A Peripheral and an Intrinsic Enzyme Constitute the Cyclic AMP Phosphodiesterase Activity of Rat Liver Plasma Membranes

Robert J. MARCHMONT and Miles D. HOUSLAY
Department of Biochemistry, University of Manchester Institute of Science and Technology, P.O. Box 88, Manchester M60 1QD, U.K.

(Received 31 October 1979)

1. Approx. 10% of the rat liver cellular cyclic AMP phosphodiesterase activity was associated with a plasma-membrane fraction. 2. Lineweaver–Burk plots of this activity were clearly non-linear, yielding extrapolated $K_m$ values of 0.7 and 60.6 $\mu$M. 3. Treatment of these membranes with high-ionic-strength NaCl solutions apparently released 80% of this activity assayed at 0.4 $\mu$M-cyclic AMP, and 15% of the activity assayed at 1$\mu$M-cyclic AMP. 4. The high-salt-solubilized enzyme gave a non-linear Lineweaver–Burk plot. 5. The cyclic AMP phosphodiesterase activity of the washed high-salt-treated membranes exhibited a linear Lineweaver–Burk plot, yielding a $K_m$ of 60 $\mu$M. 6. The high-salt-solubilized enzyme exhibited a single peak of activity upon polyacrylamide-gel electrophoresis, a single peak upon sucrose-density-gradient centrifugation (3.9 S) and decayed as a single exponential upon heat-treatment (half-life 1 min at 55°C). 7. The activity of washed high-salt-treated membranes decayed as a single exponential upon heat-treatment (half-life 42 min at 55°C), and was solubilized in the detergent Triton X-100. 8. Cytosol-derived cyclic AMP phosphodiesterase activity could bind to washed high-salt-treated plasma membranes, but was totally eluted by washing with 1$\mu$M-KHCO$_3$, unlike the high-salt-solubilized enzyme, which required high salt concentrations to elute it. 9. We suggest that the cyclic AMP phosphodiesterase activity of rat liver plasma membranes can be resolved into two components: a single peripheral protein exhibiting apparent negative co-operativity, that is distinct from cytosol forms, and an intrinsic protein exhibiting normal Michaelis kinetics.

Cyclic AMP has been identified as the intracellular mediator of the action of a number of hormones. Cyclic nucleotide concentrations within a tissue will depend not only on the rate of its formation, catalysed by hormone-sensitive adenylate cyclase (EC 4.6.1.1), but also on the rate of degradation. Since the hydrolysis of cyclic AMP to 5'-AMP catalysed by the cyclic AMP phosphodiesterase (EC 3.1.4.17) is the only physiological mechanism known to terminate the action of the cyclic nucleotide, it represents a potential site for the regulation of tissue cyclic AMP concentration.

There is an apparently complex pattern of cyclic AMP phosphodiesterase activity associated with rat liver. Kinetic analysis of fresh homogenates implies the existence of two enzyme forms (Spears et al., 1973; Arch & Newsholme, 1976), DEAE-cellulose chromatography of a 100 000g × 60 min supernatant derived from a sonicated homogenate resolves three forms, and treatment of the 100 000g × 60 min pellet from an homogenate with low-ionic-strength buffer releases another form (Loten et al., 1978). The derived patterns of activity in homogenates and 100 000g supernatants may well be complicated by the presence of both soluble activator and inhibitor proteins (see, e.g., Lyn et al., 1974, 1975; Teo & Wang, 1973; Kakiuchi et al., 1973; Wang & Desai, 1977).

As the site of the glucagon-stimulated synthesis of cyclic AMP occurs at the plasma membrane and because insulin binding to cell-surface receptors may decrease intracellular cyclic AMP concentration by interaction with cyclic AMP phosphodiesterase (Sakai et al., 1974; Kono et al., 1975), we have
carried out a study of the cyclic AMP phosphodiesterase activity associated with the liver plasma membrane.

Materials and Methods

Materials

Ophiophagus hannah venom and Dowex 1 anion-exchange resin were from Sigma, Kingston upon Thames, Surrey, U.K. Cyclic AMP, adenosine, triethanolamine hydrochloride and all enzymes were from Boehringer (U.K.), Lewes, East Sussex, U.K. Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of AR quality from BDH Chemicals, Poole, Dorset, U.K.

Plasma-membrane preparation

Rat liver plasma membranes were prepared from male Sprague–Dawley rats, weighing 200–300g, by a modified (Houslay et al., 1976) method of Pilkis et al. (1974). Membranes were either used fresh or after storage in liquid N₂ in 1mM-KHCO₃ buffer, pH 7.2, at protein concentrations of 5–20mg/ml. No differences in results were noted.

