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Purification and Characterization of the Catalytic Subunit of Adenosine 3′:5′-Cyclic Monophosphate-Dependent Protein Kinase from Bovine Liver

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1. The catalytic subunit of bovine liver cyclic AMP-dependent protein kinase (EC 2.7.1.37) was purified essentially by the method of Reimann & Corbin [(1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1384]. 2. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, sedimentation-velocity centrifugation and sedimentation-equilibrium centrifugation showed that the catalytic subunit was monodisperse. Polyacrylamide-gel isoelectric-focusing electrophoresis revealed the presence of at least three isoenzyme forms of catalytic subunit activity with slightly different pI values (6.72, 7.04 and 7.35). 3. Physical properties of the catalytic subunit were determined by several different methods. It had mol.wt. 39000–42000, Stokes radius 2.73–3.08nm, $s^0_{20.w}$ 3.14S, f/f_0 1.19–1.23 and, assuming a prolate ellipsoid, axial ratio 4–5. 4. Amino acid analysis was performed on the catalytic subunit. It had one cysteine residue/molecule which was essential for activity. Inhibition by thiol-specific reagents was partially prevented by the presence of ATP–Mg^{2+}. 5. The circular-dichroic spectrum showed the catalytic subunit contained 29% α-helical form, 18% β-form and 53% aperiodic form. Near-u.v. circular dichroism showed the presence of aromatic residues whose equivalent molar ellipticity was greatly altered by the addition of ATP–Mg^{2+}. 6. Kinetic experiments showed that the catalytic subunit had an apparent K_m for ATP of 7μM. 5′-Adenylyl imidodiphosphate inhibited competitively with ATP with a K_i of 60μM. The kinetic plot for histone (Sigma, type II-A) was biphasic showing 'high'- and 'low'-K_m segments. Under assay conditions the specific activity of the catalytic subunit was 3×10^6 units/mg of protein. Of various metal ions tested, the catalytic subunit was most active with Mg^{2+}. 7. When assayed with histone (Sigma, type II-A) as substrate, the activity of the catalytic subunit was increased by non-ionic detergents or urea. No such activation was observed with casein as substrate.

In mammalian tissues, the only well-defined effect of cyclic AMP and hence, indirectly, of certain hormones is to dissociate cyclic AMP-dependent protein kinase¶ (EC 2.7.1.37) into its catalytic (C) and regulatory (R) subunits (Soderling *et al.*, 1973; Keely *et al.*, 1975) according to the equation (Brostrom *et al.*, 1971; Rubin *et al.*, 1972; Rosen *et al.*, 1973; Bechtel & Beavo, 1974):

$$R_2C_2 + 2 \text{ cyclic AMP} \rightleftharpoons R_2(\text{cyclic AMP})_2 + 2C$$

When the catalytic subunit is combined with the regulatory subunit in the holoenzyme, its catalytic activity is inhibited. However, when the holoenzyme is dissociated by cyclic AMP, the catalytic subunit is able to catalyse ATP-dependent phosphorylation of certain enzymes (and possibly other proteins) and thereby cause alterations in their enzymic activities (see Krebs, 1972; Rubin & Rosen, 1975; Corbin *et al.*, 1976, for reviews). The catalytic subunit is therefore important in the regulation of the activity of certain enzymes and in the manifestation of some hormonal effects *in vivo*. In the present paper, we describe the purification to homogeneity of the catalytic subunit of a 'type II' isoenzyme (see, Corbin *et al.*, 1975; Sugden & Corbin, 1976) of a protein kinase from bovine liver essentially by the method of Reimann & Corbin (1976). Some of its physical and kinetic properties are also described.

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¶ Abbreviations: in this paper, the term protein kinase refers to cyclic AMP-dependent protein kinase holoenzyme. PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene; c.d., circular dichroism.

Materials and Methods

Materials

Carrier-free [^{32}P]phosphoric acid was obtained from I. C. N. Pharmaceuticals, C and R Division, P.O. Box 80739, Los Angeles, CA 90080, U.S.A. [γ-^{32}P]ATP was prepared by the method of Glynn & Chappell (1964) as modified by Walsh *et al.* (1971). Inorganic chemicals and ethylenediamine were from Fisher Scientific Co., St. Louis, MO 63132, U.S.A. Reagents for electrophoresis and Bio-Gel HTP hydroxyapatite were from Bio-Rad Laboratories, Richmond, CA 94804, U.S.A. Whatman DE-11 DEAE-cellulose was from H. Reeve-Angel, Clifton, NJ, U.S.A., and was washed with 0.5M-NaOH and water until the pH was 8, then 0.5M-HCl and water until the pH increased to 5, before equilibration with buffer. Gel-filtration materials were from Pharmacia Fine Chemicals, Piscataway, NJ 08854, U.S.A. All other chemicals and enzymes were from Sigma, St. Louis, MO 63178, U.S.A. except for the following: 4,4'-bis(dimethylaminodiphenylcarbinol) was from Pierce, Box 117, Rockford, IL 61105, U.S.A.; bovine haemoglobin and serum albumin were from N.B.C., Cleveland, OH 44128, U.S.A.; cyclic AMP was from Calbiochem, La Jolla, CA 92037, U.S.A.; human transferrin was from Miles Laboratories, Elkhart, IN 46514, U.S.A. 5'-Adenylyl imidodiphosphate was from I.C.N. Pharmaceuticals, Cleveland, OH 44128, U.S.A., and was purified as its Na^+ salt essentially by the method of Yount *et al.* (1971). Histone F_{2b} from calf thymus was prepared by the method of Johns (1964). Bovine liver and calf thymus were obtained immediately *post mortem* from a local slaughterhouse and stored in ice during transport. All potassium or sodium phosphate buffers were prepared by mixing equimolar solutions of K_2HPO_4 and KH_2PO_4 or Na_2HPO_4 and NaH_2PO_4 to give the desired pH. EDTA was neutralized to pH6.8 with NaOH before use.

Methods

Protein kinase assay. The protein kinase assay was based on the phosphorylation of histone and carried out as a routine essentially as described earlier (Corbin & Reimann, 1974). The reaction was initiated by the addition of 20μl of suitably diluted enzyme solution to 50μl of a mixture containing 17mM-potassium phosphate (pH6.8), 0.33mM-[γ-^{32}P]ATP (25–30c.p.m./pmol), 6mM-magnesium acetate and 0.5mg of histone (Sigma, type II-A). The reaction mixture was incubated at 30°C for various times after which the reaction was terminated by pipetting a 50μl sample on to a rectangle (1cm×2cm) of Whatman 3MM filter paper, which was immediately dropped into ice-cold 10% (w/v) trichloroacetic acid (10ml/filter paper). The filter papers were washed and dried by the method of Wastila *et al.* (1971) and counted for radioactivity in 10ml of a fluor containing 2.88 litre of toluene, 0.96 litre of ethylene glycol monomethyl ether, 16g of PPO and 0.4g of dimethyl-POPOP. In some experiments, protamine (7.1mg/ml) was used as substrate in the reaction mixture. In this case, the reaction was terminated with 20% (w/v) trichloroacetic acid. Protein kinase activity is expressed in terms of units where one unit of enzyme activity catalysed the incorporation of 1pmol of ^{32}P from [γ-^{32}P]ATP into histone per min at 30°C. Enzyme solutions were diluted when necessary immediately before assay in 50–110mM-potassium phosphate buffer, pH6.8, containing 0.1mM-dithiothreitol.

