squirrel BAT revealed only one peak of enzyme activity, eluting at 130 mM in a 0–300 mM KCl gradient (with a very minute shoulder at 195 mM). By contrast, CM-cellulose chromatography of catalytic subunit derived from a homogenate of multiple ground squirrel tissues gave two strong peaks of activity, one eluting at 130 mM and the other at 170–180 mM, and also a minor peak at 240–250 mM. Thus, some ground squirrel tissues clearly contain both holoenzyme types and 2 or 3 forms of catalytic subunit, whereas BAT contains, virtually exclusively, only one holoenzyme and one catalytic subunit type. The presence of a single catalytic subunit in BAT was also supported by the very sharp, single peak of activity on isoelectrofocusing (IEF) (pI = 7.8), whereas a comparable analysis of liver showed 2 isoforms (pI 6.5 and 7.2) (Fig. 3). Two forms of catalytic subunit were also found in bovine cardiac muscle type II PKA (pI values approximately 7.5 for Cα and 8.0 for Cβ) (Kinzel et al. 1987). Thus, we are confident that the purified BAT PKAc studied here represents a single isoform of the catalytic subunit. Given the organ distribution of the catalytic subunits in other mammals, it is most likely that the BAT PKAc is an alpha isoform. The alpha isoform also predominates in skeletal muscle, by about 6:1 (Uhler et al. 1986), so the comparison of BAT PKAc with the enzyme from rabbit skeletal muscle is valid.

The purification procedure developed for the PKAc from ground squirrel BAT was highly reproducible. Generally, an approximately 200-fold purification was achieved resulting in a final specific activity of 266 ± 10 ($n = 3$) nmol phosphate transferred per min per mg protein at 22 °C. The enzyme was judged to be homogeneous by SDS-PAGE. The subunit molecular weight of *S. richardsonii* BAT PKAc, 41 kDa, was similar to the enzyme isolated from other mammalian sources (Kinzel et al, 1987; Olsen and Uhler 1989; Taylor et al. 1990).

Differential temperature effects on the properties of ground squirrel PKAc may benefit enzyme function in activating thermogenesis. One such effect was on cAMP-mediated dissociation of the holoenzyme. Although the $K_a$ for cAMP dissociation of the ground squirrel holoenzyme was unaltered by temperature change between 37 °C and 5 °C, the relationship between cAMP concentration and holoenzyme dissociation shifted from near-hyperbolic at 37 °C ($n_H = 1.1$) to sigmoidal at 5 °C ($n_H = 2.2$). This would increase the sensitivity of enzyme dissociation to small changes in cAMP concentration and would permit a rapid increase from a low percentage of PKAc (1–3% in the steady state) to maximum PKAc over a very small range of cAMP concentration. Thus, when arousal is initiated by β-adrenergic signals, which in turn activate adenylyl cyclase to enhance cAMP production, the sigmoidal response of PKA to cAMP at low body temperature would result in a very rapid increase in the amount of free catalytic subunit in BAT, that could in turn facilitate an equally rapid increase in the phosphorylation of PKAc target proteins.

The kinetic constants determined for *S. richardsonii* BAT PKAc were similar to those reported for the PKA catalytic subunit from other mammalian sources, although some minor differences were apparent that could facilitate enzyme function in the hibernator. The apparent $K_m$ values for both Mg.ATP and Kemptide of the ground squirrel enzyme were 2–10 times higher than those reported for other mammalian sources (Kinzel et al. 1987; Whitehouse et al. 1983). However, the $K_m$ values for all three substrates (Mg.ATP, Kemptide, histone IIA) decreased at 5 °C which could maintain or enhance responsiveness of the enzyme at the low body temperature of the hibernating state. The total adenylate pool in tissues of hibernating animals frequently falls during torpor to only about 50% of the values in the euthermic animal (Storey 1997). Hence, the low temperature effect in reducing $K_m$ ATP of PKAc would effectively "self-adjust" the ratio between $K_m$ and in vivo ATP concentration. This is not uncommon for enzymes of ectothermic animals (Hochachka and Somero 1984) and in hibernators could allow a similar relative activity of the enzyme to be maintained at high and low body temperatures. The greater affinity for both model phosphate acceptors (Kemptide and histone) at low temperature also suggests that the enzyme might have greater affinity for various protein substrates in vivo at low body temperature and this could be another mechanism that would aid the rapid initiation of thermogenesis when arousal begins. The rabbit enzyme, by contrast, showed $K_m$ values that were largely temperature insensitive.