Distribution of cyclic AMP phosphodiesterase activity between total particulate and plasma-membrane fractions

Three rat livers were homogenized in 1mM-KHCO₃, pH 7.2, as described by Pilkis et al. (1974). Half of this homogenate was taken for the preparation of a plasma-membrane fraction (Pilkis et al., 1974) and the rest was centrifuged for 1h at 100000gₛ, to yield a supernatant and total particulate fraction.

Cyclic AMP phosphodiesterase assay

A two-step radioassay described in detail by Rutten et al. (1973) was used to determine activity in the liver homogenate, 100000g x 60 min supernatant and total particulate fraction. For routine assay of the activity associated with the liver plasma membrane, and for the activity of the high-salt-solubilized enzyme, a two-step radioassay procedure based upon that described by Thompson & Appleman (1971) was used. In the first stage, 100μl of a reaction mixture containing (final concentrations) 5mm-MgCl₂, 3.75mm-2-mercaptoethanol, cyclic [³H]AMP (approx. 100000c.p.m.), 40mm-Tris/HCl buffer, final pH 7.4, and an appropriate amount of enzyme was used. During kinetic analysis the substrate concentration range was from 4 x 10⁻⁷ to 1 x 10⁻³M. The reaction mixture was 'whirlimixed' and incubated at 30°C for an appropriate time interval, usually 30min before termination by boiling for 2min. After the reaction mixture had cooled to 4°C, 25μg of Ophiophagus hannah venom (1mg/ml) was added, and incubation of this mixture was carried out for 10min at 30°C to attain complete conversion of 5'-AMP to adenosine. After this stage, 0.4ml of a resin slurry [Dowex 1; 200–400 mesh; Cl⁻ form as resin/water (1:2, v/v)] was added to the reaction mixture. This mixture was vortex-mixed several times over a 15min period before being centrifuged at 14000gᵥ, in a Jobling 320 microfuge for 4min to sediment the resin thoroughly. Samples (150μl) of the supernatant were removed for counting to determine the [³H]adenosine in solution after removal of cyclic [³H]AMP by the resin. Corrections were made for the cyclic [³H]AMP not removed by the resin by using a boiled enzyme preparation as a blank.

Standard deviations for assays are given with (n – 1) degrees of freedom.

Dowex resin preparation

Dowex 1 (200–400 mesh; Cl⁻ form) was prepared as described by Pichard & Cheung (1976), except that 1m-NaOH and 1m-HCl were used.

Treatment of plasma membranes with NaCl solutions

To 1.5ml of a 'cocktail' containing final concentrations of 20mm-2-mercaptoethanol, various NaCl concentrations and 40mm-Tris/HCl, final pH 7.4, was added 1.5ml of the plasma-membrane preparation (4mg/ml in 1mM-KHCO₃, pH 7.2). This mixture was incubated for 45min before 1.0ml portions were loaded on to 3ml of 60% sucrose in 10mm-Tris/HCl buffer, pH 7.4. Centrifugation was carried out for 30min at 300000gᵥ at 4°C. The clear supernatant fractions were collected and tested for cyclic AMP phosphodiesterase activity (high-salt-solubilized enzyme). The membranes collecting at the interface were removed and washed twice with 1mM-KHCO₃, pH 7.2, by centrifugation at 14000gᵥ for 5min at 4°C, before final resuspension in the same buffer (washed high-salt-treated membranes). Both the high-salt-treated enzyme and the high-salt-treated membranes could be stored in liquid N₂ without affecting the results, although normally these fractions were prepared fresh for immediate use.

Routinely, 0.4m-NaCl was used in these experiments.

Treatment of washed high-salt-treated membranes with detergent

Plasma membranes after treatment with 0.4m-NaCl and subsequent washing as described above were resuspended at 10mg of protein/ml. Then 1.5ml of this preparation was rapidly mixed with an equal volume of a solution containing 1% (v/v) Triton X-100, 10mm-2-mercaptoethanol and 80mm-Tris/HCl, pH 7.4. The mixture was incubated on ice for 30min before 1ml portions were taken and
centrifuged as described above for the treatment of plasma membranes with NaCl solutions. The supernatant fractions were taken as a soluble Triton X-100 extract of washed high-salt-treated plasma membranes.

Polyacrylamide-gel electrophoresis

This was carried out on 3 or 5% gels in a continuous buffer system of 0.0746 M-glycine/10 mM-Tris/KOH, final pH 8, as described by Davis (1964). Gels were run at 1mA per tube for 90–120 min at 4°C.

Some gels were stained for protein with Amido Black [1% (w/v) in 7% (v/v) acetic acid], and excess stain was removed by soaking in 7% (v/v) acetic acid. Other gels (7.5 cm long) were cut into 1.5 mm slices and left for 24 h at 4°C in 100 μl of 5 mM-2-mercaptoethanol/40 mM-Tris/HCl buffer, pH 7.4, to elute proteins from the polyacrylamide-gel matrix before samples were taken for assay of cyclic AMP phosphodiesterase activity.