Analytical ultracentrifugation. Sedimentation-equilibrium experiments were carried out by the short-column method (Van Holde & Baldwin, 1958) at 15000rev./min and the meniscus-depletion method (Yphantis, 1964) at 28000rev./min at 7.1°C by using a Beckman model E ultracentrifuge equipped with photoelectric scanner optics and electronic speed control. The catalytic subunit was dialysed overnight at 4°C against 2000vol. of 20mM-potassium phosphate/100mM-KCl, pH6.5, before centrifugation. An AN-H rotor with 12mm Kel-F centrepiece cell assembly was used. FC-43 oil provided a visible lower meniscus. At equilibrium, two to four scans were made at the slowest scan speed. The data were analysed by calculating the cell weight-average and z-average molecular weights and the point average weight-average molecular weight at 0.15mm intervals within the cell. The entire $\ln(E_{280})$ against r^2 curve was also fitted to a parabola in r^2 and it was determined whether the coefficient of the $(r^2)^2$ term was significantly different from zero at a 95% confidence level in order to detect curvature.

Sedimentation-velocity experiments were carried out at 56000rev./min at 20°C in an AN-H rotor and a 12mm Kel-F centrepiece cell assembly. Scans were made at 8min intervals until the plateau region vanished. Sedimentation coefficients were calculated from least-squares slopes of plots of $\ln(r)$ against time. In addition, the expected plateau protein concentration, as estimated from the radial square dilution law, was plotted against the actual plateau concentration as a function of the time of sedimentation.

Sucrose-density-gradient centrifugation. Sedimentation coefficients were determined by the method of Martin & Ames (1961). Sucrose gradients (5–20%, w/v) were formed in 5mM-Tris/HCl/1mM-EDTA at pH7.5. Bovine haemoglobin (50μl of 15mg/ml) and rabbit muscle phosphorylase *b* (50μl of 5mg/ml) were used as standards. Centrifugation was carried out in a Beckman L5-65 ultracentrifuge in a Beckman SW 41 rotor at 190000*g* (at $r_{av.}$ = 10.93cm) at 4°C for 18–24h. Fractions (0.6ml) were collected and assayed for phosphorylase *b* (Cori *et al.*, 1943) and catalytic subunit activity. Haemoglobin was

determined from its E_{411}. Sedimentation coefficients of 8.2S for phosphorylase (Keller & Cori, 1953) and 4.6S for haemoglobin (Schachman & Edelstein, 1966) were used to calculate sedimentation coefficients.

Gel filtration. Stokes radii were determined by chromatography on a column (2.6cm×58cm) of Sephadex G-100 (superfine grade; void volume 107ml) equilibrated with 350mM-potassium phosphate, pH6.8. Samples (1ml) were placed on the column in 350mM-potassium phosphate, pH6.8, containing 10% (w/v) sucrose, and fractions (2–4ml) were collected by downward flow (rate 6ml/h). The column was standardized with the following proteins for which Stokes radii had been calculated by standard equations (see, Siegel & Monty, 1966): bovine serum albumin (Stokes radius 3.5nm; Creeth, 1952); bovine serum albumin dimer (4.75nm; Hughes, 1950; Creeth, 1952); soya-bean trypsin inhibitor (2.45nm; Rackis *et al.*, 1962); bovine pancreas ribonuclease (1.92nm; Rothen, 1940); cytochrome *c* (1.7nm; Margoliash & Lustgarten, 1962); ovalbumin (2.95nm; Kegeles & Gulter, 1951; Castellino & Barker, 1968). Exclusion and inclusion volumes were determined with Blue Dextran and Bromophenol Blue respectively. All protein standards were applied to the column separately as described above at a concentration of 10mg/ml and were determined spectrophotometrically. Data were plotted by the method of Laurent & Killander (1964); see also Siegel & Monty (1966).

Circular dichroism. C.d. spectra of the catalytic subunit were taken in a Cary 60 spectropolarimeter with model 6002 CD attachment. The cell temperature was regulated at 25°C by means of a jacketed cell holder and circulating-water bath. Catalytic subunit was in 20mM-potassium phosphate/100mM-KCl at pH6.5. Cells with path-lengths of 1.0cm for near-u.v. measurements and 0.05cm for far-u.v. measurements were used. The mean residue weight was taken to be 114.9. For each spectrum, two to three scans were made for the sample and for the baseline. The far-u.v. c.d. spectrum was analysed for the secondary structure content of the catalytic subunit protein by eqn. (1) and the c.d. reference data for the α-helical, β-form and aperiodic conformation were given by Chen *et al.* (1974):

$$[\theta]^{\lambda} = f_{\alpha}[\theta]^{\lambda}_{\alpha} + f_{\beta}[\theta]^{\lambda}_{\beta} + f_{R}[\theta]^{\lambda}_{R} \quad (1)$$

where $[\theta]^{\lambda}$ is the equivalent molar ellipticity. The experimental $[\theta]^{\lambda}$ from 243nm to 204nm was used with eqn. (1) to estimate f_{α}, the fraction of residues in the α-helical conformation and f_{β}, the fraction of residues in the β-sheet conformation by using the criterion of least squares, subject to the constraint that $1-f_{\alpha}-f_{\beta}=f_{R}$, where f_{R} is the fraction of residues in the aperiodic structure.

The near-u.v. c.d. spectrum for the catalytic subunit was also recorded in the presence of 20mM-potassium phosphate, 100mM-KCl, 0.033mM-ATP and 6mM-magnesium acetate at pH6.5. At this concentration of protein (0.81mg/ml) and ATP (0.033mM), the protein-binding site for ATP–Mg^{2+} was approx. 75% saturated, as estimated from the apparent K_m for ATP–Mg^{2+} and assuming that the apparent K_m for ATP was a true dissociation constant. The c.d. difference spectra (in terms of molar ellipticity) were calculated by using mol.wt. 41700. The observed ellipticity of 0.033mM-ATP–Mg^{2+} was found to be less than one-tenth of the increase in ellipticity observed for the catalytic subunit on addition of the ATP–Mg^{2+} and therefore the c.d. difference spectrum was not corrected for the ellipticity of ATP–Mg^{2+} itself.

Gel electrophoresis. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was done at pH7.2 in 100mM-sodium phosphate buffer containing 0.1% (w/v) sodium dodecyl sulphate at room temperature (21°C) at 6mA/tube. Gels (0.5cm×11cm; 7.5%) were prepared by the method of Weber & Osborn (1969) with solutions degassed under vacuum and were pre-electrophoresed at 6mA/tube for 30min before use. Electrophoresis was carried out in a Buchler Instruments Division (Fort Lee, NJ 07024, U.S.A.) electrophoresis apparatus. Protein samples were heated at 65–70°C in 10mM-sodium phosphate, 0.1% (w/v) sodium dodecyl sulphate, 140mM-2-mercaptoethanol, 10% (v/v) glycerol, 0.002% (w/v) Bromophenol Blue at pH7.2 for 10min and applied to the gels. Gels were standardized with 10–20μg of the following proteins of known molecular weight: bovine serum albumin (mol.wt. 68000); bovine serum albumin dimer (mol.wt.136000); pepsin (mol.wt. 35000) (see Weber & Osborn, 1969); human transferrin (mol.wt. 88000; Schulze *et al.*, 1955).