Protein kinases in general are highly flexible enzymes. Large rotational movements of the conserved kinase lobes as well as movements of flexible loops and domains accompany binding of different protein substrates, cofactors, auto-inhibitory domains or interacting proteins, either during catalysis or for regulatory purposes (Scott 1993; Taylor and Radzio-Andzelm 1994). The break in the Arrhenius plot at 9 °C implies that a conformational change also occurred in *S. richardsonii* BAT PKAc below this temperature (Fig. 4A) that resulted in a 2.3-fold increase in the activation energy of the enzyme over the lower temperature range. The $Q_{10}$ of the enzyme reaction at temperatures below this break were slightly higher than the $Q_{10}$ of the enzyme reaction above the break, 2.4 versus 1.8 respectively. The break in the Arrhenius plot (Fig. 4B) for the rabbit enzyme appeared at a considerably higher temperature (17 °C), and distinguished two sections of the plot. Velocities of the rabbit enzyme above this temperature increased with a $Q_{10}$ value (1.7) that approximated that of the ground squirrel enzyme; however, the $Q_{10}$ value (4.9) for enzyme velocities below this point was significantly larger than that determined for the ground squirrel enzyme. The differences in the temperature sensitivities of the two enzymes suggests a difference in their primary structures that is also consistent with the different kinetic properties of the two enzymes. Body temperatures below 10 °C frequently occur during hibernation and the smaller $Q_{10}$

value at low temperatures for the reaction catalyzed by the hibernator enzyme would maintain a good potential for enzyme activity at lower body temperatures. This could enhance the ability of the hibernator kinase to propagate the signal for the initiation of brown fat NST during arousal from the torpid state. However, the implied changes in ground squirrel PKAc conformation with temperature that are suggested by the Arrhenius data were not mirrored by the fluorescence data. Both the emission intensity and the wavelength of maximum fluorescence responded to changing temperature in a linear manner throughout the temperature range examined (3–55 °C). It should be noted, however, that the fluorescence data were obtained from the protein alone in solution, whereas the Arrhenius data come from measurements of enzyme activity in the presence of substrates that may change protein conformation. Since the two methods revealed different trends in conformational change with changing temperature, those different behaviors are likely to be substrate-related. Thus, it would appear that temperature effects on the conformation of the hibernator protein are minimal in the absence of substrates and slightly more substantial when substrates are present.

The influence of temperature on the pH optimum of ground squirrel PKAc was dramatic; the optimum shifted from about 6.8 at 37 °C, to 8.6 at 5 °C. This change cannot be accounted for by a temperature effect on buffer pH, for the assays were done in phosphate buffer which shows only a very minor change in pH with temperature (0.0016 pH unit increase per 1 °C decrease in temperature or 0.05 pH unit for the 32 °C change in assay temperature). Furthermore, the pH optimum of rabbit PKAc was virtually unaffected by temperature change, being 6.8–7.5 at both assay temperatures; therefore, another specific difference between the enzymes of the hibernating and nonhibernating species could be indicated. The two enzymes also differed in the shape of the pH versus activity curve, with the hibernator enzyme showing high activity (> 75% of maximum) over a broad range of pH values from pH 5–9; the rabbit enzyme retained less than 40% of maximum activity at pH 6 and fell to only 60–70% of $V_{max}$ at pH 8.2. The broad optimum range of the hibernator enzyme could allow enzyme function over a wide range of intracellular pH values; this could also benefit enzyme function, as intracellular pH changes over cycles of hibernation and arousal, typically by 0.2–0.4 pH units (Malan 1988; McArthur et al. 1990). Hence, the basic shift in pH optimum at hibernating body temperatures, as well as the generally broad pH range over which high activity is retained, could facilitate PKAc activity and allow for expression of high enzyme activity no matter what the body temperature was.