Sucrose-density-gradient centrifugation

To 100 μl of a marker-enzyme system containing 100 μg of cytochrome c (horse heart), 20 μg of malate dehydrogenase (EC 1.1.1.37; pig heart), 70 μg of lactate dehydrogenase (EC 1.1.27; rabbit skeletal muscle), 12 μg of fumarase (EC 4.2.1.2; pig heart) and 350 μg of catalase (EC 1.11.1.6; bovine liver) was added 100 μl of the cyclic AMP phosphodiesterase preparation (100 μg of protein of the high-salt-soluble enzyme or 1 mg of protein of the 100000 g × 60 min supernatant from a rat liver homogenate). This mixture was carefully layered on to 3.6 ml of a continuous linear sucrose density gradient (15–30%). The gradients were prepared from equal volumes of 15 and 30% (w/v) sucrose solutions in either 10 mM-Tris/HCl buffer, final pH 7.4, or 0.4 M-NaCl/10 mM-Tris/HCl buffer final pH 7.4. After centrifugation in an MSE 6 × 4.2 ml swingout rotor at 58000 rev./min (gav., 450000) for 20 h at 4°C, samples (150 μl) were collected from the bottom of the tube. Portions (40 μl) were taken for cyclic AMP phosphodiesterase assay. Assays for the marker enzymes were carried out as described in full previously (Houslay & Tipton, 1973). The marker proteins did not interfere with the sedimentation of the cyclic AMP phosphodiesterase activity.

The κ20w values of the marker proteins used were those published previously (see Haga et al., 1977) and were: cytochrome c, 1.91 S; malate dehydrogenase, 4.3 S; lactate dehydrogenase, 7.3 S; fumarase, 9.1 S; catalase, 11.3 S.

Protein determination

Protein determinations were carried out by the microbiuret method of Goa (1953), with bovine serum albumin as standard, as modified by Houslay & Palmer (1978).

Heat-stability

Portions (100 μl) of enzyme containing either 100 μg of protein of high-salt-solubilized enzyme or 1 mg of protein of either native plasma membranes or washed high-salt-treated membranes or the 100000 g × 60 min supernatant of a rat liver homogenate were used. These were incubated in a thermostatically controlled water bath for various time intervals at various temperatures before removal into an ice/water mixture at 0–4°C. The fractions were subsequently assayed for cyclic AMP phosphodiesterase activity.

Parenchymal-cell preparation

Isolated intact hepatocytes were prepared from 24 h-starved 200–300 g male Sprague–Dawley rats by the method of Elliott et al. (1976).

Computer-aided curve-fitting procedures

Estimates of Km and Vmax, for the intrinsic enzyme and Km, Vmax, and n (the Hill coefficient) for the peripheral enzyme activity in isolated rat liver plasma membranes were achieved by a curve-fitting procedure. A Nottingham Algorithm Group Library program (NAGFLIB: 1326/427: Mk5: Dec. 75) designated EO4 GA/AF was used. This found a least-squares solution of M non-linear equations in N variables by minimizing the sum of the squares of:

\[ F(X) = \sum_{i=1}^{M} R_i(X)^2, \quad M \geq N \]

where \( X = (X_1, X_2, \ldots, X_N)^T \) by a procedure (Fletcher, 1971) based on the method of Marquardt (1963). Subprograms for calculation of the residuals were prepared and the appropriate partial differentials calculated.

Binding of cyclic AMP phosphodiesterase activity to washed high-salt-treated plasma membranes

Washed high-salt-treated membranes and high-salt-solubilized enzyme from native plasma membranes (6 mg of protein) and a high-speed supernatant of an homogenate (100000 g × 60 min) were prepared as described above.

Washed high-salt-treated membranes (derived from 6 mg of native membrane protein) were divided into two portions (100 μl). To one portion was added 100000 g × 60 min supernatant (6 mg of protein in 400 μl containing 6 munits of enzyme activity at 1 mM-cyclic AMP substrate concentration). To the other portion was added high-salt-solubilized enzyme (0.5 mg of protein) and a sufficient volume of 1 mM-KHCO3, pH 7.2, to achieve an overall 4-fold dilution in ionic strength (from 0.4 to 0.1 M-NaCl) (400 μl overall). There was no apparent loss in the
enzyme activity of the high-salt-solubilized protein on reduction of ionic strength, and no activity sedimented under the centrifugation conditions used. Both membranes plus soluble protein mixtures were vortex-mixed and incubated for 1h at 4°C before centrifugation at 14000g for 10min at 4°C and the supernatant and membrane pellet fractions collected. The pellet was resuspended with 100μl of 1mM-KHCO₃, pH 7.2, and samples from both fractions (25μl) were assayed for cyclic AMP phosphodiesterase activity.