Polyacrylamide-gel isoelectric-focusing electrophoresis was performed by a method similar to that of Catsimpoolas (1968). To 6.25ml of acrylamide (292g/l) containing methylenebisacrylamide (7.5g/l) the following were added in order: 1.25g of glycerol, 10ml of water, 1.25ml of Biolyte 3/10 carrier ampholytes (Bio-Rad), 0.5ml of riboflavin 5′-phosphate (0.2g/l) and 6ml of water. Gels (10cm) were overlayered with water and formed overnight in tubes (0.5cm×11.5cm) by irradiation under a desk lamp with a fluorescent tube. Electrophoresis was carried out in a Buchler Instruments Division electrophoresis apparatus. Samples (100μl) were applied in solutions containing 25% (w/v) sucrose and overlayered with 100μl of 20% (w/v) sucrose and 100μl of 10% (w/v) sucrose. The gel tubes were then filled with 5% (v/v) H_3PO_4. The upper electrolyte was 5% (v/v) H_3PO_4 and the lower electrolyte was 5% (v/v) ethylenediamine. The anode was in the upper reservoir. Electrophoresis was carried out for 15h at constant voltage (150–200V) in the cold-room with ice-cold water circulating through the cooling jacket. The lower

electrolyte was stirred with a magnetic stirrer. Bovine haemoglobin (1 mg) was applied to a separate gel as a control in all electrophoreses. After completion of electrophoresis, gels were sliced with a Bio-Rad model 190 electrophoresis gel slicer, and catalytic subunit activity was eluted overnight in 200 μl of 55 mM-potassium phosphate buffer, pH 6.8, containing 0.1 mg of bovine serum albumin/ml, 0.1 mM-dithiothreitol. Duplicate gels were sliced and eluted with 100 μl of water for pH determinations with a MI-410 micro-combination pH probe (Microelectrodes, Grenier Industrial Village, Londonderry, NH 03052, U.S.A.).

Protein was stained by immersion of sodium dodecyl sulphate/polyacrylamide gels in 0.1% (w/v) Coomassie Brilliant Blue R-250 in ethanol/acetic acid/water (5:1:5, by vol.) for 1 h. Gels were destained in a diffusion destainer (Bio-Rad model 172) in ethanol/acetic acid/water (5:1:5, by vol.). Protein was measured at 580 nm in an Instrumentation Specialities Co. model UA-5 absorbance monitor with a model 659 gel-scanner attachment.

Amino acid composition. Amino acid composition of catalytic subunit (0.5 mg) in 80 μl of 350 mM-potassium phosphate, pH 6.8, was determined in a Beckman model 121 amino acid analyser after a 20 h hydrolysis with 6 M-HCl (2 ml) at 110°C in a sealed tube. Tryptophan content was determined similarly after a 22 h hydrolysis in 4 M-methanesulphonic acid (2 ml) at 115°C. Cysteine in catalytic subunit was determined by the method of Rohrbach *et al.* (1973) in 40 mM-sodium acetate/6 M-guanidinium hydrochloride, pH 5.1. Under such conditions, ε of 4,4′-bis (dimethylaminodiphenylcarbinol) was 113125 litre·mol^{-1}·cm^{-1} at 614 nm. Values for $\bar{v}$ and the extinction coefficient of catalytic subunit were calculated from the amino acid composition by the methods of Cohn & Edsall (1943*a*) and data given in Sober (1968).

Protein determination. Protein was determined essentially as described by Lowry *et al.* (1951), with crystalline bovine serum albumin as a standard, or by measurements of E_{280}.

Results and Discussion

Purification of the catalytic subunit of bovine liver protein kinase

The catalytic subunit of cyclic AMP-dependent protein kinase was purified in high yield essentially by the method of Reimann & Corbin (1976). The results of purification are shown in Table 1.

(i) *DEAE-cellulose chromatography.* Unless otherwise stated, all operations were carried out at 0–4°C. Fresh bovine liver (6 kg) was homogenized in a Waring Blendor in 0.25kg batches for five 10s intervals in a total of 12 litres of 10 mM-potassium phosphate buffer, pH 6.8, containing 1 mM-EDTA and 0.1 mM-dithiothreitol. The homogenate was centrifuged at 10000*g* (at $r_{av.}$ = 9.8 cm) for 30 min. The resulting supernatant was filtered through glass wool and added to 4 litres of packed DEAE-cellulose (Whatman DE-11) equilibrated with homogenization buffer. The resulting slurry was left for 1 h with frequent stirring and was then poured into a column (7.5 cm × 90 cm). The column was washed with 55 mM-potassium phosphate buffer, pH 6.8, containing 1 mM-EDTA and 0.1 mM-dithiothreitol until the E_{280} of the eluate fell to 0.1 (about 55 litres was required). It was then washed with 12 litres of 45 mM-potassium phosphate buffer containing 0.1 mM-dithiothreitol and 0.1 mM-cyclic AMP at pH 6.8. The flow rate was approx. 1 litre/h. Fractions (30–35 ml) were collected until the E_{254} of the fractions was greater than 1, indicating that cyclic AMP had started to be eluted and elution of the catalytic subunit was complete.

(ii) *Hydroxyapatite chromatography.* Fractions containing catalytic subunit activity were pooled and applied by downward flow (flow rate approx. 200 ml/h) to a column (5 cm × 5 cm) of hydroxyapatite (Bio-Rad Bio-Gel HTP) previously washed repeatedly with water to remove fines, and equilibrated with 50 mM-potassium phosphate/0.1 mM-dithiothreitol, pH 6.8. The column was washed with 100 mM-potassium phosphate/0.1 mM-dithiothreitol, pH 6.8 (400 ml), and eluted with a linear gradient (1 litre) of

Table 1. *Purification of catalytic subunit from bovine liver*

Step	Volume (ml)	Protein (mg/ml)	Activity (units/ml)	Specific activity (units/mg of protein)	Yield (%)
Whole homogenate	18000	60	3750	63	(100)
10000*g* supernatant	12000	41	5980	146	106
DEAE-cellulose chromatography and elution with cyclic AMP	7450	0.034	6400	188000	71
Hydroxyapatite chromatography I	400	0.095	106000	1116000	63
Hydroxyapatite chromatography II	16	1.92	3088000	1608000	73
Sephadex G-100 chromatography	20	0.47	864000	1838000	26
Hydroxyapatite chromatography III	5	1.62	3000000	1852000	22

100–350mM-potassium phosphate buffer, pH6.8, containing 0.1mM-dithiothreitol. Fractions (5ml) were collected and assayed for catalytic subunit activity, which was eluted at about 200mM-potassium phosphate, and protein. Fractions containing catalytic subunit activity were pooled, diluted 1:1 with 5mM - potassium phosphate / 0.1mM - dithiothreitol, pH6.8, and applied to a second hydroxyapatite column (2.6cm×2cm) equilibrated as described above. The column was washed with 350mM-potassium phosphate/0.1mM-dithiothreitol, pH6.8 (30ml), and fractions (0.8ml) were collected.

(iii) *Sephadex G-100 chromatography.* Fractions containing catalytic subunit activity were pooled and applied to a column (2.6cm×58cm) of Sephadex G-100 (superfine grade) equilibrated with 350mM-potassium phosphate, pH6.8. The flow rate was about 10ml/h. Fractions (4ml) were collected and those containing catalytic subunit activity were pooled, diluted with 2vol. of water and applied to a column (0.9cm×1.5cm) of hydroxyapatite (Bio-Gel HTP washed as above) equilibrated with 100mM-potassium phosphate, pH6.8. Catalytic subunit was eluted with 350mM-potassium phosphate, pH6.8 (20ml), and fractions (1ml) were collected.

The simple method described above is particularly useful for tissues containing 'type II' protein kinase isoenzymes (Corbin *et al.*, 1975) that are eluted from DEAE-cellulose at relatively high (>0.15M) ionic strengths. Although there is no evidence that the catalytic subunits of 'type I' and 'type II' protein kinase isoenzymes differ (Corbin *et al.*, 1976), the 'type I' isoenzyme would not be retained by DEAE-cellulose by the method of purification described above. The availability of large quantities of catalytic subunit should prove useful in investigations of the role of protein phosphorylation *in vitro*. Studies of this type are being done in a number of laboratories. The catalytic subunit could also be used to prepare affinity columns for purification and binding studies of protein kinase subunits and protein substrates.