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## References

Beebe SJ, Oyen O, Sandburg M, Froysa A, Hansson V, Jahnsen T (1990) Molecular cloning of a tissue specific protein kinase (C gamma) from human testis – representing a third isoform for the catalytic subunit of cAMP-dependent protein kinase. Mol Endocrinol 3: 465–475

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Brooks SPJ, Storey KB (1992a) Mechanisms of glycolytic control during hibernation in the ground squirrel *Spermophilus lateralis*. J Comp Physiol B 162: 23–28

Brooks SPJ, Storey KB (1992b) Bound and determined: a computer program for making buffers of defined ion concentrations. Anal Biochem 201: 119–126

Brooks SPJ, Storey KB (1992c) A simple computer program with statistical tests for the analysis of enzyme kinetics. BioTechniques 13: 906–911

Cannon B, Nedergaard J (1985) The biochemistry of an inefficient tissue: brown adipose tissue. Essays Biochem 20: 110–164

Cannon B, Jacobsson A, Rehnmark S, Nedergaard J (1996) Signal transduction in brown adipose tissue recruitment: noradrenaline and beyond. Int J Obesity 20 Suppl 3: S36–S42

Cheng HC, Kemp BE, Pearson RB, Smith AJ, Misconi L, Van Patten SM, Walsh DA (1986) A potent synthetic peptide inhibitor of the cAMP dependent protein kinase. J Biol Chem 261: 989–992

Chijiwa T, Mishima A, Hagivara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, Hidaka H (1990) Inhibition of the forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. J Biol Chem 265: 5267–5272

Corbin JD, Reimann EM (1974) Assay of cyclic AMP dependent protein kinases. Methods Enzymol 22: 389–412

Corbin JD, Keely SL, Park CR (1975) The distribution and dissociation of cyclic adenosine 3′:5′-monophosphate-dependent protein kinases in adipose, cardiac and other tissues. J Biol Chem 250: 218–225

Dicker A, Cannon B, Nedergaard J (1996) Stimulation of nonshivering thermogenesis in the Syrian hamster by norepinephrine and ß-selective adrenergic agents: a phenomenon of refractoriness. Comp Biochem Physiol C 113: 37–43

Eftink MR (1994) The use of fluorescence methods to monitor unfolding transitions in proteins. Biophys J 66: 482–501

El Hachimi Z, Tijane M, Boissonnet G, Benjouad A, Desmadril M, Yon JM (1990) Regulation of the skeletal muscle metabolism during hibernation of *Jaculus orientalis*. Comp Biochem Physiol B 96: 457–459

Gamm DM, Baude EJ, Uhler MD (1996) The major catalytic subunit isoforms of cAMP-dependent protein kinase have distinct biochemical properties in vitro and in vivo. J Biol Chem 271: 15736–15742

Helmerhost E, Stokes GB (1980) Microcentrifuge desalting: a rapid quantitative method for desalting small amounts of protein. Anal Biochem 104: 130–135

Hochachka PW, Somero GN (1984) Biochemical Adaptation. Princeton University Press, Princeton

Horwitz BA (1989) Biochemical mechanisms and control of cold-induced cellular thermogenesis in placental mammals. In: Wang LCH (ed) Advances in comparative and environmental physiology, vol 4. Springer, Berlin Heidelberg New York, pp 83–116

House C, Kemp BE (1977) Protein kinase C contains a pseudosubstrate prototype in its regulatory domain. Science 238: 1726–1728

Jansky L (1973) Non-shivering thermogenesis and its thermoregulatory significance. Biol Rev 48: 85–132

Kase H, Iwahaski K, Nakanishi S, Matsuda Y, Takahashi M, Murakata C, Sato A, Kaneko M (1987) K-252 compounds,

novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. Biochem Biophys Res Commun 142: 436–440

Kemp BE, Graves DJ, Benjamini E, Krebs EG (1977) Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. J Biol Chem 252: 4888–4894