Membrane fractions from this stage were divided into two further portions (approx 1.5mg of protein each). One portion was incubated in a high-salt (0.4M-NaCl)-solubilization ‘cocktail’ and centrifuged at 300000gav, for 30min at 4°C as described above. The second portion was incubated in 1mM-KHCO₃, pH 7.2, and centrifuged in an identical manner.

The supernatant and membrane fractions resulting from centrifugation were collected and assayed for cyclic AMP phosphodiesterase activity at 0.4μM-cyclic AMP. The activity of the endogenous integral (high-Km) enzyme of the membranes was subtracted in all cases, and was more than 95% of the initial activity at the end of the experiment after removal of the absorbed activities.

All these fractions were prepared fresh before use. One unit of enzyme activity is defined as the amount hydrolysing 1μmol of cyclic AMP/min.

Results

Assay of cyclic AMP phosphodiesterase

It has been demonstrated that 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, causing an underestimation of enzyme activity (Lynch & Cheung, 1975). We can demonstrate that the amount of [3H]adenosine bound by the resin is significantly affected by the concentration of cyclic AMP used in the assay. For the range of cyclic AMP concentrations normally used in kinetic studies (4 × 10⁻⁴ to 1 × 10⁻³ M), the fraction of [3H]adenosine bound can vary from 50 to 41% (Fig. 1). If one fails to correct for this occurrence, then rates at low cyclic AMP concentrations would be apparently greater than reality, which could lead to a diagnosis of apparent negative co-operativity.

Furthermore it has been demonstrated (Rutten et al., 1973) that, if tissue preparations are contaminated with adenosine deaminase, then the adenosine produced in the assay system can be converted to inosine, which can become absorbed to the resin, leading to serious underestimates of the rates of reaction. To prevent the resin binding of inosine, the assay method of Rutten et al. (1973) was used to assess the cyclic AMP phosphodiesterase activity of the homogenate, total particulate fraction and 100000g × 60 min supernatant. Plasma membranes contained no detectable adenosine deaminase activity (≤0.02 μ-unit/mg of protein), and the two-step procedure of Thompson & Appleman (1971) was used routinely; this compares with an activity of 8.2 munits/mg in the 100000g × 60 min supernatant.

In all of the experiments detailed, great care was taken to assess that the rates of reaction were linear over the times of incubation. Routine incubations were carried out so that less than 10% of the cyclic AMP was degraded, and under such circumstances enzyme activity was linear for as long as 2h at 30°C.

Liver plasma membranes

Preparation of a liver plasma-membrane fraction shows that there was cyclic AMP phosphodiesterase associated with it. This comprised some 8% of the total cell cyclic AMP phosphodiesterase activity when assayed at 0.4μM-cyclic AMP and 5% at 1μM-cyclic AMP (Table 1). Although our plasma-membrane fraction appeared to be free of endoplasmic reticulum (glucose 6-phosphatase activity <0.5% of the total) and mitochondria (succin-
Table 1. Distribution of cyclic AMP phosphodiesterase activity between plasma-membrane and total particulate fractions of a rat liver homogenate

For details see the Materials and Methods section. Results are means ± S.D. for three determinations.

<table>
<thead>
<tr>
<th>Cyclic AMP assay concentration</th>
<th>Specific activity (nmol/min per mg of protein)</th>
<th>Recovery (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homogenate</td>
<td>Particulate</td>
</tr>
<tr>
<td>1 mM</td>
<td>2.92 ± 0.09</td>
<td>2.42 ± 0.08</td>
</tr>
<tr>
<td>1 µM</td>
<td>0.062 ± 0.004</td>
<td>0.056 ± 0.006</td>
</tr>
</tbody>
</table>

Table 2. Kinetic parameters of the cyclic AMP phosphodiesterase activity of rat liver