Criteria of purity

Catalytic subunit migrated as a single band of protein on sodium dodecyl sulphate / polyacrylamide-gel electrophoresis (Fig. 1). From sedimentation-velocity centrifugation, the plateau concentration of catalytic subunit was level and decreased with time of sedimentation strictly according to the radial square dilution law (see, Bowen, 1970) within experimental error (results not shown). The derivative of the E_{280} of the protein boundary was symmetrical throughout the experiments (results not shown). Such criteria indicate monodispersity.

By sedimentation-equilibrium centrifugation, both at 15000 and 28000 rev./min, the catalytic subunit was monodisperse as judged by two criteria. First, the point weight-average molecular weight was invariant with increasing concentration. Secondly, for both the high-speed (see, e.g., Fig. 2) and low-speed equilibrium runs, the overall plots of $\ln(E_{280})$ against r^2 appeared to be linear within experimental error, as judged by the finding that the coefficient of the $(r^2)^2$ term was not statistically different from zero when the data were fitted to a parabola in r^2, i.e. $\ln(E_{280}) = a_0 + a_1 r^2 + a_2 (r^2)^2$. Further, for the low-speed equilibrium run, the z-average and weight-average molecular weights were equal within experimental error.

A single symmetrical peak of catalytic subunit activity was obtained after Sephadex G-100 chromatography, hydroxyapatite chromatography or sucrose-density-gradient centrifugation (results not shown). Previous studies have shown that a single symmetrical peak of activity was eluted on phosphocellulose ion-exchange chromatography of catalytic subunit from

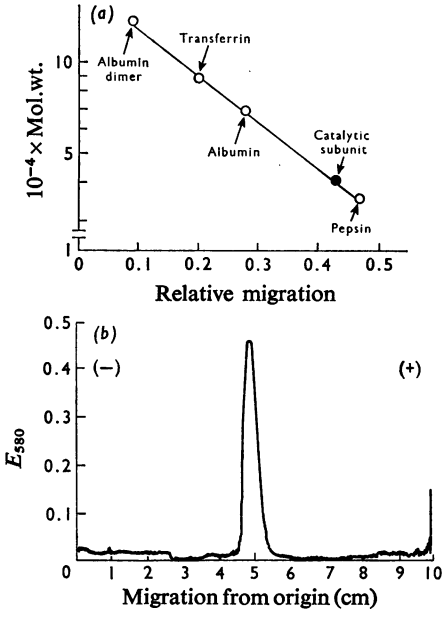

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of catalytic subunit*

Gel electrophoresis of catalytic subunit (5μg) was performed as described in the Materials and Methods section. A standard curve of molecular weight against relative migration (ratio of migration from top of the gel of protein-band front relative to Bromophenol Blue front) was constructed (*a*). From this curve, the molecular weight of catalytic subunit was calculated. A scan of a gel of catalytic subunit (stained and scanned as described in the Materials and Methods section) is shown in (*b*). Albumin refers to bovine serum albumin.

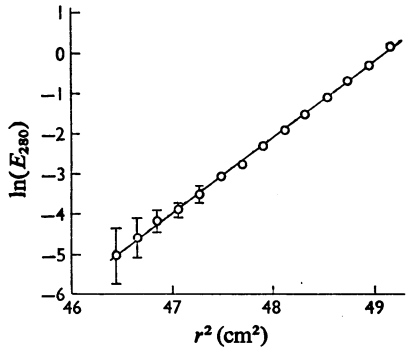

Fig. 2. *Sedimentation-equilibrium centrifugation of catalytic subunit by the method of Yphantis* (1964)

Sedimentation was performed at 28000rev./min and 7.1°C. Molecular weight was determined from a plot of ln(E_{280}) against r^2 and the least-squares line corresponded to mol.wt. 39592. The error bars represent the S.D. for ln(E_{280}) at the various values of r^2.

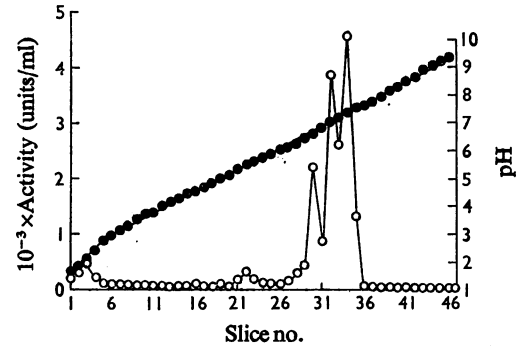

Fig. 3. *Polyacrylamide-gel isoelectric focusing of catalytic subunit*

Samples (25–50μg) of catalytic subunit were applied to gels and electrophoresed as described in the Materials and Methods section. Gels were sliced and catalytic subunit activity (○) and pH (●) in the slices determined as described in the Materials and Methods section. Slice 1 is the top (anode end) of the gel and slice 47 is the bottom (cathode end) of the gel.

rat tissues (Corbin *et al.*, 1976). Thus the catalytic subunit appeared to be monodisperse by these criteria.

Polyacrylamide-gel isoelectric focusing

Three major bands of catalytic subunit activity were resolved by polyacrylamide-gel isoelectric focusing (Fig. 3). The pI value for each peak, with activity as percentage of total activity recovered in parentheses, was 6.72 (20%), 7.04 (33%) and 7.35 (36%). Preincubation of catalytic subunit with 70mM-potassium phosphate, 2mM-ATP and 20mM-magnesium acetate at room temperature for 1 h before isoelectric focusing did not significantly alter the activity profile (results not shown). Since the catalytic subunit isoenzymes differed in pI values by less than 0.6 pH unit, they may differ only slightly in their amino acid composition. Because the enzyme was monodisperse by other criteria (see above) any difference in molecular weights of the isoenzymes must be small. The significance, if any, of these multiple catalytic subunit isoenzymes *in vivo* is not known, although they may differ in protein substrate specificity or some other important property.

Stability of catalytic subunit

Catalytic subunit activity was stable for at least 3 months when stored in 350mM-potassium phosphate buffer, pH6.8, at 4°C. However, on storage, it was noticed that the enzyme no longer migrated as a single protein on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, but that one or two faint bands of higher molecular weight appeared (results not shown). Such bands disappeared after Sephadex G-100 chromatography and are thought to be caused by polymerization of the catalytic subunit into higher-molecular-weight forms. At low catalytic subunit (<5μg/ml) and/or phosphate (<50mM) concentrations, the enzyme was relatively unstable and was assayed as rapidly as possible after dilution.

Reassociation of catalytic subunit with regulatory subunit

Catalytic subunit reassociated with and was inhibited by the freshly prepared regulatory subunit of protein kinase from bovine liver (see, Sugden & Corbin, 1976).

Physical properties

The physical properties of the catalytic subunit are shown in Table 2.

(i) *Molecular weight by sedimentation-equilibrium centrifugation.* From the slope of the plot of ln(E_{280}) against r^2 for the meniscus-depletion experiment at 28000rev./min (Fig. 2), a value for mol.wt. of 39592 was obtained. From the sedimentation-equilibrium data obtained at 15000rev./min, a value of 43796 was calculated by eqn. (2) (see Van Holde & Baldwin, 1958):

$$\bar{M}_w \text{ cell, mass} = \frac{C_b - C_a}{C_0} \cdot \frac{1}{b^2 - a^2} \cdot \frac{1}{A} \qquad (2)$$

where $\bar{M}_w$ is weight-average molecular weight, C_a is protein concentration at upper meniscus, C_b is protein concentration at lower meniscus, C_0 is initial protein concentration, a is radial distance to upper meniscus, b is radial distance to lower meniscus and A is

Table 2. *Physical parameters of the catalytic subunit*

The parameters were calculated as described in the Materials and Methods and the Results sections. Values for $s^0_{20,w}$ are the means of two separate determinations at two protein concentrations. Values for molecular weight from sedimentation-equilibrium centrifugation are the means of determinations at two different rotor speeds. All other values are ±S.E.M. with the numbers of separate determinations in parentheses.