Kinzel V, Hotz A, Konig N, Gagelmann M, Pyerin W, Reed J, Kubler D, Hofmann F, Obst C, Gensheimer HP, Goldblatt D, Shaltiel S (1987) Chromatographic separation of two heterogeneous forms of the catalytic subunit of cyclic AMP-dependent protein kinase holoenzyme type I and II from striated muscle of different species. Arch Biochem Biophys 253: 341–349

Klaus S, Casteilla L, Bouillaud F, Ricquier D (1991) The uncoupling protein UCP: a membraneous mitochondrial ion carrier exclusively expressed in brown adipose tissue. Int J Biochem 23: 791–801

Laemmli UK (1970) Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227: 680–685

Malan A (1988) pH and hypometabolism in mammalian hibernation. Can J Zool 66: 95–98

McArthur MD, Hanstock CC, Malan A, Wang LCH, Allen PS (1990) Skeletal muscle pH dynamics during arousal from hibernation measured by $^{31}$P NMR spectroscopy. J Comp Physiol B 160: 339–347

Nedergaard J, Cannon B (1990) Mammalian hibernation. Phil Trans R Soc Lond B326: 669–686

Nicholls DG, Locke RM (1984) Thermogenic mechanisms in brown fat. Physiol Rev 61: 1–64

Nicholls DG, Cunningham S, Rial E (1986) The bioenergetic mechanisms of brown adipose tissue. In: Trayhurn P, Nicholls DG (eds) Brown adipose tissue. E. Arnold, London, pp 52–85

Olsen SR, Uhler MD (1989) Affinity purification of the Cα and Cβ isoforms of the catalytic subunit of cAMP-dependent protein kinase. J Biol Chem 264: 18662–18666

Qi M, Zhuo M, Skalhegg BS, Brandon EP, Kasndel ER, McKnight GS, Idzerda RL (1996) Impaired hippocampal plasticity in mice lacking the Cbeta1 catalytic subunit of cAMP-dependent protein kinase. Proc Natl Acad Sci USA 93: 1571–1576

Reimann EM, Walsh DA, Krebs EG (1971) Purification and properties of rabbit skeletal muscle adenosine 3′,5′-monophosphate-dependent protein kinases. J Biol Chem 246: 1986–1995

Rubin CS, Erlichman J, Rosen OM (1974) Cyclic AMP dependent protein kinase from bovine heart muscle. Methods Enzymol 38: 308–315

Scott JD (1993) Cyclic nucleotide dependent protein kinases. In: Taylor CW (ed) Intracellular messengers. Pergamon, London, pp 137–166

Storey KB (1987a) Regulation of liver metabolism by enzyme phosphorylation during mammalian hibernation. J Biol Chem 262: 1670–1673

Storey KB (1987b) Investigations of the mechanisms of glycolytic control during hibernation. Can J Zool 65: 3079–3083

Storey KB (1997) Metabolic regulation in mammalian hibernation: enzyme and protein adaptations. Comp Biochem Physiol A 118: 1115–1124

Taylor SS, Radzio-Andzelm E (1994) Cyclic AMP-dependent protein kinase. In: Woodgett JR (ed) Protein kinases. Oxford University Press, London, pp 1–29

Taylor SS, Buechler JA, Yonemoto W (1990) cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Ann Rev Biochem 59: 971–1005

Uhler MC, Chrivia JC, McKnight GS (1986) Evidence for a second isoform of the catalytic subunit of cAMP-dependent protein kinase. J Biol Chem 261: 15360–15363

Vesterberg O (1971) Isoelectric focusing of proteins. Methods Enzymol 22: 389–412

Wang LCH (1989) Ecological, physiological and biochemical aspects of torpor in mammals and birds. In: Wang LCH (ed) Advances in comparative and environmental physiology, vol 4. Springer, Berlin Heidelberg New York, pp 361–401

Whitehouse S, Feramisco JR, Casnellie JE, Krebs EG, Walsh DA (1983) Studies on the kinetic mechanism of the catalytic subunit of the cAMP-dependent protein kinase. J Biol Chem 258: 3693–3701

Wunder BA (1979) Hormonal mechanisms. In: Underwood LS, Tieszen LL, Callahan AB, Folk GE (eds) Comparative mechanisms of cold adaptations. Academic Press, New York, pp 143–158

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