Purification and properties of the insulin-stimulated cyclic AMP phosphodiesterase from rat liver plasma membranes

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The peripheral high-affinity cyclic AMP phosphodiesterase from rat liver plasma membranes was purified to apparent homogeneity. The procedure used involved the initial purification of liver plasma membranes and the solubilization of the enzyme by using a high-ionic-strength medium. This was followed by chromatography of the enzyme on DEAE-cellulose, Affi-Gel Blue, a novel affinity column and Sephadex G-100. A 9500-fold purification of the enzyme with a 24% yield was achieved by this procedure. The purified enzyme was apparently monomeric ($M$, 52000), as it exhibited identical molecular weights on analysis by gel filtration, sedimentation and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. It is suggested that the non-Michaelis kinetics exhibited by the enzyme are due to it obeying a mnemonical mechanism, where it displays $K_m$ 0.7 $\mu$M, $V_{max}$ 9.1 units/mg of protein and Hill coefficient ($h$) 0.62. Cyclic GMP acts as a poor substrate for the enzyme, with $K_m$ 120$\mu$M and $V_{max}$ 0.4 unit/mg of protein, and also as an inhibitor of the enzyme, with $I_{50}$ (concentration giving 50% inhibition) 150$\mu$M when assayed at 0.4 $\mu$M-cyclic AMP. Inhibition by 5'-AMP is unlikely to be of physiological importance, as it is only a weak inhibitor of the enzyme ($I_{50}$ 47 mM assayed at 0.4 $\mu$M-cyclic AMP).

Cyclic AMP is degraded intracellularly by the action of cyclic AMP phosphodiesterase (EC 3.1.4.17), which is believed to exist in multiple forms within the cell (Appleman et al., 1973; Wells & Hardman, 1977; Thompson & Strada, 1978). The number, nature and localization of these species is as yet ill-understood.

Theoretical studies indicate that multiple forms of cyclic AMP phosphodiesterase with distinct intracellular locations, activities and kinetic mechanisms may play an important role in modulating intracellular cyclic AMP. Indeed the control of their activity may be as important as the effect of hormonal activation of adenylate cyclase (Fell, 1980; Erneux et al., 1980). It is only recently that any cyclic AMP phosphodiesterases have actually been purified to homogeneity. These are the calmodulin-activated, low-affinity soluble enzyme from brain (Klee et al., 1979; Morrill et al., 1979; Sharma et al., 1980) and a calmodulin-insensitive, high-affinity, soluble enzyme from dog kidney (Thompson et al., 1979). Both of these soluble enzymes display normal Michaelis-Menten kinetics.

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We have identified (Marchmont & Houslay, 1980a) a high-affinity enzyme in rat liver that exhibits apparent negative co-operativity. This peripheral enzyme is associated with the plasma membrane through predominantly electrostatic interactions. Insulin can apparently activate this enzyme by a mechanism that is dependent on both cyclic AMP and ATP (Marchmont & Houslay, 1980b,c,d). This paper describes the purification of this membrane-bound cyclic AMP phosphodiesterase to apparent homogeneity.

Materials and methods

Venom from the snake Ophiophagus hannah, Dowex 1 anion-exchange resin, trypsin, trypsin inhibitor and 1,5-diaminopentane were from Sigma, Kingston-upon-Thames, Surrey, U.K. Cyclical AMP, cyclic GMP, ATP, AMP and all enzymes were from Boehringer (U.K.), Lewes, East Sussex, U.K. Isobutylmethylxanthine and 8-chlorotheophylline were from Aldrich Chemical Co., Gillingham, Dorset, U.K. Kieselgel H was from Camlab (Glass) Ltd., Cambridge, U.K. Sephadex G-100 and CNBr-
activated Sepharose 4B were from Pharmacia Fine Chemicals, Uppsala, Sweden. Affi-Gel Blue, which is made by coupling Cibacron Blue F3G A to cross-linked agarose beads, and chemicals for electrophoresis were from Bio-Rad (U.K.) Laboratories, Watford, Herts., U.K. DEAE-cellulose was from Whatman Biochemicals (U.K.), Maidstone, Kent, U.K. Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of A.R. quality from BDH Chemicals, Poole, Dorset, U.K. Bovine brain calmodulin was kindly given by Dr. R. Grand, Department of Biochemistry, University of Birmingham, U.K., and was prepared by the method of Grand et al. (1979).