Fitting methods are: E, extrapolated lines from linear portions of the curved Lineweaver-Burk plots; CF1, computer curve fitting to the two enzyme species (see Materials and Methods section); H, Hill plot; W, method of Wilkinson (1961). Errors are ± S.D. on the averages of the calculated values for the preparations. (1) and (2) refer to the 'low-Km' and 'high-Km' enzymes respectively.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>No. of determinations</th>
<th>Fitting method</th>
<th>Km (1) (µM)</th>
<th>Km (2) (µM)</th>
<th>Vmax (1) (pmol/min per mg)</th>
<th>Vmax (2) (pmol/min per mg)</th>
<th>Hill coefficient (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native plasma membranes</td>
<td>8</td>
<td>E</td>
<td>0.9 ± 0.1</td>
<td>68.6 ± 5.2</td>
<td>108 ± 6.3</td>
<td>2542 ± 161</td>
<td>—</td>
</tr>
<tr>
<td>Hepatocyte plasma membranes</td>
<td>4</td>
<td>E</td>
<td>0.8 ± 0.1</td>
<td>70.2 ± 6.8</td>
<td>108 ± 6.3</td>
<td>2648 ± 182</td>
<td>0.48 ± 0.12</td>
</tr>
<tr>
<td>High-salt-solubilized enzyme</td>
<td>6</td>
<td>E</td>
<td>0.9 ± 0.1</td>
<td>71.8 ± 5.3</td>
<td>126 ± 18.2</td>
<td>2755 ± 121</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>Washed high-salt-treated membranes</td>
<td>6</td>
<td>W</td>
<td>—</td>
<td>58 ± 6.2</td>
<td>—</td>
<td>1321 ± 102</td>
<td>—</td>
</tr>
<tr>
<td>Triton-solubilized enzyme from washed-high-salt-treated membranes</td>
<td>4</td>
<td>W</td>
<td>—</td>
<td>72 ± 8.3</td>
<td>—</td>
<td>1133 ± 210</td>
<td>—</td>
</tr>
</tbody>
</table>

Cyclic AMP phosphodiesterase activity was proportional to protein concentration, and Lineweaver-Burk plots of this activity were clearly biphasic (Fig. 2), yielding extrapolated Km values of 0.7 and 60.6 µM. A similar result was obtained with a plasma-membrane fraction derived from isolated hepatocytes (Table 2).

Treatment of liver plasma membranes with high-salt solutions

Liver plasma membranes were treated with NaCl solutions of various ionic strengths (0.02–0.4 M) as described in the Materials and Methods section. This treatment effected the release of a fraction of the total cyclic AMP phosphodiesterase activity of the membranes. At any particular salt concentration this release was time-dependent, exhibiting a plateau at around 30–40 min. Routinely we carried out the incubations for 45 min, and Fig. 3(a) demonstrates the percentage release of activity over this time period at various concentrations of NaCl. The amount of cyclic AMP phosphodiesterase activity released reached a plateau at I > 2.5. Clearly the apparent fractional activity released depends upon the assay concentration of cyclic AMP used, being 80% at 0.4 µM-cyclic AMP and only 12% at 1 mM-

Vol. 187 N
cyclic AMP, suggesting the resolution of kinetically distinct forms.

Interestingly, the addition of Ca$^{2+}$ to these incubations inhibited the release of this cyclic AMP phosphodiesterase activity. This is shown in detail for one particular ionic strength in Fig. 3(b).

Lineweaver–Burk plots of the cyclic AMP phosphodiesterase activity of the washed high-salt-treated membranes were clearly linear over a substrate range $4 \times 10^{-7}$–$1.0 \times 10^{-3}$M-cyclic AMP (Fig. 4), yielding a single $K_m$ of 60$\mu$M (Table 2). The enzyme activity was proportional to protein con-
centration, and change in protein concentration had no effect on the form of the Lineweaver–Burk plot.

The cyclic AMP phosphodiesterase activity released from the plasma membrane by high-ionic-strength treatment yielded a non-linear Lineweaver–Burk plot (Fig. 4) indicative of either two enzyme forms or apparent negative co-operativity. Extrapolated $K_m$ values of 0.8 and 16 µM could be obtained (Table 2). As with the total activity in the plasma membrane, the activity of the high-salt-solubilized enzyme was proportional to protein concentration, and change in protein concentration had no effect on the form of the Lineweaver–Burk plot. The presence of 0.1 M-NaCl (after dilution into the assay) did not appear to alter the kinetics of the high-salt-solubilized enzyme, since dialysis had negligible effect on enzyme activity, and no effect on the form of Lineweaver–Burk plots and the kinetic constants obtained.

With hepatocyte plasma membranes as the enzyme source, similar results could be obtained for release of enzyme by high salt concentrations, the forms of the Lineweaver–Burk plots and the kinetic parameters for the various activities (Table 2), indicating that both of the enzymes are found in this single cell type.

This high-salt-solubilized enzyme was not released or leached from the membranes during the three washings with 1 mM-KHCO$_3$, involving 30 min incubations on ice, used in their preparation.

The $100000\times g \times 60$ min supernatant from a rat liver homogenate

The activity of the cyclic AMP phosphodiesterase in this fraction was clearly not linear with respect to protein concentration, and the form of the plot depended upon the cyclic AMP concentration used in the assay. At high protein concentrations (200 µg/ml), Lineweaver–Burk plots were clearly non-linear (concave down; results not shown), producing extrapolated apparent $K_m$ values of 2.7 and 44 µM (Table 2). At low protein concentrations (10 µg/ml), a linear plot resulted (results not shown), exhibiting a single $K_m$ of 44 µM (Table 2). In all cases the initial rates were obtained from linear time courses.