Experimentally determined parameters

Parameter	Measured by:	
$s_{20,w}$ (S)	Sucrose-density-gradient centrifugation	3.6±0.1 (4)
$s^0_{20,w}$ (S)	Sedimentation-velocity centrifugation	3.14±0.05
Stokes radius (nm)	Gel filtration	2.73±0.04 (4)
Mol.wt.	Sedimentation-equilibrium centrifugation	41700±2000
Mol.wt.	Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis	39000±600 (3)
$E^{0.1\%}_{1cm}$ at 280nm (litre·g^{-1}·cm^{-1})	Spectrophotometry and amino acid analysis	1.42

Calculated parameters

Parameter	Calculated from:	
Stokes radius (nm)	$s^0_{20,w}$ and mol.wt. by sedimentation-equilibrium centrifugation	3.08
Mol.wt.	Amino acid analysis	41823
Mol.wt.	$s_{20,w}$ and Stokes radius	42000
$\bar{v}$ (cm^3·g^{-1})	Amino acid analysis	0.739
f/f_0	$s_{20,w}$ and Stokes radius	1.19
f/f_0	$s^0_{20,w}$ and mol.wt. by sedimentation-equilibrium centrifugation	1.23
Axial ratio	$s_{20,w}$ and Stokes radius	4–5
Axial ratio	$s^0_{20,w}$ and mol.wt. by sedimentation-equilibrium centrifugation	4–5
$10^7 \times D_{20,w}$ (cm^2/s)	$s_{20,w}$ and Stokes radius	7.99
$10^7 \times D^0_{20,w}$ (cm^2/s)	$s^0_{20,w}$ and mol.wt. by sedimentation-equilibrium centrifugation	7.09
$E^{0.1\%}_{1cm}$ at 280nm (litre·g^{-1}·cm^{-1})	Amino acid analysis	1.24–1.42

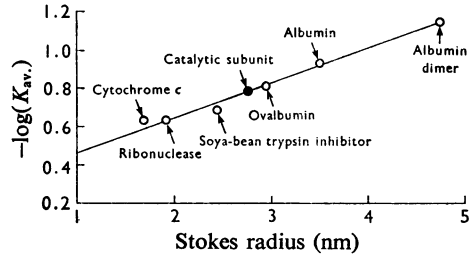

Fig. 4. *Determination of Stokes radius of catalytic subunit*

Chromatography of catalytic subunit [25μg in 1ml of 350mM-potassium phosphate and 10% (w/v) sucrose at pH6.8] on a column of Sephadex G-100 (superfine grade) was carried out as described in the Materials and Methods section. Albumin refers to bovine serum albumin.

$(1-vp)\omega^2/2RT$. For subsequent calculations, molecular weight was taken to be the average of these two determinations (41700). The initial E_{280} of the catalytic subunit was 0.3 at both rotor speeds.

(ii) *Sedimentation coefficients.* Sedimentation-velocity centrifugation at an E_{280} of 0.63 or 0.17 gave values for $s_{20,w}$ (±S.D. for 21 time-points) of 3.16±0.02S and 3.11±0.02S respectively. Since there appeared to be no significant effect of protein concentration on $s_{20,w}$ at these low concentrations, $s^0_{20,w}$ was simply taken to be the average of these two determinations, 3.14±0.05S. Sucrose-density-gradient centrifugation gave an $s_{20,w}$ (3.6S) that was slightly higher than that obtained by sedimentation-velocity centrifugation. The difference between these values may be caused by one or more of several factors, e.g. differences in the conformational state of the protein at 4° and 20°C, problems with the concentration-dependence of $s_{20,w}$ of proteins in sucrose-gradient centrifugation and inaccuracies in the reported sedimentation coefficients for the standard proteins.

(iii) *Calculated parameters.* From values of mol.wt. (41700±2000), $s^0_{20,w}$ (3.14±0.05S), $\bar{v}$ (0.739) (Cohn & Edsall, 1943*a*) and an assumed hydration of 0.2g of water per g of protein, a value of f/f_0 of 1.23±0.08 was calculated. By using the model of a prolate ellipsoid, this would correspond to an axial ratio of 4–5 (see, Cohn & Edsall, 1943*b*). From $s^0_{20,w}$, molecular weight and $\bar{v}$, a Stokes radius of 3.08nm was obtained. This compares with a value of 2.73nm obtained by gel filtration (Fig. 4).

Table 3. *Amino acid analysis of catalytic subunit*

Amino acid analysis was performed as described in the Materials and Methods section. From the analysis, a mean residue weight was calculated. From the molecular weight determined by sedimentation-equilibrium centrifugation (41 700) and the mean residue weight, the total number of residues per molecule was calculated. By multiplication of the latter by the residues per 100 residues, the number of individual residues per molecule was calculated.

Amino acid	Residues per 100 residues	Residues per 364 residues	Assigned residues per molecule
Lysine	10.23	37.1	37
Histidine	2.75	10.0	10
Arginine	4.31	15.6	16
Tryptophan	1.48	5.5	6
Aspartic acid	10.30	37.4	37
Threonine	4.23	15.4	15
Serine	4.75	17.2	17
Glutamic acid	11.91	43.2	43
Proline	4.17	15.1	15
Glycine	7.02	26.1	26
Alanine	7.16	26.0	26
Half-cystine	0.17	0.6	1
Valine	4.44	16.1	16
Methionine	1.68	6.1	6
Isoleucine	4.46	16.2	16
Leucine	9.56	34.7	35
Tyrosine	3.62	13.1	13
Phenylalanine	7.58	27.5	28

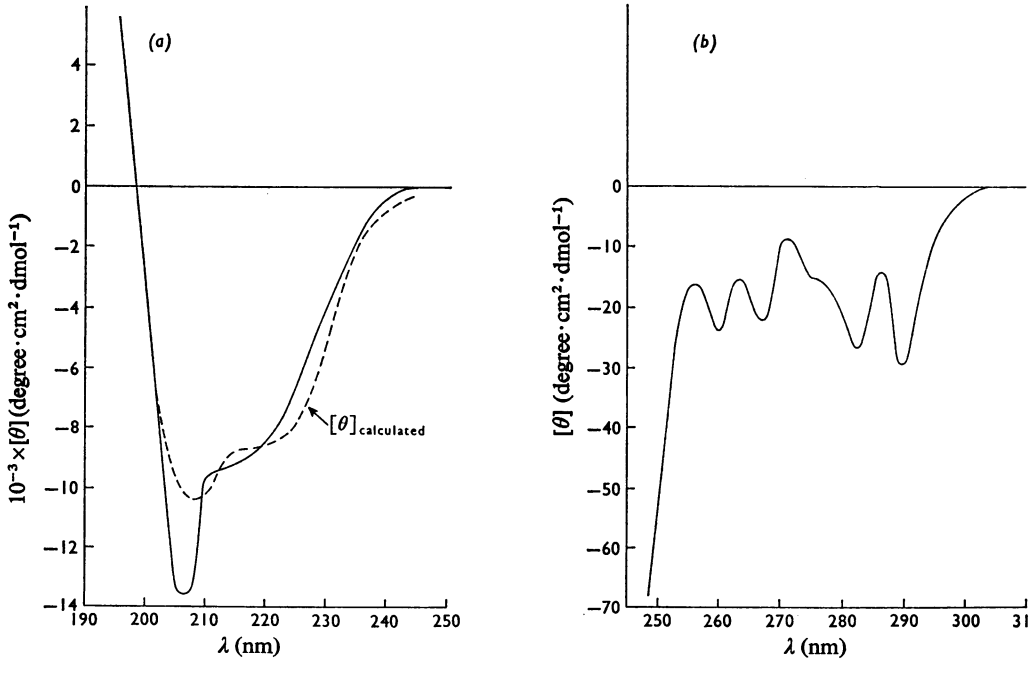

Fig. 5. *C.d. spectra of catalytic subunit*

C.d. spectra for the catalytic subunit are shown in the far-u.v. region (protein concn. 0.19mg/ml) in (*a*) and in the near-u.v. region (protein concn. 0.81mg/ml) in (*b*). Catalytic subunit was in 20mM-potassium phosphate and 100mM-KCl at pH6.5 at 25°C. Results are expressed in terms of $[\theta]$, the equivalent molar ellipticity. (*a*) ----, $[\theta]$ calculated from the data of Chen *et al.* (1974) for a protein consisting of 29% α-helical form, 18% β-form and 53% aperiodic conformation.

