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Submitochondrial localization and asymmetric disposition of two peripheral cyclic nucleotide phosphodiesterases

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There are two distinct cyclic AMP phosphodiesterases associated with the liver mitochondrion: one with the outer membrane and one with the inner membrane. No activity is associated with the lysosomal fraction. Both of the enzymes are peripheral proteins and can be released from the membranes by high-ionic-strength treatment. Treatment of intact mitochondria with trypsin and insoluble trypsin localizes these enzymes to the cytosol-facing surface of their respective membranes. The enzymes differ in regard to sedimentation coefficient, thermostability and susceptibility to inactivation by trypsin. Both enzymes degrade cyclic AMP and cyclic GMP. Whereas the outer-membrane enzyme displays Michaelis kinetics and appears to be a low-affinity enzyme, the inner-membrane enzyme displays kinetics indicative of apparent negative co-operativity.

Cyclic AMP plays a crucial regulatory role in cell metabolism and function in the liver and in many other organs (Robison *et al.*, 1971).

A considerable amount is known about adenylate cyclase, the enzyme responsible for the intracellular production of cyclic AMP, whose activity is closely regulated by hormones, neurotransmitters, guanine nucleotides and membrane fluidity (Ross & Gilman, 1980). However, with the exception of certain cells that can actively extrude cyclic AMP (Brunton & Mayer, 1979), the hydrolysis of cyclic AMP to AMP by the cyclic AMP phosphodiesterase activity within the cell provides us with the only known mechanism of lowering intracellular cyclic AMP concentrations. Although it has been recognized for many years that tissues contain multiple forms of this enzyme, their number, properties, location and regulation are as yet ill-understood (Appleman *et al.*, 1973; Wells & Hardman, 1977; Thompson & Strada, 1978). Indeed it is only more recently that certain soluble (Klee *et al.*, 1979; Morrill *et al.*, 1979; Thompson *et al.*, 1979) and membrane-bound (Marchmont *et al.*, 1981) forms have been purified to homogeneity.

In the main, studies on cyclic AMP phosphodiesterases have focused on either the activity in total membrane fractions or that in total soluble fractions (see Thompson & Strada, 1978). However, in order to appreciate the role of these enzymes within the cell and their regulation by hormones, it is necessary to define in a precise fashion the subcellular localization of the multiple forms of cyclic AMP phosphodiesterase. This is of particular importance, if, as is suggested by theoretical studies (Fell, 1980), discrete pools of membrane-bound cyclic AMP phosphodiesterase could lead to gradients of cyclic AMP concentrations within the cell. These might provide a means for the local modulation of metabolic processes occurring in different intracellular compartments.

By using the approach of purifying individual membrane fractions we have shown that associated with the liver plasma membrane are two cyclic nucleotide phosphodiesterases capable of hydrolysing cyclic AMP (Marchmont & Houslay, 1980*a*). These are a high-K_m integral enzyme and a low-K_m peripheral enzyme. The peripheral enzyme has been purified to homogeneity (Marchmont & Houslay, 1980*b*, 1981). In the present study we have attempted to identify the cyclic nucleotide phosphodiesterase activity associated with rat liver mitochondria.

Materials and methods

Materials

Cyclic AMP, cyclic GMP, guanosine, triethanolamine hydrochloride, NAD^+ and $NADP^+$ were all obtained from Boehringer, Lewes, East Sussex, U.K. Snake venom (*Ophiophagus hannah*), Dowex 1 anion-exchange resin, ADP, 2,4-dinitrophenol, ammonium persulphate, trypsin inhibitor (soya-bean, type 1-S), insoluble trypsin attached to polyacryl-

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amide beads, trypsin (bovine pancreas, type 1) and all marker enzymes and assay substrates were from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. Acrylamide, bisacrylamide and *NNN'N'*-tetramethylethylenediamine were from Bio-Rad Laboratories, St. Albans, Herts., U.K. Cyclic [8-^{3}H]AMP, cyclic [8-^{3}H]GMP and [8-^{3}H]guanosine were from The Radiochemical Centre, Amersham, Bucks., U.K. Bovine brain calmodulin was kindly given by Dr. R. Grand, University of Birmingham, and trifluoperazine was kindly given by Smith, Kline & French, Welwyn Garden City, U.K. All other chemicals were of AnalaR quality from BDH Chemicals, Poole, Dorset, U.K.

Assay of cyclic nucleotide phosphodiesterase activity

The routine assay of cyclic nucleotide phosphodiesterase activity of rat liver mitochondria and mitochondrial subfractions was performed by a two-step radioassay procedure based on that described by Thompson & Appleman (1971), as described by us in detail previously (Marchmont & Houslay, 1980*a*). Appropriate corrections were made for the binding of [^{3}H]adenosine to the resin under various assay conditions (Marchmont & Houslay, 1980*a*). Cyclic AMP and cylic GMP were used as substrates and the effect of cyclic GMP on the binding of guanosine by the Dowex 1 anion-exchange resin was determined by a procedure outlined previously by us for adenosine (Marchmont & Houslay, 1980*a*). It was found that 60% of the total guanosine present in the incubation was bound to the resin. This fraction remained constant even though the cyclic GMP concentration was varied from 1mM to 0.4μM in the incubation mixture. To determine the phosphodiesterase activity of crude liver homogenates the two-step radioassay method described by Rutten *et al.* (1973) was used so as to obviate the difficulty caused by contaminating adenosine deaminase. Protein determinations were made by a modified micro-biuret method (Houslay & Palmer, 1978), with bovine serum albumin as standard.