---

**Fig. 5. Sucrose-density-gradient centrifugation of the high-salt-solubilized enzyme**

Cyclic AMP phosphodiesterase activity was assayed at 0.4 µM (△) and 1 mM (●) cyclic AMP. The experiment was also performed with 0.4 M-NaCl in the gradient (▲) and assayed at 0.4 µM-cyclic AMP. The inset demonstrates the marker-enzyme distribution: a, cytochrome c; b, malate dehydrogenase; c, lactate dehydrogenase; d, fumarase; e, catalase; the arrow marks the position of the cyclic AMP phosphodiesterase activity. Fraction numbers were normalized against marker-protein distribution for direct comparison of runs with NaCl present and those without.
Sucrose-density-gradient centrifugation

The activity profile of the high-salt-solubilized plasma-membrane enzyme after sucrose-density-gradient centrifugation showed a single symmetrical peak of activity when fractions were assayed at either 0.4 μM or 1.0 mM cyclic AMP (Fig. 5). As demonstrated in the inset, the sedimentation coefficients of our known standards plotted against fraction number gave a linear result, allowing us to estimate the sedimentation coefficient of the high-salt-solubilized enzyme as 3.9S (±0.15, s.d., n = 4). In case some degree of aggregation had occurred because of the low-ionic-strength conditions, we ran a similar experiment with 0.4 M NaCl included, obtaining an identical result.

When a fraction of the 100000g x 60 min super-

---

**Fig. 6. Polyacrylamide-gel electrophoresis of the high-salt-solubilized enzyme**

Cyclic AMP phosphodiesterase activity at 1 mM (●) and 0.4 μM (▲) cyclic AMP concentrations from individual gel slices is shown.

**Fig. 7. Heat-treatment of cyclic AMP phosphodiesterase**

These were carried out as described in the Materials and Methods section, using cyclic AMP concentrations of 0.4 μM (▲, △) or 1 mM (●, ○) in the assays. (a) Native plasma-membrane fraction at 55°C; (b) high-salt-solubilized enzyme at 55°C (▲, ●) and 45°C (△, ○); (c) washed high-salt-treated membranes at 55°C (△, ○) and 60°C (▲, ●); (d) 100000g x 60 min supernatant of a rat liver homogenate at 55°C. Errors are given as ± S.D. (n = 6) for three different membrane preparations.
natant of a rat liver homogenate was run in the same manner, two peaks of enzyme activity were found. These had sedimentation coefficients of 3.55 S (±0.16, s.d., n = 5) and 6.8 S (±0.1, s.d., n = 5). Both activity peaks were observed at low (0.4 μM) or high (1.0 mM) cyclic AMP assay concentrations, although the relative proportions of activity were dependent on the substrate concentrations employed.

The rates of reaction of cyclic AMP phosphodiesterase activity were linear with respect to time for all of these experiments.

**Polyacrylamide-gel electrophoresis of high-salt-solubilized enzyme**

Treatment of this enzyme by polyacrylamide-gel electrophoresis and elution of the enzyme activity from gel segments, clearly demonstrated a single symmetrical peak of activity whether the enzyme was assayed with 0.4 μM- or 1.0 mM-cyclic AMP (Fig. 6). In all cases rates were taken from linear time courses.

**Heat inactivation of cyclic AMP phosphodiesterase activity**

Semi-logarithmic plots of percentage of enzyme activity against time for native membranes incubated at 55°C are shown in Fig. 7(a). These plots were clearly biphasic whether 0.4 μM- or 1.0 mM-cyclic AMP was used as the assay substrate. At the low substrate concentration there was an initial rapid loss of 85–90% of the total activity, followed by a slow decay of the remainder (Fig. 7a). With 1 mM-cyclic AMP, the initial rapid loss of activity accounted for only 10–15% of the total, the remainder decaying at a slower rate (Fig. 7a). From both plots one could estimate the half-life of the first component to be about 1 min and that of the second component to be 44 min (Table 3).

The cyclic AMP phosphodiesterase activity of the washed salt-treated membranes (Fig. 7c) decayed exponentially with time, irrespective of the substrate concentration used. At 55°C the half-life obtained was 42.5 min.

When the high-salt-solubilized enzyme was heat-treated at either 55°C or 45°C, the semi-logarithmic plots were linear (Fig. 7b), even when more than 90% of the activity was destroyed, yielding half-lives of 1.0 min and 6.1 min respectively. These results were identical irrespective of whether the assay cyclic AMP concentration was 1.0 mM or 0.4 μM (Fig. 7b and Table 3).