**Plasma-membrane preparation**

Rat liver plasma membranes were prepared by a modification (Houslay et al., 1976) of the method of Pilkis et al. (1974). This involved using a Teflon pestle and glass homogenizing vessel, making the crude low-speed pellet 60% (w/v) in sucrose with solid sucrose and 1 mM-KHCO₃, pH 7.4, washing the membranes in 1 mM-KHCO₃, pH 7.4, by recentrifugation, and storing the membranes at 10 mg of protein/ml in 1 mM-KHCO₃, pH 7.4, in liquid N₂.

**Assay of cyclic AMP phosphodiesterase**

This was carried out by a modification of the two-step method of Thompson & Appleman (1971) as described by us in detail previously (Marchmont & Houslay, 1980a). The [³H]adenosine produced in the first step of the assay by the action of 5'-nucleotidase can become bound to the Dowex 1 resin used in the second step of the assay, causing an underestimation of activity. This can have serious consequences for kinetic analyses if corrections are not made, especially as the fraction bound can be influenced by the cyclic AMP concentration. In all instances the appropriate corrections were made as detailed in Marchmont & Houslay (1980a). Routine incubations were carried out so that less than 10% of the cyclic AMP was degraded in the assays. Linearity of cyclic AMP degradation was assessed in every instance and initial rates were measured. All assays were carried out in duplicate at pH 7.2 at 30°C; errors are given as s.d. with n—1 degrees of freedom; computation and curve fitting was as described previously (Marchmont & Houslay, 1980a).

**Protein determination**

On the membrane preparations, protein was determined by a modification (Houslay & Palmer, 1978) of the micro-biuret method of Goa (1953), with bovine serum albumin as the standard. Fractions containing soluble proteins were assayed with the Bio-Rad Protein Assay Kit (see Bradford, 1976; Spector, 1978).

**Polyacrylamide-gel electrophoresis**

Non-denaturing polyacrylamide-gel electrophoresis was carried out in 5%-acrylamide gels as described previously (Marchmont & Houslay, 1980a). Some gels were stained for protein with either Amido Black or Coomassie Blue and others were sliced and eluted for assay of enzyme activity as before (Marchmont & Houslay, 1980a).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the reduction and carboxymethylation of samples and calibration was performed as described by us in detail previously (Marchmont & Houslay, 1980b,c). Gels were stained with Coomassie Blue and after destaining were scanned with a Gilford model 2400 spectrophotometer with gel-scanning attachment.

**Affinity-column matrix production**

This involved reaction of 8-chlorotheophylline with 1,5-diaminopentane and isolation of a theophylline derivative which presumably contained aminopentane at the 8 position, 8-{3'-aminopentamino}-1,3-dimethylurine-2,6-dione. This product was then linked to CNBr-activated Sepharose 4B, presumably through the 3'-amino group to provide an affinity matrix (theophylline-Sepharose).

1,5-Diaminopentane (2.18 g), 8-chlorotheophylline (0.4 g) and ethanol (20 ml) were sealed in a thick-walled glass tube and incubated at 140°C for 20h, in a contained oven. The reaction mixture was then analysed by t.l.c. on a 0.25 mm layer of Kieselgel H silica on glass plates developed in a ethanol/0.5 M-ammonium acetate (5:2, v/v). The product, which was subsequently isolated, was the only one present that gave a spot that both fluoresced under u.v. light and also reacted with ninhydrin. This spot had Rₚ of 0.47 in the above system. The only other u.v.-absorbing spot migrated as did theophylline, and the only other spot staining with ninhydrin migrated as 1,5-diaminopentane.