Preparation of mitochondria

(1) *Isolation.* Rat liver mitochondria were prepared from male Sprague–Dawley rats, weighing 200–300g. The rats were killed by decapitation and the livers rapidly removed into ice-cold 0.25M-sucrose/20mM-Tris/HCl buffer containing 5mM-$MgCl_2$, final pH7.4. The liver was chopped into pieces, rinsed with the ice-cold sucrose/Tris/$MgCl_2$ buffer and diluted with 6vol. of ice-cold buffer. The liver suspension was homogenized at 750rev./min in a pre-cooled Potter–Elvehjem homogenizer and Teflon pestle, six up and six down strokes being used once the liver had been dispersed. The homogenate was centrifuged at 600*g* for 10min at 4°C. The pellet was discarded and the supernatant was centrifuged at 10000*g* for 10min at 4°C to obtain the mitochondrial pellet. This was gently resuspended in the centrifuge tube, with the cooled homogenizer pestle, to half the original volume with ice-cold sucrose/Tris/$MgCl_2$ buffer and centrifuged at 10000*g* for 15min. The supernatant was discarded and the washing procedure repeated twice. After each wash a 0.5ml sample of the supernatant and pellet was retained and checked for lactate dehydrogenase contamination from the cytosol. Either the washed mitochondrial pellet was resuspended in the ice-cold sucrose/Tris/$MgCl_2$ buffer, pH7.4 (approx. 0.90mg/100μl), and assayed immediately for phosphodiesterase activity, or the mitochondria were used in the mitochondrial subfractionation procedure. In some instances the resuspended washed mitochondrial fractions were stored frozen in liquid N_2.

(2) *Isolation of mitochondrial subfractions.* The subfractionation procedure used was the 'shrink–swell' method described by Sottocasa *et al.* (1967), which, unlike other methods, does not utilize detergent or digitonin.

(3) *Preparation of mitochondrial and mitochondrial inner-membrane freeze–thawed supernatant fraction.* The mitochondrial resuspension (approx. 0.90mg/100μl) or the mitochondrial inner-membrane preparation (0.60mg/100μl) was frozen and thawed twice by immersion into liquid N_2 with slow thawing at room temperature. The membranes were pelleted on to a layer of 60% (w/v) sucrose in 40mM-Tris/HCl buffer, pH7.4, by centrifugation at 300000$g_{av.}$ for 1h at 4°C. The supernatant was tested for succinate dehydrogenase contamination and either used immediately or stored in liquid N_2.

Thermal denaturation procedure

Samples (100–200μl) of the mitochondrial outer-membrane fraction (approx. 0.10mg/100μl), mitochondrial inner-membrane frozen–thawed supernatant (approx. 0.40mg/100μl) or total mitochondrial fraction (0.90mg/100μl) were incubated at various temperatures over a range of time intervals, then cooled to 4°C before being assayed.

Preparation of 'total mitochondrial' and 'total lysosomal' fractions

Fractions were prepared as described by Bergeron *et al.* (1973). The final 'total mitochondrial' and 'total lysosomal' pellets were resuspended in ice-cold 0.25M-sucrose/20mM-Tris/HCl buffer containing 5mM-$MgCl_2$, final pH7.4, and assayed for succinate dehydrogenase, β-galactosidase and cyclic AMP phosphodiesterase (1mM-cyclic AMP) activities.

Treatment of mitochondrial membranes with high- and low-ionic-strength solutions

Mitochondrial outer-membrane and inner-membrane fractions were treated with either high- or low-ionic-strength solutions consisting of 0.4 M-NaCl in 10 mM-Tris/HCl buffer, final pH 7.4, or 1 mM-$KHCO_3$, pH 7.4, respectively as described by Marchmont & Houslay (1980*a*).

Sucrose-density-gradient centrifugation

This was performed with the use of various markers as described by Marchmont & Houslay (1980*a*) with a 15–30% (w/v) continuous linear sucrose gradient.

Polyacrylamide-gel electrophoresis

This was performed under non-denaturing conditions as described previously by us (Marchmont & Houslay, 1980*a*).

Treatment with trypsin

Soluble trypsin (final concentrations of 76.8, 140.8 and 281.6 units/ml) and insoluble trypsin bound to polyacrylamide beads (final concentrations of 6.25, 1.25 and 0.625 units/ml) were used in the experiments. Insoluble trypsin was washed three times in ice-cold 0.25 M-sucrose/20 mM-Tris/HCl buffer containing 5 mM-$MgCl_2$, final pH 7.4, by centrifugation in a Jobling micro-centrifuge at 14000 $g_{av.}$ for 10 min at 4°C before use in order to remove any trypsin that may not have been attached to the polyacrylamide beads. A 1 ml sample of the enzyme preparation, i.e. intact mitochondria (approx. 0.9 mg/100 μl), mitochondrial outer membrane (0.10 mg/100 μl) or mitochondrial inner-membrane frozen–thawed supernatant (0.40 mg/100 μl), was added to the trypsin preparation. This was then incubated at 30°C with continuous stirring. Portions (70 μl) of the incubation mixture were at various time intervals immediately mixed with 10 μl portions of trypsin inhibitor (1.0 mg/ml final concentration) and kept on ice before being assayed. Control blanks containing trypsin together with inhibitor were used. No changes in activity occurred with control systems.