From the $s_{20,w}$ value from sucrose-gradient-centrifugation, the Stokes radius from gel filtration (Fig. 4) and $\bar{v}$, the molecular weight, f/f_0 and axial ratio may be calculated (see, Cohn & Edsall, 1943*b*; Siegel & Monty, 1966). Such parameters showed close similarity to the parameters obtained by sedimentation-equilibrium and -velocity centrifugation. The molecular weight calculated from sucrose-density-gradient-centrifugation and gel-filtration data agreed closely with that obtained by sedimentation-equilibrium centrifugation, supporting the validity of the technique of Siegel & Monty (1966).

Physical properties of the catalytic subunit (Table 2) were similar to those obtained previously for the bovine heart catalytic subunit (Erlichman *et al.*, 1973; see also, Rubin & Rosen, 1975). Comparison of molecular-weight data from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and other techniques indicate that the catalytic subunit, as isolated, was a monomer.

Amino acid analysis

Amino acid analysis of catalytic subunit is shown in Table 3. No glucosamine or galactosamine was detected. By the method of Rohrbach *et al.* (1973), it was shown that catalytic subunit contained 1.09 mol of thiol group/mol of enzyme and on this basis a value of 1 cysteine residue/molecule was assigned. Such an analysis did not preclude the possibility that the enzyme contained a cystine residue(s), since a large loss could have occurred during analysis.

From the amino acid analysis, a value of 0.739 $cm^3 \cdot g^{-1}$ was calculated for $\bar{v}$ [see Cohn & Edsall (1943*a*) and Table 2]. A value of $E^{0.1\%}_{1cm}$ at 280 nm (Table 2) was calculated from data in Sober (1968) and the amino acid composition. The value of 1.24 litre $\cdot g^{-1} \cdot cm^{-1}$ was calculated by assuming that all aromatic residues were exposed to the aqueous solvent, and a value of 1.42 litre $\cdot g^{-1} \cdot cm^{-1}$ was calculated by assuming that all aromatic residues were not exposed to solvent (taken from spectral data for amino acids in ethanol).

Extinction coefficient of catalytic subunit

A value of 1.42 litre $\cdot g^{-1} \cdot cm^{-1}$ was obtained for $E^{0.1\%}_{1cm}$ at 280 nm. Protein concentration was calculated from the amino acid composition after measuring alanine, glycine, asparagine and glutamine residues. The empirical value of $E^{0.1\%}_{1cm}$ agrees closely with the calculated value (see above).

Circular dichroism

The far- and near-u.v. c.d. spectra of the catalytic subunit are shown in Fig. 5. The dashed curve in Fig. 5(*a*) gives the calculated spectrum for $f_\alpha = 0.29$, $f_\beta = 0.18$ and $f_R = 0.53$. The root mean square derivation between the experimental and theoretical curves was 1171 degree $\cdot cm^2 \cdot dmol^{-1}$. The catalytic subunit thus appears to have a considerable amount of secondary structure. In general, the fit of the least-squares theoretical spectrum was fairly good, except for the 204–210 nm region. The disparity between experimental and theoretical curves in this area may result from the far-u.v. c.d. of the aromatic chromophores. With 13% of the residues being tryptophan, tyrosine and phenylalanine residues, the combined contribution of the aromatic residues to the far-u.v. c.d. spectrum of the catalytic subunit could be as large as 2000–3000 degree $\cdot cm^2 \cdot dmol^{-1}$ in the 200–220 nm wavelength region (Sears & Beychock, 1973; Holladay & Puett, 1976). This contribution may well account for some of the lack of fit between the calculated and the theoretical c.d. spectra.

The near-u.v. c.d. spectrum (Fig. 5*b*) appears to have contributions from tryptophan residues (peaks at 283 and 290 nm) and phenylalanine residues (peaks at 260 and 267 nm). The peak at 290 nm indicates that at least some of the tryptophan residues are in a relatively hydrophilic environment. If each of the five or six tryptophan residues contributes equally to the 290 nm peak, a value of about −1900 degree $\cdot cm^2 \cdot dmol^{-1}$ for each tryptophan residue results. In

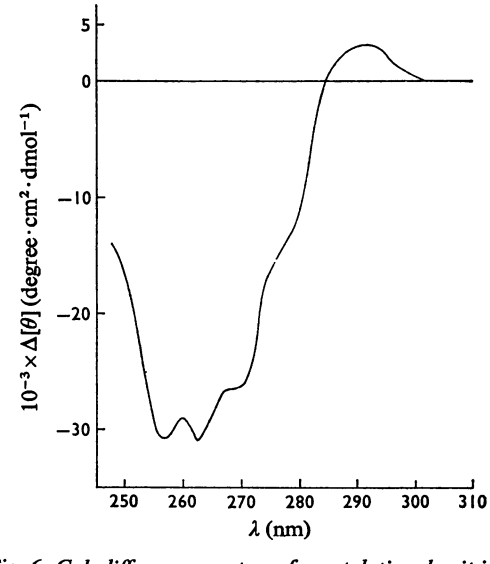

Fig. 6. *C.d. difference spectrum for catalytic subunit in the presence of ATP–Mg^{2+}*

The c.d. difference spectrum was calculated by subtraction of the c.d. spectrum in Fig. 5(*b*) from that of catalytic subunit in the presence of 0.033 mM-ATP and 6 mM-magnesium acetate, and multiplying by the number of residues to give $\Delta[\theta]$ in molecular ellipticity.

absolute magnitude, this value is about twice that observed for tryptophan residues in short peptides (Holladay *et al.*, 1976). The 13 tyrosine residues do not appear to contribute appreciably to the spectrum in the 260–285nm region.

The c.d. difference spectrum resulting from the addition of ATP–Mg^{2+} to the catalytic subunit is shown in Fig. 6. The spectrum was not corrected for the spectrum of ATP–Mg^{2+} alone since the contribution to $[\theta]$ was small. There is a complete lack of similarity in the difference spectrum to the c.d. spectrum of ATP–Mg^{2+} (Heyn & Bretz, 1975). At least part of the increase in ellipticity between 260 and 285nm which occurred on binding ATP–Mg^{2+} may result from one or more tyrosine residues. The changes observed in the spectrum in the 300–285nm wavelength region on ATP–Mg^{2+} binding are small. These results suggest that the orientation and/or environment of tyrosine and phenylalanine residues are more affected by binding of ATP–Mg^{2+} than are tryptophan residues.