This product was then purified chromatographically. A glass column (50 cm x 3 cm) was packed with a slurry of Kieselgel H silica in ethanol/ammonium acetate (1:1, v/v) to a height of 35 cm. The reaction mixture (4 x 20 ml) was applied to the top of the column and eluted under gravity with the ethanol/ammonium acetate solvent mixture. The eluent was monitored for absorbance at 280 nm and collected in 2 ml fractions. During 100 h of elution the first peak of u.v.-absorbing material, which was eluted behind the solvent front and did not react with ninhydrin, was discarded. The second u.v.-absorbing peak reacted with ninhydrin and had Rₚ of 0.47 in the system described above. All the fractions in this second peak were pooled. The resulting solution was...
then evaporated on a rotary evaporator. This yielded a yellow oily residue. The addition of a little (4 vol.) ethanol caused the oil to gel. The resulting supernatant was decanted before the gel was dissolved in excess of ethanol. Addition of diethyl ether then resulted in the formation of a white precipitate of the product, which was removed and was washed by filtration by using a little diethyl ether. The product was then rotary evaporated in order to remove any remaining traces of solvent before it was attached to CNBr-activated Sepharose 4B.

CNBr-activated Sepharose 4B (20 ml) was mixed at 20°C with 10 ml of 0.1 M-Na₂CO₃/NaHCO₃ buffer, pH9.5, containing 45 mg of 8-(5'-amino-pentamino)-1,3-dimethylpurine-2,6-dione. The gel slurry was then gently shaken for 20 h at 4°C. After this the slurry was washed with 250 ml of water and then free CNBr groups were allowed to react with 0.1 M-ethanolamine (250 ml) for 1 h. The affinity-gel matrix was then washed with (a) 0.1 M-Na₂CO₃/NaHCO₃ buffer, pH9.5 (125 ml), (b) water (250 ml), (c) 0.5 M-NaCl (250 ml) and (d) water (250 ml) before use.

Purification of the peripheral cyclic AMP phosphodiesterase from rat liver plasma membranes

Step 1: high-salt solubilization. Plasma membranes (1.5 g of protein) were treated with the high-ionic-strength (0.4 M-NaCl) solubilization mixture for 1 h at 4°C as described in detail previously (Marchmont & Houslay, 1980a). They were then centrifuged at 300000 x g, for 30 min at 4°C. The supernatant (150 ml), which contained the solubilized enzyme (Marchmont & Houslay, 1980a), was dialysed overnight against two changes of 5 litres of buffer A (final concentrations: 0.1 M-NaCl, 5 mM-MgCl₂, 2 mM-2-mercaptoethanol and 20 mM-Tris/HCl, final pH 7.4).

All subsequent operations were carried out at 4°C.

Step 2: DEAE-cellulose chromatography. The dialysed enzyme preparation was applied to a column (2.2 cm x 30 cm) of DEAE-cellulose equilibrated in buffer A at a rate of 0.5 ml/min. The column was then washed with buffer A at no appreciable quantities of protein appeared in the eluate (5 bed vol.). The bound cyclic AMP phosphodiesterase was then stepwise eluted with 50 ml of buffer A containing a final concentration of 0.5 M-NaCl. Fractions (3 ml) were collected and those containing the highest specific activity of cyclic AMP phosphodiesterase were pooled and dialysed overnight against 5 litres of buffer A.

Step 3: Affi-Gel Blue chromatography. A column (10 cm x 1.5 cm) of Affi-Gel Blue was equilibrated with buffer A. The dialysed DEAE-cellulose eluate was applied to the column at a rate of 0.25 ml/min and then the column was washed with buffer A until no detectable protein was eluted, i.e. there was no difference between the A₂₈₀ of the eluate and buffer A. At this point the enzyme activity was stepwise-eluted with buffer A containing 0.5 M-NaCl; 1 ml fractions were collected. The fractions with the highest specific activity were pooled.

Step 4: Affinity chromatography. A small column (11 cm x 0.5 cm) of theophylline-Sepharose was equilibrated with buffer A containing 0.5 M-NaCl. The pooled fractions were then slowly (0.25 ml/min) added to the column. The column was then washed with buffer A containing 0.5 M-NaCl until no protein appeared in the eluate, i.e. A₂₈₀ of eluate = A₂₈₀ of buffer A containing 0.5 M-NaCl. After this the column was treated with 15 ml of buffer A containing 0.5 M-NaCl and 500 μM-isobutylmethylxanthine. Fractions (1 ml) were then collected and dialysed separately overnight at 4°C against 1 litre of buffer A containing 0.5 M-NaCl. The fractions containing the highest specific activities of cyclic AMP phosphodiesterase were then pooled and freeze-dried. The freeze-dried powder was then resuspended in 1 ml of buffer B (5 mM-MgCl₂/20 mM-Tris/HCl, pH 7.4).