Determination of the mitochondrial coupling state

This was determined by the method of Chance & Williams (1955). The degree of coupling of mitochondria isolated in the 0.25 M-sucrose/20 mM-Tris/HCl buffer containing 5 mM-$MgCl_2$, final pH 7.4, was tested. Coupling was also tested after incubation of the mitochondria for 30 min at 30°C in either the phosphodiesterase assay medium or the 'coupling assay' medium and subsequent pelleting and resuspension in 0.25 M-sucrose/Tris/HCl buffer, pH 7.4, containing 5 mM-$MgCl_2$.

Other enzyme assays

Adenylate kinase activity was assayed by the modified method of Haslam & Mills (1967), succinate dehydrogenase activity by the modified method of Bachmann *et al.* (1966), monoamine oxidase activity as described by Houslay (1980), glucose 6-phosphatase as described by Houslay & Palmer (1978) and adenosine deaminase activity as described by us previously (Marchmont & Houslay, 1980*a*).

Results

Cyclic nucleotide phosphodiesterase assay conditions

In all the experiments, the reaction rates were taken from linear initial rates. Reaction velocity was linearly related to protein concentration. All results are averages of duplicate or triplicate determinations.

The presence of adenosine deaminase in the enzyme preparations can result in underestimations of the phosphodiesterase reaction rates (Rutten *et al.*, 1973). No detectable adenosine deaminase activity (<0.02 μunit/mg of protein) was found in the mitochondrial preparations, compared with an activity of 9.4 munits/mg of protein in the 100000 $g \times$ 60 min supernatant fraction of rat liver cells. The mitochondrial fractions were washed before use as described in the Materials and methods section, until the cytosolic lactate dehydrogenase contamination present in the first mitochondrial pellet fell from 0.46 μunit/mg of protein to an undetectable value (<0.2 nunit/mg of protein). Contamination by the endoplasmic reticulum and lysosomes, as detected by glucose 6-phosphatase and β-galactosidase assays respectively, showed that <2% of the total cellular endoplasmic reticulum and <8% of the total cellular lysosomes were associated with the mitochondrial preparations. The 'total mitochondria' and 'total lysosomal' fractions obtained by the procedure of Bergeron *et al.* (1973) showed that the mitochondrial fraction contained >99% of the sum of the phosphodiesterase activities of the 'total mitochondrial' and 'total lysosomal' fractions.

Distribution of phosphodiesterase and marker-enzyme activity in mitochondrial subfractions

Mitochondrial subfractions were isolated. These consisted of a supernatant fraction that contained the enzymes in the intermembrane space, a fraction containing the outer membranes and one containing mitochondrial inner-membrane vesicles partially deprived of outer membranes. The distribution of cyclic AMP phosphodiesterase activity and that of the marker enzymes is presented in Table 1.

The specific-activity distribution of the marker enzymes monoamine oxidase, succinate dehydro-

Table 1. *Distribution of phosphodiesterase and marker enzyme activities in submitochondrial fractions*
For full experimental details see the text. Cyclic AMP phosphodiesterase activity was assayed at 1 mM-cyclic AMP. Similar results were obtained with 1 mM-cyclic GMP. N.D., Not detected (<1%); —, not determined.

	Activity (% of total activity)			Specific activity (nmol/min per mg of protein)		
Enzyme	Supernatant	Outer membranes	Inner membranes	Supernatant	Outer membranes	Inner membranes
Cyclic AMP phosphodiesterase	1.8	12.8	74.6	0.36	0.97	1.39
Malate dehydrogenase	3.6	3.6	83.7	270	90	730
Succinate dehydrogenase	N.D.	N.D.	90.2	—	—	11.5
Adenylate kinase	12.6	34.2	41.4	9.8	7.0	2.8
Monoamine oxidase	N.D.	43.2	44.1	—	4.27	1.39

genase, malate dehydrogenase and adenylate kinase show that we have resolved the outer-membrane fraction, inner-membrane-plus-matrix fraction and intermembrane-space fraction respectively. The monoamine oxidase distribution indicates that, on average, 49% of the outer membranes have been released, and the succinate dehydrogenase distribution shows that these are free of contamination by inner membranes. The presence of adenylate kinase activity in the outer-membrane fraction is a contaminant from the supernatant fraction (see Sottocasa *et al.*, 1967) and could be removed (>96%) by washing with low-ionic-strength buffers (see the Materials and methods section), whereas the phosphodiesterase activity was not so removed (<5% eluted).

On the basis of the monoamine oxidase marker-enzyme distribution pattern, the phosphodiesterase activity associated with the inner membranes was determined by subtracting the phosphodiesterase activity contributed by the contaminating outer membranes from the total phosphodiesterase activity of the inner-membrane fraction. Thus, at 1 mM-cyclic AMP substrate concentration, 81% of the total mitochondrial phosphodiesterase activity was found to be associated with the inner membranes, and the remaining 19% was located in the mitochondrial outer membranes.