Inhibition of catalytic subunit by thiol-specific reagents

Because amino acid analysis indicated 1mol of cysteine/mol of enzyme, it was decided to investigate whether that residue was essential for enzymic activity. It was found that catalytic subunit was inhibited by iodoacetamide (Fig. 7*a*). Similar results were observed with the other thiol-specific reagents *p*-hydroxymercuribenzoate and *N*-ethylmaleimide (results not shown). Histone F2b was used as substrate in this series of experiments, since it does not contain free thiol groups (Iwai *et al.*, 1970) and hence problems of thiol-specific reagents alkylating the substrate may be avoided. Inhibition was dependent both on time and inhibitor concentration. Similar results (not shown) were obtained when the substrate was protamine, which also does not contain free thiol groups (Ando & Watanabe, 1969). ATP–Mg^{2+} partially protected catalytic subunit activity against inhibition by iodoacetamide (Fig. 7*b*) or *N*-ethylmaleimide (results not shown). Mg^{2+} or ATP plus EDTA did not protect significantly. Such results indicate that binding of ATP–Mg^{2+} to the enzyme prevents access of thiol-specific reagents to the sensitive thiol group either by a conformational change in the enzyme structure or

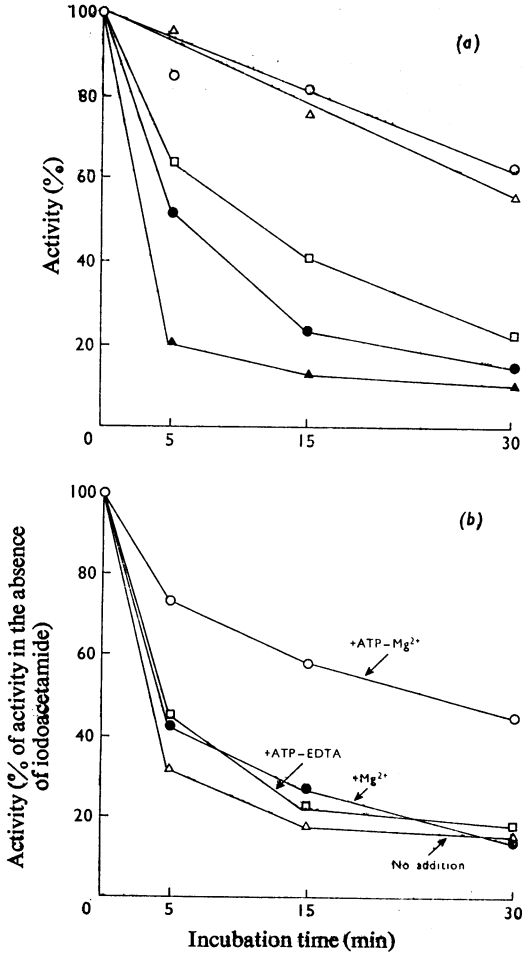

Fig. 7. *Inhibition of catalytic subunit activity by iodoacetamide and protection against inhibition by iodoacetamide by ATP–Mg^{2+}*

(*a*) Catalytic subunit (5μg) was preincubated at 0°C in 10mM-Tris/HCl, pH8.5, containing the following concentrations of iodoacetamide: 0.5mM (△), 2.5mM (□), 5mM (●) and 12.5mM (▲). The final volume was 2ml. A control in the absence of iodoacetamide was run concurrently (○). After each of the indicated preincubation times, a sample (20μl) was withdrawn and assayed for catalytic subunit activity by incubation for 5min at 30°C with a 50μl sample containing 24mM-potassium phosphate, 8.4mM-magnesium acetate, 0.35mM-[γ-^{32}P]ATP (specific radioactivity 20–30c.p.m./pmol) and 0.5mg of histone F2b at pH6.8. The incorporation of ^{32}P into histone was determined as described in the Materials and Methods section. Activity is expressed as a percentage of a zero-time control performed in the absence of inhibitor. (*b*) Catalytic subunit (1.25μg) was preincubated at 0°C with 10mM-Tris/HCl and 12.5mM-iodoacetamide at pH8.5 containing 0.1mM-ATP and 10mM-magnesium acetate (○), 0.1mM-ATP and 1mM-EDTA (□) or 10mM-magnesium acetate (●); △, no addition. The final volume was 0.5ml. After each indicated preincubation time, a sample (20μl) was withdrawn and assayed for catalytic subunit activity as described in (*a*). Results are expressed as a percentage of the appropriate controls preincubated at 0°C for the same time and with the same additions as the tests but in the absence of iodoacetamide.

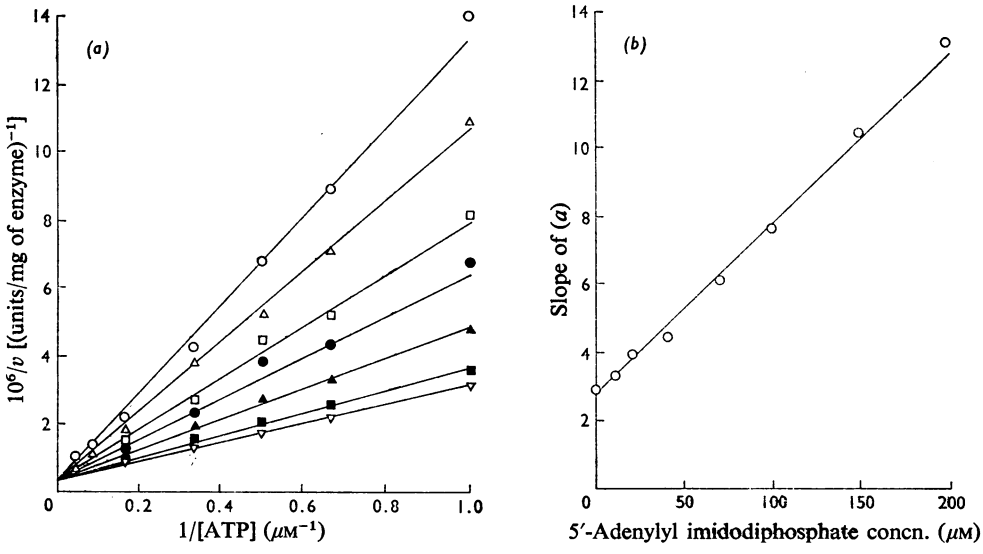

Fig. 8. *K_m of catalytic subunit for ATP and inhibition of catalytic subunit by 5'-adenylyl imidodiphosphate*

(*a*) A Lineweaver & Burk (1934) plot is shown for various concentrations of ATP at a fixed concentration of histone (Sigma, type II-A, 7.1 mg/ml) (▽). The specific radioactivity of [γ-^{32}P]ATP was 500–1000 c.p.m./pmol. Assays were run for 2 min at 30°C in the presence of 6 mM-magnesium acetate. Assays were initiated by the addition of 7.25 ng of catalytic subunit (20 μl) in 55 mM-potassium phosphate, pH 6.8. Otherwise methodology was as described in the Materials and Methods section. Activity was also measured at the various concentrations of ATP in the presence of the following concentrations of 5'-adenylyl imidodiphosphate: 200 μM (○), 150 μM (△), 100 μM (□), 70 μM (●), 50 μM (▲) and 10 μM (■). (*b*) Slopes of the lines in (*a*) were plotted against 5'-adenylyl imidodiphosphate concentration [a point at 20 μM-5'-adenylyl imidodiphosphate which is not shown in (*a*) is included] and from this secondary plot, a value of K_I for 5'-adenylyl imidodiphosphate was calculated.

Table 4. *Dependence of catalytic subunit activity on metal ions*

All metal ions were added as their Cl^- salts (except for Ni^{2+}, which was added as $NiSO_4$) to produce a concentration in the assay of 6 mM. Activities are expressed as a percentage of the activity in the presence of 6 mM-$MgCl_2$.