Step 5: Sephadex G-100 gel filtration. The enzyme preparation was applied to a column (30 cm x 2.2 cm) of Sephadex G-100, equilibrated with buffer B, at a rate of 20 ml/h. Fractions (2 ml) were collected, and those with the highest specific activity were pooled. In order to concentrate the enzyme, the pooled fraction was freeze-dried and rehydrated in 1 ml of buffer B. The enzyme could be stored either freeze-dried or frozen in liquid N₂, or frozen at -20°C, with no loss in activity for at least 2 months.

Results

Purification of the peripheral liver plasma-membrane cyclic AMP phosphodiesterase

Starting with 1.5 g of liver plasma-membrane protein, the purification scheme produced 30 μg of apparently homogeneous enzyme. This scheme typically effected a 9500-fold purification with a 24% yield (Table 1). Fig. 1 demonstrates an example of the elution profiles of both protein and enzyme activity from the various columns. We used batch-elution methods rather than salt gradients to produce a relatively tight band of eluted activity and so increase our yield. When gradients were used, a rather smeared activity elution profile was obtained.

Gel electrophoresis both under non-denaturing and denaturing conditions yielded a single protein-staining band (Fig. 2). Under non-denaturing conditions the single protein-staining band coincided with the cyclic AMP phosphodiesterase activity (Fig. 3).
Table 1. *Purification of the peripheral rat liver plasma-membrane cyclic AMP phosphodiesterase*

Specific activity was assayed at 0.4μM-cyclic AMP.

<table>
<thead>
<tr>
<th>Step</th>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Specific activity (nmol/min per mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membranes</td>
<td>1500</td>
<td>0.04</td>
<td>(100)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>High-salt extract</td>
<td>380</td>
<td>0.125</td>
<td>85</td>
<td>3.1</td>
</tr>
<tr>
<td>2</td>
<td>DEAE-cellulose</td>
<td>35</td>
<td>1.136</td>
<td>66.5</td>
<td>28.4</td>
</tr>
<tr>
<td>3</td>
<td>Affi-Gel blue</td>
<td>1.2</td>
<td>24.9</td>
<td>50</td>
<td>623</td>
</tr>
<tr>
<td>4</td>
<td>Affinity column</td>
<td>0.058</td>
<td>308.1</td>
<td>30</td>
<td>7700</td>
</tr>
<tr>
<td>5</td>
<td>Gel filtration</td>
<td>0.030</td>
<td>380.2</td>
<td>24</td>
<td>9500</td>
</tr>
</tbody>
</table>

(Molecular size of the purified enzyme)

The molecular weight of the enzyme by gel filtration (Fig. 1d) was estimated as 52000 ± 4000 (n = 3), from sucrose-density-gradient sedimentation as 53000 ± 4500 (n = 3) (see Marchmont & Houslay, 1980a) and from the reduced and carboxymethylated enzyme on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as 52300 ± 2500 (n = 3). This suggests that the enzyme is monomeric.

(Kinetic properties)

The purified enzyme exhibited non-linear kinetics (Fig. 4), which yielded \( K_m = 0.71 ± 0.15 \mu M \), \( V_{max} = 9.14 ± 0.13 \) units/mg or protein, and a Hill coefficient \( (h) = 0.62 ± 0.04 \) for three determinations with different enzyme preparations and duplicate assays. The limiting values for \( K_m \) and \( V_{max} \) are given in Table 2. The catalytic-centre activity of this enzyme is 38749, assuming a single active site per molecule \( (M_r 52000) \) of protein. These parameters were not affected by calmodulin \( (0.1-200 \mu g/ml) \) when added in the presence of \( \mathrm{Ca^{2+}} (100 \mu M) \).