Effect of high- and low-ionic-strength solutions and freeze–thawing on the mitochondrial outer and inner membranes

To determine whether the phosphodiesterase enzyme in the outer membrane was a peripheral rather than an integral enzyme, the membranes were treated with high- and low-ionic-strength solutions. When assayed at 1 mM-cyclic AMP, >98% of the total phosphodiesterase activity remained associated with the outer-membrane pellet after a low-ionic-strength wash. However, after treatment of the membranes with the high-ionic-strength solution, >96% of the total outer-membrane phosphodiesterase activity was solubilized and found to be present in the supernatant fraction.

Treatment of the mitochondrial inner-membrane fraction gave similar results. Again none of the activity (<3%) was released by the low-ionic-strength wash, but >95% of the activity was released from the inner-membrane fraction after high-ionic-strength treatment. Since the distribution of monoamine oxidase, a marker enzyme for the outer membrane, showed that 50% of the outer membranes were still associated with the inner-membrane fraction, we can calculate that only 11% of the total phosphodiesterase activity in this fraction was contributed by the outer membranes. If only the outer-membrane enzyme were peripheral, then only 11% of the total mitochondrial inner-membrane phosphodiesterase activity would be expected to be released. This clearly was not the case, and thus the inner-membrane enzyme must also be peripheral.

In contrast with the similar responses of these activities to release effected by high-ionic-strength solutions, freeze–thawing of the outer-membrane and inner-membrane fractions in liquid N_2 released <5% of the cyclic AMP phosphodiesterase activity from the outer-membrane fraction and >80% of the activity of the inner-membrane fraction. This indicates, and is confirmed by our later trypsin- and heat-denaturation studies, that freeze–thawing selectively releases the inner-membrane peripheral enzyme.

Asymmetrical distribution

To determine the sidedness of these enzymes on the membranes, intact mitochondria, outer membranes and an inner-membrane frozen–thawed supernatant fraction were treated with either a soluble trypsin preparation or an insoluble trypsin preparation where the enzyme was attached to polyacrylamide beads. The intact mitochondria were tested for adenylate kinase (intermembrane space) and malate dehydrogenase (matrix) latency both

Table 2. *Sensitivity of mitochondrial cyclic nucleotide phosphodiesterase activity to trypsin*
For full experimental details see the text. Enzyme assays were performed with 1 mM-cyclic AMP. Three different experiments were conducted with duplicate assays. Means ± S.D. ($n = 3$) are given. The 'fast' decaying component is due to the inner-membrane enzyme, and the 'slow' decaying component is due to the outer-membrane enzyme. Abbreviation: IMEFTS, mitochondrial inner-membrane freeze–thawed supernatant.

Trypsin used	Preparation	Final concn. of trypsin (units/ml)	Half-life (min)	
			Fast component	Slow component
Soluble trypsin	Mitochondria	76.8	8.50 ± 0.04	30 ± 1.3
		140.8	6.50 ± 0.06	21 ± 0.9
		281.6	4.50 ± 0.03	10 ± 1.4
	Outer membranes	76.8	6.50 ± 0.03	
		140.8	5.25 ± 0.02	
		281.6	4.25 ± 0.01	
	IMEFTS	76.8		28.5 ± 1.00
		140.8		19.0 ± 0.05
		281.6		6.8 ± 0.01
Insoluble trypsin	Mitochondria	0.625	27.5 ± 0.91	
		1.25	11.0 ± 0.60	100 ± 6.3
	Outer membranes	0.625	29.0 ± 0.30	
		1.25	13.0 ± 0.50	
	IMEFTS	0.625		49.0 ± 2.1
		1.25		26.0 ± 0.80

before and after trypsin treatment to assess mitochondrial integrity.

The cyclic AMP phosphodiesterase activity associated with all fractions was inactivated by soluble trypsin (Table 2 and Fig. 1). The linear first-order decay plots observed with either outer membranes or the inner-membrane freeze–thawed supernatant indicated that each contained a single distinct species of cyclic AMP phosphodiesterase. The initial rapid rate of decrease in enzyme activity seen for whole mitochondria closely follows that observed with isolated outer membranes, whereas the activity exhibiting a lower sensitivity to trypsin, in the second part of the curve, identifies the action of trypsin on the inner-membrane enzyme (Table 2). From the activity decay curves obtained with whole mitochondria (Fig. 1) it is possible to make an estimate of the relative proportions of the two mitochondrial phosphodiesterase enzymes. This shows that some 20% of the total mitochondrial phosphodiesterase activity is associated with the outer membrane and 80% with the inner membrane.

At all three concentrations of soluble trypsin used, adenylate kinase activity was detected in the mitochondrial supernatants, indicating that soluble trypsin had disrupted the mitochondrial outer membrane. However, soluble trypsin did not disrupt the inner membrane, for not only was malate dehydrogenase latency (>95%) preserved but there was no change (<4%) in total malate dehydrogenase activity, whereas in disrupted mitochondria under similar conditions it was decreased by some 50% over the time of this incubation.