Metal ion	Activity (%)
No added metal	<1
Cd^{2+}	2
Ba^{2+}	29
Ni^{2+}	23
Co^{2+}	52
Zn^{2+}	1
Mn^{2+}	7
Ca^{2+}	<1
Cu^{2+}	<1
La^{3+}	<1
Li^+	<1
Fe^{3+}	<1
Sr^{2+}	<1
Mg^{2+}	(100)

some other form of steric hindrance. Such a conclusion is supported by c.d. experiments (see above). Such a conformational change could explain, at least in part, the stimulation by ATP–Mg^{2+} of reassociation of free regulatory and catalytic subunits (Brostrom *et al.*, 1971), inhibition by ATP–Mg^{2+} of the dissociation of protein kinase by high ionic strength or histone (Corbin *et al.*, 1976) and the decreased affinity of 'type I' protein kinase for cyclic AMP in the presence of ATP–Mg^{2+} (Bechtel & Beavo, 1974).

Kinetics of catalytic subunit with ATP

A Lineweaver & Burk (1934) plot for ATP is shown in Fig. 8(*a*). The apparent K_m of the catalytic subunit for ATP ± S.E.M. was 7.6 ± 0.7 μM (four determinations). The $V_{max.}$ ± S.E.M. was $3.05 \times 10^6 \pm 0.05 \times 10^6$ units/mg of enzyme (four determinations). Since the kinetic plots for ATP were linear, it is unlikely that the catalytic subunit isoenzymes have significantly different K_m values for ATP. The catalytic subunit was inhibited by 5'-adenylyl imidodiphosphate. Inhibition was competitive with ATP as shown by plotting data (Fig. 8*a*) by the method of Lineweaver & Burk (1934) and production of a linear secondary plot (Fig. 8*b*). From the secondary plot a value of K_I of 60 μM was calculated for 5'-adenylyl imidodiphosphate. This compound may be useful in studying the

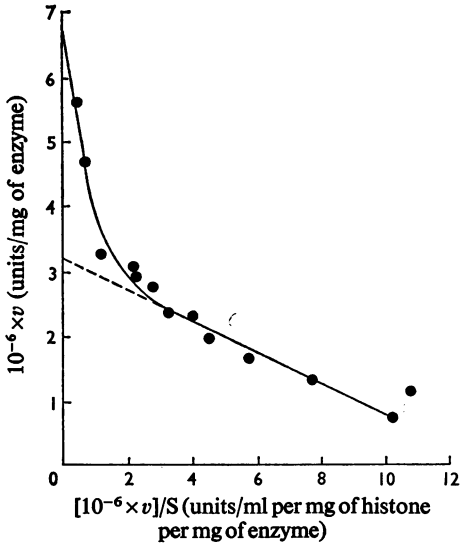

Fig. 9. *Eadie* (1942) *plot for catalytic subunit at various histone concentrations*

Methodology was generally similar to that described in Fig. 8(*a*) in the absence of 5'-adenylyl imidodiphosphate. Mixed histone (Sigma, type II-A) was the substrate. The [γ-^{32}P]ATP concentration was 0.25mM and the specific radioactivity was 100–200c.p.m./pmol.

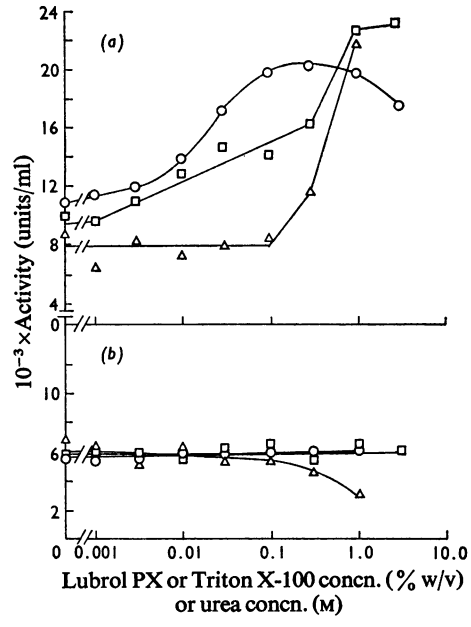

Fig. 10. *Effects of detergents and urea on catalytic subunit activity*

Catalytic subunit activity was assayed as described in the Materials and Methods section with (*a*) histone (Sigma, type II-A) or (*b*) casein as substrate. Triton X-100 (○), Lubrol PX (□) or urea (△) was added to the assay mixture before the addition of enzyme. Casein was dissolved by the method of Reimann *et al.* (1971) and its concentration in the assay was 7.1mg/ml.

binding properties of the catalytic subunit and also in affinity chromatography.

Metal ion requirement

The dependence of activity on total Mg^{2+} concentration of catalytic subunit was investigated (results not shown). Activity is half-maximum at 0.8mM-Mg^{2+} and maximum at 6–8mM. Other metal ions partially replaced the Mg^{2+} requirement (Table 4). Of the metal ions (other than Mg^{2+}) tested, Co^{2+} was most effective.

Protein substrates of catalytic subunit

Mixed histone (Sigma, type II-A), histone F2b, casein, protamine and glycogen synthetase I were substrates for the catalytic subunit (results not shown). Glycogen synthetase I form was converted into the D form by incubation with catalytic subunit and ATP–Mg^{2+} with concomitant incorporation of phosphate into glycogen synthetase.

An Eadie (1942) plot for mixed histone (Sigma, type II-A) is shown in Fig. 9. The plot is biphasic showing 'low-K_m' and 'high-K_m' components. The 'low-K_m' component showed an apparent K_m of 0.25mg of histone/ml and extrapolated to a $V_{max.}$ of 3.2×10^6 units/mg of protein. The 'high-K_m' component showed an apparent K_m of 3.1mg of histone/ml and extrapolated to a $V_{max.}$ of 6.65×10^6 units/mg of protein. Such a biphasic plot has several interpretations: (*a*) the enzyme displayed negative co-operativity (unlikely as the enzyme appeared to be a monomer, see above) or substrate activation; (*b*) there were two separate enzymes present with different affinities for the histone substrate; (*c*) the histone substrate used was heterogeneous. Although (*c*) is possibly the likeliest alternative, alternative (*b*) should not be ignored because of the presence of catalytic subunit isoenzymes in the preparations.

When assayed with histone (Sigma, type II-A), catalytic subunit activity was significantly activated (about twofold) by the non-ionic detergents Triton X-100 or Lubrol PX or by the presence of urea in the assay medium (Fig. 10*a*). Activation was maximum at a Triton X-100 concn. of 0.1–1.0% (w/v) or a Lubrol PX concn. of 1.0–3.0% (w/v). However, when

catalytic subunit was assayed with casein as substrate, no stimulation of activity was observed (Fig. 10*b*). Such an observation is technically important, since some investigators have used histone (Sigma, type II-A) as substrate and Triton X-100 to solubilize catalytic subunit bound to particulate fractions (Korenman *et al.*, 1974). An activation of protein kinase-catalysed histone phosphorylation by NaCl has been observed (Corbin *et al.*, 1972). It is suggested that these agents (detergents, urea, NaCl) alter histone conformation so as to increase its rate of phosphorylation.

Note Added in Proof (Received 9 September 1976)

Since completion of the c.d. experiments in the presence of ATP–Mg^{2+}, it has been discovered that the catalytic subunit may possess ATPase activity. In the c.d. experiments, it is possible that a considerable proportion of the ATP may be hydrolysed to ADP. Although this does not materially affect the experimental conclusions, it should be noted that changes in the c.d. spectra may be being caused by the binding of ATP–Mg^{2+} and/or ADP.

We thank Miss Lila Clack for performing amino acid analyses, Dr. Roger A. Johnson for purification of 5′-adenylyl imidodiphosphate, Dr. Thomas R. Soderling for performing the experiments of glycogen synthetase, and Dr. Charles R. Park for his interest and encouragement. This work was supported by Research Grants AM 15988 and 15611 from the National Institutes of Health, United States Public Health Service. P. H. S. acknowledges the receipt of a U.K. Science Research Council–NATO Post-doctoral Fellowship. J. D. C. is an investigator of the Howard Hughes Medical Institute.

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