The product of the reaction, 5'-AMP, acted as an inhibitor (Fig. 4), with increasing concentrations of AMP causing a decrease in the value of \( h \). In the presence of 1mM- and 3mM-AMP, the value of \( h \) was 0.50 ± 0.06 and 0.43 ± 0.04 respectively for three determinations. The concentration \( (I_50) \) of AMP required to achieve 50% inhibition of the activity of the enzyme was 47 ± 8 mm when assayed at 0.4μM-cyclic AMP and 3.0 ± 0.5 mm when isobutylmethylxanthine is used to elute the enzyme from the column and a Bio-Rad protein assay was performed. (d) Step 5: gel filtration on Sephadex G-100. Key as for (a). The inset shows the calibration of the column with (a) cytochrome c, (b) ovalbumin, (c) bovine serum albumin and (d) pig heart malate dehydrogenase, with molecular weights from Darnall & Klotz (1972). The arrow marks the cyclic AMP phosphodiesterase activity.
Purification of cyclic AMP phosphodiesterase

Fig. 2. Polyacrylamide-gel electrophoresis of the purified cyclic AMP phosphodiesterase
(a) Non-denaturing conditions; (b) after treatment with sodium dodecyl sulphate. For further details see the Materials and methods section.

Fig. 3. Polyacrylamide-gel electrophoresis of the cyclic AMP phosphodiesterase activity
The gel as in Fig. 2(a) was scanned with a Gilford model 2400 spectrophotometer and the results are shown against an equivalent gel which was sliced up, eluted and assayed for cyclic AMP phosphodiesterase activity as in Marchmont & Houslay (1980a).

Fig. 4. Inhibition of activity by AMP
Double-reciprocal plots of cyclic AMP hydrolysis in the absence of AMP (●) and in the presence of final concentrations of 1 mM-(○) and 3 mM-(■) AMP. Data are shown from a typical experiment using duplicate assays.

Fig. 5. Action of cyclic GMP on the purified enzyme
(a) Double-reciprocal plot of cyclic GMP hydrolysis by the enzyme; (b) double-reciprocal plot of cyclic AMP hydrolysis in the absence (●) and in the presence of final concentrations of 20 µM-cyclic GMP (○) and 50 µM-cyclic GMP (■).

assayed at 5 µM-cyclic AMP (three determinations). The limiting values for $K_m$ and $V_{max}$, during product inhibition are given in Table 2. During these
Table 2. Limiting values of the kinetic constants of the native, purified peripheral cyclic AMP phosphodiesterase from rat liver plasma membranes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_1$ (μM)</th>
<th>$V_1$ (μmol/min per mg of protein)</th>
<th>$K_2$ (μM)</th>
<th>$V_2$ (μmol/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.71 ± 0.15</td>
<td>1.24 ± 0.32</td>
<td>39 ± 4</td>
<td>9.17 ± 0.20</td>
</tr>
<tr>
<td>1 mM-AMP</td>
<td>0.65 ± 0.01</td>
<td>1.15 ± 0.20</td>
<td>45 ± 4</td>
<td>9.02 ± 0.10</td>
</tr>
<tr>
<td>3 mM-AMP</td>
<td>0.55 ± 0.06</td>
<td>0.63 ± 0.10</td>
<td>54 ± 6</td>
<td>8.96 ± 0.43</td>
</tr>
<tr>
<td>0.02 mM-cyclic GMP</td>
<td>0.78 ± 0.15</td>
<td>1.18 ± 0.2</td>
<td>40 ± 6</td>
<td>9.08 ± 0.20</td>
</tr>
<tr>
<td>0.05 mM-cyclic GMP</td>
<td>0.91 ± 0.20</td>
<td>1.17 ± 0.3</td>
<td>51 ± 7</td>
<td>9.06 ± 0.4</td>
</tr>
</tbody>
</table>

experiments it was observed that the snake venom nucleotidase was able to effect the complete hydrolysis of 5'-[3H]AMP in the presence of added non-radioactive 5'-AMP.

Cyclic GMP acted as a poor substrate for the enzyme. Over the range of concentrations tested it yielded an apparently linear Lineweaver–Burk plot (Fig. 5), with $K_m$ 120 ± 11μM and $V_{max}$. 0.40 ± 0.11 unit/mg of protein for three determinations. Cyclic AMP also inhibited the hydrolysis of cyclic AMP by this enzyme (Fig. 5), with an $I_50$ of 150 ± 20 μM-cyclic GMP, when assayed at 0.4 μM-cyclic AMP. However, it did not alter $h$ for the reaction, as did 5'-AMP. The effect of cyclic GMP on the limiting values of $K_m$ and $V_{max}$ is shown in Table 2.