The phosphodiesterase activity of both the outer-membrane and the inner-membrane freeze–thawed supernatant was inactivated by insoluble trypsin (Fig. 1 and Table 2). As in the experiments with soluble trypsin, the outer-membrane phosphodiesterase enzyme shows a greater trypsin-sensitivity, i.e. has a shorter half-life, than the inner-membrane enzyme (Table 2).

A high concentration (1.25 units/ml) of insoluble trypsin yielded very similar effects to the soluble enzyme (Fig. 1 and Table 2), showing that the outer membrane had become leaky, with a release of >90% of the adenylate kinase activity, whereas the inner membrane remained intact, with >95% latency of malate dehydrogenase. Again >80% of the phosphodiesterase activity could be accredited to the inner-membrane enzyme. However, treatment of intact mitochondria with a low concentration (0.625 unit/ml) of insoluble trypsin showed that, after an initial rapid decrease, the phosphodiesterase activity remained constant at 80% of the initial enzyme activity (Fig. 1 and Table 2). In this case full latency was retained by both adenylate kinase (>95%) and malate dehydrogenase (>95%), demonstrating that both membranes remained intact under these conditions.

In all of these experiments the total malate dehydrogenase and adenylate kinase activities were assessed in the mitochondrial fraction both at the beginning and at the end of the experiment by treatment with 1% Triton X-100. Latency was calculated from the amount of enzyme remaining in the supernatant after centrifugation of the mitochondria at 10000 *g* for 15 min. In no instance was there any loss in total malate dehydrogenase activity

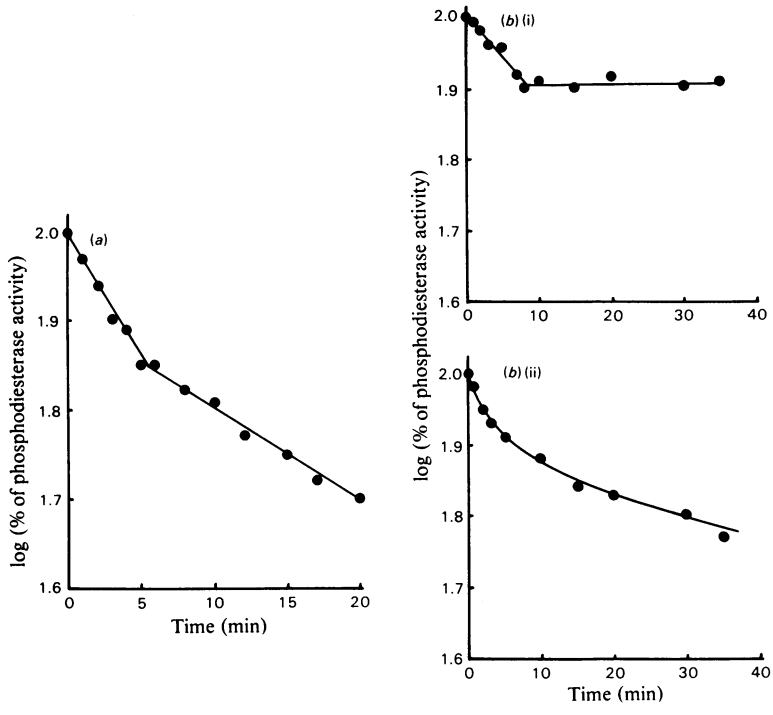

Fig. 1. *Trypsin inactivation of mitochondrial cyclic nucleotide phosphodiesterase*
(*a*) Effect of soluble trypsin at 76.8 units/ml, and (*b*) effect of insoluble trypsin at (i) 0.625 unit/ml and (ii) 1.25 units/ml. For full experimental details see the text. Activity was followed with 1 mM-cyclic AMP as substrate. The results represent averages of three different mitochondrial experiments with duplicate assays.

Table 3. *Thermal inactivation of mitochondrial cyclic nucleotide phosphodiesterase activity*
For full experimental details see the text. Cyclic AMP phosphodiesterase activity was measured at 1 mM substrate concentration. The values given represent the means for duplicate experiments. Biphasic plots were separated into fast and slow components representing the two enzymes present in whole mitochondria. Their respective contributions to the total enzyme activity at 1 mM-cyclic AMP substrate concentration are given after extrapolation from linear portions of the plot. Between 17 and 19 time-points were tested over a 50 min period for the biphasic and slow-decaying activities. Seven time-points were tested for the fast-decaying components. Abbreviation: IMEFTS, mitochondrial inner-membrane freeze–thawed supernatant.