With the 100000 g, x 60 min supernatant of rat liver homogenate as the source of enzyme, heat-treatment at 55°C indicated two forms of the enzyme, with half-lives of 3.2 min and 10.4 min (Fig. 7d) with 1 mM-cyclic AMP.

**Triton X-100 treatment of washed high-salt-treated plasma membranes**

Exposure of the washed high-salt-treated plasma membranes with increasing concentrations of the non-ionic detergent Triton X-100 clearly resulted in the liberation of the residual cyclic AMP phosphodiesterase activity (Fig. 8). This solubilized enzyme yielded linear Lineweaver–Burk plots with $K_m$ 72 μM, a slight increase over that of the membrane-bound form (Table 2).

**Rebinding of cyclic AMP phosphodiesterase activity**

Table 4 shows the results of a typical rebinding experiment of cyclic AMP phosphodiesterase activity from solutions of either the high-salt-solubilized enzyme or the 100000 g, x 60 min super-

---

**Table 3. Heat-stability of the cyclic AMP phosphodiesterase**

Errors are given as ± s.d. obtained on average slopes of six decay plots derived from three different enzyme preparations.

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Incubation temperature (°C)</th>
<th>Assay concentration of cyclic AMP</th>
<th>Half lives of components (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>Native plasma-membrane fraction</td>
<td>55</td>
<td>0.4 μM</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>1.0 mM</td>
<td>1.1 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>0.4 μM</td>
<td>10 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>1.0 mM</td>
<td>10 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.4 μM</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>1.0 mM</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td>High-salt-solubilized enzyme</td>
<td>55</td>
<td>0.4 μM</td>
<td>42.5 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>1.0 mM</td>
<td>42.5 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.4 μM</td>
<td>12 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.0 mM</td>
<td>12 ± 0.5</td>
</tr>
<tr>
<td>Washed high-salt-treated membranes</td>
<td>55</td>
<td>0.4 μM</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>1.0 mM</td>
<td>10.2 ± 0.3</td>
</tr>
<tr>
<td>100000 g, x 60 min supernatant of an homogenate</td>
<td>55</td>
<td>1.0 mM</td>
<td>3.1 ± 0.3</td>
</tr>
</tbody>
</table>

Vol. 187
nant of a rat liver homogenate (cytosol-derived enzyme). Substantial portions of the activity added became bound to the membranes during the in-

Fig. 8. Treatment of washed high-salt-treated membranes with detergent
A washed high-salt-treated plasma-membrane fraction (1 mg of protein/ml) was treated with various Triton X-100 concentrations, and, after centrifugation, the released and residual activities were analysed. The cyclic AMP assay concentration was 1 mM. Protein concentration was 5 mg/ml. ●, Activity remaining in membranes; ○, activity solubilized.

cubation. The recoveries of 90% indicated that little activity was lost during the procedure.

Upon washing the membrane pellet containing the bound enzyme activity with 1 mM-KHCO₃, pH 7.2, which was part of our routine in plasma-membrane preparation (Houslay et al., 1976), all of the cytosol-derived enzyme was eluted from the membrane, whereas little if any of the high-salt-solubilized enzyme was released.

Under conditions where both membrane pellets were washed with the high-salt-(0.4 M-NaCl) solubilization 'cocktail', all of the bound cytosol-derived enzyme and all of the bound high-salt-solubilized enzyme was eluted.

In performing these experiments with the high-salt-solubilized enzyme, the conditions used were not optimized for binding. We used a final NaCl concentration of 0.1 M, conditions under which we could only have expected a maximum of 44% of the enzyme to be bound (see Fig. 3a) compared with the 30% obtained, and also we used a dilute protein solution. The purpose of this was to avoid any self-aggregation phenomena that could lead to artifacts. Under these conditions, less than 1% of the enzyme activity could be sedimented by centrifugation at 100 000g x 60 min at 4°C (in the absence of added membranes). We noted, however, that dialysis overnight against 1 mM-KHCO₃, pH 7.2, did cause some aggregation of our high-salt-solubilized enzyme in concentrated solutions. That rebinding did occur under the conditions used and to such a high

Table 4. Binding of cyclic AMP phosphodiesterase activities to washed high-salt-treated plasma membranes
For details see the Materials and Methods section.