Discussion

The purification procedure described leads to the production of an apparently homogeneous preparation of the peripheral cyclic AMP-dependent phosphodiesterase from rat liver plasma membranes. This is achieved with a 9500-fold purification over the plasma membranes and with a yield of 24%. Our procedure thus compares very favourably with the methods which have been used to purify soluble phosphodiesterases to apparent homogeneity. The enzyme from brain has been purified between 2000- and 3000-fold with a 2–12% yield (Klee et al., 1979; Morrill et al., 1979; Sharma et al., 1980), and that from dog kidney 124-fold with a 5% yield (Thompson et al., 1979). Indeed, if 1.2% of the total protein in the hepatocyte corresponds to the plasma membrane (Lauter et al., 1972; Toda et al., 1975), then our purification of the enzyme over the homogenate is in fact about 790000-fold. However, because our yield of plasma membranes is only 35% (Marchmont & Houslay, 1980a), our overall yield of the peripheral enzyme would fall to about 8% on this basis.

The peripheral liver plasma-membrane enzyme may be grouped with the so-called 'high-affinity' types of cyclic AMP phosphodiesterases (see Thompson et al., 1979; Thompson & Strada, 1978). Typical of these is the dog kidney enzyme (Thompson et al., 1979), which like the peripheral enzyme is monomeric ($M_r$ 61000), has a high affinity for cyclic AMP, which has a high specificity for cyclic AMP relative to cyclic GMP $K_m$ (cyclic GMP)/$K_m$ (cyclic AMP) 140:1 for the kidney enzyme and 170:1 for the liver enzyme, cyclic AMP hydrolysis is inhibited by cyclic GMP and the enzyme is not activated by calmodulin. However, the kidney enzyme has a relatively low $V_{max}$ (95 min/100 μg of protein) for cyclic AMP hydrolysis compared with that we observe for the peripheral liver plasma-membrane enzyme. Furthermore, unlike the kidney enzyme, the liver enzyme exhibits non-Michaelis kinetics. We have made no attempt to undertake a detailed kinetic analysis of this enzyme, although we note that schemes have been suggested whereby monomeric enzymes could exhibit kinetics indicative of either negative or positive co-operativity (Rabin, 1967; Whitehead, 1970; Ainslie et al., 1972; Ricard et al., 1974). Detailed analysis of our initial rates gives no indication of any hysteretic behaviour, and so we tend to favour the 'mnemonic transition' concept proposed by Ricard et al. (1974). A prediction (Ricard et al., 1974) of this model is that the addition of product to the assays should cause an increase in the extent of, in this instance, negative co-operation. Indeed, 5'-AMP does apparently decrease the Hill coefficient ($h$). Furthermore, the one-substrate–one-product mnemonic mechanism (Ricard et al., 1974) predicts that product should act as a competitive inhibitor of the low-affinity state of the enzyme and as a non-competitive inhibitor of the high-affinity state. Although curved double-reciprocal plots make kinetic evaluation difficult, some information can be obtained on observation of the limiting values for both $K_m$ and $V_{max}$. (Table 2). The values for the low-affinity state ($K_2$, $V_2$) indicate that 5'-AMP acts as a competitive inhibitor. The values for the high-affinity state ($K_1$, $V_1$) are more difficult to assess, but they imply either non-competitive inhibition or mixed inhibition with weakly intersecting
lines. This may be because the forward reaction is so favoured over the reverse, and so the model of Ricard et al. (1974) would predict that 5'-AMP would tend to act as an uncompetitive inhibitor of the high-affinity state. On this basis our observation of weakly intersecting lines for inhibition of the high-affinity state by 5'-AMP for the native enzyme and the phosphorylated enzyme (Marchmont & Houslay, 1981) would be entirely consistent with this. On the other hand, cyclic GMP, which is a poor substrate for the enzyme, appears to act as a simple competitive inhibitor of both states of the enzyme (Table 2). This model, which suggests a co-operation between two different conformations of the same molecule for both substrate binding and catalysis, has been successfully applied to other enzyme systems (Meunier et al., 1974; Storer & Cornish-Bowden, 1977).