		Half-life of thermal inactivation (min)		Contribution of each component to the total activity (%)	
Fraction	Incubation temperature (°C)	Fast component	Slow component	Fast component	Slow component
Mitochondria	55	1.2	Thermostable	90.6	9.4
	60	0.4	7.5	90.0	10.0
IMEFTS	55	2.5	—	100	—
Outer membranes	55	—	TS	—	100
	60	—	7.5	—	100

or in latency, whereas exposure of detergent-treated mitochondria to trypsin, at all concentrations studied, did lead to activity loss. Adenylate kinase activity was, however, activated by trypsin under the conditions used; all concentrations of trypsin used, except for the insoluble enzyme at 0.625 unit/ml, led to a rapid (<1 min) release of the enzyme. Indeed, exposure of adenylate kinase to insoluble trypsin (0.625 unit/ml) led to a 2-fold increase in activity over 20 min, whereas exposure of intact mito-

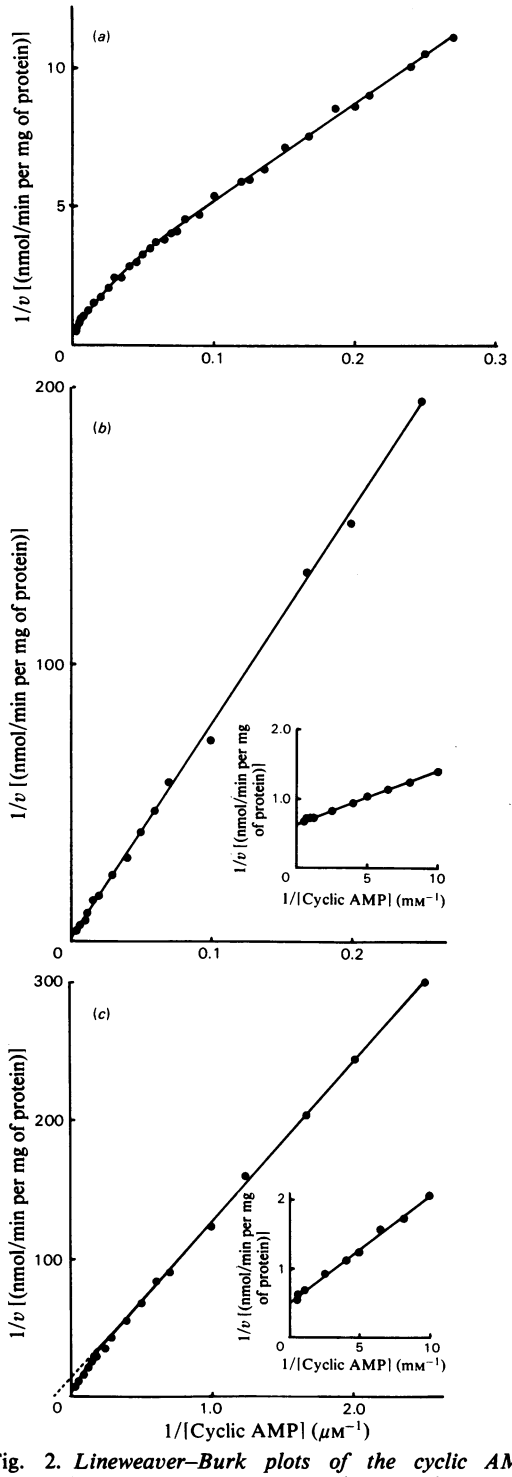

Fig. 2. *Lineweaver–Burk plots of the cyclic AMP phosphodiesterase activity of mitochondrial fractions*
(*a*) Intact, coupled mitochondria; (*b*) purified outer membranes; (*c*) inner-membrane freeze–thawed supernatants (IMEFTS). All assays were performed at 30°C under standard assay conditions. Linearity was ascertained over the entire substrate-concentration range. The inserts show an extended substrate range. Data of a similar form and quality were obtained with cyclic GMP as substrate.

chondria to this concentration of insoluble trypsin did not alter the total adenylate kinase activity present.

Thermal inactivation of phosphodiesterase activity

Table 3 summarizes the results obtained from thermal-inactivation studies performed at 55°C on the cyclic AMP phosphodiesterase activity in mitochondria, outer membranes and the inner-membrane freeze–thawed supernatant. These results show that in whole mitochondria there are two phosphodiesterase enzymes, which decay with distinctly different half-lives. However, linear thermal-inactivation plots were obtained with outer membranes or the inner-membrane freeze–thawed supernatant, implying that they each contained a single type of cyclic AMP phosphodiesterase enzyme.

The half-life for the outer-membrane activity is similar to the second, long-lived, component of the biphasic curve observed with whole mitochondria, whereas the half-life for the inner-membrane freeze–thawed supernatant is similar to the rapidly decaying component (Table 3). Interestingly, the sensitivities of the inner-membrane enzyme to both trypsin and thermal denaturation are similar (Tables 2 and 3) when the enzyme is either soluble or membrane-bound.

In the supernatants obtained on freezing and thawing the mitochondrial or the mitochondrial inner-membrane fractions, the long-lived outer-membrane enzyme could not be detected. However, sonication of mitochondria followed by freezing and thawing results in the release of both the outer-membrane and inner-membrane enzymes (results not shown).

The percentage contributions of the two phosphodiesterase enzyme components to the activity of whole mitochondria were obtained by extrapolation (Table 3) and were in good agreement with that observed from the trypsin experiments.

Kinetic analysis

With either cyclic AMP or cyclic GMP as substrate for the phosphodiesterase activity, the Lineweaver–Burk plots of intact mitochondria and the inner-membrane freeze–thawed supernatant were downwardly curving (Fig. 2). In contrast, that for the outer-membrane activity was linear. Table 4 gives the Hill coefficients, and limiting values for both K_m and $V_{max.}$ with cyclic AMP and cyclic GMP as substrates. The 'true' $V_{max.}$ values of the two phosphodiesterase enzymes calculated on the basis of total mitochondrial protein are also shown.