<table>
<thead>
<tr>
<th>Assay concentration of cyclic AMP</th>
<th>High-salt-solubilized enzyme</th>
<th>100 000g x 60 min supernatant of an homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubation of soluble enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>with washed high-salt-treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>membranes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bound (%)</td>
<td>1 μM</td>
<td>30</td>
</tr>
<tr>
<td>Remaining in solution (%)</td>
<td>1 mM</td>
<td>27</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>1 μM</td>
<td>60.2</td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>60.5</td>
</tr>
<tr>
<td>% of bound remaining bound</td>
<td>1 μM</td>
<td>90.6</td>
</tr>
<tr>
<td>% of bound being released</td>
<td>1 mM</td>
<td>87.1</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membranes washed in 1 mM-KHCO₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>buffer, pH 7.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of bound remaining bound</td>
<td>1 μM</td>
<td>96</td>
</tr>
<tr>
<td>% of bound being released</td>
<td>1 mM</td>
<td>100</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membranes washed in high-salt-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>solubilization 'cocktail'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of bound remaining bound</td>
<td>1 μM</td>
<td>&lt;1.5</td>
</tr>
<tr>
<td>% of bound being released</td>
<td>1 mM</td>
<td>2.3</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of bound remaining bound</td>
<td>1 μM</td>
<td>&lt;1.5</td>
</tr>
<tr>
<td>% of bound being released</td>
<td>1 mM</td>
<td>&gt;98</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1980
extent (68% of the theoretical) indicates a high affinity of this membrane for this enzyme.

Discussion

We have demonstrated that in a purified plasma-membrane preparation there are two forms of cyclic AMP phosphodiesterase activity. These are an intrinsic membrane enzyme, which exhibits normal Michaelis kinetics and requires detergent to solubilize it, and a peripheral enzyme, which exhibits kinetics indicative of negative co-operativity and is solubilized under conditions of high ionic strength. The peripheral enzyme is apparently a single enzyme species, as it sedimented as a single peak upon continuous-sucrose-density-gradient centrifugation, migrated as a single species upon polyacrylamide-gel electrophoresis and decayed as a single exponential upon thermal denaturation. The form of the Lineweaver–Burk plots may then indicate apparent negative co-operativity, although, considering the small size of the enzyme (3.9 S), it is possible that it may turn out to be a monomeric enzyme exhibiting a mnemonic mechanism (see e.g. Ricard et al., 1974; Storer & Cornish-Bowden, 1977).

The peripheral enzyme is not an adsorbed species of the cytosol enzyme, for our re-binding studies demonstrate quite clearly that, although cytosol enzyme can be adsorbed to membranes treated with high-ionic-strength solutions, it was efficiently removed after a low-ionic-strength wash similar to that used in the preparation of plasma membranes, whereas the resorbed high-salt-solubilized enzyme was not. Furthermore, the cytosol enzymes exhibited heat sensitivities and $s$ values unlike those of the peripheral enzyme. This peripheral enzyme is also distinct from the EDTA-released enzyme from a 100 000 g × 60 min pellet characterized by Loten et al. (1978), which exhibited very different $K_m$ and $s$ values and sensitivity to thermal denaturation. Presumably the enzyme of Loten et al. (1978) was either an adsorbed cytosol species or one associated with other intracellular membranes.

Interestingly the Arrhenius-plot behaviour of the plasma-membrane cyclic AMP phosphodiesterase activity assayed under conditions in which more than 90% of the activity reflected that of the intrinsic protein has suggested that, although this enzyme is firmly bound to the liver plasma membrane in both hamsters and rats, it is exclusively localized on the inner half of the bilayer (Houslay & Palmer, 1978; Houslay, 1979). Its activity senses lipid-phase separations occurring in the cytosol facing half of the lipid bilayer only, and is modified by changes in bilayer fluidity that can be effected with the neutral local anaesthetic benzyl alcohol (Gordon et al., 1979).

Having resolved the cyclic AMP phosphodiesterase activity of the rat liver plasma-membrane enzyme fraction into these two forms, we can derive a $K_m$ and $V_{max}$ for both species and a Hill coefficient for the peripheral enzyme. By using a curve-fitting procedure, it is possible to calculate the kinetic parameters for the $K_m$ and $V_{max}$ of the intrinsic enzyme and the $K_m$, $V_{max}$ and Hill coefficient for the peripheral enzyme from Lineweaver–Burk plots of the cyclic AMP phosphodiesterase activity of the native plasma membranes. These data are given in Table 2, together with the estimated $K_m$ and $V_{max}$ values that can be obtained by extrapolation from 'linear' portions of the graphs. These are given purely for comparison with the calculated ones, as such a method is usually used by workers in this field (see e.g. Appleman & Terasaki, 1975).

We see also that attachment of the peripheral enzyme to the membrane has little effect on its kinetic behaviour. The reason for the attachment of this enzyme to the plasma membrane may well be to localize it or perhaps to modulate its activity by interaction with the insulin receptor.

We thank Dr. K. R. F. Elliott and Dr. J. D. Craik for preparation of rat hepatocytes. M.D.H. thanks the M.R.C. for a project grant.

References


Vol. 187