Intracellular cyclic AMP concentrations in the hepatocyte will range from about 0.3 to 4 μM, depending on the action of hormones (Blackmore et al., 1979; Smith et al., 1978), which suggests that the activity of this enzyme may be functionally relevant. Clearly its ability to act on cyclic GMP is largely irrelevant, owing to the high K_m and low V_max for action on this substrate. The range of concentrations over which we were able to study the breakdown of cyclic GMP by the enzyme was limited by solubility problems. Even though over this range the enzyme appeared to exhibit Michaelis kinetics, its K_m was such that any non-linearity, which might well be evident at higher concentrations, would have been masked.

Cyclic GMP and the product of the reaction, 5'-AMP, are weak inhibitors of this enzyme. Certainly over the range of cyclic AMP concentrations that will be encountered in vivo by this enzyme (0.3–5.0 μM) the effects of these two agents are likely to be negligible.

The peripheral enzyme is clearly quite different from the soluble, low-affinity, brain enzyme (Klee et al., 1979; Morrill et al., 1979; Sharma et al., 1980), for the brain enzyme is dimeric, consisting of two identical subunits (M_r 59 000). The soluble brain enzyme also exhibits Michaelis kinetics in the presence of its activator, calmodulin. The ratio (cyclic AMP/cyclic GMP) of the V_max of the brain enzyme for hydrolysis favours cyclic AMP breakdown (10:1), as does that of the peripheral liver enzyme (24:1). However, the brain enzyme has a much lower affinity for cyclic AMP (150 μM) and unlike the high-affinity cyclic AMP phosphodiesterases, it has a higher specificity for cyclic GMP than for cyclic AMP (K_m (cyclic GMP)/K_m (cyclic AMP) = 0.06). This enzyme has a much higher V_max (167 units/mg of protein) for cyclic AMP hydrolysis than does the peripheral enzyme. Indeed the brain enzyme appears to be typical of the low-affinity cytosol species (see Wells & Hardman, 1977), as a similar calmodulin-activated enzyme has been partially purified (80%) from bovine heart (Ho et al., 1977). This has a mol.wt. of 150000, which undoubtedly reflects a subunit structure. It also exhibits a high V_max for cyclic AMP hydrolysis (120 units/mg of protein) and has similar kinetic properties to the brain enzyme.

A third type of cyclic AMP phosphodiesterase has been identified kinetically, although it has yet to be purified to homogeneity and fully characterized. In this instance the hydrolysis of cyclic AMP normally exhibits positive co-operativity (h = 1.5). However, the presence of low concentrations of cyclic GMP (0.1–50 μM) in these assays increases the rate of hydrolysis of cyclic AMP and apparently restores Michaelis kinetics (see Wells & Hardman, 1977). This enzyme also catalyses the hydrolysis of both cyclic AMP and cyclic GMP at similar rates and has been observed in both soluble and particulate fractions from rat liver (Beavo et al., 1971). The peripheral enzyme is clearly distinct from these species.

The solubilized and purified peripheral cyclic AMP phosphodiesterase from rat liver plasma membranes may be classified as a high-affinity enzyme whose physiological role is to hydrolyse cyclic AMP rather than cyclic GMP. It bears marked resemblances to monomeric cytosol-derived high-affinity enzyme (Thompson et al., 1979), but it has distinct differences, notably in its kinetic properties, specific activity and in its ability to bind tightly to a biological membrane (see Marchmont & Houslay, 1980a). The very clear differences between all of the enzymes discussed make it extremely unlikely that the multiple forms of cyclic AMP phosphodiesterase arise from the modification of a single protein species (see Wells & Hardman, 1977, for discussion).

The purification procedure described utilizes a novel affinity column. This may be of use in the purification of cyclic AMP phosphodiesterases that are not affected by calmodulin and thus cannot be purified by using an affinity column where calmodulin is linked to Sepharose. Extremely effective purification can thus be achieved by treatments which utilize both Affi-Gel Blue and the theophylline–Sepharose columns.

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