Table 4. *Kinetic parameters of mitochondrial cyclic nucleotide phosphodiesterase activity*

Kinetic parameters for the linear plot of the outer membranes were obtained after Wilkinson (1961) analysis. For intact mitochondria and mitochondrial inner-membrane freeze-thawed supernatant the non-linear plot was extrapolated at its linear extremities to yield limiting kinetic parameters K_m^1 and K_m^2. A Hill coefficient (h) was derived for the data for the enzyme of the mitochondrial inner-membrane freeze–thawed supernatant. However, true $V_{max.}$ values for the contribution of the inner-membrane and outer-membrane enzymes (I and O respectively) could be ascertained knowing the relative purification of these enzymes in their respective fractions. Hence the true $V_{max.}$ is given as the $V_{max.}$ observed in the purified fraction $\times (P/P_m)$, where P is the total protein in the purified fraction and P_m is the total mitochondrial protein. The substrate concentration range tested was from 2mM to 0.3μM at 44 different substrate concentrations.
Abbreviation: IMEFTS, mitochondrial inner-membrane freeze–thawed supernatant.

Substrate	Preparation (no. of determinations)	K_m^1 (μM)	K_m^2 (μM)	$V_{max.}^1$ (nmol/min per mg)	$V_{max.}^2$ (nmol/min per mg)	Hill coefficient (h)	True $V_{max.}$(I) (nmol/min per mg)	True $V_{max.}$(O) (nmol/min per mg)
Cyclic AMP	Mitochondria (6)	16.9 ± 1.4	300 ± 4.0	0.2 ± 0.03	1.9 ± 0.13	—	4.6 ± 0.1	0.45 ± 0.02
	Outer membranes (4)	—	120 ± 2.4	—	1.5 ± 0.01	—	—	—
	IMEFTS (4)	22.2 ± 2.3	1052 ± 10	0.63 ± 0.03	5.9 ± 0.42	0.66 ± 0.03		
Cyclic GMP	Mitochondria (2)	5.6 ± 0.3	132 ± 8	0.24 ± 0.01	2.0 ± 0.1	—	5.6 ± 0.21	0.45 ± 0.01
	Outer membranes (2)	—	22.6 ± 1.4	—	1.45 ± 0.01	—	—	—
	IMEFTS (2)	1.9 ± 0.5	238 ± 6.4	0.23 ± 0.02	5.1 ± 0.21	0.68 ± 0.02		

The ratio of the relative contribution to the total activity of the two phosphodiesterase enzymes in whole mitochondria can be deduced from the ratios of the observed initial velocities, for the outer-membrane fraction and the inner-membrane freeze–thawed supernatant, at any given substrate concentration by applying the required correction for total mitochondrial protein, On this basis, at 1mM-cyclic AMP the inner-membrane enzyme contributes some 82% to the total mitochondrial activity, whereas the outer-membrane enzyme contributes some 18%. This is in reasonable accord with that determined by the trypsin and thermal-denaturation studies where assays were made at this substrate concentration.

With freeze–thawed mitochondria, isolated outer membranes and inner-membrane freeze–thawed supernatant assayed at both 1mM- and 1μM-cyclic AMP, there was no significant (<10%) change in activity elicited by the addition of (i) 10μM-calmodulin, (ii) 10μM-trifluoperazine, (iii) 1mM-EGTA, (iv) Ca^{2+} (1–100μM), or (v) 10μM-calmodulin + Ca^{2+} (1–100μM).

Polyacrylamide-gel electrophoresis and sucrose-density-gradient centrifugation

Polyacrylamide-gel electrophoresis of the inner-membrane enzyme results in the identification of a single symmetrical peak of phosphodiesterase activity, at R_F 0.51, assayed at 1mM-cyclic AMP.

Centrifugation of both the inner-membrane enzyme and the dialysed high-salt-solubilized enzyme from outer membranes on a 15–30% linear sucrose density gradient resolved in each case only a single symmetrical peak of activity when assayed at 1mM-cyclic AMP. The sedimentation-coefficient values of the two enzymes were determined from the linear plots of fractions against sedimentation-coefficient values of the marker enzymes, whereupon the inner-membrane enzyme yielded a sedimentation coefficient of 1.4S (S.D. ± 0.09, $n = 4$) and that of the outer-membrane enzyme was found to be 9.4S (S.D. ± 0.10, $n = 4$).

Mitochondrial coupling states

The degree of mitochondrial coupling was determined and the ADP/O ratio (P/O ratio) was found to be 1.78 ± 0.015 ($n = 4$) with sodium succinate as substrate. On suspension in the phosphodiesterase assay medium as described in the Materials and methods section, the mitochondria remained coupled at both 4°C and after 30min at 30°C, retaining a P/O ratio of 1.74 ± 0.02 ($n = 4$) for sodium succinate.

Discussion

Rat liver lysosomes do not exhibit a cyclic nucleotide phosphodiesterase activity, whereas mito-

chondria appear to contain two distinct cyclic nucleotide phosphodiesterase enzymes, one of which is attached to the outer membrane and the other to the inner membrane. The outer-membrane enzyme is unlikely to be due to contamination with cytosol species, as the washing procedure removed any associated lactate dehydrogenase activity, and the kinetic characteristics, thermal stability and sedimentation coefficient differ from those of the cytosol activity (Marchmont & Houslay, 1980*a*). Furthermore, either freeze–thawing together with sonication or high-ionic-strength treatment was necessary to liberate this enzyme from mitochondrial outer membranes. These activities do not apparently accrue from any contamination with endoplasmic reticulum, as the activity in such membranes is all integral and exhibits different kinetics and thermostability from those of the enzymes discussed here (B. Cercek, unpublished work).

Both of the mitochondrial cyclic nucleotide phosphodiesterases are peripheral enzymes, that is they are associated with the membrane primarily via electrostatic interactions and can hence be solubilized by high-ionic-strength treatment or by freeze–thawing (together with sonication in the case of the outer-membrane enzyme). This is in contrast with integral enzymes, which are firmly embedded in the bilayer, interacting by extensive hydrophobic interactions and requiring detergent treatment for solubilization (Houslay, 1981).

The cyclic nucleotide phosphodiesterase activity associated with both the mitochondrial inner and outer membranes is supplied by distinct single enzymes, as indicated by thermal- and trypsin-denaturation (protein stability), sedimentation (size) and polyácrylamide-gel-electrophoresis (charge) studies. As both enzymes are degraded by soluble trypsin under conditions where malate dehydrogenase exhibits full latency yet adenylate kinase latency is lost, we can conclude that the peripheral enzyme associated with the mitochondrial inner membrane is located on the cytosol (C)-side, which faces the intermembrane space. Such a localization would be consistent with our lack of observation of any latent cyclic nucleotide activity, for we would not expect the cyclic AMP substrate to cross the inner membrane of the fully coupled mitochondria used in our assays. The use of a low concentration (0.625 unit/ml) of insoluble trypsin shows clearly that the outer-membrane peripheral enzyme is attached to the external surface of the membrane. In this case its activity remained susceptible to the action of trypsin, whereas that on the inner membrane was protected under conditions where both adenylate kinase and malate dehydrogenase exhibited full latency.

The outer-membrane peripheral enzyme exhibited normal Michaelian kinetics, with a lower K_m for cyclic GMP than for cyclic AMP, but with $V_{max.}$ values (Table 4). The hydrolysis of cyclic AMP by this enzyme is inhibited by cyclic GMP, rather than activated (B. Cercek, unpublished work), and thus the kinetic characteristics are typical of the 'low-affinity' species of cyclic nucleotide phosphodiesterases (Wells & Hardman, 1977). On the other hand the inner-membrane peripheral enzyme displays kinetics indicative of apparent negative co-operativity. Owing to the small size of the single enzyme responsible for this activity, it is possible that the kinetic pattern arises from a monomeric enzyme obeying a mnemonic kinetic mechanism in a similar fashion to the peripheral plasma-membrane enzyme (Marchmont *et al.*, 1981), although we cannot rule out the possibility that it has two substrate-binding sites exhibiting distinct K_m values. Neither of the enzymes appears to be Ca^{2+}/calmodulin-sensitive. The form of the kinetics is such that at 1 mM-cyclic AMP the inner-membrane enzyme is responsible for 80% of the hydrolysis of cyclic AMP. Furthermore, extrapolation of initial-velocity data points over an effective physiological substrate concentration range (0.5–10 μM; see Marchmont & Houslay, 1980*b*) yields a much lower apparent K_m value for cyclic AMP (21 μM), implying that this enzyme may be of some importance in determining cyclic AMP concentrations in the vicinity of the mitochondrion. Indeed Erneux *et al.* (1980) have postulated that such apparent negative co-operativity in a cyclic AMP phosphodiesterase has important regulatory advantages in the cell. However, the value of the limiting K_m for the low-affinity expression of this enzyme activity is unusual in that it is much greater than has been observed for other species exhibiting apparent negative co-operativity (see, e.g., Wells & Hardman, 1977; Loten *et al.*, 1978; Thompson & Strada, 1978; Marchmont & Houslay, 1980*a*).

The molecular size of these enzymes is in the range that has been reported for various species in a number of tissues (see Pichard & Cheung, 1976).

The purpose of having such a variety of different enzymes capable of hydrolysing cyclic nucleotides within a given tissue is unclear at present. It may well be that such enzymes can lead to the local control of cyclic AMP concentrations at sites within the cell. We might thus expect that these enzymes would be regulated individually, and indeed, for example, insulin does appear to activate specific enzymes (Houslay *et al.*, 1982). The resolution and identification of enzymes associated with specific membranes should enhance our appreciation of such controlling influences. Indeed, as cyclic AMP can modulate the release of Ca^{2+} from mitochondria under physiological conditions (Arshad & Holdsworth, 1980), then it is possible that the activity of these enzymes could influence such a process.

Furthermore, the liver plasma-membrane peripheral enzyme has been shown to bind to a single class of protein sites on the membrane (Houslay & Marchmont, 1981) that serve both to localize the enzyme and to regulate its activity. An analysis of the interaction of the mitochondrial peripheral proteins with the membrane may well aid in understanding their function.

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